Tissue Engineering for Artificial Organs

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Regenerative Medicine, Smart Diagnostics and Personalized Medicine

Edited by Anwarul Hasan

Volume 1

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Foreword

With the rapid advances that are seen nowadays in the area of tissue engineering, and the lack of manuscripts dealing with the state of the art of the field, this book presents a comprehensive review of tissue engineering in its various applications. Although a relatively new field, tissue engineering has seen huge progress in the past few decades, and scientists from various backgrounds are jointly working on making it a reliable method not only for organ replacement but more recently for *in vitro* disease models as well.

The book presents a collection of basic topics related to tissue engineering, which are organized in a progressive manner. Starting with a brief historical background on the development of tissue engineering as a substitute for organ transplantation, the current state of the art and the future prospects are also pointed to. The different pillars of the field are then discussed: biomaterials, stem cells, biosensors, biomanufacturing, and bioreactors are all necessary topics to consider when trying to engineer tissue constructs for organ replacement. For a new researcher entering the field, this first part gives an idea of the multidisciplinary aspect of tissue engineering. In fact, a combination of efforts is needed from experts in engineering, cell biology, materials, chemistry, and even microfluidics to be able to construct a scaffold for the engineered tissue, seed it with cells, and ensure proper attachment and functioning of the cells inside it. In the second part of the book, specific organs are treated in more detail, and different tissues are considered, from simple skin substitutes to the more complex organs such as lungs, and even to the nervous system with neural tissue engineering. Examples are cartilage, bone, musculoskeletal, cardiac, and heart vessel tissue engineering. Other organs are also treated, such as the liver, kidney, and pancreas. In a more recent advance, the final chapter of the book deals with the use of bionics in tissue engineering, which is an area with great future prospects.

The structure of the book should make it easy for the reader to follow the progression of tissue engineering from its basic elements (materials, cells, etc.) to

the full reconstruction of organs. In the second part, a whole chapter is dedicated to each type of tissue reconstruction in order to discuss all of its details and challenges.

In summary, the book offers a detailed and clear perspective of tissue engineering in its recent forms and applications. Given the variety of topics discussed in the context of recent advances, it could serve both as an introductory step into the field for new researchers in tissue engineering and as a reference for more advanced readers interested in learning about a particular topic.

February 2017

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Preface

Tissue engineering is a new interdisciplinary field that aims at developing or regenerating artificial tissues and organs by using a combination of cells, biomaterials, engineering techniques, and suitable physicomechanical and biochemical stimuli to improve or replace biological functions. It is a relatively young field, as it emerged as a properly defined academic field only in the 1980s. However, it has progressed rapidly in terms of striking advances in the use of cells, biomaterials, and mechano-biochemical stimuli for the purpose of artificial organ generation.

The motivation for tissue engineering research emerges from the fact that millions of people worldwide go through various biomedical implant surgeries every year and the total cost for organ replacement surgeries is in billions of dollars annually. The currently available options for these transplants are autologous grafts, allografts (donor/cadaveric), xenografts, and artificial prostheses. All of these currently available options and technologies for organ replacement have severe limitations. The use of autografts and allografts, for example, are limited because of the lack of tissue donors, previous harvesting, or anatomical variability, as well as the concerns about their long-term functionality. Xenografts suffer from their relatively short life span, poor control over their physical and mechanical properties, inflammation, and calcification. Synthetic prosthetics often get rejected within a few months to a few years by the immune system of the body.

Tissue engineering has emerged to resolve these issues by generating implantable artificial tissues and organs. The ability to repair or regenerate damaged human tissue has already been a tremendous leap forward in the field of medicine. The goals and aims of tissue engineering have expanded rapidly from growing engineered skins for replacing the skin of burn victims and repairing cartilage joints of arthritis victims to re-creating tissues of more complex human organs such as the liver, kidneys, and heart, as well as regenerating lost neurons in patients with Alzheimer's disease and growing engineered blood vessels for cardiac bypass grafts. The targeted applications of engineered tissues have also expanded from implantable organs to *in vitro* models of tissues for the development of various *in vitro* disease models that could revolutionize the new therapeutics for numerous diseases. Furthermore, the progress of patient-specific smart diagnostics and personalized medicine will greatly benefit from the development of functional healthy or diseased models of engineered

tissues on microfluidic platforms. As a result, billions of dollars have been poured into bioengineering research over the last two decades, particularly focusing on organ regeneration. This has been resulting in exciting advancements and progress in the field.

However, the number of textbooks on tissue engineering for artificial organs have remained very limited, most of which are also not up to date. This book is intended to fill in a large part of this gap, reporting the major recent advances in tissue engineering and regenerative medicine. Tissue engineering for these applications requires the understanding of various fields of science and engineering. Biology, chemistry, microfluidics, as well as structural and material engineering are just some of the fields that help in understanding the concept of replacing and regenerating body tissues. Various biomaterials, their biocompatibility with *in vivo* conditions, and understanding the chemical makeup and engineering structure of the engineered tissue to fulfill its task are also important. Therefore, this book is divided into two parts. The first part aims to give an introduction to tissue engineering in general. The history of tissue engineering, the recent breakthroughs, and the future perspectives are discussed in Chapter 1. The major topics associated with tissue engineering, namely biomaterials, stem cell sources, induced pluripotent cells, biosensors, and bioreactors are presented in Chapters 2–6, respectively.

The second part of this book delves deeper into a more detailed discussion of various tissues and organs of the body. From skin, cartilage, bone, and musculoskeletal tissue to blood vessel, cardiac, heart valve, lungs and tracheal tissue as well as pancreas, kidney, liver, neural tissue, and bionics, the book presents the challenges that are faced in engineering each type of tissue. Also, it shows how to successfully engineer a tissue that does the function of its natural and biological counterpart.

This book has a slightly different focus from other available books on tissue engineering in that here the focus is on the application of tissue engineering and regenerative medicine to smart diagnostics and personalized medicine in addition to the generation of implantable tissues and organs.

Publication of this book would not have been possible without the tireless efforts of all the contributors. I would therefore like to extend my thanks and sincere gratitude to all the authors from across the globe for the successful completion of this work.

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Part I

Fundamentals

1

Introduction to Tissue Engineering

Rami Mhanna and Anwarul Hasan

1.1 Introduction

Tissue engineering is an interdisciplinary field that utilizes cells, biomaterials, biochemical (e.g., growth factors) and physical (e.g., mechanical loading) signals, as well as their combinations to generate tissue-like structures [1]. The goal of tissue engineering is to provide biological substitutes that can maintain, restore, or improve the function of damaged tissues [2]. Although the first tissue-engineered skin products were introduced in the late 1970s and early 1980s giving rise to modern tissue engineering, the term "tissue engineering" was coined only in 1987 [3–6].

In fact, the use of prosthesis (e.g., gold for tooth replacement and wood for limbs and toes) was employed as early as ancient Egyptians. However, these treatments were all based on nonliving materials, which provided some structure and function but were very far from the original tissue. Medical development led, in the middle of the twentieth century, to the possibility of replacing an entire organ with an organ from a donor, known today as *organ transplantation* [7]. Although this is widely practiced today and is known to be the ultimate solution for organ failure, the need for organs always surpasses the number of available donated organs [8]. The limited donor availability and rejection of the grafts by the immune system drove the concept of *in vitro* grown tissues. The success in tissue engineering of skin grafts boosted the interest in applying similar concepts to other tissues and organs [9]. However, the relatively simple structure, the limited vascular demands of skin, and the ease of growing keratinocytes *in vitro* are not common to most tissues. The dream of regenerating tissues *in vitro* faced major hurdles associated with the engineering of complex, three-dimensional (3D), vascularized multicellular tissues.

In this chapter, we provide a brief introduction to tissue engineering. The clinical needs for tissue engineering, the history, the fundamentals, and the applications of tissue engineering are discussed in brief. The recent advancements in the

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field, as well as some of the major challenges and the future of tissue engineering, are also briefly discussed.

1.2 Clinical Need for Tissue Engineering and Regenerative Medicine

The clinical need for tissue engineering and regenerative medicine is the result of our urge to treat defective tissues. Regardless of how such defects occurred (congenital or acquired), traditional medical tools are not yet capable of completely or efficiently fixing them. In fact, traditional medicine has severe limitations in delivering solutions for numerous health problems. Injuries and diseases are traditionally treated using pharmaceuticals, whereas prosthetic devices and organ transplantation are used in more severe conditions. While pharmaceuticals may be useful for the treatment of numerous conditions, they cannot cure a number of deadly diseases (e.g., several forms of cancers, strokes, diabetes, etc.) or diseases at their advanced stages (e.g., Alzheimer's, Parkinson's, osteoarthritis, etc.). On the other hand, prosthetic devices are not capable of restoring normal function, and the number of organ donors is always way less than required. Tissue engineering can be used to treat diseases that cannot be cured with regular pharmaceuticals and to provide natural, living, functional organs to overcome the need for donors and prosthetics.

The main goal of tissue engineering is the development of functional substitutes for damaged tissues [2]. It is estimated that the majority of tissue engineering products are used for the treatment of injuries and congenital defects, while tissue engineering products used for the treatment of diseases are less common. The worldwide tissue engineering and cell therapy market has been estimated in 2014 at about $15 billion and is expected to grow up to $32 billion by 2018. The dominant market is in the orthopedic, musculoskeletal, and spine areas followed by the skin, nervous tissues, and other organs [10]. Skin was the first tissue to be engineered; this is because of the relatively simple structure of the tissue (can be prepared using two-dimensional (2D) culture and has easy access to culturing medium). Skin is also an important tissue engineering target because of the high demand especially resulting from war burns. Skin damage can cause disfigurement and disability, which may lead to further serious infections and psychological damage to patients. All these factors made skin one of the first clinical tissue engineering targets. Tissue engineering and regenerative medicine solutions can also be applied for any tissue, although the levels of complexity would differ between targets. Examples include the heart, kidneys, cornea, nervous tissues, liver, intestines, pancreas, lungs, bone, muscle, and so on. The ultimate goal is that tissue engineering and regenerative medicine would one day be able to overcome the need for organ transplantation. The medical need for tissue engineering and regenerative medicine can be emphasized in the donor waiting list, which is always increasing at a higher pace than the number of organ donors. The ability to engineer such organs or help them regenerate would represent a great leap in the history of the health care field.

1.3 History of Tissue Engineering and Regenerative Medicine

Generating new tissues and restoring body parts or organs are ideas that were embedded in humans' imaginary world from the dawn of history. The revolution of the human race enabled these imaginary notions to become well-practiced findings all over the years. In the case of the ancient Egyptians, restoring body parts was reasoned by the importance of reuniting and reassembling the body to enable revitalization in the Afterlife, as inscribed in spells known as the "Pyramid Texts" (2375 BC) [11]. It is believed that the first dental prosthesis was constructed from gold in Egypt around 2500 BC [12]. Nerlich and colleagues account for an ancient Egyptian false big toe believed to be the oldest limb prosthesis (950–710 BC), Figure 1.1a [13]. Interestingly, this prosthesis was recently found to improve function and walking, which indicates the possibility that the purpose of these designs was not only for the Afterlife [14].

The use of nonliving materials enabled the restoration of the structure, shape, and function to some extent. However, living tissues would be needed to achieve a full recovery. History notes the miraculous leg transplantation by Saints Cosmas and Damien (about 287, Figure 1.1b). In the sixteenth century, Gaspaire Tagliacozzi Bologna, Italy, was the first to write a book on plastic surgery where he first described the nose reconstruction from the forearm flap. Tagliacozzi made a great revolution at that time when alterations in body appearance were religiously prohibited [15].

The progress in anesthesia and infection prevention in the nineteenth century helped in the rapid development of surgical procedures. This development allowed the first applications of living tissues and organs to recover malfunction [16]. Skin grafts were the first tissue-based therapies, and the introduction of techniques to preserve cells and tissues enabled allograft skin banking [17–19]. Shortly thereafter, the first successful complete organ transplantation of a kidney

Figure 1.1 Some random images showing the development of regenerative medicine throughout different eras in history. (Nerlich 2000 [13]. Reproduced with permission of Elsevier.) (a) 2500 BC: false big toe developed in ancient Egypt. (b) 278 AD: Saints Cosmas and Damian performing a leg transplant from a deceased donor onto a patient with an amputated leg. (Zimbler 2001 [15]. Wikipedia, public domain, https://commons.wikimedia.org/wiki/File:Fra_Angelico_064.jpg.) (c) In 2013, Chinese doctors saved a man's severed hand by grafting it to his ankle before later reattaching it to the patient's arm. (Gordon 2006 [21]. Reproduced with permission of John Wiley and Sons.)

between identical twins could be achieved [20]. Limited donor availability and rejection of the grafts by the immune system drove the concept of *in vitro* grown tissues, giving rise to the field of "tissue engineering."

The success of engineering skin grafts boosted interest in applying similar concepts to other tissues and organs. However, the relatively simple structure, limited vascular demands of skin, and the ease of growing keratinocytes *in vitro* are not common to most tissues. Tissue engineering first raised immense public awareness and media interest in 1997 when the BBC documented the potential of engineering an ear (Figure 1.3) [22]. The so-called Vacanti mouse represented the promise that tissue engineering holds for tissue recovery becoming known by millions around the globe. Despite the innovative and exciting nature of the Vacanti experiment, it represented only the beginning of the tissue engineering journey and the organ engineering "proof of concept." The engineered tissue in the Vacanti experiment had many limitations that make it difficult if not impossible for the system to be translated to a clinical scenario without major alterations. The engineered ear, which was intended for a three-year-old boy, was prepared using a polyglycolic acid (PLA) scaffold seeded with bovine chondrocytes and implanted in an immunodeficient mouse for culture. If such a tissue is implanted in a human, it would result in a strong immune response not only due to the mouse where it had been grown but also due to the cultured bovine cells. The ideal replacement for the mouse would be a human, most ideally the ear receiver, to totally reduce immune rejection. On the other hand, the replacement for the bovine cells would be autologous chondrocytes, which are very limited in supplies. Alternatively, other cell sources may be used, all presenting their advantages and disadvantages, which will be discussed in more details in the following sections. A second limitation is the skin coverage of the engineered tissue, which would either be missing if only the scaffold/cell structure is extracted or would have immune and structural limitations if it is removed together with the mouse skin. Fixing the skin coverage limitation might be possible using skin grafting, but it would highly increase the complexity of the system. A third limitation is concerned with the control over the growth of the ear during the mouse culture period and after transplantation. Further limitations concerning the mechanical and chemical properties of the engineered tissue resulting from the scaffold and culture conditions are all issues that face tissue engineering even today. The dream of regenerating tissues *in vitro* faced and is still facing major hurdles associated with the engineering of complex three-dimensional (3D) vascularized multicellular tissues.

1.4 Fundamentals of Tissue Engineering and Regenerative Medicine

1.4.1 Tissue Engineering versus Regenerative Medicine

Tissue engineering and regenerative medicine are often used interchangeably. However, tissue engineering typically involves the construction of a tissue *in vitro*, while regenerative medicine refers to tools for helping the body

regrow a damaged tissue *in vivo* in the patient. The need for cell sources in tissue engineering was a major limiting factor in the advancement of the field. This shortage of cell sources ignited the use of renewable cells such as stem cells and progenitors, leading to the term "regenerative medicine." Regenerative medicine is mostly based on understanding morphogenesis and natural, inherent self-repair mechanisms, and, as such, regenerative medicine typically involves the use of stem cells and progenitors. Tissue engineering and regenerative medicine, often abbreviated as "TERM," are today complementary. There is an increased interest in the use of various stem cell sources and a need to reduce culture times for engineered tissues, which consequently results in a shorter waiting period and lower prices. This will eventually result in strengthening the bonds between tissue engineering and regenerative medicine, which are likely to become inseparable.

1.4.2 The Triad of Tissue Engineering

Tissue engineering applications typically involve the combination of three pillars: cells, signals, and scaffolds, which represent the "triad of tissue engineering" (Figure 1.2). Although many of the claimed tissue engineering applications might lack one of these pillars, their combination appears to be essential for the success of tissue engineering applications. Current advances in tissue engineering involve developments in all elements of the triad. In this section, major advances in cell

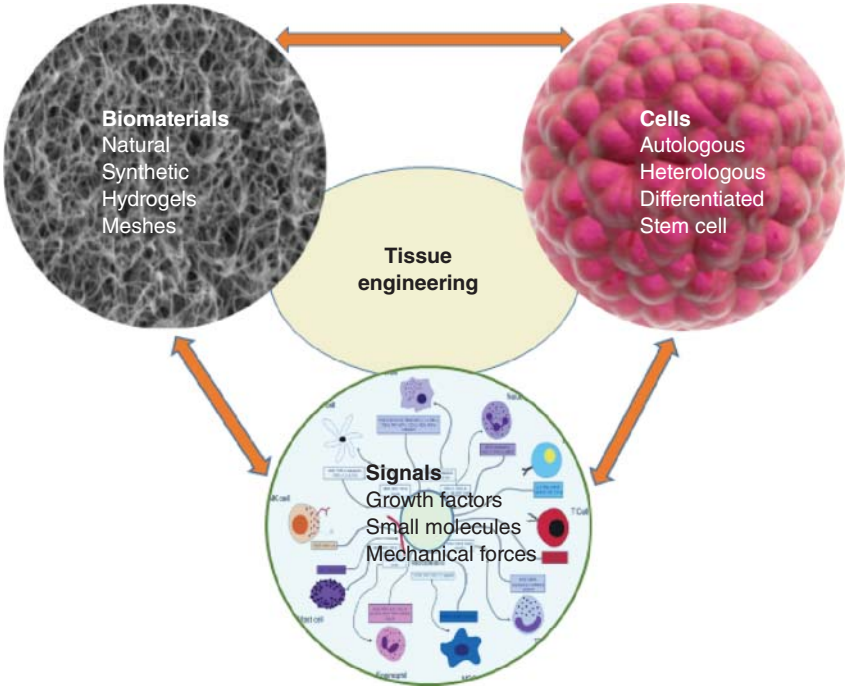

Figure 1.2 The triad of tissue engineering. The combination of cells, scaffolds, and signals is used to engineer functional tissues.

sourcing, scaffold production, signaling in tissue-engineered structures, and their combinations to create functional tissues and organs will be presented.

1.4.3 Approaches in Tissue Engineering

Incorporating the three elements of tissue engineering needs a good scaffolding technique. Over the years, different approaches have been developed, resulting in scaffolds that can support the cells and encourage tissue growth after implantation (Figure 1.3).

The most common approach is the use of a pre-made porous scaffold. Using raw materials – which can be either natural or synthetic – a porous scaffold is created through one of the different fabrication technologies currently available. The diverse possibilities of biomaterials to use and the ability to design the scaffold in a way to control its physicochemical properties make this method especially advantageous. Examples of manufacturing techniques that are used include porogens or fiber-based techniques as well as new solid free-form technologies. Once the supporting scaffold is ready, cells can be seeded inside or on top of the scaffold. A disadvantage of this method is that the post-fabrication cell seeding is both time consuming and not very efficient [23].

Instead of seeding the cells in the scaffold after it is fabricated, another strategy is to encapsulate the cells during scaffold formation. While the number of biomaterials that can be used to create this type of scaffolds is more limited, an advantage is the possibility of delivering the cells in a liquid precursor *in vivo*. Hydrogels (natural or synthetic) are usually used as a scaffold material for encapsulation given their biocompatibility and mild gelation conditions [24]. However, given the poor mechanical properties of hydrogels used in this application, this scaffolding approach is rarely used for tissues having load-bearing functions [23].

Another method that can be used for scaffolding is the decellularization of the extracellular matrix (ECM) from either allogeneic or xenogenic tissues. The ECM is a natural scaffold that allows cell attachment, proliferation, and differentiation. When seeded with the proper cells, it can produce an autologuous construct without the need for extracting tissues from the patient him/herself [25]. The advantages of this method are that it is biocompatible and presents the closest natural mechanical and biological properties needed in the body. The main disadvantage of these systems is the limited supply of autologous tissues and immune responses to non-autologous tissues. Additionally, some minor problems still exist such as inhomogeneous distribution of the seeded cells and the difficulty of removing all immune-provoking material [23]. This technique has proven useful in skin, bladder, and heart valve repair. It has also produced many commercialized decellularized scaffolds with the U.S. Food and Drug Administration (FDA) approval to be used in humans [25].

A final approach is the use of cell sheets prepared using temperature-responsive culture dishes, in a technique known as *cell sheet engineering*. This method avoids the problems caused by transplanting engineered tissues based on fabricated scaffolds; in fact, after the scaffold degrades in the body, it is often replaced by autologous ECM, which can cause fibrosis. In addition, some properties of scaffolds might be undesirable for specific applications. With the development

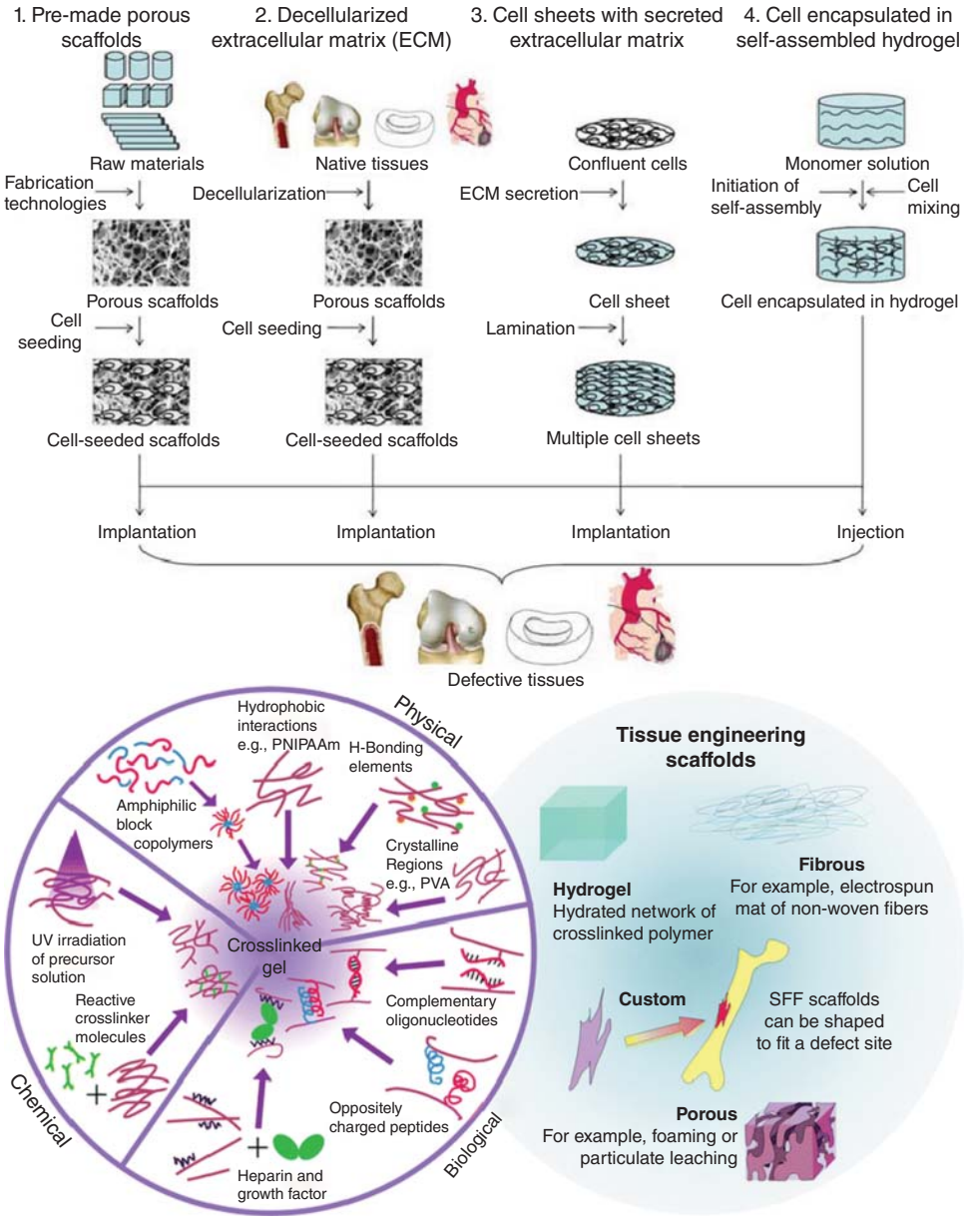

Figure 1.3 Schematic illustration showing different scaffolding approaches that are being used for tissue engineering. These scaffolds can be combined with various physiomechanical strategies to enhance tissue growth.

of regenerative medicine, the injection of a single-cell suspension showed good results replacing a scaffold, but for larger tissue reconstruction more cells are needed, which was the motivation behind cell sheet engineering [26].

1.4.4 Recent Advances in Tissue Engineering

1.4.4.1 Advances in Cell Sourcing and Cell Manipulation

Cells being the building blocks of all living tissues are the starting point for creating tissue substitutes. Growing knowledge about cell manipulation and stem cell differentiation has opened new horizons in the field, providing larger cell pools for all tissue engineering applications. Autologous cells are considered the favorite cell type for engineering tissues, as they do not evoke immune responses and thus eliminate the need for immunosuppressants and their side effects [27]. However, autologous cells are limited in supplies and require a long culture period to engineer the desired tissues. Much of current research aims to use allogeneic [28, 29] or xenogeneic [30] sources to overcome the shortage of autologous cell availability. The use of allogeneic or xenogeneic sources is, though, still associated with major obstacles, such as immune-rejection, transmission of diseases, mismatch between donor and recipient cellular microenvironment, and ethical considerations, which limit their widespread adoption in clinical applications [31].

Applications of stem cells in tissue engineering continue to grow and their use has found its way to the clinic. Although adult mesenchymal stem cells remain the dominant stem cell type used in tissue engineering, embryonic stem cells are also being used and have started to find their way into the market [27]. A major breakthrough in cell sourcing was the recent discovery by Shinya Yamanaka that adult differentiated cells could be induced to become pluripotent stem cells [32]. The discovery of induced pluripotent stem cells (iPSCs), for which Yamanaka was awarded the Nobel Prize in Physiology and Medicine in 2012, has opened unprecedented opportunities in the tissue engineering field by providing a new, large source of autologous cells. A major challenge remains in establishing standardized protocols to induce the differentiation and commitment of differentiated adult, induced, or embryonic stem cells toward the desired lineages.

1.4.4.2 Advances in Biomaterials and Scaffold Production

In the past few decades, a great number of biomaterials from natural and synthetic origins, as well as novel fabrication methods, have been proposed. Current research is focused on developing "smart biomaterials" capable of directing cell functions and/or enhancing cellular performance [33]. The role of the scaffold is to provide structural support and proper signaling cues for cells so that they can replace the scaffold with their own synthesized matrix. Synthesis of new matrix by the cells and degradation of the scaffold should be synchronized so that one process is not faster than the other.

Generally, the goal is to design a scaffold that mimics the structure and composition of the target tissue. Given the complexity of the chemical composition of natural tissues, it is often not possible to fully recapitulate them *in vitro*. Recent developments have led to the establishment of techniques such as bio-printing

[34–37] and two-photon lithography [38, 39], enabling production of precise 3D structures for tissue engineering applications. These techniques also allow the precise positioning of growth factors and recognition sequences for controlled cell behavior [40, 41]. Scaffolds can be prepared with good control over the chemical composition, allowing cells to spread and proliferate (e.g., collagen, gelatin) or inhibiting cell spreading (e.g., alginate, poly(ethylene glycol)). Scaffolds can be made to provide cells with adhesion sequences for cell attachment (e.g., RGD, GFOGER, IKVAV) and matrix metalloproteinase (MMP)-sensitive sequences for scaffold degradation [42–47]. Modifying scaffolds with small molecules, such as phosphate groups and sulfate groups, among others, has been also shown to have strong effects on cell proliferation and stem cell differentiation [48, 49]. All these studies are necessary to identify the ideal scaffolds for each individual tissue engineering application.

1.4.4.3 Advances in Cell Signaling Research and Bioreactor Development

After providing cells with a growing substrate or scaffold, cells require certain signals to survive and synthesize their own matrix that will eventually replace the carrying scaffold. Much knowledge has been acquired about cell signaling, and even more is currently being elucidated. Signals are normally generated by the surrounding cell microenvironment, sensed by receptors on the cell membrane or directly inside the cell, and translated into a variety of cell responses including proliferation, apoptosis, migration, differentiation, and matrix synthesis, among others. The most important signals sensed by cells involve oxygen levels, mechanical stimulation, growth factors, ECM molecules, and other small molecules.

It has been shown, as expected, that different tissues require different combinations of signals, and even the same tissue might require different signals at different depths or different maturation stages. For example, cells used to engineer articular cartilage, which is a relatively simple tissue known to be avascular, require relatively low oxygen levels (below 5%) for the synthesis of type II collagen (the major ECM component of articular cartilage), which in nature is synthesized in high quantities in the deeper cartilage layers. However, to engineer the superficial layer of the tissue, cells require high oxygen levels, which favor synthesis of superficial zone protein (protein mainly synthesized by chondrocytes of the superficial zone and responsible for lubrication) [50]. Moreover, physiologic tensile strain [51] and surface motion [52] are believed to promote superficial zone protein synthesis, while mechanical compression [53] and hydrostatic pressure [54, 55] have been shown to increase type II collagen synthesis (Figure 1.4). Excessive mechanical loading leads to the production of metalloproteinases and aggrecanases that degrade ECM proteins [56]. Systems have been developed for the application of tensile load, compressive load, hydrostatic pressure, shear, and perfusion [53, 57–62]. Notably, stem cells' fate can be steered through the application of phenotypic loading. To illustrate, the application of tensile loads help steer stem cells toward ligament [58], tendon [59], or bone tissue, depending on the tensile load parameters [60, 63], while shear loads can help stem cells differentiate toward cardiac muscle [64] or endothelial cells [61]. Finally, hydrostatic pressure or compression can lead to chondrogenic differentiation [65–68]. The use of bioreactors to

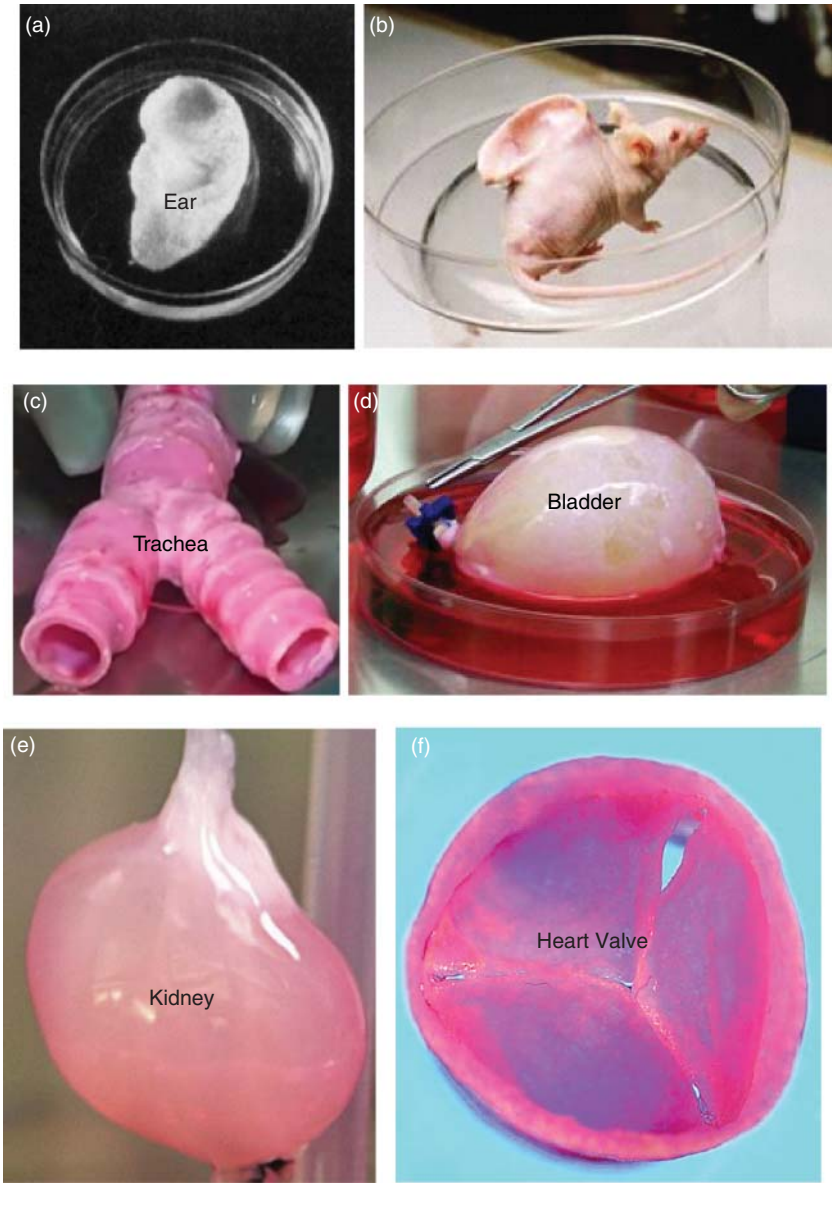

Figure 1.4 Different tissue-engineered organs. (a) Scaffold prepared from synthetic biodegradable polyglycolic acid (PLA) in the shape of a 3-year-old auricle. (b) Scaffolds implanted subcutaneously on the back of an immunodeficient mouse. (Reproduced with permission from [18].) (c) First trachea organ transplant using human's bone marrow stem cells. (d) Constructed artificial bladder seeded with human bladder cells and dipped in a growth solution. (e) Bioengineered kidney that mimics the function of a normal kidney concerning the control of the urinary system and blood filtration. (f) Tissue-engineered heart valve using human marrow stromal cells.

engineer tissues has grown exponentially in the past decade. This is the result of the understanding that tissues need to be subjected to certain forces to adopt a natural phenotype and attain physiologic matrix composition and mechanical integrity. Moreover, bioreactors improve mass transport, which is a prerequisite for engineering 3D complex tissues and organs. Another important role of bioreactors is to standardize, control, and automate the culture conditions to achieve reproducible outcomes. Reproducibility is highly critical in tissue engineering, especially when products might be clinically implemented.

Growth factors, cytokines, ECM molecules, and other small molecules have a profound effect on cell behavior. While some growth factors such as basic fibroblast growth factor (FGF-2) maintain "stemness" of stem cells [69], transforming growth factor beta (TGF-β) induces chondrogenesis [70], bone morphogenic protein (BMP) is necessary for bone formation [63, 71], and nerve growth factor (NGF) is crucial for neural differentiation [72, 73]. In tissue morphogenesis, the production of ECM molecules such as fibronectin and collagens varies depending on the development stage. Changes in the patterns of expression of the ECM molecules are associated with different processes such as stem cell condensation, cell migration, and cell differentiation [74]. Past and current research has revealed much about the function and roles of proteins and genes, but polysaccharides have not been under much focus. Polysaccharides (e.g., hyaluronic acid, chondroitin sulfate, heparin, and heparan sulfate (HS), among others) play a pivotal role in a multitude of physiological and biological processes and possess the ability to encode the function of biological entities analogous to DNA, RNA, and proteins [75, 76]. The sulfation of HS has been implicated in the repair of the central nervous system (CNS). Schwann cells exhibit higher sulfation levels of HS compared to olfactory ensheathing cells during the formation of the gliotic scar. The highly sulfated HS synthesized by Schwann cells is believed to induce a reactive astrocyte phenotype, which inhibits axon growth following CNS injuries [77]. This information can be used to improve current treatments of neural injuries or to design better neural tissue engineering products.

1.4.4.4 Engineering Complex Tissues and Organs

Tissue engineering holds strong promise of providing substitutes for damaged tissues and organs. However, the field that is less than 50 years old can be considered to be still in its infancy. Tissue engineering has found initial success with the production of simple tissues such as skin [5, 78] and cartilage [22]. Over the past few years, more complex multicellular tissues and organs have been engineered, including urethras [79], tracheas [80], blood vessels [81, 82], airways [85], and bladders [9, 86]. Advances in the tissue engineering field have also reflected in high economic returns for tissue engineering, which grew from $7.5 billion in 2010 to about $15 billion in 2014 and is expected to reach $32 billion by 2018 [10]. So far, tissues were mainly engineered using membranes with one cell type cultured on each side; therefore, they were based on 2D culture techniques. However, engineering of more complex 3D tissues is still limited by several factors affected by all elements of the tissue engineering triad. The most important challenge facing the development of 3D complex tissues

is mass transport that governs access of nutrients and secretion of wastes in engineered tissues [87, 88]. Circulation of nutrients and wastes in natural tissues *in vivo* is controlled by blood vessels. In tissue-engineered structures, mass transport can be achieved by using bioreactors, as mentioned previously, or by inducing the formation of new blood vessels. Efforts have focused on developing scaffolds with certain patterns or coatings to induce neovascularization, cell manipulation to induce differentiation, or secretion of vascular endothelial growth factors (VEGF) and proper signaling such as the addition of growth factors.

1.5 Applications of Tissue Engineering

Tissue engineering is a young field that utilizes cells, biomaterials, physical signals (e.g., mechanical stimulation), biochemical signals (e.g., growth factors and cytokines), and their combinations, to engineer tissues. The most common application of tissue engineering is to create tissues that can be used to repair or replace tissues in the body suffering partial or complete loss of function. However, tissue engineering has started to find new applications such as the development of extracorporeal life support units (e.g., bioartificial liver and kidney), *in vitro* disease models, tissues for drug screening, smart diagnosis, and personalized medicine. These applications will be discussed in more details in the following sections. Figure 1.5 depicts some of these applications.

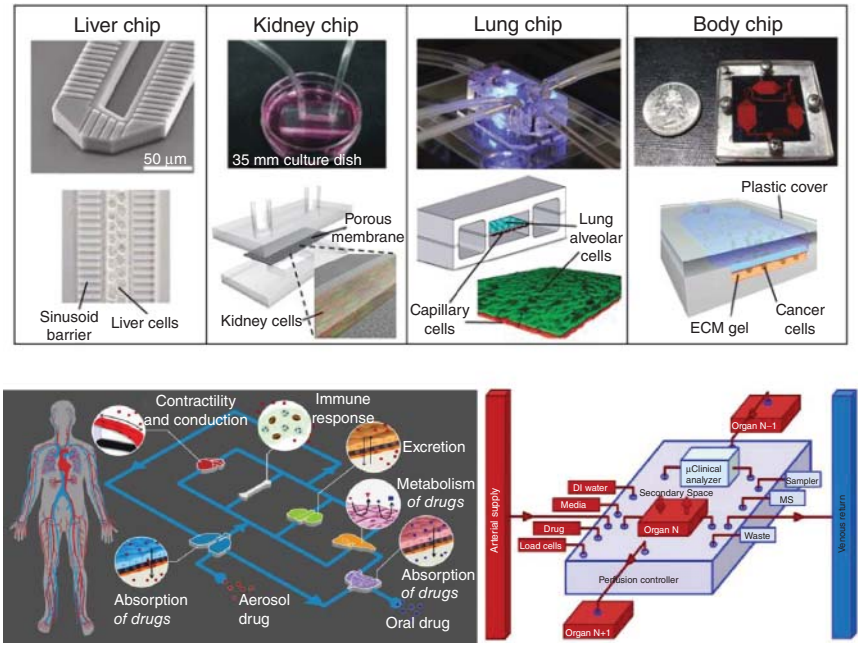

Figure 1.5 Recent advances in the applications of microfluidics in developing "organs-on-chips" models for *in vitro* investigations of engineered tissues and organs.

1.5.1 Implantable Tissues and Organs

Tissue engineering came to being as a solution to the partial or complete loss of organ functions due to congenital failure, disease, or injury. Tissue engineering may be used to restore different tissues including connective (e.g., bone, cartilage, blood), muscle (e.g., cardiac, skeletal), epithelial (e.g., skin, linings of the digestive tract), and nervous tissues (e.g., central nervous tissue, peripheral nervous tissues).

Skin epidermal tissue was the first tissue to be investigated for the purpose of skin replacement [89]. The earliest attempts to grow Keratinocytes *in vitro* were based on explant or organ cultures, which soon appeared to be overgrown with fibroblasts and exhibited limited proliferation [90, 91]. The discovery of Puck *et al*. [92] in 1956 that lethally irradiated epithelial cells can provide mitogens without proliferation opened the way for the use of these cells as a feeder layer for a cocultured cell layer. The feeder layer concept was used in 1975 by Green and Rheinwatd to grow human epithelial cells, leading to the first product of tissue engineering [3, 4]. In their work, Green and coworkers describe methods to grow skin epidermis using a skin biopsy from the patient. The harvested biopsy is digested to retrieve autologous keratinocytes, which are then cocultured with a feeder layer of mouse mesenchymal stem cells for several weeks to reach sufficient cell numbers. This approach was then commercialized under the name "Epicel" to produce autologous sheets of keratinocytes used to treat patients suffering from burn accidents. Subsequent research focused on improving the culture medium by adding calcium and hormones, enabling the growth of stratified keratinocyte sheets that do not require feeder layers. However, these sheets are very fragile and prone to damage at any stage from the laboratory to the patient. Therefore, large efforts have been made to overcome stability issues, such as the use of polyurethane backing materials and, more recently, the use of aerosol systems to spray keratinocytes directly onto the wound [93, 94].

Another tissue that was under focus in tissue engineering was cartilage. The first attempts to use cell-based techniques to repair cartilage defects were made by Peterson and coworkers in the late 1980s [95]. The technique later known as *autologous chondrocyte transplantation* (ACT) was described in humans by Brittberg *et al.* in 1994 [96]. In ACT, chondrocytes are isolated from a biopsy of healthy autologous cartilage and expanded *in vitro* for several weeks to reach sufficient numbers. A periosteal flap large enough to cover the lesion is harvested from the proximal medial tibia and sutured to the cartilage surface leaving a small gap for cell injection. The spaces between the sutures are filled with fibrin glue to prevent cell leakage. The injected chondrocytes are then expected to form a new cartilage that will be able to integrate with the surrounding tissue and withstand daily loads [97–99]. Although ACT presented a major advancement in cartilage therapy, periosteal hypertrophy encouraged the research for improved repair strategies [100, 101]. In order to address the above problem, a membrane based on type I/III porcine collagen has been developed and is used to replace the periosteal flap in ACT procedures [102–104]. The membrane, commercialized as ChondroGide®, has a porous surface on the side that faces the defect, which

allows cell attachment, and a smooth compact surface that prevents cell leakage. Although ACT and its variants represented a major step in cartilage therapy, they still face several challenges. The most pronounced challenge is the loss of the cartilage phenotype during *in vitro* monolayer expansion, known as *chondrocyte dedifferentiation* [105]. Chondrocyte dedifferentiation is associated with morphological and gene expression changes where cells behave more as fibroblasts [106, 107]. Dedifferentiated chondrocytes produce type I collagen rather type II collagen, and thus cells implanted in ACT procedures often produce fibrous tissue and not hyaline cartilage [108]. Today's research focuses on developing a variety of methods to either prevent dedifferentiation during serial expansion or to restore the cartilage phenotype of cells before or after their delivery to the defect site [109, 110]. Another challenge is to reproduce the stratified cartilage structure that is formed of structurally and chemically distinct layers at the level of cells and matrix. This structure helps the cartilage to better withstand and respond to mechanical stresses. Except for very few studies, cartilage has been traditionally treated by tissue engineers as a single layered tissue, which is due to the fact that cartilage contains only one cell type (chondrocytes). A very critical component in the engineering of cartilage tissue is to select the proper scaffold, which can be made of synthetic or natural materials and may have different mechanical, chemical, and physical properties. Another important component of tissue engineering is signals (mechanical or chemical). Currently available tissue engineering strategies for cartilage repair do not yet fully recapitulate the cartilage ultrastructure or the cartilage microenvironment that provides a multitude of cues necessary for cartilage homeostasis [111]. In its original form, ACT does not use all elements of tissue engineering, especially the scaffold and signaling (chemical and physical). So far, numerous scaffolds have been proposed to be used as chondrocyte carriers in ACT-like procedures. Additionally, chemical and mechanical signaling was used either before or after tissue transplantation. Moreover, various cell sources have been investigated, including chondrocytes, mesenchymal stem cells, adipose-derived stem cells, and, most recently, iPSCs. Although each of these systems has its advantages, there is not yet a consensus that verdicts one of these approaches as the ultimate cartilage tissue engineering approach. Current cartilage tissue engineering research is focused on understanding basic questions about cells, their interaction with their matrix, and response to mechanical stimulation in health and disease to produce an ideal engineered cartilage. The knowledge gained in the coming years in the tissue engineering triad will enable the engineering of chemically and mechanically functional cartilage.

In addition to skin and cartilage, tissue engineering and regenerative medicine has made significant steps toward bone repair. Bone tissue naturally remodels and may repair itself in case of small fractures. However, natural repair does not often occur with severe bone injuries such as non-union fractures or when extensive bone removal is performed in case of malignancy, infections, and reconstructive operations. In these cases, bone grafting is performed mainly using autologous grafts, but also possibly using allograft and xenografts. To overcome possible immunogenicity from the use of nonhuman grafts, bone extracts have been proposed, such as the use of demineralized bone matrix (DBM) [112]. Despite the

various protocols applied in the preparation of nonhuman grafts, immunogenicity remains an issue. Apart from the use of bone tissue and DBM, collagen and porous ceramics including phosphate- and calcium-based ceramics have been also employed [113]. Nevertheless, the best repair is usually observed with autografts, which, however, present some critical drawbacks such as the limited availability, surgery complications, donor site morbidity, and pain. The limitations associated with the use of autografts encourage the search for alternatives, of which tissue engineering might be the most ideal.

Tissue engineering and regenerative medicine strategies used for bone repair follow the basic tissue engineering methods and therefore rely on the combination of scaffolds, cells, and signals. The engineered implant should support one or more of the following properties: osteoconduction (implant allows good integration with host tissue and bone spreading), osteoinduction (implant encourages bone formation by inducing cell differentiation toward the bone lineage, e.g., DBM and uroepithelium) [114–116], or osteogenesis (bone formation by specific osteoprogenitors) [117]. The main role of scaffolds in bone tissue engineering and regenerative medicine is to serve as a mechanical support that structurally fills the defective bone area. This silent mechanical role can be improved by adding biologically/chemically active components that further enhance or accelerate bone repair (e.g., cells, growth factors, enzymes, and attachment moieties). The success of the scaffold relies on a number of parameters, including mechanical properties (e.g., compressive modulus), structural properties (e.g., porosity), biocompatibility (does not evoke toxic or inflammatory reactions), and biodegradability (scaffold degrades slowly to be replaced by newly formed bone). Scaffolds that mimic the inorganic bone component such as tricalcium phosphate (TCP) and hydroxyapatite (HA) are biocompatible, with the former being highly biodegradable and the latter being nondegradable. Many other biodegradable scaffolds have been investigated for bone tissue engineering, including poly(L-lactic acid) (PLLA), poly(glycolic acid) (PGA), poly(DL-lactic-*co*-glycolic acid) (PLGA), and poly(ε-caprolactone) (PCL). Additionally, polymers such as polyorthoester (POE), polyanhydrides, and poly(propylene fumarate) (PPF) have shown good biocompatibility in animal models [113, 118]. The biocompatibility of bone grafts can be improved by modification with PEG, while biodegradability can be increased by incorporating MMP-sensitive peptides. The modification of scaffolds with ECM molecules (e.g., collagen) or peptides (e.g., Arg-Gly-Asp (RGD), and more recently Gly-Phe-Hyp-Gly-Glu-Arg (GFOGER)) improve cell attachment and consequently enhance osteoconductivity. A second important pillar in bone tissue engineering and regenerative medicine is growth factors, which play fundamental roles in bone repair and bone formation. BMPs, which comprise over 20 different isoforms, have been shown to induce bone formation especially BMP-2 and BMP-7 [119, 120]. In addition to BMPs, other growth factors such as TGF-β [121], VEGF [122], fibroblastic growth factor (FGF), insulin-like growth factor (IGF), and platelet-derived growth factor (PDGF) are involved in bone repair and bone formation [123]. Finally, cells can be included in bone tissue engineering strategies to increase the healing efficiency of the implant or to initiate bone formation *in vitro* prior to implantation. Bone marrow stromal

cells (BMSCs) are the most commonly used types, which are able to differentiate toward the osteogenic lineage [63, 117, 124]. However, other cells have also been shown to possess the potential to form bone-like tissue such as adipose-derived stem cells [125], muscle-derived [126], dermal-derived [127], placenta-derived stem cells [128], embryonic stem cells [129], and recently induced pluripotent stem cells [130]. The combination of some or all the pillars of tissue engineering described above could lead to the successful bone formation.

TERM has made significant steps toward the repair of almost every human tissue. Peripheral nerve injuries and spinal cord injuries can be treated using silicon- or collagen-based nerve guides (hollow tubes), which can be combined with matrices, scaffolds, growth factors, and/or cells to improve repair. In urogenital tissues, engineering the bladder has been achieved with a good degree of success in dogs by seeding urothelial cells and smooth muscle cells on acellular matrices [131]. Atala and coworkers took the procedure one step further by seeding autologous epithelial and smooth muscle cells on biodegradable collagen or collagen/PGA bladder-shaped scaffolds and implanting them in patients with myelomeningocele with high pressure or poorly compliant bladders [86]. Urethral tissue has been engineered and clinically tested in animal models using a strategy similar to the above, where autologous epithelial and smooth muscle cells isolated from a bladder biopsy were seeded in the lumen and outer surface, respectively, of a collagen matrix [132]. Atala and coworkers used similar techniques to engineer uterus and vaginal tissues as well [133]. Attempts towards engineering a kidney – the first organ to be transplanted – have also been made despite the complexity of the tissue. Renal tissue can be engineered by growing renal cells on tubular polycarbonate membranes to be used as extracorporeal dialysis units or to be implanted to replace injured kidneys [134]. Testicle tissue engineering has been attempted, where Leydig cells were encapsulated in alginate–poly-L-lysine spheres, and shown to maintain normal testosterone levels [135]. Penile tissues such as corporal tissue have been engineered using autologous rabbit collagen matrices seeded with autologous smooth muscle cells and endothelial cells. The engineered corporal tissue was implanted in experimental rabbits, which enabled normal erection, mating, and conceiving [136]. Liver tissues have been engineered by combining hepatocytes with PGA/PLGA scaffolds, and vascularization was supplied either by using a vascular bed or using porous scaffolds that allow angiogenesis [137]. Bioartificial patches for tracheal replacement have been developed by seeding autologous muscle cells and fibroblast cells on porcine collagen matrices. The patch was able to create an airtight cover for a tracheal opening, enabling neovascularization, and was covered with viable ciliated respiratory epithelium [85, 138]. Pancreas tissue engineering involves mostly employing islet β-cells or insulin-producing cells (e.g., differentiated stem cells, progenitors, or genetically engineered somatic cells) delivered alone or within a matrix such as calcium alginate/poly-L-lysine/alginate (APA) beads for immunoprotection [139]. Finally, TERM of bowel tissues including the intestines and the stomach has witnessed significant advancements. In 2003, Vacanti and coworkers engineered intestinal tissue by seeding cells harvested from intestinal organoids on a tubular-shaped collagen-coated PGA scaffold [140, 141]. The engineered

structures were implanted in the omenta of animals and exhibited several normal intestine characteristics (e.g., epithelium submucosa and muscular layers) in addition to good angiogenesis from omental vessels. Different biomaterials have been used for gut tissue engineering applications, including synthetic biomaterials (e.g., PGA, PLGA, and PLA), natural materials (e.g., collagen, fibrin), and acellular scaffolds (e.g., small intestine submucosa). Scaffolds can be engineered to release specific growth factors relevant for intestinal development. Moreover, different cell sources have been investigated, including stem cells and genetically modified cells (Rocha and Whang, 2004).

Furthermore, extensive research is being done on cardiac tissue engineering. It aims to create functional tissue constructs that can reestablish the structure and function of injured myocardium.

1.5.2 *In Vitro* Models for Disease Studies

While most currently available engineered tissues are used for restoring organ functions in the body, new applications are currently being considered for using these tissues in disease models. The aim of this technique is to be able to control and ideally to cure many diseases that are still incurable. To test the effects of certain pharmaceuticals, scientists have been using animal models with some gene alterations to represent the human diseases. While this approach has been highly useful, some mechanisms can actually differ between animals and humans. With the impossibility of direct testing on humans and the failure of simple human cell cultures to mimic the disease behavior at the organ or tissue level, tissue engineering came in as the best option to model human diseases [142].

Tissue engineering for disease models aims to mimic the natural properties available *in vivo* such as architecture, environment, growth factors, and biomechanics [143]. In this case, the tissue is just an intermediate step toward the development of the actual treatment. This is why it is usually simple and small in size to minimize the regulatory requirements such as oxygen supply [144]. That being said, a variety of methods are used to produce the required tissues for disease models; these methods combine stem cell biology and the recent advances in tissue engineering, such as the use of scaffolds, bioreactors, organ-on-chip systems, and even 3D printing technologies [142]. The result can be a 2D network of neural cells from human iPSCs, a 3D structure of complex organs like heart valves based on valvular endothelial cells, or organ-on-chip models that rely on microchip manufacturing technology.

Although this approach is still in its earliest stages, many successful usages of engineered tissues for modeling diseases have been noted already. The first example is that of skin equivalents (SE), which have been developed as models that mimic the human skin. These engineered tissues have been useful to study the normal and altered behavior of the skin. They started off as a collagen matrix with dermal fibroblasts inside, and then were subject to more optimization so they would be able to mimic the actual *in vivo* characteristics. The new models were suitable to study many human disease processes such as the skin response to early cancer development or to wound healing [145].

Heart diseases are also being modeled through tissue constructs of human myocardium. These tissues are recently being produced using cardiomyocytes derived from human embryonic stem cells and from human iPSCs; and among the different heart diseases that are modeled are myocardial fibrosis, cryoinjury-induced myocardial infarction, dilated cardiomyopathy, and LEOPARD syndrome [146].

Other than skin and heart diseases, many others are also being modeled. For example, an *in vitro* model of Parkinson's disease has been created using a microfluidic channel with a concentration gradient of neurotoxins [147]. Moreover, engineered tissues of the liver are currently being developed to model liver diseases and try to find new treatment modalities [148]. Lungs, cartilage, intestine, kidney, bone marrow, and vascular diseases also have their share in tissue engineering models, as well as those related to the endocrine and nervous systems. Even cancer and infectious diseases are increasingly being modeled through 3D tissues, although the field is still in its infancy. For a more comprehensive review of these various models, we refer the reader to [142].

It is good to mention that, despite the huge progress that is happening within the field of disease modeling, it is still very complex to model human diseases with their complexity; this is why instead of aiming to solve the problem in its complexity, scientists are focusing more on simpler models that can replicate the basic structure and function of tissues, before dealing with more complex models of organs and systems [142].

1.5.3 Smart Diagnosis and Personalized Medicine

Personalized medicine is a novel approach that takes into consideration the unique characteristics of each patient and his or her individual response to various drugs. While it is still an emerging area of scientific investigations, it is very likely that it will govern the future of medicine [149].

The development of tissue engineering has helped the advancement of this field on many levels, the first being related to drug testing. In fact, since a 3D model of a patient's organ can be engineered by seeding the person's cells into a scaffold, it is possible to test the efficacy of different treatments on it. The human body utilizes many drug-metabolizing enzymes and drug transporters to deal with the drugs in the body. An example is cytochrome P450 (CYP), which participates in the metabolic process of many drugs. Since the CYPs genotype variation is thought to affect the individual's response to a particular drug, it would be helpful to use the engineered tissues to test the drugs for different patients [150].

Another way tissue engineering can pave the way for personalized medicine is through tailoring the tissue construct itself to fit the needs of a specific patient. For the artificial scaffold to be properly functional, it should not only allow the cells' survival but it should also be compatible with the cells' microenvironment and with the host tissue's mechanical, physical, and chemical properties. Personalized therapy has been used, for example, in tissue engineering of urethras using the patients' own cells. Because these scaffolds were compatible with the body

they were implanted in, the grafts ended up developing a normal architecture and a proper functioning [151].

Another promising application of tissue engineering is in the diagnostics field. Instead of using the traditional medical imaging techniques to provide information about the patients' internal organs, sometimes having a physical prototype is more useful. Using manufacturing techniques and rapid prototyping, many studies have created anatomical models replicating organs and tissues to make testing easier. On the other hand, the development of lab-on-a-chip devices as micro-engineered tissues has made testing procedures such as extracting blood or DNA samples much more effective [152].

1.6 Challenges in Tissue Engineering

Despite all the advances in the field of tissue engineering, many challenges persist, which are related to three elements of cells, scaffold, and signals. Starting with the cells, the sources to get them and then seed them in the scaffold are numerous. In fact, autologous, allogeneic, and xenogeneic cells are all potential sources, and each of these can be subdivided into stem cells (adult or embryonic) or differentiated cells. Since they all have their own advantages and disadvantages (immune reaction, differentiation, etc.), the choice of the right source for the cells and their culture is a challenge by itself [153].

The choice of scaffold biomaterials is not an easier task either. The scaffolds must actually respond to both the structural and functional requirements of the body. It must be biocompatible and should be able to communicate with the ECM while at the same time providing the needed mechanical support [154]. While natural materials have better biocompatibility and biodegradability, synthetic ones usually present stronger mechanical properties. This is why the use of composite materials is sometimes required, which also allows the scaffold to have its required porous structure [155].

Another important challenge in tissue engineering is related to the transportation of nutrients and waste secretion in the engineered tissue [87]. Since the majority of tissues rely on blood vessels to transport oxygen and nutrients, the 3D engineered tissue needs to be vascularized with a vascular capillary network [88]. This is not an easy task; after the implantation of the scaffold inside the body, the oxygen available is directly consumed and new vessels are formed only after several days [153]. Alternative methods to angiogenesis are thus necessary, and many techniques for prevascularization of the engineered tissues have been suggested based on subtractive, additive, and hybrid methods [156].

Finally, a major challenge is still present, namely mass production and commercialization of the engineered tissues. Specific manufacturing conditions and quality control strategies need to be ensured. In addition, answering the exact needs of the patients (demand) and providing long-term storage and shipping facilities while ensuring that the structure and function of the tissues are intact are also of great importance [154].

1.7 The Future of Tissue Engineering

The last few decades have witnessed major steps in health care, leading to improved surgical procedures and better management of diseases. All in all, the advances in the health care have raised life expectancy, augmenting vulnerability to diseases and organ failure. Consequently, the aforementioned advancements have led to an increased demand for tissues and organs. The ultimate goal of tissue engineering is to bridge the constantly growing gap between organ demand and availability by producing complete organs [157]. This area is expected to become increasingly applied as a valid clinical solution.

Stem cells will continue to be investigated for their differentiation potential, and more applications will be developed in the future. The major challenge for stem cells, whether induced, embryonic, or adult, is to achieve commitment to the desired lineages. It is expected that more applications using stem cells will reach clinical trials in the near future. Furthermore, gene therapy (silencing and activation of target genes) and drug delivery are both expected to be used to help maintain the desired cell phenotype. The ultimate goal would be to engineer immune-transparent stem-like cells with clear protocols, enabling their committed differentiation to targeted tissues. Developments in basic and applied science related to the fabrication of tissue engineering scaffolds will be a major future target. High-throughput screening techniques might prove useful to determine combinatorial effects of molecules and materials on various cell types. Decellularized tissues are also expected to remain an important source of scaffolds given their high abundance as well as their right chemical and structural composition. Potential limitations of such scaffolds will always be the shortage of supplies (e.g., scaffolds from allogeneic sources), potential immunoreactions, and ethical concerns (e.g., scaffolds from xenogeneic origins). In future, it is expected that new biomaterials will be developed incorporating selected molecules to address targeted tissues. Moreover, many basic science studies will be conducted to identify the effects of molecules on cells and determine the right degradation rate and material properties (porosity, mechanical properties, and structural properties) suitable for each tissue engineering application. An ultimate goal would be to combine scaffolds and cells to engineer tissues *in vitro*, which can be decellularized to produce customizable off-the-shelf tissue sources for various engineering applications. Future research will continue to reveal the roles of ECM molecules to define ideal recipes to engineer constructs that most closely resemble natural tissues.

The mechanisms through which cells perceive load and react to their surrounding environment are only starting to be revealed and comprise stretch-activated ion channels and integrins [158, 159]. Understanding these mechanisms will provide the basis for developing new tissue engineering tools and bioreactors, and possibly discovering new useful molecules for the treatment of sick organs and tissues. Future bioreactors will be able to perform complex combinatorial tasks in order to engineer full organs. For example, bioreactors can be designed to deliver varying oxygen levels to varying parts of the engineered tissue or different mechanical stimulation regimes, or to deliver growth factors and molecules

at predefined time points during culture. Finally, bioreactors may be made to be used on site (e.g., in the hospital) to minimize contamination risks and reduce the surgery time.

1.8 Conclusions

The field of tissue engineering has witnessed tremendous development in the past few decades, which has brought to the clinics solutions once believed to fall under science fiction. Although the application of tissue engineering principles is not widespread in clinics, a very bright future is expected for the field where more tissues will join the list of "clinically applicable tissue engineered constructs." A combination of immune-transparent cells with an off-the-shelf scaffold cultured in a complex bioreactor that delivers tailored signals for the target tissue is probably expected to become possible in the future. However, reaching the stage of clinically relevant off-the-shelf body parts still requires significant basic and applied scientific research.

Future efforts will focus on developing novel biomaterials for the different tissue engineering and regenerative medicine applications. The structure and mechanical properties of the biomaterials will be engineered to better suit the tissue of interest. These biomaterials should be capable of addressing the current major limitations of the field, especially mass transport. Moreover, the developed biomaterials are expected to be better tailored to maintain the phenotype of cultured cells and deliver on demand the optimal cocktail of growth factors and cytokines. Research should also focus on materials that would reduce implant complexity such as injectability or flexibility that allows minimally invasive surgical procedures. Finally, materials that have better integration or stability in the implant site should be designed. Biomaterials with muscle-adhesive proteins and other gluing interfaces may be investigated, or using covalent bonding based on natural residues of tissues and engineered residues on the scaffold. Future research will also focus on cell manipulation (e.g., transfection and silencing) to induce better repair or regeneration. Further understanding at the basic science level of cell behavior, both *in vitro* and *in vivo*, in tissue engineering systems including cell–cell interactions and cell–scaffold interactions will be required. Additionally, the effect of different growth factors as well as ideal amounts and timing of supplementation should be determined for the various tissue engineering applications. *In vitro* culture techniques should also be revised, particularly the switch from 2D to 3D systems and oxygen levels to match the *in vivo* situation of thick tissues. Perhaps, systematic studies that compare current *in vitro* culture systems used in tissue engineering and the *in vivo* situation will shed light on the biological effects of the currently adopted culturing techniques. This knowledge can be used to improve current cell culture techniques to achieve better tissue repair. Finally, efforts should be made toward optimizing current regulatory and ethical considerations that would pave the way for easier and safer introduction of tissue engineering and regenerative medicine solutions into the clinic.

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2

Biomaterials in Tissue Engineering

Samad Ahadian, Rahaf Rahal, Javier Ramón-Azcón, Raquel Obregón, and Anwarul Hasan

2.1 Introduction

Tissue engineering (TE) aims to re-create native tissues *in vitro*, to be used as replacement for diseased or damaged tissues in the body [1]. TE is an exciting multidisciplinary research field in life sciences, which benefits a large number of patients waiting for new tissues or organs. Cells, scaffolds, and soluble factors are the key components for the regeneration of tissues *in vitro* [2]. The scaffolds mimic the extracellular matrix (ECM) of the cells in the body. The ECM consists of highly organized and functional proteins and sugar-based macromolecules, and modulates tissue morphogenesis and maintains the tissue structure and function. There is a great diversity in the composition and morphology of ECM in different tissues, providing specific functions and properties for the corresponding tissue. ECM plays a crucial role in the growth and development of tissues. In addition, the dynamic nature of ECM mediates signaling pathways from soluble factors and other sources to regulate different cellular behaviors, such as migration, adhesion, proliferation, and differentiation [3] (Figure 2.1). Scaffolds serve as the artificial ECMs to engineer tissue constructs. Therefore, the design and fabrication of suitable scaffolds are important in TE to provide the appropriate microenvironment for the cells and thereby regulate the cellular behavior and tissue formation [5]. In particular, the scaffolds provide mechanical integrity and stability, the adhesive substrate, and soluble factors for the cells in newly formed tissues [6]. Biomaterials have widely been used as scaffolds for different TE applications [7].

For each TE application, a specific biomaterial scaffold is required. In general, these biomaterials should be biocompatible, degradable, and without producing immunogenic responses in the body. They should have the desired surface properties, porosity, and mechanical strength [8, 9]. The mechanical properties of biomaterials are important design parameters in TE applications because a biomaterial as the TE scaffold should develop and maintain a space for tissue formation and functionality. Moreover, the adhesion and differentiation of cells are closely related to the mechanical properties of the biomaterials [10]. A controllable degradation of biomaterial scaffolds is also crucial in the development

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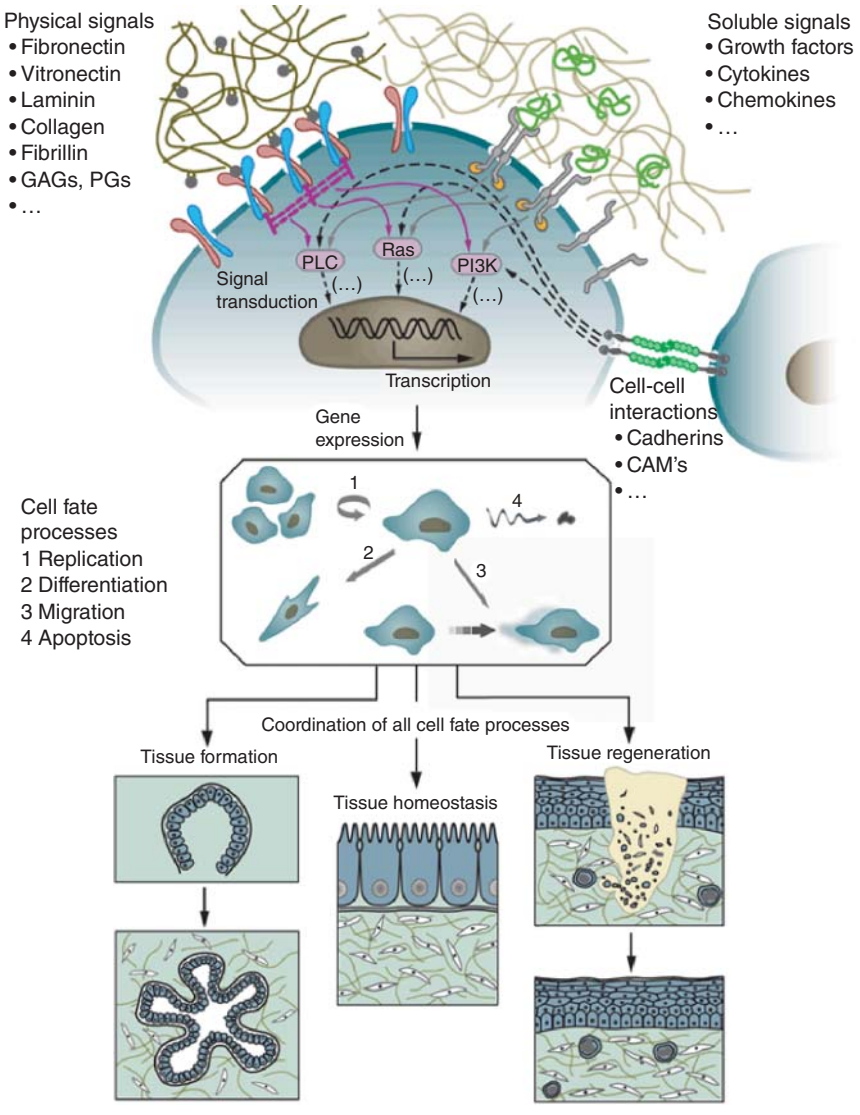

Figure 2.1 Both cells and multicellular tissues communicate with their surroundings through intricate and reciprocal molecular interactions. This cell–cell interaction creates signals inside the extracellular environment in addition to the soluble and physically bound signals that it contains. When the signals bind with the receptors on the cell surface, intracellular signaling occurs, which leads to the regulation of gene expression, homeostasis, regeneration, and tissue formation. (Lutolf 2005 [4]. Reproduced with permission of Nature Publishing Group.)

of tissues and creating their desired functionality. The degradation rate of biomaterial scaffolds may be due to enzymes, hydrolysis, or the dissolution of the biomaterial. There could be a relationship between the mechanical properties of biomaterials and their degradation rate. However, these properties of the scaffold can be decoupled in some cases; for example, scaffolds with defects would result

in the formation of softer materials with longer degradation times compared to the same scaffolds with no defects [11]. Cell–biomaterial interactions significantly affect cell adhesion and consequently its migration and differentiation. Cell-specific binding sites on the biomaterials mainly regulate these processes, and inappropriate cell–biomaterial interactions could lead to undesirable cellular responses and tissue malfunction [12]. In this chapter, after introducing the different criteria that affect the choice of biomaterials for a certain application, we briefly review and discuss the synthesis as well as the chemical and physical characteristics of commonly used biomaterials in engineering tissues.

2.2 Biomaterial–Tissue Interactions

Before getting into the different types of biomaterials used in TE, it is a good idea to consider first the properties that a biomaterial needs to have. In fact, choosing the best biomaterial for a certain application is a complex process by nature, and it is made harder by the fact that the biomaterial cannot be considered without its interactions with the tissue itself. As already mentioned, TE relies on scaffold biomaterials that will host cells from the body and, with the help of growth factors or a bioreactor, will hopefully lead to tissue regeneration [13]. These biomaterials are therefore expected to mimic the ECM functions and interactions with the cells and the surrounding tissues. These interactions have now moved beyond a simple interaction with the biological systems to influence the biological processes that can lead to tissue regeneration [13].

The first step in biomaterial–tissue interaction is the initial adsorption of proteins onto the surface of the biomaterial, which, in combination with the physical and chemical properties of the surface, is thought to lead to subsequent cellular behaviors such as adhesion, spreading, and proliferation. It is not clear how it occurs; however, according to the literature, natural polymers are more efficient than synthetic ones in controlling cellular behavior. A combination of both types is even more desirable to further tune the biological and physicochemical properties of biomaterials [14].

In general, the nature of the interaction between biomaterials and tissues in the body can be described by two main processes. The first one is the foreign body response (FBR), which is especially triggered in the case of an implantable device in order to protect the body from the foreign material. Figure 2.2 shows the different reactions that are involved during FBR. Once the biomaterial device or scaffold is implanted in the body, a tissue/biomaterial interface directly forms and causes a series of inflammatory responses [15].

FBR starts with an acute phase that can last up to several days [15]. During this phase, leukocytes are activated to the implantation site, and some growth factors and cytokines are produced. Monocytes are also recruited, which differentiate into macrophages [16]. After this period, which is the case when the biomaterial is implanted in the body, a chronic foreign body reaction is triggered. This is when lymphocytes and macrophages appear, in addition to the development of blood vessels that are necessary for wound healing and tissue regeneration [15].

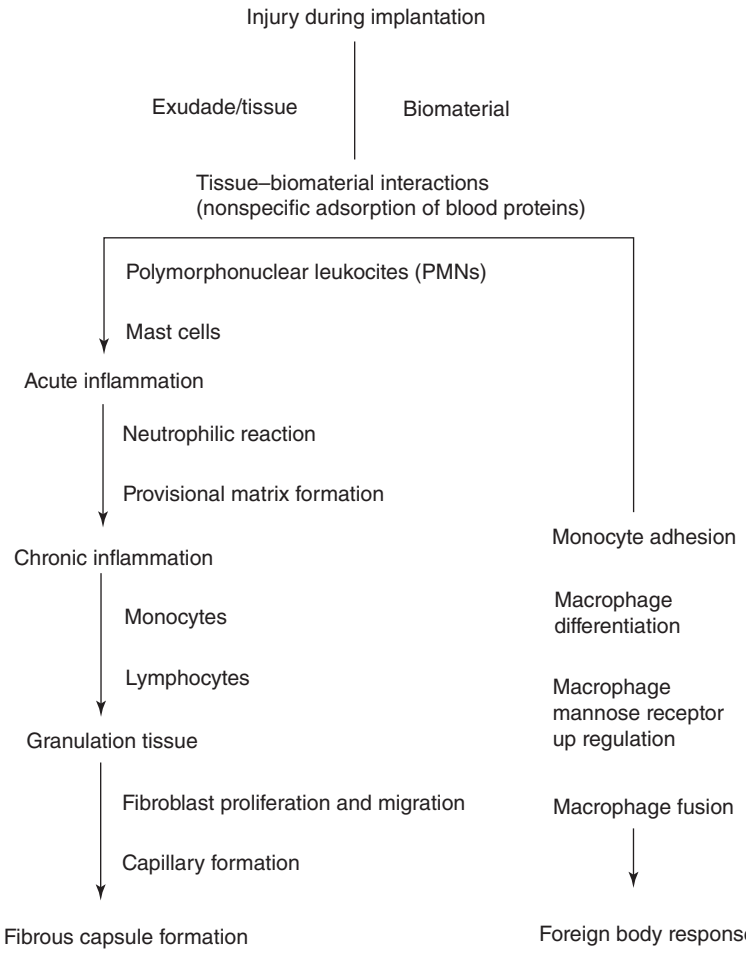

Figure 2.2 Different steps that are part of the foreign body response to an implantable device. Simultaneous occurrence can take place between the different events. (Reproduced from Ref. [15].)

In addition to the inflammatory response at the biomaterial–tissue surface, there are other reactions that are triggered in the body, such as systemic and remote effects, thromboembolic complications, and tumorigenesis. Figure 2.3 [17] shows these diverse effects that act in both directions: on the host tissues by the implant, and vice versa.

Eventually, after the two phases of foreign body reactions, the ECM starts to replace the original scaffold [15]. This cellular microenvironment, which is naturally developed by cells starting in their early embryonic stage, is made of many types of molecules, and contains different substances, such as collagen, elastic fibers, and glycosaminoglycan (GAG), which create different types of scaffolds. Cells adhere to the ECM and receive the information originating there through different binding sites and receptors. These signals play an important role in regulating the different cellular activities, such as migration, adhesion,

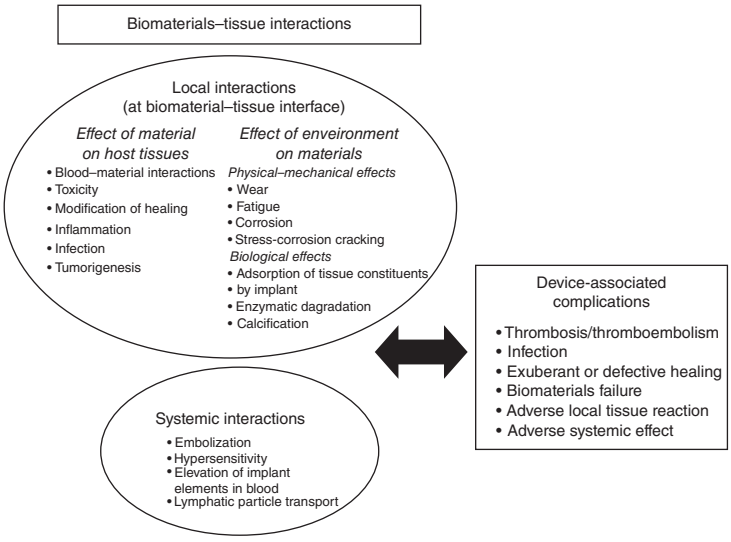

Figure 2.3 Biomaterial–tissue interactions. (Reproduced from Ref. [17].)

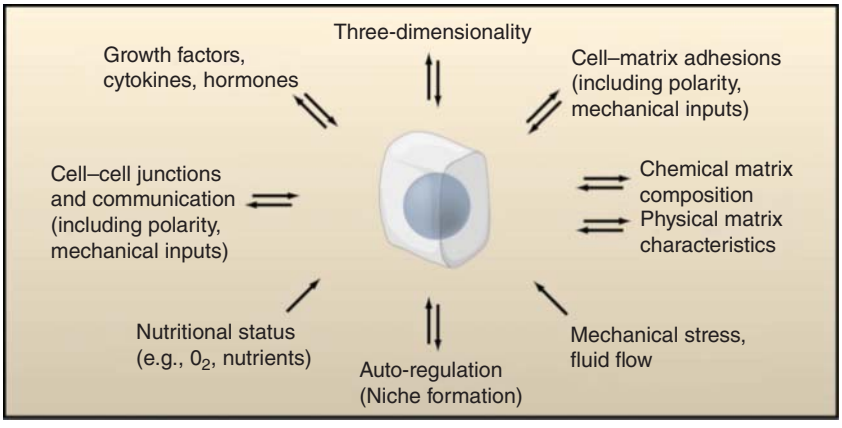

Figure 2.4 Cell biology is governed by a complex series of interactions with the ECM. (Reproduced from Ref. [18].)

differentiation, proliferation, and gene expression [3]. Figure 2.4 shows the different interactions between the cell and the ECM [18]. Before the natural ECM starts to replace the scaffold, the engineered tissue needs to provide this proper interaction with the cells. For example, photopolymerizable poly(ethylene glycol) (PEG)-based hydrogels modified with the required signals can accomplish this function similar to the natural ECM, especially in the transmission of bioactive signals in the control of cell phenotype and tissue formation [19].

So, how can the cells regulate these functions before the cells produce their own ECM? This question brings us to the next form of biomaterials–tissue interaction that we need to take into consideration: biocompatibility [15]. Biocompatibility can simply be defined as the low inflammation as a result of

the biomaterial insertion in the body [17]. Biocompatibility is not restricted to biosafety only. It does not only imply the absence of mutagenesis, carcinogenesis, and cytotoxicity but also require the material to be able to perform the task it was meant for [15] and to allow the cells to properly communicate with their microenvironment. In other words, biomaterials should be able to mimic the native cell microenvironment and to control the spatiotemporal secretion of growth factors and morphogens [20].

So, as a conclusion, for the material–cell hybrids to perform in optimal conditions, an appropriate foreign body reaction should be triggered. In addition to that, the biomaterial needs to ensure a biomimetic environment for the cells and be able to control cell behaviors, such as migration and orientation [21].

2.3 Properties of Biomaterials

Depending on the tissue to be engineered, a material can be selected based on its mechanical, physical, chemical, and biological properties [22]. For example, the use of degradable polymeric materials for fixing fractures or filling bones has been justified by their adequate mechanical properties, degradation rates, and interaction with the surrounding living tissues [23]. Figure 2.5 summarizes the use of different types of biomaterials for different applications. So, what are the various properties of biomaterials that influence these choices?

In the field of TE in particular, biomaterials are used to construct the scaffold that will be inserted in the body. This scaffold is produced based on many key considerations: it should be biocompatible, which means that it should be able to support the cells, function normally, and not trigger any immune reaction or inflammatory response; it should also be biodegradable since the objective of TE is to eventually let the body replace the implant and function by itself [13].

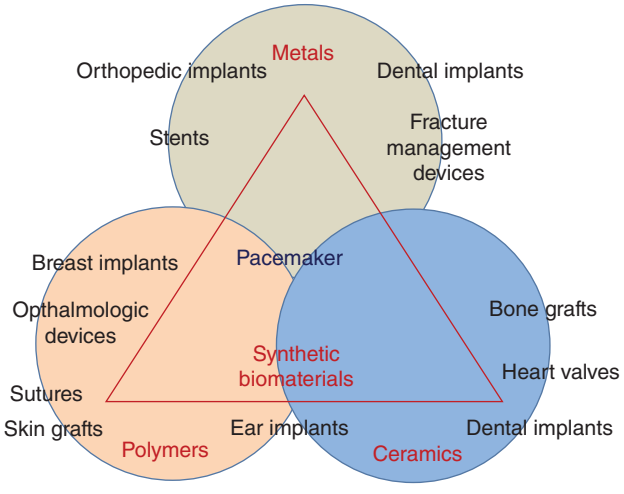

Figure 2.5 Applications of biomaterials in different devices. (Reproduced from Ref. [22].)

Table 2.1 Key mechanical properties of biomaterials and relevant characterization techniques [24].

Property	Characterization techniques
Mechanical	
Tensile strength	Tensile test
Compressive strength	Compression test
Flexural strength	three- and four-point bend test
Elastic (Young's) modulus	Calculation from stress–strain curve
Ductility	Calculation from strength test
Toughness	Single-edge notched beam
	Indirect measurement
Hardness	Indentation
Fatigue	Cyclic stress test
Creep	Constant stress test

In addition to these biological properties, many mechanical characteristics are crucial in the choice of a certain biomaterial for a TE application. Table 2.1 summarizes the different mechanical properties that should be checked for in any material to be used in TE. While those properties depend on the anatomical site itself, the general criteria are tensile and compressive strength, elasticity, toughness, and fatigue [24]. Not only should these properties help the biomaterial mimic the structure of the original tissue but they should also ensure that the biomaterial is properly communicating with the cells and helping them achieve their functions. Cells are actually equipped with surface receptors that convert the mechanical stimuli from the environment to chemical signals that influence cell functions. In addition, an ideal biomaterial should mimic different structural features of the natural ECM, such as pores and fibrils [4]. From a broader perspective, research has shown that the ideal would be a balance between the mechanical properties and a certain porous architecture, which would make vascularization and cell infiltration possible [13].

Physical and chemical properties are also important in the design and fabrication of biomaterials. The thermal properties [25] of the substance, for example, are important since they can affect its behavior inside the human body. As for the chemical composition of the material, it can have a great impact not only on its different properties, such as toxicity and degradation time, but also on the different cell interactions with it [13]. Just as the mechanical interactions in the ECM can alter the cell functions, changes in chemical signals, such as temperature and pH, affect in turn the mechanical stability of the cell. Physical properties have a similar effect on cell functions, and this is why solutions relying on physical attributes (e.g., size, shape, and mechanical properties) are increasingly being proposed. Table 2.2 shows the effect of the main physicochemical properties on cellular behavior [26].

Table 2.2 Major physicochemical parameters and their influence on cellular behavior [26].

Physicochemical parameter	Cell line	Cellular response	Example
Pore size	Chondrocyte Osteoblasts Fibroblasts	Affects cellular affinity and viability by influencing cellular movement, binding and spreading, intracellular signaling, and transport of nutrients and metabolites	Optimal pore size for chondrocytes and osteoblasts are in the range 380–405 μm whereas for fibroblasts it is in the range 290–310 μm for better bone in-growth
Porosity	Osteoblasts	Governs the maximum possible accommodation of cell mass in the scaffold	*In vitro*, low porosity enhances osteogenesis by suppressing cell proliferation and forcing cell aggregation Increased porosity compromise structural integrity and mechanical properties
Stiffness	Fibroblasts Epithelial cells Smooth muscle cells	Affects cellular adhesion, motility, survival, and differentiation	Cell contact with the substrate diminishes on increasingly soft substrates Cells migrate from softer region to stiffer region when exposed to substrate stiffness gradient
Wettability/ contact angle/surface free energy	Fibroblasts Epithelial cells Smooth muscle cells	Sigmoid relationship between spread area of cells and surface free energy	Hydrophilic biomaterial with high surface free energy promotes good cell spreading Optimal fibroblast growth was reported on hydrophilic patterns with a water contact angle of ~17°
Charge	Osteoblasts Fibroblasts	Affects cell uptake and spreading	Positively charged resin beads promote osteoblast flattening; negatively charged beads adopt "strand off" configuration Preferential adhesion and proliferation of 3T3 and 3T12 fibroblasts take place on more positively charged hydroxyethyl methacrylate polymers than on negative ones

(Continued)

Table 2.2 (Continued)

Physicochemical parameter	Cell line	Cellular response	Example
Topography and roughness	Fibroblasts Epithelial cells	Affect cellular orientation and contact guidance	Special surface topography promotes in-growth of bone Prevents epithelial down-growth along transcutaneous implants
Surface chemistry	Mesenchymal stem cells	Long-term functional differentiation of cells	Induces changes in the conformation of serum proteins such as fibronectin, leading to alteration in binding of specific integrin receptors

To illustrate the importance of the various properties we have mentioned, let us consider hydrogel as an example. Hydrogel has been increasingly used in TE for its permeability, biodegradability, as well as its physical properties and structural support to the engineered tissue [27]. These properties are made possible by the hydrophilic surface of the hydrogel, the cross-links between its chains that contribute to its elastic behavior, and the labile bonds in biodegradable hydrogels. Figure 2.6 shows the different chemical properties of the two types of hydrogels (physical and chemical). The difference between the two is basically the bond type between the molecules [28].

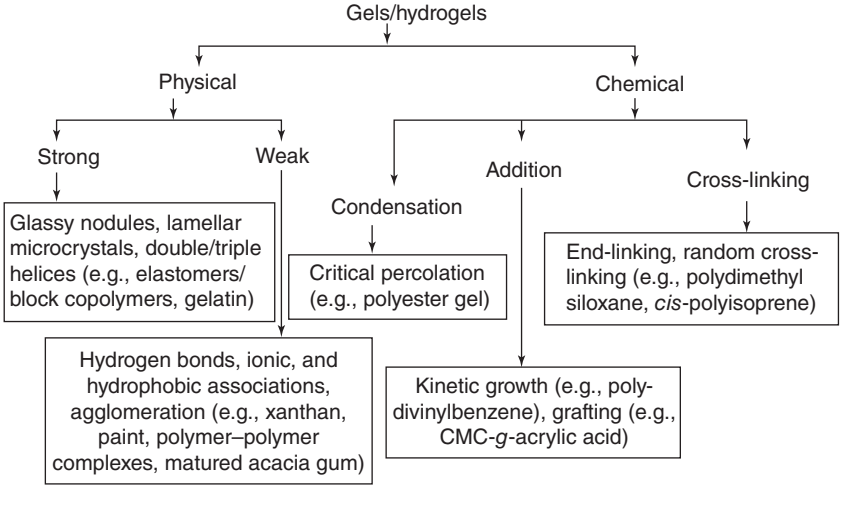

Figure 2.6 Classification of gelation mechanism and relevant examples. (Reproduced from Ref. [28].)

2.4 Scaffold Requirements for Specific Tissues

In order to choose the best biomaterial for a certain application, it is important to take into consideration the application where the engineered tissue will be used because each tissue in our body has its own properties. Some properties are obvious; for example, neural tissues should certainly be able to transmit electrical signals, or else they will not be mimicking the actual nerves' functions. Other properties, such as biocompatibility, biodegradability, and proper porosity are also necessary, and they are shown in Figure 2.7 [29]. In what follows, we will be discussing the most relevant properties of some tissues in our body that need to be present in the corresponding engineered scaffold.

Starting with orthopedic tissue, the ideal characteristics for a bone graft would be high angiogenic and osteoinductive potentials, low patient morbidity, biological safety, no restrictions on the size, access to surgeons, long shelf-life, and, of course, reasonable cost. Eventually, the scaffold should aim at restoring the normal biomechanical role of the orthopedic tissue. It should also provide the different biological and physical signals that simulate the remodeling mechanism in a natural environment. Most importantly, an engineered bone construct should have the right mechanical properties to bear the loads that the original bone was carrying [30].

Tissue-engineered vascular grafts (TEVGs) have other functions to mimic: they should integrate into the neighboring blood vessels and should be able to allow blood flow without any leakage or failure due to the blood pressure. For this to occur, specific mechanical properties are required, such as the strength to prevent rupture and elasticity to sustain cyclic loading. And, just as for all other materials,

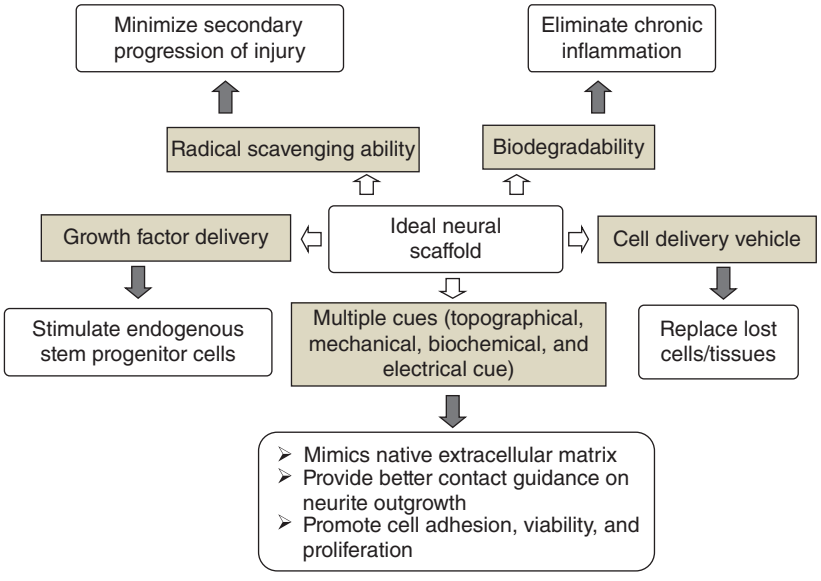

Figure 2.7 Ideal properties of a scaffold for neural tissue engineering. (Reproduced from Ref. [29].)

biocompatibility is another necessary property especially with respect to the adjacent vessels [31].

Moving to a more complex organ, such as the lung, the main priority shifts from strength to elasticity and proper adsorption kinetics. If the engineered lung tissue is not as elastic as the normal one, it will cause a restrictive condition similar to the restrictive scar tissue formation in patients having idiopathic pulmonary fibrosis or sarcoidosis. In addition to that, when implanting the engineered lung tissue, it is necessary for it to remain long enough in the body until the growth of the cells and the development of natural tissue. Porosity and surface area are also important parameters; so in order to provide all of the needed properties, sometimes hybrid scaffolds made of more than one material must be used [32].

Each type of tissue also has its own requirements in terms of scaffold properties. Cardiac muscle constructs should, for instance, account for syncytoid tissue formation, myocyte differentiation *in vitro*, and electrophysiological properties of the heart and its contractile function [33]. Skin tissue, on the other hand, needs a material that can restore the epidermal barrier function and participate in wound healing. It must also be durable and aesthetically acceptable [34]. So as a conclusion, it is very important to take into account the specific properties desirable for a certain application when choosing the best biomaterial or manufacturing process.

2.5 Classification of Biomaterials

Biomaterials used in TE can be categorized into two parts according to their origin: natural or synthetic [35]. Natural biomaterials can be found in natural tissues, such as collagen and fibrin, and they have been used as implant materials for many years. Natural biomaterials have some benefits compared to synthetic ones, such as biodegradability, desirable biological properties, and cellular responses akin to those observed in natural tissues [36]. Natural biomaterials often interact with cells through cell-binding motifs in these materials and thereby regulating various cellular behaviors. However, these materials often cause an immune response in the body most likely due to their high interactions with cells. In addition, they have low mechanical properties, stability, and reproducibility. On the other hand, synthetic biomaterials consist of biocompatible polymers. They do not generally have immunogenic responses in the body and can be produced on large scales with tunable mechanical and chemical properties. However, synthetic biomaterials do not generally interact with cells, and they may give rise an inflammatory response after implantation [37]. In what follows, the commonly used natural and synthetic biomaterials are discussed, emphasizing their applications in TE.

2.5.1 Natural Biomaterials

2.5.1.1 Collagen

Collagen can be found in the most mammalian tissues. It is responsible for maintaining the structural integrity of most connective tissues in animals [38]. Human

body is a rich source of type I–IV collagen among more than 20 different collagen types found in the body. However, the chemical structure of almost all collagens is similar, which is composed of three peptide chains wrapped around each other to form a rope-like structure [39]. Interesting properties of collagen for TE applications are their low antigenicity, natural and enzymatic biodegradability, and cell-adhesive domains; however, their use is limited because of rapid degradation, low mechanical properties, and high cost [37]. Collagen gels are thermally reversible with limited mechanical properties. However, chemically cross-linked collagen gels with glutaraldehyde [40] or diphenylphosphoryl azide [41] have improved mechanical properties compared to pure collagen. However, these gels are still expensive and partially lose their immunogenicity and biocompatibility compared to pure collagen.

Zhao *et al*. used collagen for culturing hepatocytes for the liver TE [42]. They showed that rat hepatocytes remained viable in the cylindrical collagen gels, and the aggregated hepatocytes in the collagen hydrogels produced albumin (a liver-specific protein) by 10 days of culture. Furthermore, they engineered 3D vascularized hepatic units and successfully implanted them *in vivo*. Their suggested approach is useful to make large and thick liver tissue constructs. Liver tissue plays a crucial role in the body controlling various physiological activities, such as storage and release of proteins, carbohydrates, and vitamins. In addition, liver inactivates and repels dangerous substances in the body [43]. Once liver cells (hepatocytes) are removed from the body, they proliferate poorly and lose their functions. Design of a suitable scaffold is important to restore hepatocyte functions [44]. Collagen gels have also been combined with other biomaterials such as poloxamine [45] and chitosan [46] to increase their mechanical properties for liver TE. The collagen–poloxamine hybrid biomaterials showed a higher Young's modulus compared to the pure poloxamine as well as an increase in cell adhesion [45]. The physical structure of collagen–chitosan sponges remained unchanged for 50 days of culture in enzyme solutions [46], suggesting the promising use of these biomaterials for liver TE. Berthiaume *et al*. [47] demonstrated that the sandwich-like structure of collagen had a significant effect on the organization and liver-specific functions of hepatocytes cultured in it because of mimicking the *in vivo* structure of liver tissues. However, the proposed structure is difficult to make on a large scale and allows only a limited transport of nutrients. In general, the micro and nano topographies of biomaterials as the scaffold have a significant effect on various cellular behaviors, such as migration, adhesion, and proliferation [48]. Therefore, it is important to design and fabricate structurally biomimicking biomaterials for TE applications.

Collagen can naturally be found in cartilage tissue and regulates chondrocyte adhesion and stem cell differentiation to chondrocytes through molecular interactions between the protein ligands and the cells [49]. Therefore, collagen has been widely used for cartilage TE. Since cartilage has only limited regenerative ability, it is required to fabricate engineered tissues for cartilage defects [50]. In an animal study, the autologous chondrocytes were cultured in type I–III collagen bilayer gels and then implanted in chondral defects [51]. Recently, a jellyfish collagen scaffold with high porosity was also used for cartilage TE [52]. The human

mesenchymal stem cells (MSCs) did not show any cytotoxicity in jellyfish collagen scaffolds. Furthermore, human MSCs differentiated into the cartilage lineage as confirmed by the gene expression analysis of chondrogenic genes (i.e., SOX9, collagen II, and aggrecan) and the formation of sulfated GAGs. Type VI collagen was shown to induce the proliferation of chondrocytes as a soluble factor and was used to engineer the 3D and biomimetic cartilage tissue constructs [53]. Collagen has also been combined with synthetic biomaterials to make suitable scaffolds for cartilage TE. For instance, the collagen microsponges were introduced into poly(lactic-*co*-glycolic acid) (PLGA) [54]. The chondrocytes within the composite biomaterial showed a natural morphology, homogeneous distribution, and a large amount of cartilaginous ECM proteins as implanted in the mouse models because of the cell anchoring sites of collagen, which were chemically conjugated to PLGA. A single biomaterial may not generally be sufficient to make a desirable and biomimetic microenvironment for cells in TE applications. Therefore, a combination of biomaterials is often required to provide a suitable milieu for cell culture and tissue fabrication. Most synthetic biomaterials lack cell-binding sites. Combination of natural biomaterials such as fibrin or collagen with synthetic biomaterials is a popular approach to provide natural cell-binding sites and hydrophilicity in the composite biomaterials. In addition, synthetic biomaterials generally increase the mechanical properties and can tune the degradation rate of composite biomaterials [55].

Collagen has been used for bone TE [56]. Type I collagen is the dominant component in the native bone scaffold and plays an important role in bone formation from stem/progenitor cells through a developmental cascade. Therefore, type I collagen is a suitable biomaterial to fabricate biomimetic scaffolds for bone TE. Osteoblasts cultured on the type I collagen differentiated into the bone cells as shown by immunostaining and gene expression analysis [57]. A dense 3D collagen gel modified with the polypeptides was also used for the osteogenic differentiation of MSCs [58] (Figure 2.8).

Type I collagen is the major component of dermis ECM, and because of that it has extensively been used for skin regeneration. There are some commercially available products of type I collagen for skin TE, such as Integra [59] (a product of Integra Life Sciences) and Apligraf [60] (a product of Organogenesis). It is hoped that next generations of skin substitutes would be able to release drugs in a controllable manner [61]. The porous scaffolds made of collagen have also been used for cardiac [62], vascular [63], and neural [64] TE applications. For instance, the collagen scaffold enriched with the stem cells was used to repair a heart defect in a mouse model [65]. Interestingly, the collagen was specifically labeled with a specific antibody (Sca-1) to attract more cardiac stem cells.

2.5.1.2 Hyaluronic Acid

Hyaluronic acid or hyaluronan is a natural material that is widely found in connective tissues. It is one of the simplest GAG components in the ECM and plays an important role in wound healing in the body. Hyaluronic acid can be degraded by hyaluronidase derived from serum and cells [66]. However, hyaluronic acid needs a precise purification procedure to remove impurities and endotoxins that

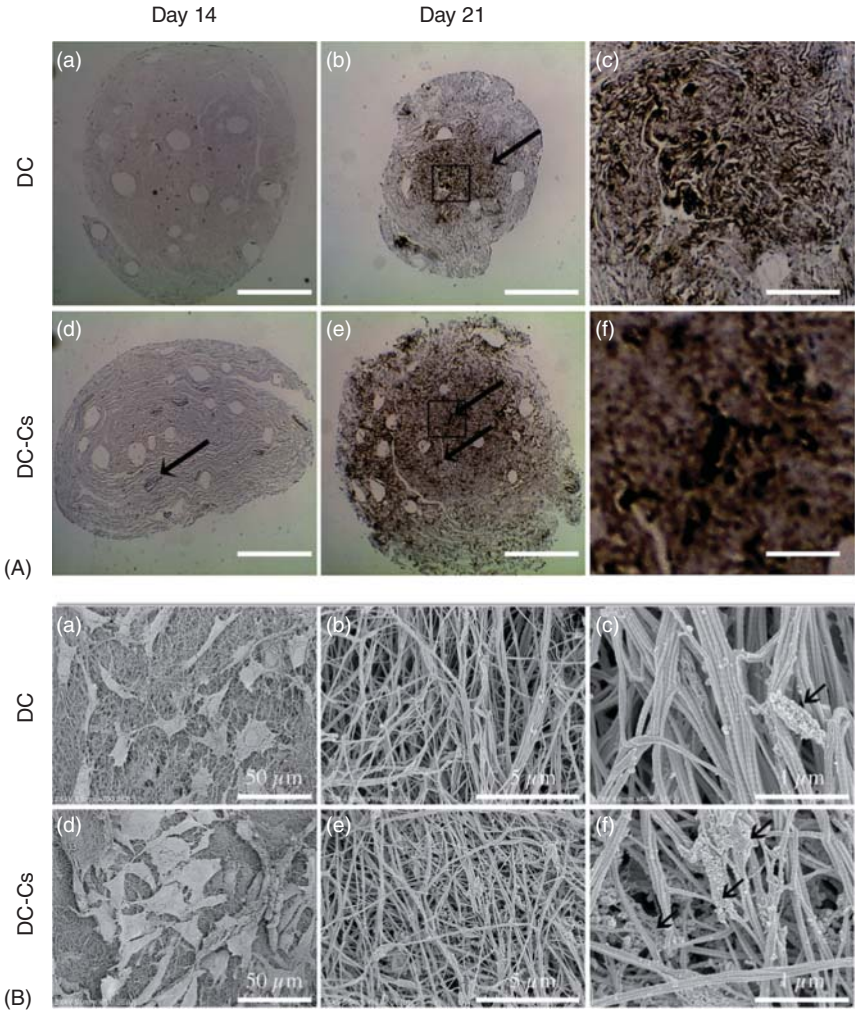

Figure 2.8 Mineralization of dense collagen (DC) and polypeptide-conjugated dense collagen (DC-Cs) rolled gels seeded with MSC. (A) (a–f) Samples of the Von Kossa staining for phosphate species taken at days 14 and 21 from the culture in the osteogenic medium. Blue spots represent cells and darker regions the mineralized collagen matrix. It is obvious that at day 14, mineralization nodules (black arrows) were only present in DC-Cs (d). DC and DC-Cs gels were both positively stained at day 21 (b and e), but the mineralization of DC-Cs structures looks more homogeneous. Note the scale bar difference between the different images. (B) Scanning electron microscopy (SEM) micrographs of the MSCs taken after 14 days from their culture in osteogenic medium. In both cultures in DC (a–c) and in DC-Cs (d–f) rolled gels are shown. The micrographs taken at increasing magnification displayed cell-mediated mineralization of the collagenous matrices; this is revealed in the higher magnification images (c and f) by black arrows. From a qualitative point of view, MSC-seeded DC-Cs gels present a higher mineral content. (Marelli 2014 [58]. Reproduced with permission of Royal Society of Chemistry.)

may cause an immune response or disease. In addition, hyaluronate gels generally have low mechanical properties, limiting their wide applications in TE [67]. However, adding some functional groups to hyaluronic acid can provide a more hydrophobic material capable of chemical cross-linking with other polymers such as PEG to control its mechanical properties [68].

In an animal study, MSCs were cultured in a hyaluronic acid sponge and implanted to repair the osteochondral defects of a rabbit [69]. Histologic results showed that the cartilage tissue was successfully formed in the treated region. Hyaluronic acid has been chemically modified for the ease of processing. The esterification of hyaluronic acid is the most commonly used approach to modify hyaluronic acid [70]. Hayff-11 is a benzyl ester of hyaluronic acid that has been commercialized and clinically approved. Human chondrocytes cultured on Hyaff-11 scaffolds were able to treat chronic cartilage lesions [71]. However, chemical modification of hyaluronic acid may have an adverse effect on its biocompatibility. Hyaluronic acid has also shown great promise in bone TE. For example, Kim *et al*. used human MSCs cultured in hyaluronic acid with the bone morphogenetic protein-2 for the recovery of a rat calvarial defect model [72]. In another study, the photo-cross-linkable hyaluronic acid containing the growth and differentiation factor 5 was used for bone TE *in vitro* and *in vivo* [73]. Hyaluronic acid has also been commercialized for skin TE (e.g., Hyalograft-3D) [74].

Hyaluronic acid has been used in combination with other biomaterials to simultaneously engineer bone and cartilage tissues. For instance, Schek *et al*. made a biphasic scaffold of polylactic acid (PLA) and hyaluronic acid [75]. A thin layer of poly(glycolic acid) (PGA) was placed between two biomaterials to avoid the cell migration between them. The cartilage tissue was obtained by the culturing porcine articular chondrocytes on the PLA layer, while the human gingival fibroblasts were cultured on hyaluronic acid to make the bone tissue. Upon implantation of the tissues in mice, the cartilage tissue produced GAGs while the blood vessels were formed in the bone tissue. In addition, mineralization was often observed at the interface between tissues. Kon *et al*. developed a multilayered gradient of hyaluronic acid and type I collagen [76]. They defined three different regions on a single scaffold (i.e., bone, cartilage, and interface regions) and the compositions of hyaluronic acid and type I collagen were different in each region. The ratio of hyaluronic acid to type I collagen was 70 : 30, 0 : 100, and 40 : 60 in the bone, cartilage, and interface regions, respectively. Following the transplantation of this composite system in an osteochondral defect model, significant enhancement was observed in the treatment of the defect. A recent review describes biphasic scaffolds to engineer cartilage tissues, highlighting the fabrication techniques and clinical studies of such scaffolds [77].

Wang *et al*. summarized the applications of hyaluronic acid scaffolds in neural TE [78]. Zhong *et al*. used thiol-modified heparin and hyaluronic acid–gelatin as a carrier for neural progenitor cell delivery as a translational treatment for stroke recovery [79]. The results demonstrated a decrease in glial scar formation and inflammation and an improvement in the cell survival compared to the cells delivered in the buffer only. Human glioblatoma cells cultured on a

methacrylated hyaluronic acid scaffold were used for neural tissue regeneration [80]. Applications of hyaluronic acid to engineer vascular [81] and muscle [82] tissues have also been reported.

2.5.1.3 Alginic Acid (Alginate)

Alginic acid or alginate is the main structural component of seaweed and is obtained from brown algae [83]. Alginates are a type of polysaccharide block copolymers containing monomers of sequential β-D-mannuronic acid and α-L-guluronic acid, which are distributed in the polymer chain [84]. Alginate has wide applications in TE and drug delivery due to its low toxicity, relatively low cost, and ease of chemical modification and gelation [85]. Despite such advantageous characteristics, alginate has the drawback of degradation through an uncontrollable and unpredictable process to release divalent ions into its surrounding medium. Covalent cross-linking of alginate gels with different molecules is a useful approach to control the swelling and mechanical properties of gels [86]. Another drawback of alginate gels in cell-encapsulation studies is the lack of cellular interactions because alginate prevents protein adsorption on itself and therefore it is not able to efficiently interact with the cells. However, modified alginate gels with some proteins, such as lectin, have high cellular binding and interactions [87]. Such modified alginate gels have been used to culture muscle cells and make muscle tissues [88]. In addition, it is feasible to incorporate soluble factors into alginate scaffolds and release them in a desired temporal and spatial manner [89]. Some applications of alginate as the TE scaffold are summarized as follows.

Glicklis *et al*. cultured the hepatocytes within the 3D porous alginate sponges (diameters of 100–150 μm) and showed that >90% hepatocytes were aggregated within 24 h of cell culture because of the non-adherent nature of alginate. In addition, the hepatocytes secreted a high amount of albumin within 1 week of culture [90]. Alginate has also shown a great promise as an *in vivo* implantable biomaterial to repair liver injuries [91]. It has also been used as an injectable material to treat myocardial infarction of the cardiac muscle [92]. Yang *et al*. galactosylated alginate by adding the amine-modified lactobionic acid to the gel and assessed the functions of hepatocytes encapsulated in the galactosylated alginate microcapsules [93]. They demonstrated that asialoglycoprotein receptors (ASGPRs) were responsible for the attachment of hepatocytes to the galactosylated alginate microcapsules and that the liver function of hepatocytes was significantly increased because of the spheroid formation of hepatocytes in the microcapsules.

Alginate has been widely used for cartilage TE for a long time [94]. Popa *et al*. recently reviewed the *in vitro* and *in vivo* applications of polysaccharide-based gels (e.g., alginate, agarose, ulvan, and carrageenan) in cartilage TE [95]. Alginate immediately converts to the hydrogel upon adding divalent cations to it, such as calcium ions. Therefore, a common approach to produce cells encapsulated in alginate beads for TE applications is to drop the cell-laden alginate solution into a calcium chloride solution, which creates ionic bridges among the polymer chains [96]. Stevens *et al*. reported a rapid-curing alginate gel for cartilage TE [97]. In addition, the proposed gel was homogeneous and mechanically stable and was suitable for *in vivo* applications. In a clinical trial, patients suffering from a lesion

in the femoral condyle were successfully treated with the autologous chondrocytes cultured in the alginate–agarose scaffolds [98]. However, alginate gels are relatively unstable in physiological media because of a decrease in mechanical properties of the gels due to the replacement of Ca^{2+} ions with Na^{+} or K^{+} ions in the polymer chains. Alginate has also been combined with other biomaterials for cartilage TE. For instance, the hybrid alginate–gelatin scaffolds were used as the cell-free and cell-encapsulated scaffolds in treating osteochondral defects in a sheep model [99]. Immunofluorescence and histological analyses revealed a high efficiency of the hybrid scaffolds to treat the defects in a 16-week follow-up.

2.5.1.4 Chitosan

Chitosan can be obtained by the deacetylation of chitin. It can act as a biomimetic biomaterial because of its similarity in structure to GAGs in native tissues. However, it can be dissolved only in dilute acids because of its high crystallinity and the presence of amino groups in its chemical structure [85]. Chitosan and its derivatives have wide applications in TE [100]. Chitosan–hydroxyapatite composites have been utilized for bone regeneration [101, 102].

The free amino groups in chitosan can be easily functionalized via carbodiimide chemistry toward a broad range of novel biomaterials for TE applications. For example, Park *et al*. made galactosylated chitosan through the reaction of the amino groups of chitosan with lactobionic acid [103]. The hepatocytes were able to attach to the galactosylated chitosan through the galactose-specific sites of the scaffold and ASGPR of hepatocytes. Feng *et al*. observed that the hepatocytes seeded on electrospun galactosylated chitosan nanofibers resulted in flat and spheroidal hepatocytes with a higher functionality compared to those cultured on galactosylated chitosan films [104]. Generally, the topology of scaffolds is an important factor impacting various cell behaviors, such as morphology, orientation, and function [72]. Electrospinning is a facile and versatile fabrication technique by which micro- and nanofibers of various natural and synthetic polymers can be made. In the electrospinning process, a high electric field is applied to give an electric charge to the underlying polymer solution. The polymer is then going through a nozzle and deposited on a receptor with a low electric field. The electrospun fibers of the polymer are formed upon evaporation of the polymer solvent or solidification of the polymer melt [105]. Electrospun fibers have found wide biomedical applications, particularly in the fabrication of biomimetic scaffolds for TE applications [106].

Chitosan is a favorite scaffold material in articular TE applications because of its similar structure to GAGs in cartilage-stimulating chondrogenesis [107]. In an animal study, a chitosan–glycerol phosphate solution was combined with the autologous blood sample from a sheep model and used to heal the cartilage defects [108]. A 6-month follow-up showed a significant improvement in the healing process of cartilage defects. The primary amino groups at the C2 position of chitosan can be protonated at pH values below 6.5. This property is convenient for the processing of chitosan under mild conditions. Using the latter protocol, an injectable chitosan gel was proposed for the *in situ* treatment of cartilage defects [109]. The proposed gel was biocompatible when exposed to both rat and human MSCs. The chitosan microspheres were made by adding the anionic

tripolyphosphate to the chitosan emulsion containing the transforming growth factor-1 (TGF-1) for engineering articular cartilage [110]. Controlled release of TGF-1 from the chitosan microspheres was observed, leading to the enhanced proliferation and ECM production of porcine chondrocytes cultured on them. Injectable biomaterial scaffolds in general form *in situ* after injection, and they are minimally invasive for therapeutic applications. They can be adaptable to any shape of host tissues or surroundings of defective tissues. In addition, one can easily incorporate cells or soluble factors in them.

Several studies have been carried out using chitosan for skin regeneration because it has significant effects on wound healing [111]. Other soft tissues, such as nerve and blood vessel tissues, have also been regenerated using chitosan scaffolds. For instance, Yuan *et al*. showed that the Schwann cells got attached, migrated, and proliferated well on chitosan fibers similar to the Büngner bands in the neural system [112]. In another study, Itoh *et al*. proposed hydroxyapatite-coated chitosan tubes with the laminin peptides as the scaffold for peripheral nerve reconstruction [113]. Madihally *et al*. fabricated heparin-modified chitosan tubes for vascular TE applications [114]. Heparin has an important impact on blood vessel formation because of its anti-thrombogenic property. It can stop the proliferation of vascular smooth muscle cells and protect and release soluble angiogenesis factors [115].

2.5.1.5 Gelatin

Gelatin is a thermoresponsive, natural biomaterial extracted from collagen via breaking its triple-helix structure into single-strand molecules. It is more cost effective and less antigenic than collagen. Gelatin has attracted much attention in biomedicine because of its biocompatibility, ease of gelation, and biodegradability [116].

Hong *et al*. fabricated galactosylated gelatin by adding the lactobionic acid to gelatin and used it to culture hepatocytes [117], which showed a higher viability compared with those cultured on the type I collagen films. In addition, some liver functions (e.g., urea synthesis and albumin secretion) were regulated for the hepatocytes cultured on the galactosylated gelatin because of the specific binding of hepatocytes with the galactose sites in galactosylated gelatin. In a recent study, hepatocytes encapsulated in galactosylated gelatin were shown to possess high cell viability, proliferation, and hepotocyte gene markers upon 21 days in culture [118]. Gelatin hydrogels have also been used for cardiac TE. For instance, McCain *et al*. recently cultured rat cardiac myocytes and human induced pluripotent stem cell (iPSC)-derived cardiomyocytes on gelatin micromolds and fabricated functional and contractile cardiac tissues using them [119]. Methacrylated gelatin is also a chemically modified gelatin with methacrylic anhydride, synthesized by our research group, which can be polymerized upon the application of UV light [120]. The mechanical and structural properties of methacrylated gelatin are tunable by changing the degree of methacrylation or concentration of the gel precursors. Methacrylated gelatin has been used for different TE applications, such as skeletal muscle [121], cardiac [122], and vascular [123] tissue regeneration.

Gelatin gels have been introduced as a carrier of biomolecules for the vascularization of tissues [124] and other TE applications [125]. Gelatin parameters, such

as its isoelectric point and cross-linking density, can be tuned to optimize kinetic release of biomolecules loaded onto gelatin. Recent research works have focused on chemical or physical modifications of gelatin to fabricate gelatin carriers for different TE and drug delivery applications. For instance, gelatin carriers in the form of gels and spheres have widely been used for the delivery of biomolecules in bone TE [126]. Another example is the use of electrospun gelatin mats encapsulating the human vascular endothelial growth factor to induce vascularization in the engineered tissues [127].

2.5.1.6 Fibrin

Fibrin comprises a network of fibrous proteins. The structural unit of fibrin polymer is fibrinogen, which can be derived from blood coagulated as a result of injury. Fibrin has been used as an adhesive for surgical wounds because it has a significant role in natural wound healing. There is no report of inflammatory reactions or toxic degradation caused by this natural biomaterial in the body. Fibrin gels are formed at room temperature through the enzymatic polymerization of fibrinogen using thrombin [128], and its degradation rate can be controlled by aprotinin or tranexamic acid [129]. Fibrin has been utilized as a scaffold for the cell encapsulation studies [49]. However, a major drawback of using fibrin is its shrinkage *in vivo*. In addition, fibrin gel has limited mechanical strength particularly after implantation *in vivo* [130].

Widespread clinical applications of soluble factors *in vivo* have been restricted because of the high rate of metabolism of these factors in the body. In this regard, fibrin gels provide a suitable platform for soluble factors by avoiding their rapid degradation and thereby making them to be released at an optimum rate. Many proteins can attach to fibrin either covalently or noncovalently. In addition, fibrin gels can be modified to tune the release rate of soluble factors encapsulated in them using the plasmin degradation sequences, bi-domain peptides, and heparin incorporation [131]. For instance, the transforming and platelet-derived growth factors were incorporated in fibrin gels to enhance cell migration, proliferation, and matrix synthesis [132]. Recently, Montgomery *et al*. reported the use of fibrin gels encapsulating differentiation-induced soluble factors for the neural differentiation of iPSCs [133].

Fibrin gels have been used to fabricate skeletal muscle [134], smooth muscle [135], liver [136], and cartilage tissues [137]. In an animal study, the autologous adipose-derived stem cells (ADSCs) were seeded in the fibrin gel to treat cartilage defects of rabbits. Immunostaining, gene analysis, and Western blotting experiments showed a higher healing effect of ADSCs in the fibrin compared with the control group. In particular, cartilage-specific genes (e.g., Type II collagen and aggrecan) were revealed in the repaired tissues. However, there are some concerns about the immune response of autologous fibrin gels [138]. Therefore, additional time and effort are required for the collection and processing of fibrin gels derived from blood.

2.5.1.7 Silk

Silk is a natural biomaterial produced by spiders. It has been studied intensively for a long time as a biomaterial because of its low inflammatory response,

biodegradability, tunable structural and chemical properties, and high mechanical properties [139]. Silk can be obtained from different sources, such as silkworms, mites, spiders, scorpions, and flies. In particular, silkworm silk has widely been used for biomedical applications and can be produced in bulk in the textile industry [140].

Silk has been used in ligament TE because of its biocompatibility and unique mechanical properties [141]. Galactosylated silk fibroin was synthesized by the reaction of silk with lactose using cyanuric chloride as the coupling spacer [142]. Hepatocytes were able to attach to the galactosylated-silk-coated substrates by approximately eightfold higher than those attached to the untreated substrates. Gotoh *et al*. prepared 3D galactosylated silk fibroin scaffolds for hepatic TE [143]. Human hepatocellular-carcinoma-derived FLC-4 cells seeded in the scaffolds formed multicellular spheroids with high cellular viability and albumin expression. The shrinkage of the scaffolds resulted in the maintenance of spheroids within 3 weeks of culture.

Human chondrocytes cultured in silk fibroin was demonstrated to have good attachment and viability and produced cartilage-specific ECM [144]. In another study, silk fibroin sponges increased the population of chondrocytes and GAG formation compared to collagen gels [145]. A similar behavior was observed for bone-marrow-derived MSCs cultured on silk fibroin [146]. Particularly, a homogeneous distribution of engineered cartilage tissue was obtained only for silk scaffolds compared to collagen. However, the exogenous silk proteins in engineered tissues may cause some problems in the body as a result of the activation of the immune system [147]. Other notable applications of silk scaffolds are in bone, vascular, neural, skin, cardiac, ocular, and bladder tissue regeneration as comprehensively reviewed and discussed elsewhere [140, 148]. They are particularly useful for slow biodegradation and high mechanical properties of biomaterial scaffolds in TE applications.

2.5.1.8 Peptides

Proteins are the main constituents of all living cells. They are composed of amino acid chains. A peptide molecule is made up of more than one amino acid linked together with a peptide bond [149]. Peptides, and particularly short-length peptides, are popular materials in biomedicine because of their biocompatibility, facile synthesis, antimicrobial effects, as well as their tunable chemistry, structure, and functionality [150]. They have been used as dental implants, 3D scaffolds, and carriers for biomolecules (e.g., genes, drugs, and proteins) under *in vitro* and *in vivo* conditions.

Peptide scaffolds can be assembled as films, gels, tubes, or fibers for different TE applications with high biocompatibility, biodegradability, and immunogenicity [151]. They can release biomolecules in a controlled manner for regenerative purposes. Some TE applications of peptide scaffolds include vascular [152], neural [153], bone [154], and cardiac [155] regeneration. In general, an overall knowledge of the different self-assembly processes of peptide systems is advantageous to understand the corresponding biological processes. In particular, it can guide researchers in the design and fabrication of novel self-assembled and functional biomaterials for TE applications.

2.5.1.9 Elastin

Elastomeric biomaterials have shown great promise in fabricating elastic tissues because of their unique biological and mechanical characteristics, such as self-assembly, elasticity, and long-term stability (Figure 2.9). Elastin is one of the components in natural ECM, providing elasticity to the different tissues in body, such as the lungs, skin, and blood vessels [157]. The half-life of elastin in the body is almost 70 years, and elastic fibers are insoluble and highly stable in native tissues, preventing their ease of processing as a biomaterial. However, various soluble elastin materials, such as elastin-like polypeptides [158], hydrolyzed soluble elastin [159], and recombinant tropoelastin [160], have been proposed to make elastin-based tissues. These elastin-based biomaterials can be self-assembled under physiological conditions.

Recently, our research group has synthesized methacrylated tropoelastin gels for cardiac TE [161]. Methacrylated tropoelastin is a photo-cross-linkable hydrogel providing a 3D modular assembly of gels in a versatile and adaptable manner to control cell functions and fate. However, it still requires a complicated and long experimental procedure for synthesizing methacrylated tropoelastin prepolymers and make them cost effective for various TE applications. Generally, the use of engineered tissues using elastin-based biomaterials *in vivo* is desirable to introduce this biomaterial for clinical applications. To this end, various aspects of elastin-based biomaterials (e.g., purity, structure, and biocompatibility and degradation in animal models) should be clarified to obtain more predictable results *in vivo*.

2.5.2 Synthetic Biomaterials

2.5.2.1 Poly(*N*-isopropylacrylamide)

Poly(*N*-isopropylacrylamide) (PNIPAAm) is an attractive synthetic biomaterial for various TE applications [162]. PNIPAAm is a thermoresponsive polymer. A main advantage of using PNIPAAm for cell-encapsulation and cell-therapy applications is that one can easily encapsulate the cells in PNIPAAm at room temperature and then form the gel upon increasing the temperature. The body temperature is higher than the temperature needed for gel formation. Therefore, the cells within the PNIPAAm can easily be injected into the body for regenerative purposes. NIPAAm can be copolymerized with other molecules, such as methacrylic, acrylic, and butylmethacrylic acids, to change the gelation temperature of the final copolymer [163].

The unique temperature-dependent gelation of PNIPAAm polymers is of great advantage for cell culture and tissue regeneration. In a standard cell culture protocol, cells are harvested from cell culture flasks or dishes using protease treatments. Such treatments may damage the cellular structure and function. However, the cells are able to easily recover on PNIPAAm polymers as a cell sheet without any damage by simply decreasing the temperature and thereby reversing the gelation of PNIPAAm [164]. This so-called cell sheet technology has been used to fabricate cell sheets of different cell types, such as cardiac [165], endothelial [166], cartilage [167], bone [168], and skeletal muscle [169] cells for TE applications. Cell sheet technology has also been successful in

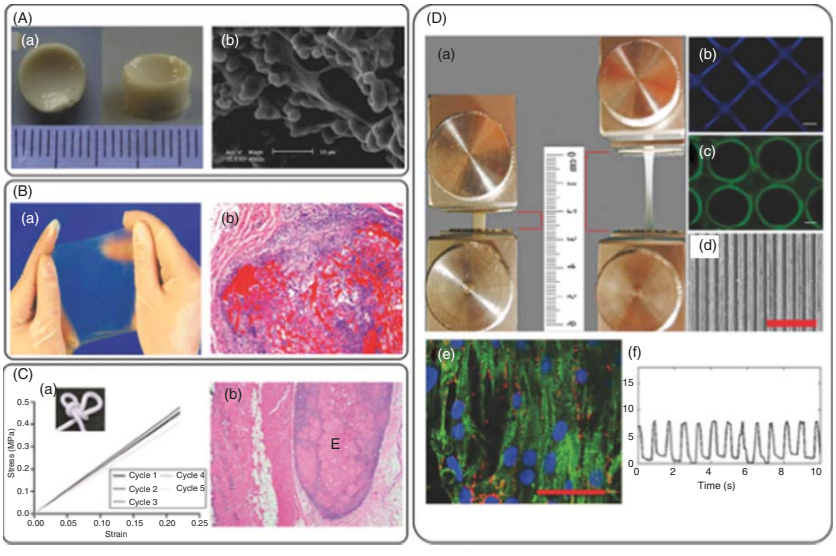

Figure 2.9 Examples of naturally derived hydrogels based on elastin. (A) Glutaraldehyde-cross-linked elastin hydrogels produced under high-pressure CO_2 (a) structure of the hydrogel after swelling and (b) SEM image of dermal fibroblast cells inside the 3D structure of the gel. (B) BS3-cross-linked elastin gel: (a) elastic hydrogel sheet and (b) hematoxylin and eosin-stained sample explanted 13 weeks after the implantation. The bright red color represents the hydrogel. (C) Physically cross-linked elastin gel: (a) stress–strain curves for five cycles; the resulting gel could be tied into a knot, which reveals its high flexibility and (b) hematoxylin and eosin-stained explant presenting the injection site, with the "E" marking the elastic. (D) Methacrylated elastin gel: (a) elastic methacrylated elastin gel before and after stretching, (b–d) formation of patterns of different geometries on the methacrylated elastin gel by using various microfabrication techniques, (e) immunostaining of cardiomyocyte markers on methacrylated elastin gel 8 days after the culture, with gel stained for sarcomeric α-actinin (green)/connexin-43 (red)/nuclei (blue) (scale bar = 50 μm), and (f) beating behavior of cardiomyocytes on micropatterned methacrylated elastin gel. (Annabi 2014 [156]. Reproduced with permission of John Wiley & Sons.)

some clinical trials to regenerate damaged or diseased tissues [170]. However, challenges ahead in this technology are the fabrication of thick and functional tissue constructs using thin cell sheets and the limited control on the chemistry and mechanics of PNIPAAm polymers, which have significant effects on cell behaviors and fate. In addition, PNIPAAm gels are nondegradable and their monomers and cross-linkers may be cytotoxic [171].

2.5.2.2 Poly(lactic acid)

Poly(lactic acid) (PLA) is a biodegradable aliphatic polyester. PLA has mostly been used for cartilage and bone TE because of its biocompatibility and high mechanical strength [50]. Synthetic biomaterials are no more favorable than natural ones for cartilage TE because acidic components are produced during the degradation of synthetic biomaterials, which decrease the pH level inside the engineered tissues causing inflammatory response and acute cell death. In addition, the attachment of chondrocytes to synthetic biomaterials is weak [172]. Therefore, synthetic biomaterials have been combined with natural ones to obtain suitable scaffolds for cartilage TE. For instance, Hu *et al*. developed an arginine-glycine-aspartic acid (RGD)-peptide-modified PLA substrate for osteoblast cell culture [173]. In addition, the alkaline phosphatase activity and calcium deposition were enhanced on the modified PLA substrates compared with the control substrates. Along with this research direction, Alvarez-Barreto *et al*. modified the surface of PLA with different functional groups, such as sulfhydryl, amines, and pyridylthiols, aiming to fabricate biomimetic PLA scaffolds [174]. They further functionalized the PLA biomaterials with the RGD-cysteine peptide to increase cell adhesion. The MSCs favorably adhered to the obtained PLA scaffolds. The attachment of whole proteins on synthetic biomaterials is difficult because they can easily denature and degrade. However, short and natural peptide motifs derived from proteins (e.g., RGD peptide) have widely been incorporated into biomaterials.

2.5.2.3 Poly(lactic acid-*co*-glycolic acid)

PLGA is synthesized by the copolymerization of cyclic dimers of glycolic and lactic acids. It is a Food and Drug Administration (FDA)-approved biopolymer and has widely been used in TE and drug delivery applications because of its biocompatibility, biodegradability, and tunable mechanical properties [175].

Yoon *et al*. prepared galactosylated PLGA substrates via the reaction of the amino groups of PLGA-PEG and lactobionic acid and used them for hepatocyte cell culture [176]. The albumin secretion of the PLGA substrates was relatively low *in vitro* compared to other commonly used substrates. However, the albumin secretion was substantially enhanced under the flow conditions because of the formation of spheroids of hepatocytes in the scaffolds. In a recent study, Roh *et al*. used the mouse bone-marrow-derived MSCs and differentiated them into hepatocyte-like cells on galactosylated PLGA substrates [177]. The proliferation of hepatocytes was increased on the galactosylated PLGA substrates compared to the control substrates. More importantly, hepatocytes formed the spheroids after 3 days of culture on the galactosylated PLGA substrates and showed liver-specific functional activities, such as urea and albumin secretions. In

another study, Fiegel *et al*. cultured rat hepatocytes in the 3D fibronectin-coated PLGA scaffolds under flow conditions [178]. Their results showed that the liver-specific functions of hepatocytes (e.g., proliferation and albumin secretion) were significantly enhanced by using the fibronectin coating of scaffolds and flow conditions. Fibronectin is a multifunctional ECM protein and plays an important role in cell attachment and migration [179]. Fibronectin's ability to enhance the cell adhesion is based on its RGD peptide [180]. However, the use of fibronectin in TE applications has generally been limited because of its high cost. The pore size of PLGA scaffolds is an important parameter to make 3D aggregates of hepatocytes for liver TE. Ranucci and Moghe fabricated porous PLGA scaffolds for liver TE and found that scaffolds with a pore size of ~3 μm induced 2D hepatocyte aggregations, whereas increasing the pore size of scaffolds to ~67 μm enhanced the 3D aggregations of cells because of an increase in cell–cell communications [181].

Hybrid PLGA biomaterials have been used for bone TE (e.g., see Figure 2.10). Natural biomaterials have commonly been employed in combination with PLGA to enhance osteoblast adhesion to PLGA and regulate the physiological activities of bone cells. For example, PLGA combined with collagen–apatite microsponges was used as a 3D scaffold for bone TE [183]. In another investigation, the PLGA substrates were coated with collagen, chitosan, and *N*-succinyl-chitosan, and the adhesion, proliferation, and differentiation of rat calvaria stromal cells on the modified PLGA substrates were evaluated [184]. The results showed that collagen enhanced cell adhesion and proliferation. However, the chitosan and *N*-succinyl-chitosan decreased the adhesion and proliferation of cells.

A study revealed that PLGA scaffolds having hydroxyapatite on their surfaces promoted bone tissue regeneration *in vivo* [185]. In another study, the osteogenesis of MSCs was enhanced on the PLGA-hydroxyapatite surfaces compared to the pure PLGA surfaces [186]. In particular, the expression of osteogenic genes (i.e., type I collagen and osteocalcin) and alkaline phosphatase activity were increased on the PLGA-hydroxyapatite compared to the pure PLGA. In addition, bone regeneration was enhanced for the PLGA-hydroxyapatite implants *in vivo*. The incorporation of apatite into PLGA scaffolds is usually obtained using gas-foaming [187] or apatite-coating [188] techniques. The exposure of PLGA-hydroxyapatite scaffolds to the bone-like apatite in simulated body fluid could further coat the surface of the scaffolds with the apatite [188].

PLGA has been used for skin TE for a very long time [189]. Some PLGA-based products for skin TE have even been commercialized. For instance, Dermagraft is a commercially available skin tissue, which includes bioresorbable PLGA (i.e., polyglactin 910) [190]. PLGA scaffold has also been used for the blood vessel formation with a tunable structure [191].

2.5.2.4 Poly(ε-caprolactone)

Poly(ε-caprolactone) (PCL) is a biodegradable polyester [192]. PCL was proposed as a biomaterial because it can be degraded using microorganisms [25] even under physiological conditions [193]. However, its degradation rate is less than that of PGA and PLGA because of five hydrophobic $—CH_2$ moieties in its polymer chain [194]. The scaffolds should generally be nonpermanent

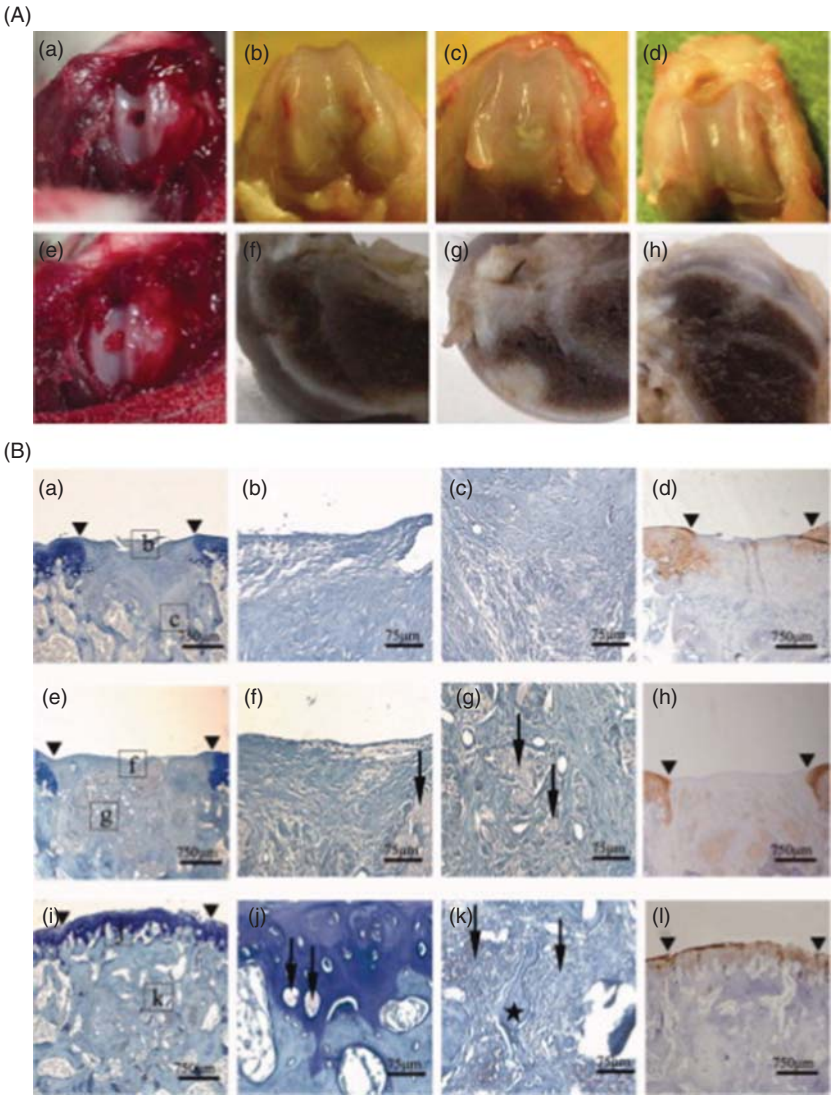

Figure 2.10 (A) Photographs showing articular cartilage defects directly after their creation (a) and their treatment with the scaffolds (e). The cartilage 12 weeks after being repaired in the control group (b,f), PLGA group (c,g), and PLGA/hydroxyapatite group (d,h). Panels (b–d) represent the front view while panels and (f–h) represent the cross-sectional view. (B) Histology of the regenerated tissue 12 weeks after the operation. Toluidine blue staining of the repaired tissue in the control group (a–c), PLGA group (e–g), and PLGA/hydroxyapatite group (i–k). Immunohistochemical staining for collagen type II in the repaired tissue in the control group (d), PLGA group (h), and PLGA/ hydroxyapatite group (l). Arrowheads indicate the location of the repaired area while the arrows refer to the remaining scaffolds; the star symbol in panel (k) represents a newly formed bone. (Xue 2010 [182]. Reproduced with permission of John Wiley & Sons.)

and degrade over time to be replaced by natural ECM of new tissue. The low degradation rate of PCL scaffolds is useful for some TE applications that need high robustness of the scaffolds during the healing process.

Despite the hydrophobicity of PCL, micro- and nanofibers of PCL have extensively been used as a scaffold for various TE applications, such as skin, cartilage, bone, and cardiac regeneration [195]. However, the mechanical properties and biocompatibility of PCL may not be sufficient for some TE applications, such as bone TE. Therefore, PCL has been combined with other biomaterials to meet the requirements of a suitable scaffold for TE applications. Hybrid PCL and chitosan scaffolds have been shown to have higher mechanical properties compared to the pure PCL scaffolds [196]. The physical properties of hybrid PCL and chitosan scaffolds were tunable by varying the ratio of PCL to chitosan in the composite material. Scaffolds with 80% PCL and 20% chitosan showed the optimum physical properties and desired cellular response. Other natural biomaterials such as silk fibroin [197] and gelatin [198] have also been combined with PCL for different TE applications. PCL has also been combined with synthetic biomaterials mainly because of its slow degradation rate compared to other synthetic biomaterials. For example, when PCL was mixed with PLGA, the fibroblasts were able to attach and proliferate on the electrospun PCL–PLGA composite scaffolds [199].

2.5.2.5 Poly(ethylene glycol)

PEG or poly(ethylene oxide) is a synthetic and hydrophilic polymer with the formula of $OH–(CH_2CH_2O)_n–CH_2CH_2OH$. PEG is an FDA-approved polymer with wide biomedical applications because of its biocompatibility and low toxicity [200]. PEG has been widely used as an inert synthetic biomaterial, which can be chemically modified using different ways to incorporate different biomolecules. For instance, PEG hydrogels have been modified with the peptide sequence Ala-Pro-Gly-Leu to make them susceptible to enzymes in the body [201]. Other proteins and peptides can also be combined with PEG to increase the number of cell-adhesive sites. It is believed that PEG hydrogels provide a suitable microenvironment for cells because of high their water content and mechanical properties. Photopolymerizable PEG hydrogels were used as scaffold for chondrocyte [202], liver [203], and muscle cells [204] by providing *in situ* gelation using UV light at physiological conditions. A synthetic and injectable hydrogel block copolymer of poly(propylene fumarate-*co*-ethylene glycol) has also been proposed for blood vessel formation [205]. This copolymer is biodegradable and can be cross-linked either chemically or using UV light.

PEG has often been functionalized or used in combination with other natural or synthetic biomaterials to render more useful composite biomaterials in biomedicine. In particular, tight control on the molecular weight and chemistry of PEG hydrogels is a useful approach to tune the physicochemical properties of other biomaterials. For example, Lopina *et al*. prepared galactose-functionalized PEG hydrogels by the reaction of 1-amino-1-deoxy galactose with the PEG hydrogel [206]. The molecular weight and water content of PEG gels remained constant after the chemical modification of the hydrogels. Hepatocytes were able to attach to the galactose-modified PEG gels with spreading morphology [207]. In another study, cartilage regeneration was achieved using PEG–chitin–chitosan

scaffolds [208]. The proposed scaffold had high porosity (>90%), high stiffness, and moderate degradability. The silicate-modified PEG–chitosan nanocomposites were also suggested for osteoblast adhesion and mineralization [209]. Adding PEG to the alginate–chitosan electrospun fibers decreased the conductivity and increased the chain entanglements of the composite biomaterial [210]. Interestingly, PEG can be easily removed using water after the formation of porous scaffolds.

2.5.2.6 Poly(vinyl alcohol)

Poly(vinyl alcohol) (PVA) can be synthesized from poly(vinyl acetate) through hydrolysis, alcoholysis, or aminolysis [211]. The hydrophilicity of PVA can be changed by controlling its molecular weight and degree of hydrolysis. PVA hydrogels can be formed through chemical reactions with glutaraldehyde [212] or epichlorohydrin [213]. To ensure biocompatibility of PVA gels and avoid the leaching problem of cross-linking chemicals, electron beam irradiation or freez/thaw is carried out upon gel formation [214]. However, the obtained PVA gels using these methods are not biodegradable under physiological conditions. Therefore, they have been utilized as permanent scaffolds with long stability. PVA hydrogels have mostly been used in cartilage [215] and bone [216] TE applications.

2.6 Fabrication Methods of Biomaterials

After presenting the different types of biomaterials (both natural and synthetic), it is also important to introduce the different fabrication techniques that are used to produce the biomaterial scaffolds. These methods are basically divided into conventional and advanced methods, as discussed in the following.

2.6.1 Conventional Fabrication Methods

In order to process the scaffold materials into the porous structures used in TE, some conventional techniques have been developed. Examples include solvent-casting and particulate-leaching, gas-foaming, phase separation, melt-molding, and freeze-drying (Figure 2.11). While these methods can produce scaffolds with a continuous, porous structure, they are not as effective in allowing precise control over the pore size, geometry, spatial distribution, or interconnectivity. It is difficult to make the internal channels within the scaffold using these methods [218]. In addition, cells are usually bound to migrate no more than 500 μm from the surface of the scaffolds, and the lack of proper nutrient and oxygen supply is also discernible (Figure 2.12) [219].

2.6.2 Advanced Fabrication Methods

In comparison to the conventional fabrication techniques of scaffolds, advanced techniques allow precise control of different scaffold parameters as well as the development of an artificial vascular system [219]. This way, scaffolds play a role

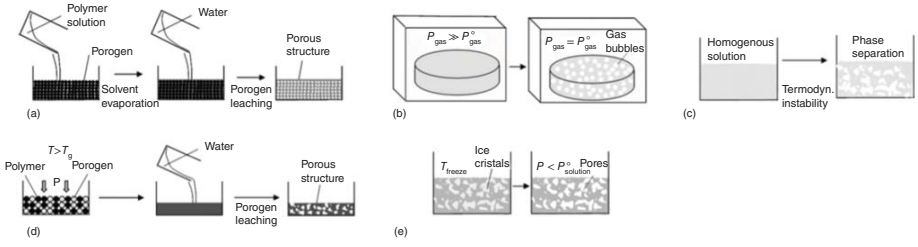

Figure 2.11 Conventional scaffold fabrication techniques: (a) solvent-casting and particulate-leaching, (b) gas-foaming process, (c) phase-separation process, (d) melt-molding process, and (e) freeze-drying process. (Modified from [217].) (Zhu [218], http://www.intechopen.com/books/advances-in-biomaterials-science-and-biomedical-applications/biofabrication-of-tissue-scaffolds. Used under CC-BY-3.0, http://creativecommons.org/licenses/by/3.0/.)

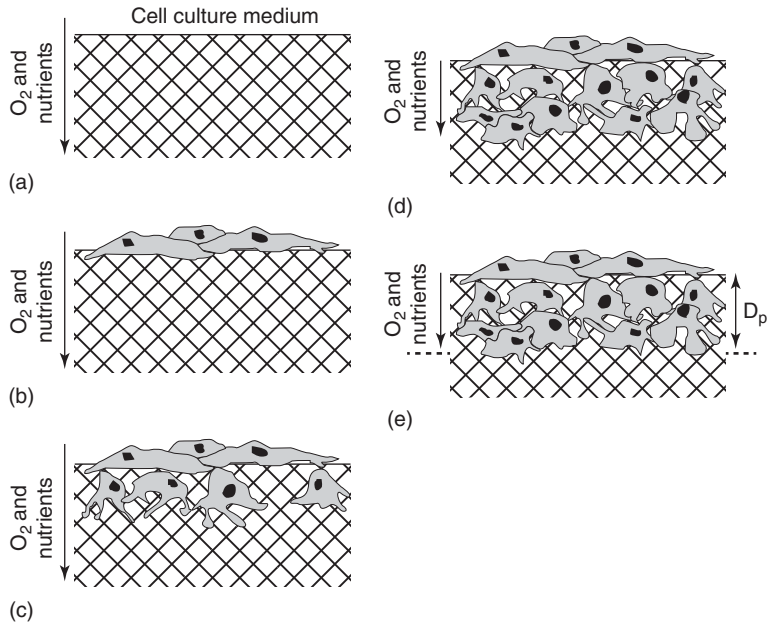

Figure 2.12 Diffusion constraints of tissue engineering scaffolds made of foam structures. (a) Tissue engineering scaffold which is an open-cell foam structure. Oxygen and nutrients are supplied from the liquid cell culture medium. (b) Cell seeding on scaffold. (c) Cells start to proliferate and migrate into the pores of the scaffold. (d) The cells fully colonize the pores and start to lay down their own ECM. (e) The top layer of cells consumes most the oxygen and nutrients in addition to limiting the diffusion of these components, thus reducing the amount available for pioneering cells migrating deep into the scaffold. Eventually, cellular migration is halted as a result of the lack of oxygen and nutrients supply. The layer of cells that can survive on the diffusion of oxygen and nutrients from the medium comprises the cellular penetration depth (D_p). (Reproduced with kind permission of Czernuszka [219].)

beyond providing a simple supporting structure, allowing the different chemical, mechanical, and biological signals as means of responding to the stimuli from the environment [218]. This is the origin of the new concept of "biofabrication", which can be defined as the production of complex living and nonliving biological products from raw materials such as living cells, molecules, ECMs, and biomaterials [220].

The main advanced scaffold fabrication techniques are electrospinning and rapid prototyping (which is also known as solid free-form fabrication (SFF)) [218].

2.6.2.1 Electrospinning

As we have already mentioned, the scaffold should not be a mere structure to support cells, but it should also have an appropriate pore size for cell migration, sufficient area for cell adhesion, growth, and differentiation, and a degradation rate that corresponds to that of the natural tissue regeneration rate. All these properties may be satisfied by the thin biomaterial fibers that are created through electrospinning process. This process can use either synthetic or natural materials

and result in meshes with fibers whose diameter is between tens of micrometers and tens of nanometers [221]. Electrospinning works by using two electrodes with opposite electric charges: one of them is placed in the polymer solution and the other works as a collector. The polymer solution is pumped as droplets, and then the generated electric field produces a force causing a jet of polymer to erupt. This is how nanofibers form and deposit on the collector node [222].

2.6.2.2 Rapid Prototyping

In contrast to the conventional machining techniques, rapid prototyping does not involve constant material removal. Instead, complex shaped scaffolds are built by adding materials layer-by-layer as dictated by a computer program. Each layer represents a cross-section of the scaffold at a certain level. The main advantage of this technique is the ability to produce parts with reproducible architecture and variable composition. This allows, in the context of TE, the control of scaffold architecture and properties. Many SFF techniques are available: some are based on laser technology, such as stereolithography, 3D printing, and selective laser sintering, while others are based on assembly technology, for example, shape deposition manufacturing and solid ground curing [223].

It is worth noting that for a scaffold to become viable both clinically and commercially, it is not enough for the fabrication technique to be appropriate in terms of making it function properly. Other considerations need to be taken into account such as cost effectiveness, scalability, storage, and delivery [13].

2.7 Testing of Biomaterials

The fabrication methods of biomaterials are not the only ones in constant development. Actually, with the importance of the properties of biomaterials in the TE process, another essential topic to consider is the testing of those materials. Testing allows us to make sure that the materials to be used in biomedical applications have the required properties.

Testing can target mechanical properties such as the response to fatigue, tensile strength, elasticity, toughness, and so on. This type of testing is not new. In 1991, for instance, Nielsen *et al.* proposed a biaxial testing procedure for elastic biomaterials to be used in membranes. The test allowed them to measure the stress–strain trend of the material, and its accuracy was assessed using finite element analysis [224].

On the other hand, testing can also target other material properties such as biodegradation and biocompatibility; biocompatibility testing is further subdivided into two different tests for cytotoxicity (cell compatibility) and hemocompatibility (blood compatibility). These tests are to be conducted on biomaterials according to ISO 10993 [225]. While adenosine triphosphate (ATP) is the parameter that is mostly measured for cytotoxicity inference *in vitro* [226], hemocompatibility check is usually done by routine blood assays for red blood cells, white blood cells, as well as platelet adhesion, activation, and coagulation [26].

Testing for the different biomaterial properties can be done both *in vitro* and *in vivo*. While *in vitro* testing is preferred because it minimizes the use of animals – thus also eliminates the differences due to the type of animals in question – it is also true that *in vivo* testing gives a much more reliable idea of what actually happens when the biomaterials are inserted into the body [227].

Both *in vitro* and *in vivo* methods were applied on the same starch-based material by Mendes *et al.* to test its potential to as a novel biomaterial for orthopedic surgery [23]. *In vivo* evaluation consisted of a surgical procedure to insert the implants in Dutch milch goats and then measure biocompatibility and biodegradation. As for the *in vitro* cytotoxicity assessment, two cell culture methods were used: minimum essential medium (MEM) extraction test and the MTT test.

In general, cell cultures are increasingly used in the *in vitro* testing for cytotoxicity and cytocompatibility [228]. With the use of these cultures, different tests for biocompatibility can be performed: the agarose-overlay test, the MTT test, or the direct cell seeding test. *In vivo* tests, on the other hand, include the chorioallantoic membrane test and different surface analysis methods (X-ray photoelectron spectroscopy or scanning electron microscopy (SEM)) [229]. While cell-based testing is particularly widespread, other testing methods are also available, as summarized in Table 2.3 [26].

2.8 Challenges for Biomaterials in Tissue Engineering

In spite of the advances that have been made in the development of biomaterials for TE applications, some challenges still exist, which must be taken into consideration whenever a new biomaterial or fabrication method is devised.

The first major challenge related to biomaterials is the difficulty of incorporating both their structural and functional requirements. As we have seen, the scaffold must have a specific architecture (pore size, geometry, etc.) that can allow cells to effectively communicate with the ECM and to achieve their basic functions, such as differentiation, migration, and adhesion. It must also have mechanical properties that can mimic those of the tissue that is being replaced in the body; in addition, it must be biocompatible and biodegradable, and should not trigger any inflammatory response in the body of the host [230].

For all of these conditions to be met, innovative biomaterial compositions and fabrication methods are constantly being developed. One way of achieving the structure–function balance is by combining synthetic and natural biomaterials. In general, combining different materials has shown advantages over using only a single biomaterial type given the mechanical, biological, and chemical properties of the composites. So the question is how to ensure their proper blending. For example, when blending collagen with synthetic polymers, how do we guarantee their solubility in a common solvent [38]? On another level, in orthopedic TE, for instance, two biomaterials with different mechanical properties are to be interfaced, which adds to the complication. How can we achieve a strong bond at the musculoskeletal interface so as to minimize stress concentration [7]? These issues have to be dealt with to ensure biocompatibility of the scaffolds and their mechanical stability in the body.

Table 2.3 Major biomaterial characterization techniques [26].

Technique	Application	Limitation
Large-scale cell-based screening	Versatile approach used to test cell responses to a wide range of scaffold properties such as surface chemistry, surface energy, surface topology, peptide ligands, pore size, and mechanical properties	Mostly limited to 2D films or surfaces despite the fact that biomaterials are frequently used to fabricate 3D scaffolds
Microarray technology	Investigating the changing cellular environment, in particular the gene and protein expression pattern, toxicity profile in response to different types of biomaterials	Limited to annotated transcripts along with cost, technical variation, and complexity of statistical analysis
Proteomic technology	Enables the profiling of a complex sample for global protein expression profile, including posttranslational modifications and subcellular localization representing the current physiological status	Poor detection of low-abundance and hydrophobic membrane proteins with 2D electrophoresis along with contamination by keratin and the autolysis of trypsin in case of mass spectrometry
Atomic force microscopy	Generates 3D profiling and imaging of the cells on materials and provides surface-adhesion force measurements	Provides only surface information and not suitable for 3D scaffolds. Cantilever mechanism cannot be used for surfaces with large changes in surface topography, like macroporous scaffolds with 600-μm-deep pores
Four-dimensional elastic light-scattering fingerprinting	Provides pinpoint precision into the variation of nanoscale architecture of living cells and tissue organization upon biomaterial interaction through real-time, noninvasive monitoring of these fingerprints	Confined to the assessment of a single process at a time in a very controlled environment
Bio-Raman spectroscopy	Monitoring the viability, cell cycle, metabolism, mitosis, differentiation, dedifferentiation, mineralization, and onset of death of single cells and cell assemblages (organoids) in real time without damage to the cells upon biomaterial interaction	Absorption of radiation from laser beam can cause sample heating and damage

Another important concern for biomaterials in TE is their vascularization. This issue is especially critical for 3D tissues of >1 mm thickness because of their metabolic and nutritional needs [230]. After they are implanted in the patient's body, the cells of the engineered tissue consume oxygen that is available for no more than a few hours; since the development of new blood vessels (angiogenesis) requires several days, an alternative strategy must be followed. For certain cells, such as hepatocytes, injecting them directly onto existing vascular beds can solve the problem of developing their own blood vessels. For others, such as bone or tendon cells, for example, this method does not work, so growth factors can be released to accelerate angiogenesis [2]. In addition to angiogenesis-based techniques, a growing approach relies on prevascularization of the engineered tissues, which allows a faster action inside the body. This can be achieved by various subtractive, additive, and hybrid methods [231].

Finally, a manufacturing challenge is also present. While many fabrication techniques are now available, the main issue is still with the commercialization of engineered tissue products for specific patient demands. Long-term storage and shipping of engineered tissues need to be done, and then their structure and function need to be evaluated [230].

2.9 Conclusions and Future Directions

TE has emerged as an exciting research field with numerous preclinical and clinical trials and products. However, one has to still design and fabricate highly complex scaffold materials [232]. There is no unique biomaterial covering all aspects of the native ECM for TE applications. Therefore, a wide variety of natural, synthetic, and natural/synthetic composite biomaterials have been developed continuously aiming to provide specific interactions with cells through their well-defined chemical (e.g., cell-adhesive sites and biofunctionality) and physical (e.g., mechanics, structure, porosity, and interconnectivity) properties [233]. Cell–biomaterial scaffold interactions have significant effects on various cellular behaviors, such as cell adhesion, organization, proliferation, differentiation, and ECM production, and, finally, tissue formation. Pursuing this research direction, rapid and inspiring advances in life sciences and technologies have greatly helped biomaterial design and fabrication processes for tissue regeneration. In particular, novel discoveries in biological processes and close collaborations between biologists, material scientists, and even medical doctors are key factors for the development of novel and functional biomaterials incorporating various biological characteristics of native ECM for TE applications.

Table 2.4 summarizes the use of natural and synthetic biomaterials for different TE applications. Controlling scaffold properties using natural and synthetic biomaterials is crucial to manipulate cell behaviors and thereby fabricate functional tissue constructs. In particular, the dynamic nature of native ECM should be considered in the design and fabrication of functional biomaterial scaffolds providing smart and dynamic scaffolds for cells to receive and respond to biological signals [234, 235]. Needless to say, incorporation of other factors, such as soluble factors,

Table 2.4 Natural and synthetic biomaterials used in TE.

Name of biomaterial	TE applications	Comments
	Natural biomaterials	
Collagen	Liver [42], cartilage [52], bone [56], skin [60], cardiac [62], vascular [63, 64], and neural [63]	Advantages: Biocompatibility, cell-binding affinity, and biodegradability Disadvantages: Initiation of immune response in body, low mechanical properties, stability, and reproducibility
Hyaluronic acid	Bone [69], cartilage [75], skin [74], neural [78], vascular [81], and muscle [82]	
Alginic acid (Alginate)	Muscle [88], liver [90], cartilage [94], and bone [99]	
Chitosan	Bone [103], liver [104], cartilage [109], skin [112], neural [113], and vascular [115]	
Gelatin	Liver [118], cardiac [123], muscle [122], and vascular [124]	
Fibrin	Neural [134], muscle [135], liver [137], and cartilage [138]	
Silk	Cartilage [146], (bone, vascular, neural, skin, and cardiac) [142, 150]	
Peptides	Vascular [154], neural [155], bone [159], and cardiac [158]	
Elastin	Cardiac [163] and vascular [159]	
	Synthetic biomaterials	
PNIPAAm	Cardiac [167], vascular [168], cartilage [169], and bone [162]	Advantages: Relatively superior mechanical properties, tunable chemistry and physical structure, high reproducibility, and absence of immunogenic responses in body Disadvantages: Low biological interactions with cells and inflammatory response after implantation
PLA	Bone [174]	
PLGA	Liver [177], bone [184], skin [190], and vascular [192]	
PCL	Skin, cartilage, bone, and cardiac [196, 200]	
PEG	Cartilage [203], liver [204], and muscle [205]	
PVA	Cartilage [216] and bone[232]	

is also important to enhance the functionality of biomaterial scaffolds. However, physical incorporation of soluble factors into scaffolds or chemical modification of scaffolds using soluble factors is not a simple task because they are highly sensitive to some experimental conditions, such as high temperature, and thereby easily lose their functionality.

There are a number of biomimetic scaffolds having multiple chemical, biological, and physical functionalities to closely mimic the natural ECM for cells. Taking cues from the nature to design and fabricate biomaterials is definitely a good way to make a suitable scaffold for engineered tissues [236, 237]. Micro- and nanotechnology approaches are extremely powerful tools to construct biomimetic scaffolds for cells aiming to control their intra- and extracellular signaling and function [238]. The use of nanomaterials in scaffold fabrication is also an asset to recapitulate the nanofeatures of native ECM [239]. However, the large numbers of developed biomaterials with different design parameters for a single biomaterial require spending much time and effort to find suitable cell–scaffold combination for a specific TE application. In this regard, high-throughput screening of cell–biomaterial interactions (e.g., high-throughput screening microarrays [240]) is useful to speed up the screening process. For instance, Anderson *et al*. [240] used a high-throughput microarray technique to screen a combinatorial library of acrylate monomers for the synthesis of novel biopolymers regulating the behaviors of human embryonic stem cells.

Ultimately, a major step toward practical and wide applications of proposed biomaterials is their successful clinical translation for humans in a safe and cost-effective manner. While a large number of excellent biomaterial scaffolds have been developed and tested to regulate cell behaviors and tissue fabrication *in vitro*, there are only a limited number of biomaterials that are approved for human use because of slow translation of these materials into clinical practice [241]. Therefore, it is required to accelerate the translation process of the developed biomaterials into clinical use using standard protocols.

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Abbreviations

3D	three-dimensional
ADSC	adipose-derived stem cell
DC	dense collagen
DC-Cs	polypeptide-conjugated dense collagen
ECM	extracellular matrix
FDA	Food and Drug Administration
iPSC	induced pluripotent stem cell

GAG	glycosaminoglycan
PCL	poly(ε-caprolactone)
PEG	poly(ethylene glycol)
PGA	poly(glycolic acid)
PG	proteoglycan
PLA	poly(lactic acid)
PLC	phospholipase C
PLGA	poly(lactic-*co*-glycolic acid)
PNIPAAm	poly(*N*-isopropylacrylamide)
PVA	poly(vinyl alcohol)
RGD	arginine-glycine-aspartic acid
SEM	scanning electron microscopy
TE	tissue engineering
TGF-1	transforming growth factor-1

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3

Harnessing the Potential of Stem Cells from Different Sources for Tissue Engineering

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3.1 Introduction

The introduction of newer and innovative treatments to cure long-persisting ailments and medical conditions has led to improved treatment and a surge in the newer forms of treatment such as stem cell therapy, tissue engineering, surgical implants, and other mechanical devices. An increase in the number of ailments, due to organ failure and subsequent dearth in the availability of transplants, has led to the development of tissue engineering, which is an interdisciplinary approach toward regenerative and translational medicine. Tissue engineering can be defined as the application of the principles of engineering in life sciences toward the fundamental understanding of the structural and functional relationships of normal cells and pathological mammalian tissues and the development of biological substitutes to restore, maintain, and improve functions. It involves large-scale production and manipulation to obtain maximum efficiency with accuracy in developing biological substitutes or offering support to the tissues and organs. This is done using biomaterials as implantable devices, by genetically manipulating cells, or altering the tissue's microenvironment. Thus tissue engineering includes techniques that help to replace, repair, maintain, enhance, and manipulate the functions of a diseased or malfunctioning tissue or organ in humans and animals. Tissue engineering finds applications in tissues such as bone, cartilage, and muscles, as well as organs such as heart, liver, lungs, kidney, and so on. This is done by understanding the complex morphology and biological tissue-specific function of the cells and tissue in the natural environment by the development of techniques *ex vivo* to mimic tissue-specific functions using natural components or biocompatible synthetic materials in order to study the interactions. Thus, tissue engineering has developed into a relevant area of study where tissue and organ failure is treated by implanting natural, synthetic, or semisynthetic mimics that are fully functional from the start or grow into the required functionality inside the human body by interacting with neighboring cells and tissues. Among the various strategies are (i) implanting freshly obtained or *in vitro* actively grown cells, (ii) implanting tissue that has been assembled *ex vivo* on scaffolds [1], and (iii) *in situ* regenerating tissues and organs [2].

Living cells form the core functional part of tissue engineering. They may be obtained from different sources, which include autologous cells, allogeneic cells,

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cadavers, xenogeneic donor cells, genetically engineered cells, and stem cells. While the use of autologous cells minimizes the use of immunosuppressive drugs, they are of little use because they are terminally differentiated cells [3]. In addition, it is inconvenient and not always possible to obtain these cells. Simultaneously, non-autologous cells require the administration of immunosuppressive drugs, which might lead to infections [4]. Stem cells, on the other hand, are known to be plastic and undifferentiated, and thus can be engineered into any desired tissue or organ. In particular, they are an attractive form of treatment, as they can grow into specific cells and be expanded *in vitro* to form a pool of different cell lineages when compared to matured cells. Apart from cells, natural, synthetic and semisynthetic or hybrid biomaterials also play an important role in tissue engineering as scaffolds, which give stability and a platform for the cells to grow in a 2D or 3D environment and remain viable. In this chapter, we explore the role of different stem cells used in tissue engineering applications [5].

3.2 Stem Cells in Tissue Engineering

Stem cells are a unique kind of the primitive, immature cells that have a remarkable capacity to develop into different kinds of cells. They serve as a continuous and natural internal repair mechanism. They are not found in abundance and are comparatively rare in our body. The significance of stem cells has been expanding ever since their first clinical trial over 30 years ago, which dealt with bone marrow transplantation with mouse embryonic stem cells (ESCs). The applications of stem cells are attributed to their three salient features: homing, restoration of function by differentiating into multiple cell types, and the secretion of bioactive growth factors [6]. Because of these, stem cells are used in a wide range of applications, which include cell-based therapy for immunomodulation, tissue-engineered scaffolds, and, more recently, as carriers for drug and gene delivery. Some of the other applications of stem cells include understanding the biology of diseases, regenerative medicine, and therapeutics. The applications of stems cells are by no means exhaustive. Some of the diseases treated by stem cells include Alzheimer's, Parkinson's, diabetes, autoimmune diseases, burns, blood, bone defects, cardiac, cartilage, kidney, lung, and liver disorders, cancers, and tumors. Nowadays stem cells are also used by scientists to screen new drugs and to understand certain congenital diseases.

3.3 Unique Properties

As mentioned previously, the rationale for the use of stem cells in tissue engineering is due to their self-renewal capacity and plasticity. Stem cells are (i) uncontrolled, self-renewing cells through repeated cell division, (ii) have the ability to differentiate into different cell lineages (plasticity), and (iii) have capacity to transform into more than one cell type. They display marked differences based on the source and state of differentiation. This is established

using various colonial and surface markers. While all stem cells are plastic, their source defines the potency. Totipotent cells and pluripotent cells are obtained from the early embryonic stages, while multipotency is a characteristic of adult stem cells (ASCs) which are more tissue- or cell-specific. Totipotency, pluripotency, multipotency, and plasticity have become synonymous with stem cells. Pluripotent stem cells include both ESCs and artificially developed induced pluripotent stem cells (iPSCs). PCSs differentiate spontaneously by forming aggregates and embryonic bodies (EBs) closely resembling embryogenesis and also help in the self-repair mechanism [7]. They can be differentiated into almost all cell lineages, whereas ASCs are more specific. Thus stem cells have opened the gates for unlimited access to engineer different kinds of cell *in vitro*.

However, differentiation plays a very important role in stem cell research. Some of the major concerns in the use of stem cells for tissue engineering include (i) the available quantity of stem cells, (ii) differentiation potential of the cells, (iii) survival of the differentiated cells in 3D environments, (iv) biomechanical compliance, and (v) combinations of all these factors. Differentiation depends on both intrinsic and extrinsic factors. The former include genetic make-up and the cells' natural capacity to grow and divide to form a particular cell type, while the latter include the cellular interaction, chemokine and cytokine signaling, and also to a great extent the microenvironment [8]. Thus it is very important to understand the factors and signals that effect the differentiation and expansion of stem cells into various cell and tissue types. Age, gender, disease condition, and ethnicity of the source cells also contribute to the differentiation potential.

3.4 Types of Stem Cells

For decades, ESCs and ASCs were the only kinds of known stem cell sources. In 1981, the mouse ESC was first isolated, and a decade later (in 1998) the human ESC was isolated from an *in vitro* cultured embryo. As the name suggests, ESCs are obtained from early embryos and can be differentiated into any cell line corresponding to the three germ layers *in vitro* and *in vivo*. They are pluripotent, while ASCs are obtained from somatic cells of adult tissues such as the bone marrow, blood, muscles, brain, and so on. All tissues contain a reservoir of stem cells, which are used to replace the cells lost during wear and tear, injury, or disease. In 2006, scientist generated a new class of stem cells called iPSCs. Based on their source, stem cells can be classified as ESCs, ASCs (also sometimes known as mesenchymal stem cells or MSCs), and iPSCs (Figure 3.1) [9].

3.4.1 Embryonic Stem Cells

On the seventh day of embryo development, a blastocyst progresses into gastrulation. The inner geometrically defined cells of the blastocyst, which have the capacity to differentiate into different germ layers, can be expanded *ex vivo* as ESCs. ESCs were initially assumed to be abnormal cells derived from a tumor-like mass, but later, through differentiation, the cells were found to be nonmalignant and normal early embryonic cells (inner mass cells) that

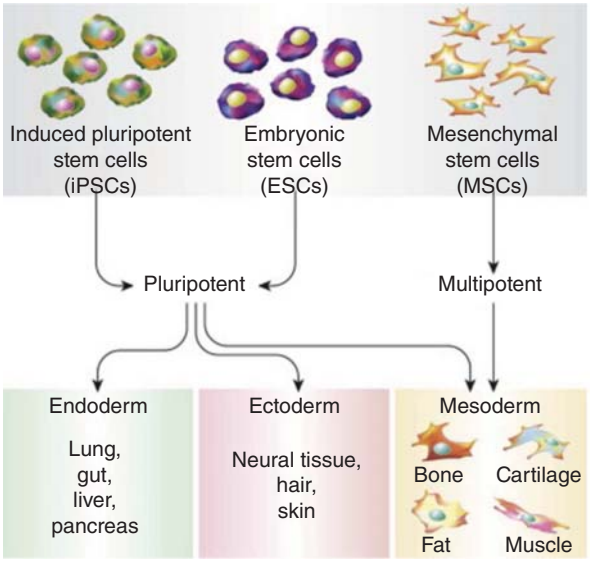

Figure 3.1 Schematic representation of the different source-specific classification of stem cells types and their differentiation potential. However, these days MSCs are also known to give rise to other cells such as nerve cells, iPSCs, and so on. (Whitworth 2014 [9]. Reproduced with permission of Elsevier.

are capable of colonizing into a complete organism. ESCs are also developed using various cloning techniques such as stomatic cell nuclear transplant, altered nuclear transfer, parthenogenesis, arrested embryo technique, single blastomeric approach, and so on. ESCs are preferred over the ASCs due to their ability to develop into any kind of body cells with unlimited cell division ability and maintain their diploid and undifferentiated nature. This self-renewal capacity and pluripotency is controlled by various factors such as the length of the telomere, transcriptional factors, cellular pathway, genes, signaling, multilineage priming, and so on. Core genes, namely Oct4, Sox2, and Nanog, are the basic factors that control the lineage-specific growth of stem cells. Concentrations of LIF (leukemia inhibitory factor) and interleukin-11 and DNA methylation are also required to prevent differentiation [10]. ESC differentiation follows the JAK/STAT (janus kinase/signal transducer and activator of transcription) pathway. Raz *et al*. suggest that the level of activated STAT3 plays a critical role in maintaining differentiation. Withdrawal of LIF spontaneously differentiates ESCs to form distinct cellular aggregates or embryoid bodies, which further contain differentiating cells of ectoderm, endoderm, and mesoderm lineage [11].

During early embryonic development, very few cells are present that develop into germ layers, and thus any genetic aberration within these cells leads to a change in the genome of the particular organism. Thus, epigenetic changes with prolonged ESC culture and chromosomal abnormalities due to laboratory manipulations are some of the problems that lead to the rejection of allogeneic ESCs [11]. The lineage specification and developmental pathways are found to be conserved among different ESCs, but the differentiation pathways vary from one organism to another. Recently, *in vitro* single-step differentiation of ESCs into MSCs and subsequent differentiation into bone, cartilage, and adiposities were achieved [12]. The most common method of differentiation includes

supplementing the medium with growth factors, hormones, or cytokines, changing the chemical composition of the medium, transfecting foreign genes, altering the cellular interactions, and coculturing. For example, the coculturing of ESCs with osteoblasts induces the ESCs to differentiate toward osteogenic lineage [13].

Extensive work with ESCs has led to the immortalization and development of bone and cardiac cells, but most work is directed toward the study of the mechanism of growth and differentiation of ESCs. Human ESCs are useful cells to study early human development, pathogenesis of congenital defects, and drug screening. Primordial germ cells are also being developed from ESCs [14]. Nonhuman primate heart [15] and mouse ESCs may form nerve cells that appear to be able partially to restore the spinal cord function in a rat model [16]. Current research in ESCs is centered on obtaining highly purified cell lineages without any teratoma formation. Recent clinical transplantation of ESCs as retinal tissues has shown no signs of hyperproliferation, tumorigenicity, or ectopic tissue formation [17]. Although ESCs contribute to donor cells for numerous clinical applications, there are major challenges, particularly ethical ones, to be overcome before ESCs can be used in clinical practice.

3.4.2 Induced Pluripotent Stem Cells

Artificially, pluripotency is harnessed by the transformation and reprogramming of adult stomatic cells into an ESC-like state by the induction of core genes central to pluripotency along with other transcriptional factors. These cells are called iPSCs. They are functionally similar to ESCs but have limited transcriptional and genetic memory and tend to jump back to their original state. iPSCs exhibits various stem cell markers including those characteristic of ESCs. The first mouse iPSCs were produced in 2006, followed by the human iPSCs (hiPSCs) in 2007. The use of iPSCs helps in overcoming various shortcomings associated with stomatic cells, and they are considered ideal for stem-cell-based therapy as they can be both autologous and allogeneic. They are an alternate source of autologous cells, as they do not elicit any immune response in livings cells. Thus they are more personalized and can be differentiated into nonimmunogenic, disease-specific cells. The major advantage of using iPSCs is that they have an uncontrolled tendency to divide, grow, and differentiate into any desired lineages that are functionally potent and stable compared to the adult cells from which they were obtained. Some of the other notable advantages are patient specificity due epigenetic similarity and an unlimited and renewable source of cells, which can be a platform for cell-based therapy, and negligible immunogenicity. The difference between ESCs and iPSCs is under debate, but clinical trials are being conducted to establish the same. iPSCs can be obtained from almost all adult cell lines including reprogrammed cancer cells. Originally, reprogramming was done using lentivirus or retrovirus. Newer methods include adenoviruses [18], the Sendai virus [19], *piggyBac* transposons [20], and episomal plasmids [21] to deliver the transgenes and, more recently, transducing the cells with synthetically modified messenger RNAs encoding reprogramming factors, small molecules, and recombinant proteins [22].

The applications of iPSCs can be broadly classified as cell-based therapy, high-throughput drug screening, tissue engineering, and medicine. It is seen that mouse ESCs and mouse iPSCs differentiate into smooth muscle cells, demonstrating the feasibility of harnessing PSCs to serve as an advanced cell source for vascular engineering [23]. It was also demonstrated that iPSC-derived cells could be used to treat cardiac disorders [24], autosomal recessive disorders like cystic fibrosis [25], high-mortality-rate diseases like spinal muscular atrophy [26], and enzymatic diseases like diabetes by using onsite reprogramming techniques [27, 28]. Some of the limitations associated with iPSCs are the possibility of their leading to tumorigenic cells due to improper gene silencing [29], undesired mutations, and faulty epigenesis. In order to prevent these shortcomings, efforts are under way with transmigration bypassing pluripotency [30].

3.4.3 Adult Stem Cells

ASCs are undifferentiated cells that are obtained from differentiated adult cells. Every organ or tissue is made up of these ASCs at very low frequency (1 stem cell per 10^5 cells). They are multipotent progenitor cells. They have the capacity to develop into various specific cell types. For example, hematopoietic stem cells (HSCs) can form different bloods cells such as red blood cells (RBCs), white blood cells (WBCs), neutrophils, macrophages, and so on. ASCs are generally dormant and are activated during adverse condition signaling for their differentiation into specific cell types. There are many different types of ASCs based on their source. The most common ones include HSCs (blood stem cells) and MSCs. Both the cell types are primarily obtained from the bone marrow. Some other ASCs originate from germ layers.

- *Endoderm origin*: epithelial stem cells, gastrointestinal-tract stem cells, pancreatic stem cells, hepatic oval cells, and mammary prostatic gland stem cells, and ovarian and testicular stem cells.
- *Mesoderm origin*: bone marrow, hematopoietic, stromal stems, and cardiac stem cells.
- *Ectoderm origin*: neural stem cells, skin stem cells, and ocular stem cells.

The hallmark feature of ASCs is their ease of induction into the specific cell types. However, they are not capable of dividing continuously over prolonged passages. Thus, some of the challenges with ASCs include the difficulty to isolate and purify and continuous culture in large scales, and their restricted differentiation potential. All these restrict the applications of ASCs in tissue engineering. Genetic modification, transdifferentiation, and reprogramming are some of the methods used to expand the horizon of research with ASCs. ASCs possess several advantages. They are easily obtainable, they do not form teratomas when injected *in vivo*, they do not need any feeder layers to grow, and they do not require sacrificing human embryos for their isolation. Thus they have an edge over ESCs. ASCs also showcase targeting, as they "home" toward the damaged cells and help in regeneration. Most ASCs are tissue-specific except bone-marrow-derived MSCs (BMSCs), which makes it extensively used in research. They are all multipotent, but exceptions are amniotic fluid- and placenta-derived stem cells and human

umbilical cord MSCs (hUCMSCs), which are able to be expanded into a wide variety of cells.

3.4.3.1 Mesenchymal Stem Cell

They are progenitor cells and the most commonly used subtype of stem cells. Wakitani *et al*. fist isolated MSCs in 1966 for cartilage repair, and over the years the work carried out by the group has suggested MSCs as an effective and safe way of treating cartilage defects. They are less differentiated compared to the other ASCs and have the capacity to grow both *in vitro* and *in vivo* into different kinds of tissues such as bone, muscle, cartilage, tendons, adipose, and so on. They can also be programmed to develop into nerve cells, cardiac muscles, hepatocytes, and so on, making clinical application of MSC-based techniques possible.

Under normal culture conditions, MSCs help in the maintenance and regeneration of connective tissues, display a fibroblast-like morphology, are adherent and colony-forming, and are known to migrate to injured or inflammatory tissues where they participate in the repair [31]. A mesengenic diagram by Caplan *et al*. explains the different pathways associated with MSCs [32]. MSCs can be either BMSCs or adipose-derived stem cells (ADSCs); their difference is in the expression of adhesion molecules. ADSCs are simple to extract and cost effective. This makes these stem cells a viable option to obtain MSCs. While BMSCs shows promising results in bone tissue engineering, ADSCs are useful for cartilage tissue engineering and, most recently, for neural tissue engineering.

3.4.3.2 Hematopoietic Stem Cells

Coexisting with MSCs in the bone marrow, HSCs aid in the formation of blood and immune cells. They reconstitute the hematopoietic system in the body, which is apoptotic and has the shortest life span in our body. They have the capacity for indefinite self-renewal compared to other ASCs, but their proliferation and differentiation depend mainly on interactions of the cells with adhesion molecules in the extracellular matrix (ECM) of the bone marrow stroma [33]. Major functions of HSCs include self-renewal, differentiation, mobilization, circulation, and apoptosis. They are of two types: long-term and short-term HSCs based on their span of proliferation. Efforts have been made to isolate these two types of HSCs, which is detrimental, as short-term HSCs are not suitable for cell therapies. However, a reliable method to obtain long-term HSCs is still elusive. HSCs are also difficult to grow *in vitro*. Attempts to expand HSCs in tissue culture with stem-cell-promoting factors have not resulted in a significant expansion of HSCs, but rather some of these induce differentiation of HSCs, which limits their application. Some of the HSC-specific surface markers include CD11b, CD14, CD34, CD45, CD79, CD19, and HLA-DR. Transient culture was seen when the cells were grown with combinations of different cytokines. Mobilized peripheral blood has replaced bone marrow as a source of HSCs [34]. Blood and bone disorders such as aplastic anemia, thalassemia, sickle cell anemia, and cancers are treated using HSC transplantation. Thus, understanding the biology and developing culture methods to establish the relation between self-renewal, differentiation, and apoptosis of HSCs would help us to apply better techniques to harness these cells for enhanced wound

healing and treatment of leukemia and lymphoma. Recently, Heidt *et al*. used HSCs to study the cross-talk within the immune system and various immune components [35].

3.4.3.3 Wharton's Jelly Stem Cells

The intervascular matrix of the umbilical cord has been named Wharton's jelly stem cells (WJSCs) or human umbilical cord perivascular cells (hUCPVCs). WJSC refers to the region surrounding the blood vessels within the umbilical cord and contains mucoid connective tissue and fibroblast-like cells [22]. Characterization of WJSCs has shown the presence of both MSC and ESC markers [36]. It shows a CD34+ subpopulation in a CD38− population, which indicates that can produce early progenitor HSCs in long-term cultures which are highly proliferative compared to BMSCs and ESCs [37, 38]. Some of the recent advances include the use of autologous hWJSCs as stromal support cells for CD34+ expansion. Rat models for cerebral ischaemia, intracerebral hemorrhage, spinal cord injury, Parkinson's disease, retinal disease, type-1 diabetes, and myogenic disease were tested with hWJSC-derived cells and tissues [38, 39]. Some of the limitations of WJSCs are that they have low yield and the fear of metaplasia; on the other hand, human WJSCs are known to be hypo-immunogenic and non-tumorigenic and have great potential for safe cell-based therapies [36, 40].

3.5 Application of Stem Cells in Tissue Engineering

3.5.1 Bone Tissue Engineering

Conventionally, MSCs are used to regenerate bones and other skeletal structures. However, MSCs can be also be used to derive liver, kidney, muscle, skin, neural, and cardiac cell lineages (Table 3.1) [40, 56]. The osteoblasts present within the bone structures help direct the differentiation of autologous MSCs into bone cell lineage; however, in certain diseases such as osteoporosis, osteoarthritis, infection, cancer, and even nonuniform fractures, they can have a negative impact on the regeneration process. Thus bone tissue engineering plays an important role in alleviating the stresses caused [57, 58]. Immunotolerance and immunomodulatory properties of stem cells have aided the progress of bone regeneration in clinical trials. This will help in the treatment of autoimmune deficiency disorders that have an erosion effect on bones [59, 60]. Bone and skeletal structures undergoing repair require mechanical and structural supports through the use of a scaffold. It is also known that these scaffolds help in the differentiation of stem cells into osteogenic lineages [61, 62]. Collagen scaffolds for adults and hydroxyapatite and polylactic scaffolds for children are examples of scaffolds used to grow MSCs [63]. In canines, it is seen that autologous or allogeneic MSCs, or MSCs combined with a porous ceramic scaffold with β-tricalcium phosphate or hydroxyapatite, significantly increased bone formation. No immunologic response was recorded in these animals. Ovine and caprine studies have also shown that tissue-engineered bone samples

Table 3.1 Summary of diverse biomedical applications of stem cells for tissue engineering and regeneration therapy.

Applications	Cell types/sources	Origin	Cell surface markers	Characteristics/remarks
Cardiac	ESC [40]	Human	VWF+, CD-31, NKX2.5, ANF Ki67+, ANF, NKX2.5	• Engineered 3D vascularized cardiac tissue. • HUVECs or hESC-ECSs contribute to vascularization of cardiac tissue. • Functional and structural properties of new cardiac tissue.
	hESC, MSC [41]	Fetal and adult human	Nkx2.5, Gata-4, mef-2c, Isl1, TroponinT	• Tissue printing of progenitor cells on scaffolds. • Specific porous structures preserves function of human cardiomyocyte progenitor cells.
Blood vessels	hiPSC [42]	Human	CD34+, KDR+, NRP1+, CD31−, CD144−	• hiPSCs form blood vessels, endothelial cells, and mesenchymal cells *in vivo* from various hiPSCs. • Functional and durable blood vessels formed from different iPSCs.
	ADSC [43]	Mice	CD13, DC73, CD9-0	• 3D adipose-derived MSC spheroids using liquid overlay technique seed onto porous polyurethane scaffolds. • Increased efficacy of future MSC vascularized based tissue engineering strategies.
Bone	hESC [44]	Human	FUNX2	• PLGA scaffolds with native bone ECM direct osteogenic differentiation of hESCs to spark bone formation. • Decellularized scaffolds encourage cell adhesion, proliferation, and osteogenic differentiation.
	BMSC [45]	Mouse	Calcein, type I collagen	• A highly porous type I collagen-hydroxyapatite scaffold was developed that retains progenitor cells. • Supports robust new bone formation after 3 weeks *in vivo*.

(Continued)

Table 3.1 (Continued)

Applications	Cell types/sources	Origin	Cell surface markers	Characteristics/remarks
Cartilage	MSC [46]	Adult goats	Type II collagen	• Quantify collagen type II protein production; • Applying hypoxic culture conditions enhances chondrogenesis
	iPSC [47]	Mice	GFP+, type II collagen, col2, Acan, col10, sox9	• Expanding iPSCs seeded in agarose effective in repairing cartilaginous matrix. • Constructs made with GFP^+ cells showed the highest integrative repair strength.
Liver	BMSC [48]	Mice	Collagen type I & IV, Laminin, AFP, CK19, G6P, TAT, GATA4	• Acellular liver scaffolds created by cryo-chemical decellularization are effective at removing cellular materials from liver while conserving the ECM components; • Hepatic-like tissues generated by re-seeding MSCs into scaffolds restore liver functions after transplantation and helps donor liver shortage.
	Hepatocytes-iPSC [49]	Human Fibroblasts	Sox17, GATA4, FOXA2, E-CAD, ALB, vWF, CD-31, ECAD, GAPDH, ALB	• Liver tissue constructs were engineered via differentiation of hepatocytes and endothelial cells from iPSCs. • Clinical use of 3D multicomponent fiber assembly liver tissue.
Lung	Fetal cells-epithelial progenitor [50] normal lung tissue	Human and mice	p63, CK5 TTF1, C (SP-C), TTF1, GATA6, ETS1	• Basal epithelial cells are not clonogenic. • Human lung stem cells give rise to different populations of epithelial cells and to pulmonary vessels • C-kit + human lung stem cells show self-renewal, clonogenicity, and multipotency.
	Type II pneumocytes – iPSC [51]	Human	Foxa2, TTF1, SPC^-, eGFP +	• Lamin proved to be the best material for PDLLA scaffolds. • *In vitro* expansion of pneumocytes.

Neural	hiPSC [52]	Used in rats	Oct3/4, SOX2, c-Myc, KLF4Nanog, Lin28, AP2, nestin, AP2, p75, HNK1 +S100B/+NuMA	• Neural crest stem cells taken from iPSCs and ESCs are similar. • Neural crust stem cells differentiated into Schwann cells facilitate myelination of axons promotes nerve regeneration. • Engineered scaffolds combined with stem cells have superior therapeutic effects.
	iPSC [53]	Human	FOXA2, LMX1A, OTX2,PITX, PITX3	• Differentiation of midbrain dopamine neurons. • Established scalable source of neurons for neural transplantation.
Skin	ADSC [54]	SD rats	FGF1, VEGF, CCL2, CXCR4, MMP1	• Self-assembled spheroids portray increased expression of paracrine factors. • Enhances skin wound healing and encourages capillary formations.
	MSC [55]	Human	Ker 10, filaggrin, involucrin	• Collagen nanofibrous scaffolds created to copy the fibrous structure of native skin ECM for epidermal differentiation of MSCs. • BMSCs were driven towards epidermal lineage.

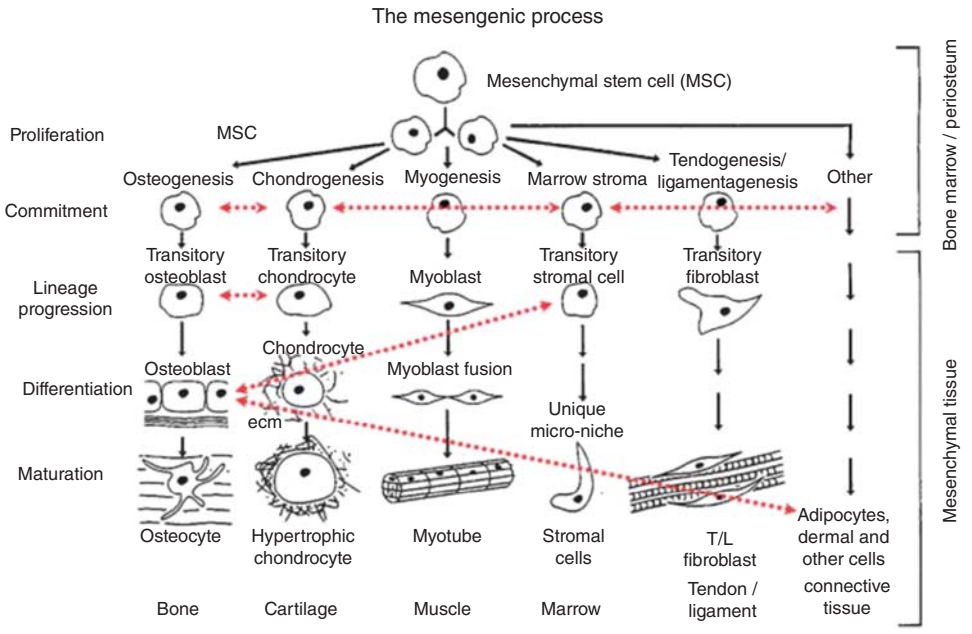

Figure 3.2 Mesengenic process diagram, with horizontal or diagonal arrows (dotted lines) depicting the plasticity of mesenchymal cells and the transdifferentiation of mature phenotypes into wholly different cell types (Caplan 2007 [32]. Reproduced with permission of John Wiley and Sons.)

retain the same strength and functionality as the original bone 32 weeks after implantation [64]. Soluble cytokines, osteoinductive factors, and other growth factors combined with MSCs help to enhance the therapeutic potential in bone formation [65]. Figure 3.2 shows the *in vivo* application of different types of stem cells in combination with calcium phosphate cement scaffold for bone regeneration therapy in a cranial defect animal model [66].

3.5.2 Cartilage Tissue Engineering

A wide variety of strategies are prevalent in the field of tissue engineering that help repair cartilage. Rheumatoid arthritis, osteoarthritis, and intra-articular fracture are a few cartilage disorders that are caused by partial or full-thickness defects; partial cartilage does not naturally have access to bone marrow MSCs and also lacks the capability to self- repair. A wide variety of strategies are used in the field of tissue engineering that help repair cartilage, resulting in a decrease of pain and inflammation in the cartilage or repair the cartilage via production/formation of fibro cartilage [67]. Chondrocytes are generally used for cartilage tissue engineering. However, over the years a newer form of therapy, which includes the use of stem cells expanded over large cultures in chondrogenic media, has been introduced. This method has the capacity to develop into cartilage lineages when implanted [68]. In order to stay localized, a uniform distribution of the stem cells is required, as well as withstanding

in vivo forces and stress, at the site of lesion from the neighboring cartilage. It is vital that the stem cells are cultured into biologically compatible materials and are also stable, as these forces and interactions play an important role in condrogenic differentiation of stem cells [67, 69]. Both autologous and allogeneic BMSCs loaded on different types of scaffolds are in various stages of clinical trials [70, 71].

3.5.3 Cardiac Tissue Engineering

Engineering cardiac tissues is of great significance, as it helps us in understanding the cardiac physiology and treat myocardial infarction, cardiac arrhythmias, valve failure, and other disorders, using minimal invasive therapies. Transplantation therapies with various functionalized cardiomyocytes hold the future for treatment of heart disorders. Also, stem cell therapy of different ischemic and non-ischemic heart failures is a field that has a great clinical significance [72]. It has been reported that non-myocyte's capability to mechanically re-create the neighboring ECM and express paracrine effects on cardiomyocytes (CM) appears to be crucial for aiding the formation of quality cardiac tissue. However, 3–17% of fibroblasts from the heart were found to be essential for PSC CMs from murine or human sources to form a syncytium when encapsulated in a 3D fibrin hydrogel matrix [73, 74]. In addition, it was found that culturing hPSCs for extended periods (120 days) or adding T3 thyroid hormone can lead to a larger cell size and advanced CM maturation with improved functionality [75]. For functionality, it is known that dense and ordered alignment of the CMs inside the ECM scaffold, as well as the formation of functioning junctions, is required to create an environment to provide intercellular current flow and force transmission. However, even with recent progress, the electrical and mechanical functionalities of tissues are inferior to those of the adult myocardium. The various techniques used to differentiate hPSC-derived CMs into cardiac tissue constructs involve scaffold-free cell sheets [76], fibrin-based [77] or collagen-based hydrogels, and porous gelatin scaffolds [74, 78–80]. Currently, hPSC-derived CMs are delivered to the heart via implantation of a tissue patch [81] or injectable hydrogels or cell suspensions. It has also been stated that such injectable materials help identify the various interaction between the cardiac and stem cells *in vitro*, apart from having better retention capacity [82, 83]. In addition, other studies have provided results that show the electrical coupling between the graft and the host, which meant that the cells were in synchronization with the ECG of the heart. hPSC-CM-engineered tissues still need to be improved for reliable use *in vitro* assays and therapeutic applications (Figure 3.3).

3.5.4 Neural Tissue Engineering

Nerve regeneration is a complex phenomenon. The nervous system is divided into two major systems: the central nervous system (CNS) and the peripheral nervous system (PNS). Damage done to the CNS leaves long-term effects, as their capacity for renewal is limited compared to that of the PNS. Autologous nerve graft is used to treat injuries to the PNS, which requires repeated surgeries. Thus tissue engineering strategies are employed to overcome these shortcomings

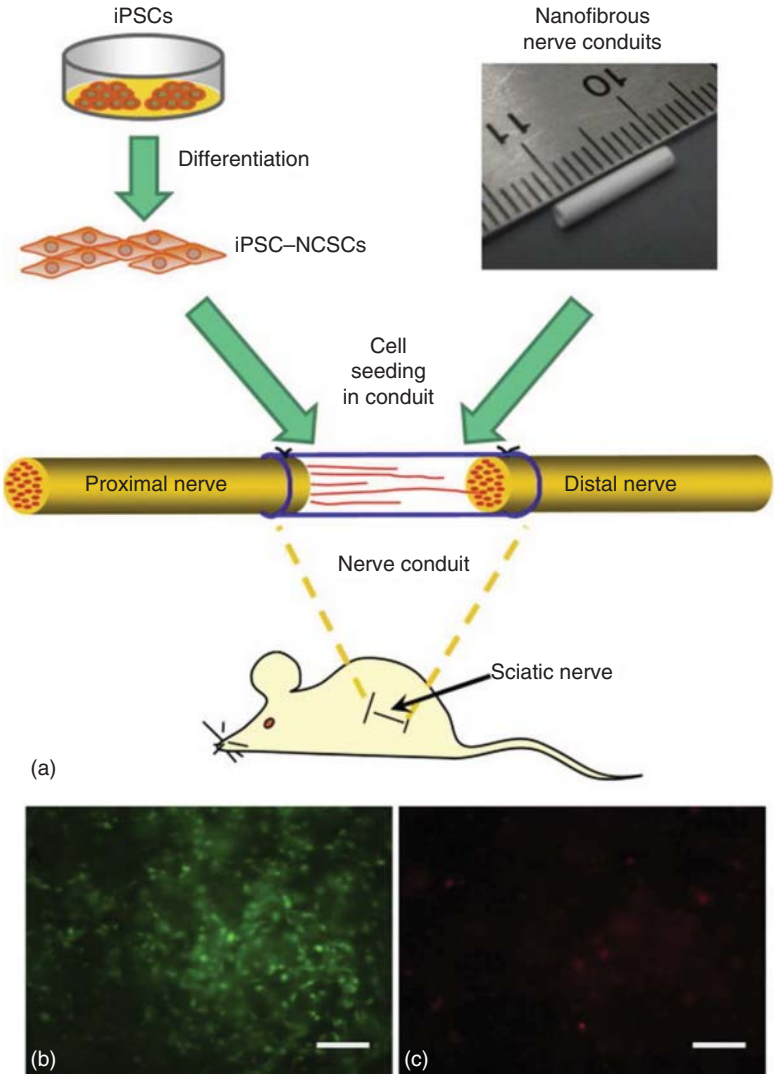

Figure 3.3 Tissue-engineered nanofibrous nerve conduits for peripheral nerve regeneration. (a) Schematic outline of tissue engineering approach by combining NCSCs and a nanofibrous nerve conduit. (b,c) The NCSCs were mixed with matrigel, injected in the nerve conduits, and cultured for 1 day *in vitro*. The viability of the cells was tested by using live/dead assay. Live cells (calcein staining, in green) are shown in (b). Dead cells (ethidium homodimer-1 staining, in red) are shown in (c). Scale bars are 100 μm. (Wang 2011 [53]. Reproduced with permission of Elsevier.)

involved with the nervous system. Currently, autologous nerves are used to derive stem cells for peripheral nerve construction. This procedure sacrifices nerve tissue, and is only met by the slow growth rate and culturing of nerve stem cells *in vitro* [84]. In these cases, adipose tissue is considered an option because of its inherent multipotency. Adipose tissue is similar to neural tissue in that both

are able to differentiate into "specialized neurospheres" under the influence of proper growth factors [85–87]. Advantages of this include easy availability, rapid expansion, and low immunogenicity, as well as differentiation *in vitro*. Previous studies have shown promise of MSCs to transdifferentiate into a neural phenotype [88]. Also, BMSCs under stimulation with 1 mmol l^{-1} β-mercaptoethanol (BME) expressed neuron-specific enolase. The study showed that the cells were morphologically transformed to develop contracted cell bodies and terminal bulbs similar to mature neurons. Following this study, Ashijan *et al*. substituted BME for insulin, indomethacin, and isobutyl methylxanthine [89]. It was found that these cells still lacked mature neural cell markers, and the study was not able to test for electrophysiological stimulation. However, Safford *et al*. was able to solve both problems in their studies. Using combinations of butylated hydroxyanisole/KCl and valproic acid/forskolin/hydrocortisone/insulin, they successfully demonstrated the expression of the mature stem cell markers GFAO, MAP2, and β-III tubulin, the presence of voltage-gated calcium channels, and the ability to upregulate the glutamate receptor [90]. Another recent study compared the difference between differentiated and undifferentiated stem cells over a period of 14 days, which was performed by reverse-transcriptase polymerase chain reaction (RT-PCR). The results showed a reduction in glial fibrillary acidic protein (GFAP) and nestin levels in the differentiated cells, while there was an increase in β-II-tubulin, neuron-specific enolase (NSE), Tau, and microtubule-associated ptotein-2 (MAP2) synaptophysin marker expressions [91]. Differentiation into neural lineage could be further promoted by adding a combination of specific growth factors to ADSCs. This increased the expression of Schwann cell markers such as S100, GFAO, p75, and β-III-tubulin [92, 93]. Further testing was done to show that transformations in the morphology of ADSCs into neural tissues occurred under various stimuli [94]. Clinical trials involving multiple animals treated canine- and murine-specific neurological conditions [95, 96]. New research has shown that electrical stimulation improved neurite outgrowth toward negatively charged cathode electrode and axonal regeneration [97, 98]. Interestingly, electrical stimulation of MSCs, when grown on a surface with high conductivity, can improve the neural differentiation potential [99, 100].

3.5.5 Tissue Engineering for Other Tissues Including Skin, Liver, Lungs, Bladder, and Pancreas

In addition to the bone, cartilage, cardiac, and neural tissue engineering that we have already covered, stem cells are being used for the creation of many other tissues, broadening the range of diseases that can be fought through the process. In this section, we discuss the use of stem cells in the engineering of skin, liver, lungs, bladder, and pancreas tissues. Skin was the first tissue to be engineered [101]. Matrix-based at the beginning, engineered skin tissues are utilizing more and more stem cells with the advances that are occurring in the field. Adult MSCs have shown to be particularly efficient in skin repair and regeneration; and because ESCs have many limitations, iPSs are also being considered as a way of creating skin substitutes (Figure 3.4).

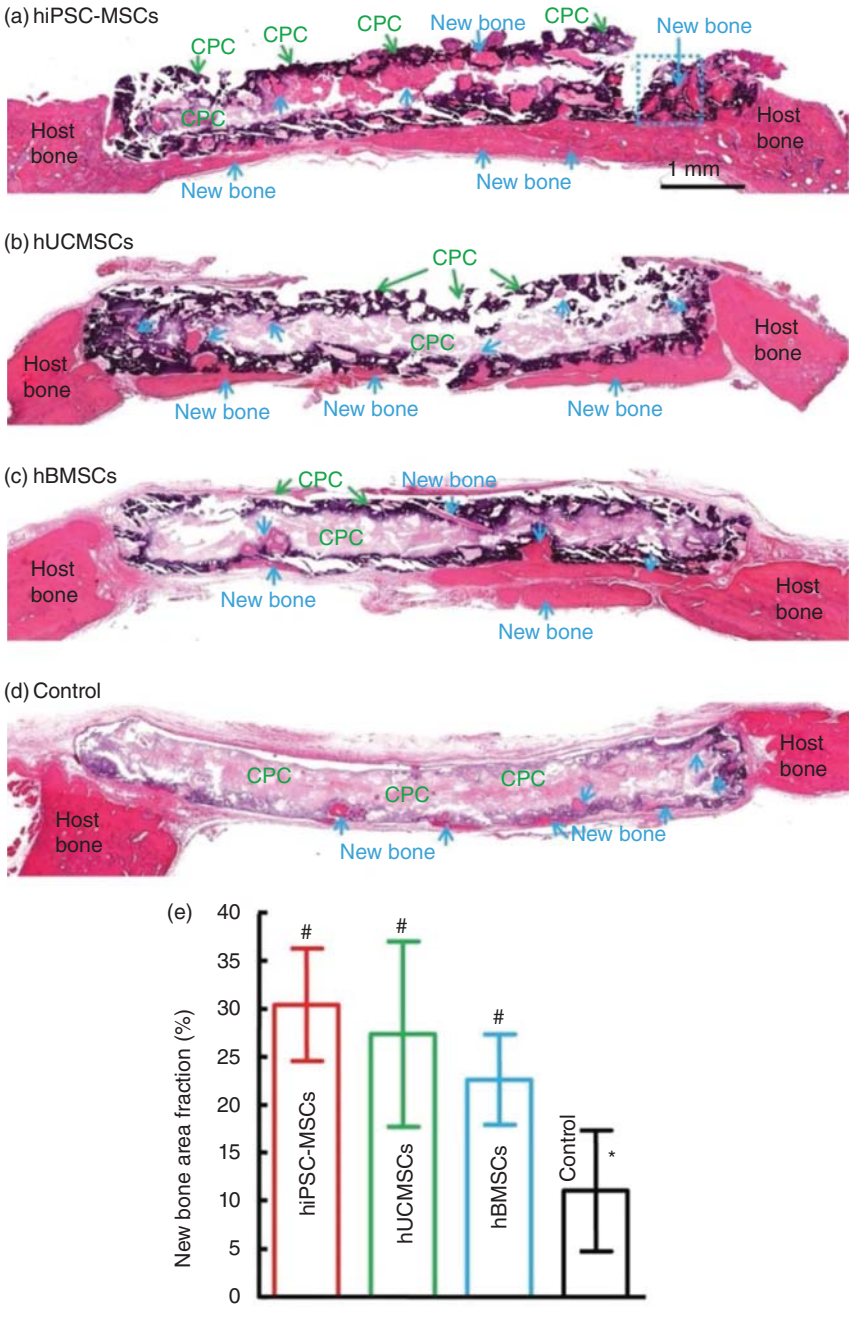

Figure 3.4 Calcium phosphate cement scaffold seeded with hiPSC-MSCs, hUCMSCs, and hBMSCs inducing osteogenic regeneration. (a–d) H&E staining of stem-cell-seeded implants in critical-sized cranial defects in nude rats. (e) Histomorphometry analysis of new bone area fraction. Bars with dissimilar marks (# and *) indicate values that are significantly different from each other ($P < 0.05$). Each value is mean ± SD ($n = 6$). Scaffolds containing hiPSC-MSCs, hUCMSCs, and hBMSCs exhibit better bone regeneration than CPC control without cells. There is no significant difference between the hUCMSC and hBMSC groups ($P > 0.1$). (Akita 2004 [65]. Reproduced with permission of John Wiley and Sons.)

According to Fiegel *et al*. [102], tissue engineering is also being introduced as a new therapeutic strategy to treat liver problems. The advantage of this method over organ transplantation is that it can overcome both the scarcity in organ donations and the needed immunosuppressive treatment that can lead to many complications over the life of the individual. Because the use of fetal liver stem cells is fraught with many ethical concerns, adult-derived HSCs and MSCs are preferred, especially because they do not present the risk of teratoma formation. MSCs were particularly shown to be able to differentiate to hepatocyte-like cells, but, to date, many challenges still persist and no fully functional hepatocytes have been obtained from stem cells [103].

The importance of using stem cells in tissue engineering also shows in the process of engineering whole lung tissue. Calle *et al*. explain that, to make lung tissue, three components are of primordial importance: the scaffold, which provides the structure and architecture of the lung; the cells; and the bioreactor that is used to cultivate the organ. Given the variety of cell types present in the lungs, stem cells are especially needed to engineer new lung tissue [104].

As for the bladder, the many obstacles that are related to the use of urothelial and smooth muscle cells in tissue engineering have made stem cells better candidates for the operation. Both ESCs and ASCs (MSCs) have shown promise in differentiating into smooth muscle cells [105].

Finally, pancreas tissue engineering is currently presented as a potential solution for many diseases, diabetes being the prime example. With the use of human endometrial mesenchymal stem cells (hEnMSCs), which were the first to be successfully isolated from the human endometrium, Niknamasl *et al*. were able to create β-cells like those of the pancreas [105].

3.6 Challenges and Future Directions

There is no doubt that stem cells have proven to be excellent candidates for use in tissue engineering, given their capacity of homing, differentiation, and secretion of growth factors. That said, it is also true that many challenges still exist and a lot more is still to be tested and discovered for the use of stem cells in tissue engineering.

An important challenge is, for instance, to be able to design a capillary network for the engineered tissue that is appropriate in terms of providing the required gas exchange, nutrition, and waste removal. This is made harder by the fact that the cells used in the scaffold are different for each tissue engineered, and therefore requires different culturing environment, dosages, and so on [106]. Another one is to find the optimal stem cell source, which is not an easy task. As already mentioned, ESCs can cause teratoma [107]. It is also the basis of many ethical and religious concerns, and because ESCs can grow indefinitely in a tissue culture, the risk increases for immune rejection, which means life-long immunosuppressant treatment and thus another inconvenience of ESCs [106]. ASCs, on the other hand, have their own drawback to be overcome in the future. One example

is related to the immunosuppressive properties of MSCs, which might lead to tumors in patients [108]. As for adult stem cells, in general – especially HSCs from blood or bone marrow – they present an additional challenge: that is, to grow them in a laboratory environment without their becoming specialized [106]. All of these challenges still need to be solved in addition to many others, such as optimizing isolation techniques for minimal contamination and preserving the required cell types after differentiation. It is also necessary to address ethical concerns early enough and to discover the optimal cell source, bioreactors, and scaffolds to use.

3.7 Conclusion

As stem-cell-based therapy is employed in the clinic, it is a struggle to clear the qualms behind the effect of transducing signals and biomechanical communications between the cells and their environment. Thus the understanding of the biomechanical properties of muscles, fluids, bones, implants, engineered cells, scaffolds, and ECMs that interact with each other also plays a regulatory role in cell growth, differentiation, and various metabolic activities. Both theoretical and computational models have been developed over the years to study these mechano-biological properties at their interface. However, further research in this direction is needed to fully understand cell–microenvironment interactions at the molecular level, which plays a vital role in regulating the stem cells' fate [107]. This includes studies on matrix stiffness which influences stem cell lineage and tumor progression [109], axonal tension which regulates presynaptic vesicle clustering [110], and stiffness gradients which guide the migration and differentiation of cells. Together, detailed *in vitro* and *in silico* investigations [112–113] hold the potential to accurately determine the suitability of stem cells for tissue engineering applications and clinical treatments.

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4

Induced Pluripotent Stem Cells in Scaffold-Based Tissue Engineering

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4.1 Introduction

Tissue engineering is a multidisciplinary field, and its prime aim is to repair and regenerate the functions of damaged tissues and organs that fail to heal spontaneously by themselves, with the help of cells and engineered matrices called scaffolds [1]. The regeneration of damaged tissues requires the existence of cells capable of proliferation and differentiation, which will contribute functionally to the reparative process of tissue remodeling. Stem cells are cells that have the remarkable potential to transform into many different tissue types of the human body. The addition of stem cells to the tissue engineering armamentarium has opened up new avenues with the potential of developing stem-cell-based constructs for the regeneration of damaged tissues. Stem cells possess a distinct ability to self-renew and differentiate, making them a more suitable option as a seeding cell source for tissue-engineered constructs in comparison to other cell types like somatic cells. Stem cells encompass a large class of cell types, which includes fate-restricted multipotent adult stem cells, embryonic stem cells (ESCs), and the recently discovered induced pluripotent stem cells (iPSCs). Among them, the latter hold significant promise for generating engineered tissues and organs owing their embryonic-cell-like state and thus have attracted the attention of the stem cell community. Human iPSCs have biological similarities with human ESCs with respect to their morphology, differentiation potential, and molecular signature, which make them a potential alternative for ESCs in therapeutic applications.

There are many characteristics that make iPSCs superior to ESCs as a cell source in tissue engineering. For instance, iPSCs have the potential for generating patient-specific cells with high pluripotency by reprogramming the patient's own cells and thereby reducing the chances for immunological rejection. Additionally, there are few or no ethical concerns associated with the iPSCs when compared to ESCs. Their use also obviates the need for invasive procedures to obtain pluripotent cells because of the vast availability of reprogrammable cell types. Because of the several merits associated with iPSC-seeded tissue-engineered scaffolds in terms of their structural and functional properties that could mimic the native extracellular matrix (ECM), these kinds of engineered scaffolds have

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applications in different areas such as bone, cartilage, cardiac, skin, and neural tissue engineering [2]. For instance, Hoveizi *et al.* have recently compared the cell adhesion and proliferation behavior of human induced pluripotent stem cells (hiPSCs) on polycaprolactone (PCL) electrospun nanofibrous scaffolds with solution-cast film scaffolds to understand the interactions between ECM-mimicking biomaterials and cells. Their results demonstrated that the nanofibrous scaffolds better support attachment and proliferation, emphasizing the sensing ability of hiPSCs with regard to the physical properties and chemical composition of the biomaterial. The potential applications of iPSCs are discussed in detail with experimental examples in Section 4.5.

Although iPSC-seeded tissue-engineered scaffolds are established and proven to be useful in *in vitro* tissue engineering, there are still a few challenges to building iPSC-based tissue constructs with biomimetic architecture and function. Though reprogramming technologies have progressed at a fast pace, still their efficiency is low with no clear knowledge of the governing mechanisms behind the process. In recent reports, however, some iPSC-derived progenies have been proven to retain the tumorigenic potential, thus hindering their clinical applications. Moreover, the lack of a practical strategy for iPSC differentiation and standard target cell purification protocols may also result in side effects after *in vivo* transplantation. In contrast to the initial beliefs about iPSCs' capability to overcome immunity-related problems, some recent reports have shown that iPSCs possess higher immunogenicity than predicted and thus they could evoke immune responses even in syngeneic recipients. Altogether, an efficient strategy to modulate the immunogenicity of iPSCs in reprogramming is indispensible for their clinical translation, which needs thorough examination before their clinical transplantation, including patient-specific iPSCs.

Considering the aforementioned impact of iPSC-based, tissue-engineered scaffolds, in this chapter we focus our attention on iPSCs as a potential cell source for scaffold-based tissue engineering and analyze their merits and demerits. For the benefit of readers, the basics of iPSCs and scaffold-based tissue engineering are also discussed, along with culture techniques, cell–scaffold interactions, and their applications in different tissue engineering fields. This is not to suggest that this is the only choice of cells available for generating engineered tissues and organs, but the key intention is to stimulate research on iPSCs in the context of scaffold-based tissue engineering and to evaluate their full potential, in terms of cellular growth and function, as an alternate cell source for tissue regenerative medicine. This chapter is expected to be useful for readers to gain insight into the impact of iPSCs in various 3D scaffold-based tissue engineering fields such as bone, cartilage, cardiac, skin, and neural tissue engineering.

4.2 Basics of Induced Pluripotent Stem Cells

Stem cells are nonspecialized cells that have the remarkable property of self-renewal and differentiation ability into specialized cell types (totipotency, pluripotency, and multipotency). Stem cells are considered an important source

of cells for tissue engineering and regenerative medicine. They are broadly classified into ESCs and somatic stem cells (also called adult stem cells). Both play unique roles in tissue regenerative medicine. iPSCs form another subclass of adult stem cells. In view of recent advancements in the field of stem cells, iPSCs have emerged as a potential clinical alternative for ESCs by overcoming the ethical concerns related to ESCs. The iPSCs are genetically reprogrammed adult cells with the characteristics and differentiation potential similar to those of ESCs.

In a path-breaking study by the Yamanaka group, murine fibroblasts were reprogrammed by retroviral transduction of a set of pluripotent-specific transcription factors (*OCT4, SOX2, KLF4,* and *c-Myc*) into mouse embryonic fibroblasts (MEFs) or tail-tip fibroblasts (TTFs), thus demonstrating the enhanced property as an excellent marker for pluripotency (Table 4.1) [3]. Later, the Thomson group employed the cellular reprogramming strategy with the lentiviral expression system to deliver four factors (*OCT4, SOX2, NANOg,* and *LIN28*) to fetal MRC5 lung fibroblasts and newborn BJ-1 foreskin fibroblasts, which resulted in high efficiency for fetal fibroblasts and 0.01% for newborn fibroblasts [5]. Thereafter, the initial methodology was redesigned to further increase the efficiency and reduce the number of integrated vector sequences for reprogramming iPSCs. Further, to avoid the ethical issues associated with the use of human embryos related to ESCs production, the resultant pluripotent cells from these experiments were derived directly from the patient's own cells. Hence, those novel stem cells were designated as hiPSCs. In addition, the differentiation of hiPSCs into functional cells is beneficial for cell-based therapy and also plays an important role in the establishment of patient-specific disease models for drug discovery and development.

iPSCs can be generated from somatic cells by various methods using different types of vectors. Retroviruses and lentivirus are the most commonly used viral vectors for delivering the transcription factors to reprogram the somatic cells. Among the viral vectors, specifically lentivirus has ability to infect nondividing and proliferating cells, which makes it suitable as a delivery vehicle. In a study

Table 4.1 Original Yamanaka factors replaced with other factors to reprogram the cells [3, 4].

Original transcription factors	Replaced with	Need for replacement
SOX2	*SOX1* and *SOX3*	*SOX2* proved to form teratomas and hence replaced with *SOX1* and *SOX3*, but are less efficient.
KLF4	*KLF1*	*KLF4* is highly tumorigenic and thus leads to tumorigenicity in iPSCs; replaced with *KLF1* which is less tumorigenic but with reduced efficiency.
c-Myc	*L-Myc* and *N-Myc*	*c-Myc* retrovirus on reactivation shows tumorigenicity in the chimeras and the progeny mice, as *c-Myc* gene is an oncogene; hence replaced with factors which show less tumorigenicity.

by Papapetrou *et al.*, four bi-cistronic lentiviral vectors were generated to encode the four reprogramming factors coexpressed with fluorescent proteins to demonstrate the impact of reprogramming factor expression for enhancing hiPSCs induction [6]. The group used reprogramming based on a "single cassette reprogramming vector" for generating iPSCs [6]. Similarly, another group performed excision of iPSC-based integrated sequences using lentiviral reprogramming vectors from patients with Parkinson's disease [7]. In contrast, yet another group used a nonintegrated virus like adenovirus as a carrier for generating iPSCs. The reprogramming efficiency increased in mouse and human cells [8]. Reprogramming of other somatic cells, such as neonatal and adult fibroblasts, blood cells, and so on, was reported to generate iPSCs by using Sendai-based reprogramming vectors. An alternative way is to initiate reprogramming by directly delivering the reprogramming proteins into the cells instead of delivering the transcription factors and then depending on their expression. Proteins play a major role in the expression of reprogramming factors, and therefore bioactive proteins in an *Escherichia coli* expression system can be used to reprogram mouse fibroblasts and human fibroblasts, but it has several limitations such as low efficiency and other technical challenges. Nonviral reprogramming methods include delivery of transcribed mRNA to efficiently express the reprogramming factors. For instance, microRNAs (miRNAs) originating from ESC-specific cell cycle-regulating family were reported to facilitate efficient reprogramming of MEFs into iPSCs [9].

In a notable study reported by the Yamanaka group, conventional plasmids were used to generate mouse iPSCs without any transgene sequence traces from MEFs [10]. Though the results showed integration in some of the iPSC lines, the procedure yielded one order less reprogramming efficiency in comparison to virus-based vector approaches. Human mesenchymal stem cells (hMSCs) have also been reported to be reprogrammed using the PiggyBac vector, but the results showed a 50-fold less reprogramming efficiency of the vector when compared with retroviral-mediated vector systems. Remarkably, small molecules (e.g., valproic acid (VPA), CHIR99021, tranylcypromine, 616452, forskolin (FSK), and 3-deazaneplanocin (DZNep)) were found to be useful in reprogramming mouse fibroblasts into iPSCs. Although the reprogramming efficiency was lower than that of conventional viral approaches, it provides an ideal chemical protocol that is more flexible and convenient than the biological protocols that depend on transcription factors. The usage of a cocktail of chemical compounds eliminates many drawbacks (e.g, tumorigenesis) of the other integration methods.

Besides the vectors, culture conditions also play a major role in the generation and expansion of iPSCs. There are two types of methods commonly used for generating iPSCs: feeder-dependent and feeder-free. The basic differences between these two methods are the medium used and the feeder layer onto which the cells adhere. Feeder-dependent protocol uses MEF as a feeder layer, while the feeder-free protocol commonly uses Matrigel or vitronectin XF. Recently, Nakagawa *et al*. developed a feeder-free culture system for generating iPSCs [11]. The authors used different matrices for coating, such as Matrigel, CellStart, and the recombinant laminin-511 E8 fragment (rLN511E8), but the cells proliferated best on rLN511E8 and not on the other matrices. Further,

they used this matrix along with the xeno-free medium for the iPSCs culture. A new xeno-free medium named "StemFit" was used – as no other medium gave good results – which used hiPSC colonies similar to the ones cultured on feeder cells. Another difference between the two methods is the medium used: the feeder-dependent method uses the basic stem cell culture medium (DMEM-F12 medium, KO-SR, glutamine, nonessential amino acids, and bFGF), while the feeder-free method uses the E8 medium (DMEM/F12, L-ascorbic acid-2-phosphate magnesium, sodium selenium, FGF2, insulin, $NaHCO_3$, transferrin, TGFβ1, or NODAL).

The use of animal-derived, undefined products for the iPSC culture and expansion is the major drawback, as it hinders the clinical use of these cells and also creates batch-to-batch variations. Therefore, this has led to the generation of defined culture conditions such as replacement of fetal bovine serum (FBS) with knockout-serum replacement (KO-SR) and also the replacement of the MEF feeder layer with human feeder cells (HFCs). Despite all these development, still stem cells are exposed to animal-derived products, which leads to the possibility of getting infections and graft rejections in humans. Therefore, there was a great need of creating xeno-free culture conditions, which led to the development of a xeno-free medium containing the base of knockout-Dulbecco's Modified Eagle's Medium (KO-DMEM) with additions of human serum albumin, amino acids, vitamins, antioxidants, trace minerals, and growth factors. After optimization, it was proven that the hESCs maintained their pluripotency and other characteristics after prolonged culture in the xeno-free medium [12].

Although the discovery of iPSCs is encouraging and has resulted in numerous successful studies, it is just beginning to be used in human clinical trials. Despite plenty of disease models based on cells that are differentiated from iPSCs, the generation of complex 3D tissues and organs for regenerative medicine is still a major challenge. There have been two encouraging clinical trials using human ESC-derived cells for therapy that have been approved by the U.S. Federal Drug Administration (FDA) [13]. Geron Corporation (Menlo Park, CA, USA) performed the first FDA-approved clinical trials using hESC-derived cells to treat a spinal cord injury [14]. The other encouraging trial was performed by Advanced Cell Technology (USA) using hESC-derived retinal pigment epithelial cells to treat macular degeneration [15]. Although no significant vision improvement was observed 4 months after transplantation, structural evidence confirmed that the cells got attached and continued to persist in the treated patients. Bulk transfection of oncogenic reprogramming factors (Oct4, Sox2, and Nanog) into somatic cells can cause abnormality in iPSCs, but it is still under investigation.

Certain tissues derived from iPSCs can elicit immune rejection response. For instance, in murine models the autologous iPSC-derived endothelial cells (iECs) were reported to elicit an immune response that resembles the one against a comparable somatic cell such as the aortic endothelial cell (AEC). The immune rejection of the autologous iECs was characterized by hallmark features of lymphocytic infiltration, excessive interferon-γ, and cytotoxic factor (granzyme-B and perforin) expression [16]. The rejection intensity induced by ESC-derived allografts differs from that by iPSCs-derived autografts due to the fact that MHC-I molecules are expressed in all allogeneic ESCs-derived cells.

Certain syngeneic iPSCs derivatives can express minor antigens if a specific autologous cell type derived from iPSCs is immunogenic, but they are capable of eliciting serious minor antigen-induced rejection of the cells. In general, we can still take better advantage of iPSCs for therapy than allogeneic ESC lines, even when immunologic issues are considered. Although significant progress has been made in understanding tumorigenicity, immunogenicity, and genomic instability in iPSCs [13], the relationship between these abnormalities and how to overcome the associated hurdles for clinical development of iPSCs are still under investigation. However, the development of integration-free reprogramming approaches, availability of disease modeling, and initiation of preclinical trials have significantly enhanced the prospects of advancing iPSC technology from bench to bedside.

Owing to the limitations associated with iPSCs such as their poor reprogramming efficiency and tumorigenic potential, iPSCs have not yet been considered as a model stem cell source, but extensive research is going on in this direction. To provide a solution to this problem, many other methods are being explored to generate iPSCs or to directly use somatic cells in relevance to clinical applications. In a recent approach, Jung *et al.* have explored chemical biology to enhance the reprogramming efficiency of the iPSCs and tried to increase the quality of the produced iPSCs [17]. They also explored the phenotype governing the biological mechanisms associated with iPSC generation [17]. The authors have emphasized the use of small-molecule modulators in the direct reprogramming of readily available cell types into clinically useful cell types (glial cells, neurons, cardiomyocytes (CMs), etc.) that can be used in translational research approaches. In another study, Baek *et al.* have revealed the effect of extremely low-frequency electromagnetic fields (EL-EMFs) on various biological processes (cell development, differentiation, etc.) [18]. Epigenetic changes that can support the effective somatic cell reprogramming were induced in the histone lysine methyltransferase MII2 as a result of EL-EMFs exposure. These approaches obviate the need for conventional cell reprogramming using vectors and provide an effective method to induce epigenetic reprogramming including the acquisition of pluripotency. There are still many problems that need to be addressed for iPSC technology to make it a promising cell source for tissue regenerative medicine applications. Owing to their potential for self-renewal, proliferation, and differentiation, iPSCs can provide an invaluable means of deriving patient-specific PSCs. For instance, in neural disorders, iPSCs have been reported to be the model cell source for the central nervous system. Therefore, iPSC-based tissue regeneration and engineering has been extensively investigated in recent years and could be used in therapeutic applications in the coming days.

4.3 Concept of Scaffold-Based Tissue Engineering

The concept of tissue engineering, in particular scaffold-based tissue engineering, involves culturing of isolated cells from the patient or donor on a scaffold that supports the growth and functioning of the isolated cells into a specific

tissue, which could then be grafted back to the defective site of the patient where tissue regeneration is required [1]. Cells, engineered matrices (also called scaffolds), and bioactive molecules are the key components of scaffold-based tissue engineering. The selection of these components is of great importance for obtaining better cell–material interactions that guide tissue regeneration. Scaffold plays a key role in tissue engineering by providing a structural support and a 3D microenvironment to the cells in order to support cell attachment and subsequent tissue development. From a biological perspective, cells in the human body reside in a complex mixture of pores, ridges, and various components of micro- and nano-featured ECM environment, which plays a vital role in facilitating cell–matrix interactions and cell–cell communications upon implantation of the engineered graft. Therefore, development of scaffolds with structure and properties matching those of the native ECM is essential in order to mimic the microenvironment of native tissue. Some of the common properties required for an ideal scaffold include structural support and physical microenvironment modulation according to the residing cells to attach, grow, migrate, and respond to the signals; to provide mechanical properties (such as elasticity, rigidity) comparable to those of the replaced tissue; provision of bioactive cues to the residing cells; biodegradability within the body environment to facilitate neovascularization; and remodeling of tissue in response to developmental, physiological, and pathological challenges during the dynamic processes of the tissue (such as morphogenesis). However, the multiple functions, complex composition, and the dynamic nature of the ECM in native tissues make it difficult to mimic it exactly. Nevertheless, many types of biomaterials are being tested worldwide as tissue scaffolding systems, including porous scaffolds, decellularized ECM-derived scaffold matrices, cell sheet secreted ECM matrix, cell-laden hydrogels, nano-fibers, and so on. Recently, Mohtaram *et al.* investigated the loop mesh and biaxially aligned microscale retinoic acid-functionalized PCL nanofibers for promoting neuronal differentiation of hiPSCs. The authors compared the effect of micro and nanoscale topographic cues on the neuronal outgrowth efficiencies of hiPSC-derived neuronal cells [19]. Their results confirmed that the topographic cue provided in the form of biaxial aligned nanofibers supported the maximum neurite outgrowth length of these cells compared to the loop-mesh topography [19]. These studies confirm the importance of the scaffolds in directing stem cell differentiation for tissue engineering applications. Besides the scaffolds, cell sources are also an important determinant for deciding the success of tissue-engineered products. The development of a viable construct involves an efficient cell source, which should be nonimmunogenic, proliferative, and easy to harvest, and possess the ability to differentiate into a variety of cell types with specialized functions. For autologous cells of patients suffering from extensive end-stage organ failure diseases, which have limited proliferative capacity in culture, stem cells have been envisioned as an alternative cell source. Cell sources utilized for tissue engineering include mature (non-stem) cells, adult stem cells or somatic stem cells, ESCs, totipotent stem cells or zygotes, and the recently introduced iPSCs. These cells can be autologous, allogeneic, or xenogenic in nature. Though a wide variety of cell sources are available, they suffer from a number of limitations.

For instance, mature cells show low proliferation and differentiation potential, whereas ESCs show unlimited self-renewal and multi-lineage potential but are restricted because of ethical and legislative issues. To overcome these limitations, iPSCs have been developed with properties of ESCs but overcoming ethical problems. iPSCs are a type of pluripotent stem cells that can be generated directly from the adult cells by reprogramming the transcriptional factors. For instance, Liu *et al*. have employed the potential of iPSCs in 3D PCL/gelatin scaffolds for chondrogenesis and articular cartilage defect restoration [20]. Their results confirmed the higher expression levels of chondrogenic markers in iPSCs *in vitro* culture than in control groups. In animal studies, enhanced gross appearance, histological improvements, and higher cartilage-specific gene expression supported the sub-chondral bone regeneration [20]. The details of iPSCs and their applications in various tissue engineering fields are discussed thoroughly in this chapter. While the selection of the appropriate cell source remains the key to the successful realization of stable and complex 3D constructs, design criteria of robust scaffolds and the mobilization of bioactive molecule that can modulate the cell–material interactions are also equally important, and thus can efficiently mimic a native physiologic environment. It has been proven that the incorporation of various growth factors, bioactive molecules, or nano-fillers as biological signals can promote the desired differentiation lineage within the seeded stem cells. In a comparative study by Klangjorhor *et al.*, co-adsorption of hyaluronan (HA) and the transforming growth factor β3 (TGF-β3) on gelatin scaffolds was found to assist the proliferation of primary human articular chondrocytes in a 3D culture [21]. The study confirmed that gelatin scaffolds on which HA and TGF-β3 were adsorbed were effective and more suitable than the conventional supplemented media method for the *in vitro* assessment of human chondrocyte 3D culture [21]. Cell–material interactions within these functional tissue constructs are critical for the effective functioning of these products. A concise understanding of cell–material interaction requires a thorough knowledge of cell biology and cell–ECM interactions in order to be extrapolated to the biomaterial's surface. In support of the importance of cell–material interaction, recently it has been reported that precise control of the internal structure of the scaffold can provide a more efficient carrier of the seeded stem cells for the regeneration of the damaged tissue [22]. For an elaborative understanding of cell–material interactions and their effect on the 3D microenvironment or synthetic niche of the seeded stem cells, see Section 4.4 with illustrations.

4.4 Cell–Scaffold Interactions

The formation of engineered tissues/organs and their physiological functions are mainly dependent on how the cells interact with engineered matrix (scaffold). The study of cell–matrix (scaffold) interaction is therefore of great importance for the success of tissue engineering, as it determines the cellular fate and function of the cultured cells and governs their differentiation potential. In

native tissue environment, cells are surrounded by ECM, which is a complex mixture of collagen fibers, proteoglycans, and multi-adhesive matrix protein supporting cells to attach and grow in a specific manner. For tissue engineering applications, the isolated cells from any source may need a suitable support matrix to grow, as most of the cells used are anchorage-dependent. For this purpose, biomaterials have been developed as scaffold materials to mimic the native microenvironment of the cells, which allows cell adhesion, migration, proliferation, and differentiation. The scaffold therefore should be designed with ECM-like features, having the general properties such as tissue compatibility and tissue-specific microenvironment properties. The scaffold materials are mostly made up of natural polymers such as gelatin, chitosan, hyaluronic acid, alginate, and so on, as well as synthetic polymers such as PCL, polyglycolic acid (PGA), polylactic acid (PLA), and so on. At present, there are a variety of materials and methods to generate scaffold specific to each cell and tissue. Among the various materials and methods, hydrogel-based cell-encapsulated scaffold [1] and nanofibrous scaffold [23] have attracted much attention of the biomaterial and tissue engineering community to generate tissue mimics. Biomaterial scaffolds act as carriers to promote cell activity and regulate cell functions in a controlled manner. They provide space for vascularization, neo-tissue formation, and remodeling to occur simultaneously along with efficient transport of nutrients and growth factors and removal of waste. The overall architecture of a tissue is determined by the scaffold structure, which can facilitate adhesion by involving cell–cell communication and cell–material interaction.

Cell–material interaction is the most important determinant for successful tissue regeneration, especially when handling anchorage-dependent cells. These interactions are purely dependent on the surface property of the scaffold. The cell seeding density should be optimized to acquire better cell–material interactions. Cells adhering to the surface of a scaffold initiate a sequence of physicochemical reactions between them and the biomaterial surface. In this process, the implant comes into contact with the cell's microenvironment and allows protein adsorption to mediate cell adhesion and signals through the cell adhesion receptors, mainly integrins. Following adhesion, cells release active compounds for signaling, responsible for ECM deposition, cell proliferation, and differentiation. The different factors that affect cell attachment include cell behavior, biomaterial surface properties (hydrophobicity, charge, roughness, softness, and chemical composition), environmental factors, and so on. In addition, mechanical factors have also been found to strongly influence the cell–material interaction and the subsequent cell behavior. In the case of mechanical interactions between the cell and the scaffold surface, the cytoskeleton of the cell is the main component, which could transmit and sustain the force at different locations within the cell. The cytoskeleton is mainly composed of microtubules, actin filaments, and intermediate filaments. Cells exhibit very different behaviors depending on the elasticity of the material they are cultured on or, equivalently, on the elasticity of the extracellular microenvironment. Other important parameters for evaluating cell–material interactions are the scaffold's biocompatibility and wettability. Studies have suggested that endogenous proteins can be rapidly adsorbed onto the material surface driven by the surface free energy, which can provide the

structural framework for initiating cellular adhesion. The main purpose of biomaterials is to restore the functionality and original morphology of the native tissue.

To further enhance the material properties for the betterment of the cell–material interactions, many surface and chemical modification techniques are being applied. Cells are able to respond to the nano-topographical features of the scaffold and can modulate their behavior in terms of adhesion, orientation, motility, surface antigen display, cytoskeletal condensation, and so on. Specifically, chemically modified material surfaces with ligands (RGD peptide or fibronectin) that can bind to specific extracellular receptors have been largely used to enhance the seeding efficiencies. Interestingly, fibronectin coating has been proven to increase the number of focal adhesions within each cell on the micropillar arrays to measure the forces exerted by the smooth muscle cells (SMCs) during adhesion. Stem cells can begin to differentiate into mature tissue cells when exposed to the intrinsic properties of the ECM, such as matrix structure, elasticity, porosity, and composition, with the help of mechano-sensitive pathways that can convert these biophysical cues into biochemical signals and commit a cell for a specific lineage. Xie *et al*. aimed at regenerating patient-specific blood vessels by using iPSC-derived SMCs and a nanofibrous porous scaffold. The porous NF poly-L-lactide (PLLA) scaffold favored a contractile phenotype of primary SMCs under *in vitro* culture conditions [24]. Mouse iPSCs were generated and then stimulated for differentiation into SMCs with retinoid acid (RA) treatment. When cultured on 3D PLLA NF scaffolds, these cells exhibited higher SMC-specific marker gene and lower pluripotent marker gene expression levels in comparison with the spontaneously differentiated cells [25].

ECM proteins including fibronectin, laminin, collagen, and vitronectin provide conditions that support cell adhesion, proliferation, and differentiation, and have also been investigated as feeder-free hiPSCs culture substrates. A recombinant form of human laminin-511, a component of the natural human ESCs niche, was enrolled as a system to culture human ESCs and iPSCs [26]. ECMs were coated on tissue culture plastic and poly-D,L-lactic acid plates, a biodegradable polymer, to improve the differentiation and maintenance of lung epithelium derived from mouse ESCs, which could be enhanced by ECM proteins such as collagen I, laminin 332, and fibronectin [27]. In a recent study, electrospun polymer matrices comprised of poly(lactic-*co*-glycolic acid) (PLGA) nanofibers were studied for feeder-free expansion of hiPSCs and human ESCs into multilayered 3D "patty-like" spheroid structures in defined xeno-free culture medium to maintain their pluripotency [28]. Hou and Huang investigated the role of ECM and cell–ECM interactions for endothelial differentiation of iPSCs by utilizing high-throughput ECM microarrays composed of circular ECM printed areas on glass slides [29, 30]. The studies confirmed the role of ECM proteins through integrin–ECM signaling in regulating the survival, proliferation, and neurite outgrowth of human ESC-derived neural progenitor cells (NPCs) in a dose-dependent manner [30]. Major ECM proteins such as integrin α6β1 and its ligand laminin regulate NPC differentiation compared to other substrates including fibronectin, collagen I, and so on. The decellularization of native

ECM derived from PSC aggregate can be used as a 3D scaffold that contains a balanced composition of biological factors to coordinate cellular events of stem cells. The ECM protein pool of laminin, collagen IV, and heparan sulfate plays a major part in regulating the balance of neuronal and glial cell differentiation; ECM with higher proportions of laminin and heparan sulfate supports neuronal differentiation. Therefore, 3D ECM scaffolds with bioactive molecules have been postulated to simulate the *in vivo* microenvironment for neural differentiation of iPSCs [31]. Hence, there is an urgent need to optimize ECM-based scaffolds for efficient cell differentiation and functional maturation. These 3D ECM scaffolds can interact with other niche factors (e.g., cytokines, accessory cells, and nutrients) and provide the physiologically relevant microenvironment. Various studies emphasize that the emerging paradigm in stem cell engineering involves the properties of the surrounding microenvironment, which can be critical regulators of cellular functions. Therefore, it is important to understand the molecular mechanism of how cells interact with the microenvironment both *in vitro* and *in vivo* for the development of physiologically functional tissue constructs.

4.5 Application of Induced Pluripotent Stem Cells

The potential of iPSCs for the repair and regeneration of the tissue defects has paved the way for tissue engineering. Culturing of iPSCs in 3D scaffolding culture systems is an emerging field of research and is widely used to engineer physiologically functional tissues/organs. The increased attention toward iPSCs can be attributed to their numerous attractive features as discussed in the earlier sections. In the preceding sections, different tissue engineering applications of the iPSCs in relation to biomaterials, their interaction, and their regenerative potential have been discussed.

4.5.1 Bone Tissue Engineering

Being a part of the skeletal system, bones provide shape, mechanical support, and protection to the body and contribute to the mineral homeostasis of the body. Bone is a dynamic tissue with the unique capability of self-regeneration or self-remodeling to a certain extent throughout their life. However, many circumstances call for bone grafting because of bone defects caused by either traumatic or nontraumatic episodes. Although conventional bone grafting techniques (autografting and allografting) are clinically available, they have their own limitations such as patient specificity, immunological rejections, limited supply for the required demand, and chances of pathogen transfer from allografts [32]. Advanced tissue engineering aims to grow patient-specific bone substitutes for reconstructive treatments. Many new types of cells and biomaterials are being continuously explored. Studies have shown that bone-specific cell types can be raised from human ESCs when cultured on osteoconductive scaffolds [33–35]; however, there are ethical concerns related to the use of human ESCs in clinical practice [36]. On the other hand, hiPSC-derived MSCs show

outstanding advantages such as donor specificity, high expansion potential, and strong self-renewal potential [37–39]. In fact, the use of hiPSCs involves no ethical concerns unlike human ESCs. hiPSC-MSCs were reported to show superior hind limb ischemia treatment and promote vascular and muscle regeneration compared to bone-marrow-derived MSCs [37]. The potential of iPSC-MSCs to regenerate bone was evaluated by transplanting iPSC-MSCs into immune-compromised mice suffering with calvaria defects. Results of this study confirmed *de novo* bone formation within the calvaria defects, and the transplanted hiPSC-MSCs were further confirmed to have participated in bone regeneration [39]. Subsequently, De Peppo *et al.* studied the induction of mesenchymal lineages from hiPSCs for engineering large bone grafts with defined geometries with mature, compact, and phenotypically stable bone substitutes by using nonintegrating reprogramming vectors [40]. Three hiPSCs cell lines derived from different sources, namely dermal fibroblasts 11c and 1013A and bone marrow cells (BC1), were induced into mesenchymal lineage using different reprogramming methods such as retroviral vectors for 11c, Sendai virus for 1013A, and episomal vectors for BC1. These mesenchymal lineages were studied for bone tissue formation in an osteoconductive scaffold perfusion bioreactor culture model. hiPSC-derived mesenchymal progenitors were reported to form dense bone-like tissue matrix in perfusion culture on osteoconductive scaffolds [40].

Bioceramics such as hydroxyapatite (HA) and other calcium phosphates (CaPs) comprise an important class of biomaterials for bone applications. CaP-based composite materials are widely used as bone grafting materials because of their bioactive potential (e.g., octeoconductivity and osteointegration). The physicochemical and mechanical properties of a nanocomposite biopolymeric matrix can be directly modulated by manipulating the amount of CaP in it (Figure 4.1). Dispersion of different amounts of (1–8%) calcium-deficient nanohydroxyapatite (d-HAp) to PLLA produced a set of nanofibrous (PLLA/d-Hap) scaffolds with similar architectures and tunable mechanical properties [41]. Electrosun PLLA/d-HAp nanocomposites were found to induce hBM-MSCs and murine pluripotent (iPSCs and ESCs) stem cell differentiation toward the osteogenic lineage without using exogeneous soluble differentiating agents [41]. Interestingly, murine pluripotent and human multipotent stem cells cultured on neat PLLA scaffold under the same culturing condition were reported to lack osteogenic differentiation. Additionally, properties of electrospun PLLA/d-HAp nanocomposites were found to be independent of the stem cell type, which therefore highlights the fact that the direct interaction of stem cell–polymeric nanocomposite matrix and the mechanical properties acquired by the PLLA/d-HAp nanocomposite play key roles in the differentiation process [41].

Another form of CaP-based scaffold is calcium phosphate cements (CPCs), which can be injected and set *in situ* to achieve intimate adaptation to complex-shaped defects. Reports of iPSCs seeded onto CPC scaffolds have supported the attachment, proliferation, and differentiation of iPSCs into osteogenic lineage and supported the formation of bone minerals [42]. In a recent study by Thein Han *et al.*, iPSC-MSCs seeded onto functionalized CPC with biofunctional agents such as fibronectin (Fn), genetically engineered proteins, fibronectin-like

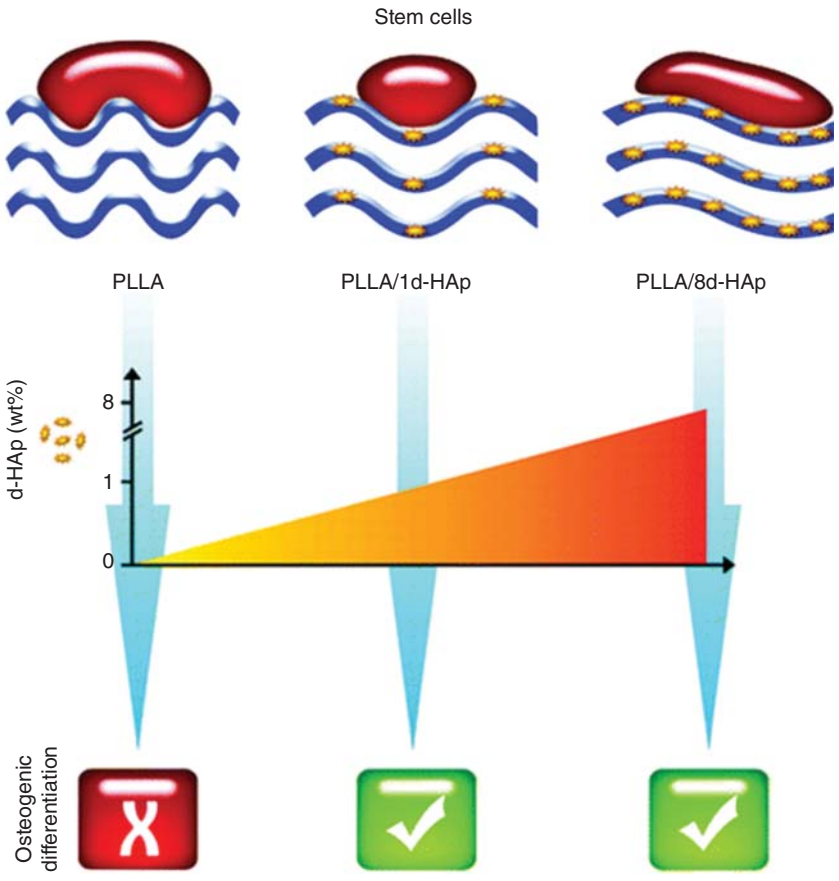

Figure 4.1 Schematic representation of tuning multi/pluri-potent stem cell's fate by electrospun PLLA/d-HAp nanocomposite. (D'Angelo 2012 [41]. Reproduced with permission of the American Chemical Society.)

engineered protein polymer (FEPP), and Arg-Gly-Asp (RGD) were shown to promote cell adhesion. Geltrex is a soluble form of the reduced growth factor basement extract consisting of laminin, collagen IV, entactin, and heparin sulfate proteoglycan. Platelet concentrates also improve cell functions, and were compared with nonfunctionalized CPC. The results obtained from this study confirmed enhanced attachment, proliferation, osteogenic differentiation, and mineral synthesis of iPSC-MSCs seeded onto functionalized CPC compared to the control CPC substrates. Mineral synthesis by iPSC-MSCs seeded on CPC-RGD, CPC-Fn, and CPC-platelets was significantly higher than on other groups ($P < 0.05$). For instance, the mineral synthesis by iPSC-MSCs on CPC platelets was nearly threefold higher than on CPC control [43].

Silk-based scaffolds have also been effectively used for bone tissue engineering using iPSCs as seeding cells. iPSCs could restore the form and function of the lost cementum, periodontal ligaments (PDLs), and alveolar bone for periodontal

regeneration [44]. The enamel matrix derivative (EMD) Emdogain® gel is an osteoconductive agent composed of proteins originating from the amelogenin family. Several reports have confirmed that EMD could increase the alkaline phosphatase (ALP) activity and matrix mineralization in human PDL cells, osteoblasts, and rodent BMSCs [45]. Transplantation of iPSCs with EMD has been reported to promote cementum regeneration [40]. In a study by Duan *et al.*, highly porous apatite-coated silk fibroin 3D scaffolds were seeded with iPSCs and EMD. The CaP coatings on the mineralized silk scaffolds were reported to reduce the fibrous encapsulation layer, enhance direct bone contact, and stimulate differentiation of stem cells toward osteogenic lineage. This kind of engineered scaffold supported iPSCs' differentiation and the formation of new alveolar bone and cementum formation with regenerated PDL in between them [46]. Thus, the results of this study confirm the potential of iPSCs combined with EMD as a valuable tool for periodontal tissue engineering.

There is continuous focus on developing biomaterials that could effectively trigger the differentiation of iPSCs into osteoblasts for safe bone regeneration. Recently, Wang *et al.* studied a bacteriophage producing a unique nanofiber matrix that could provide specific signal peptides with nano-topographical cues to stimulate native microenvironment and also to independently vary the biochemical and biophysical cues in a system [47]. The nanofiber matrix was generated from an engineered M13 phage with an ordered surface nanotopography through layer-by-layer self-assembly. Alternative immersions of the glass substrate into the cationic poly-L-lysine solution and anionic phage nanofiber solution were carried out to establish the electrostatic self-assembly of the phage matrix (Figure 4.2). The results supported the differentiation of cultured iPSCs into mesenchymal progenitor cells (MPCs) followed by osteoblasts without any osteogenic supplements. This observation was mainly attributed to the elongation induced by phage nanofibers in the matrix to support iPSC differentiation. Cell elongation and alignment were independent of the peptide

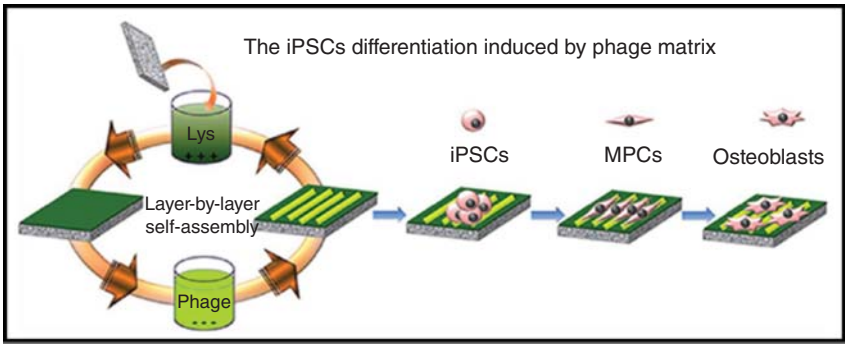

Figure 4.2 Schematic diagram showing the fabrication scheme of phage-assembled matrix and the interaction between the phage matrix and iPSCs. (Wang 2014 [47]. Reproduced with permission of the American Chemical Society.)

sequences displayed on the constituent phage nanofibers and thus were solely dependent on the ordered surface topography [47].

Besides cell-based strategy, there are promising approaches for bone regeneration with bioengineered technologies. Bone morphogenic protein 2 (BMP2) is one of the most effective osteogenic growth factors that induces osteogenic differentiation [48]. Direct delivery of BMP2 *in vivo* for bone defect repair requires a large dose of BMP2, and local delivery of BMP2 may suffer because of the instability and short half-life time, thus increasing the cost. An effective method for BMP2 delivery is gene therapy to overexpress BMP2 in certain cells [49]. Liu *et al.* demonstrated the BMP2 gene transduction of hiPSC-MSCs using lentiviral vectors to enhance the osteogenic differentiation and bone mineral production on a chitosan–CPC scaffold immobilized with RGD for bone tissue engineering [50]. BMP2 gene-modified iPSC-MSCs were seeded on chitosan–CPC scaffold immobilized with RGD for bone tissue regeneration. Results confirmed enhanced osteogenic differentiation of iPSC-MSCs with no significant adverse effects on cell growth and attachment to CPC scaffold [50].

BMP2-induced transduction of potent transcription factors such as the nuclear matrix protein SATB2 in iPSCs enhances the osteogenic differentiation of iPSCs. In SATB2 overexpression, iPSCs shows increased mineral nodule formation and elevated expression levels of the osteogen-specific genes including osteocalcin. The SATB2-overexpressing iPSCs cultured onto silk-based scaffolds were transplanted into critical-sized calvarial bone defects in nude mice. In a follow-up study after 5 weeks, the results confirmed enhanced new bone formation and mineralization in the SATB2-treated mice group [51]. These advanced modalities, which integrate iPSCs and tissue engineering strategies, will enhance the efficacy of bone regenerative medicine.

4.5.2 Cartilage Tissue Engineering

Cartilage tissue engineering is a promising technique for the repair and regeneration of human cartilage that has been damaged or injured by a trauma or disease such as osteoarthritis (OA) or rheumatoid arthritis [52]. Articular cartilage is a white, smooth, and highly organized tissue that covers the ends of the bone and comes together to form joints and permits them to glide smoothly over each other. The structure contains around 75–80% water content with a dense ECM having lightly distributed chondrocytes in it. Collagen fibers (type II collagen, about 15%) are the main components of the ECM along with proteoglycans (about 12%) [53, 54]. Unfortunately, articular tissues lack the self-renewal ability due to their low cell to matrix ratio, which in turn limits the natural regenerative potential of the tissues [55]. There are numerous surgical methods that have been developed for the restoration of the cartilage, such as drug therapy, micro-fracture, drilling, abrasion arthroplasty, autologous chondrocyte implantation, and osteochondral autograft/allograft transplantation. Despite of their wide acceptance, these conventional approaches are also associated with some limitations such as their high cost, inability to regenerate cartilage with mechanical properties matching with those of the native tissue, or sometimes providing only symptomatic relief to the patient. To overcome these limitations,

scaffold-based tissue engineering has been used to develop artificial tissue scaffolds with cells cultured in a 3D microenvironment to perfectly mimic the native tissue properties [56]. Tissue engineering procedures demand a large number of cells, which often hinders its application. For instance, it has been reported that repeated passaging can induce dedifferentiation among a chondrocyte population. To overcome these problems, iPSCs have been used, which have proved to be an ideal source for chondrocyte differentiation [57]. Diekman *et al.* have demonstrated that murine iPSCs can be induced into chondrocytes with proper differentiation and purification procedures, leading to the regeneration of the cartilage tissue in the mice suffering from OA [58]. To examine the ability of iPSC-derived cells to cure cartilage defects, an *in vitro* cartilage defect model was created with cells embedded in agarose scaffold, thereby supporting the application of iPSCs as a cell source in cartilage tissue engineering [8]. In another interesting study, Imaizumi *et al.* demonstrated the regeneration of tracheal cartilage by utilizing iPSCs [59]. iPSCs cultured in a 3D scaffold made up of collagen type I were analyzed for *in vitro* chondrogenesis and then examined for *in vivo* transplantation into nude rats suffering from tracheal defects. In 6 out of 11 implanted rats, cartilage tissue regeneration in the tracheal wall was observed, whereas no changes were observed in the control or undifferentiated iPSCs (Figure 4.3). Additionally, polymerase chain reaction

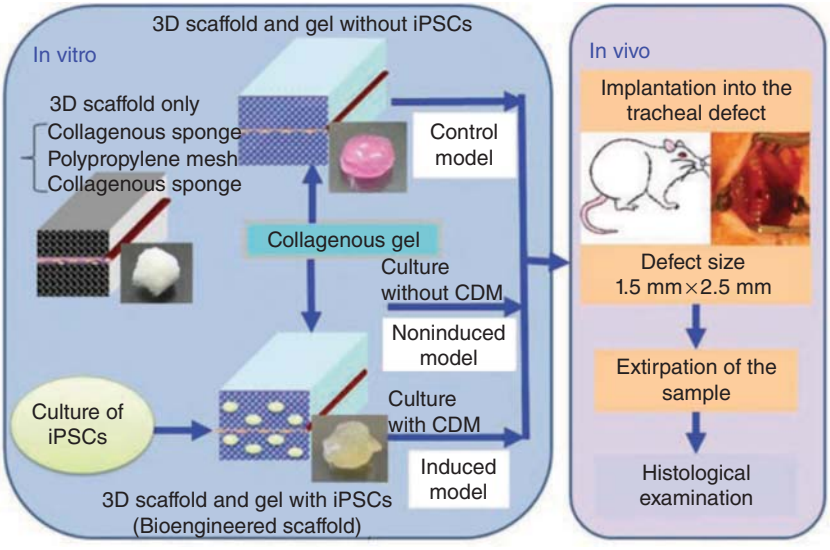

Figure 4.3 Fabrication methods of bioengineered scaffolds and their implantation technique into rats with tracheal defects. Induced pluripotent stem cells (iPSCs) were cultured in the 3D scaffold containing collagen gel with DMEM and chondrocyte differentiation medium (CDM; bioengineered scaffold model). The three-dimensional scaffold consists of collagenous sponge and polypropylene mesh. Control model: 3D scaffold without iPSCs. Noninduced model (DMEM culture): 3D scaffold with noninduced iPSCs. Induced model (CDM culture): 3D scaffold with induced iPSCs. Noninduced and induced scaffold: bioengineered 3D scaffold. (Reprinted with permission from Imaizumi *et al.* [59].)

(PCR) and laser micro-dissection (LMD) techniques confirmed the presence of iPSC-derived GFP (green fluorescent protein) gene, thereby confirming the regenerative potential of iPSCs for tracheal cartilage [59]. Furthermore, Liu *et al.* studied the chondrogenic potential of iPSCs cultured in PCL/gelatin nanofibers in order to restore cartilage defects. Here, PCL provided the mechanical strength to the scaffold. It was observed that even after 2 months no PCL fiber hydrolysis was noticed, and even though there was 40% decrease in the tensile strength of the degraded fibers, the Young's modulus remained unchanged. *In vivo* studies showed higher collagen II, aggrecan, and SOX9 levels specific to the cartilage along with sub-chondral bone regeneration. Therefore, because of the nanofiber morphology and hydrophilicity, the cultured iPSCs showed increased chondrogenesis [20]. Overall, it is evident that the biomaterials, in the form of scaffold, in combination with iPSCs, have a tremendous potential for inducing regeneration of cartilage within the damaged tissues. Therefore, iPSCs hold great promise for cartilage regeneration.

4.5.3 Cardiac Tissue Engineering

Cardiovascular diseases are among the leading causes of mortality and morbidity worldwide. Stem cells play a key role in engineering cardiac tissue. In the recent days, iPSCs have emerged as a vital cell source for various cardiovascular tissue engineering methods owing to their unlimited regenerative capacity [60]. The assembly of a large number of cells including CMs and other cell types (e.g., vascular cells, cardiac fibroblasts) is required to fully compensate a damaged human heart [61]. Continuous interaction of CMs with their surrounding ECM highlights their dynamic behavior [62]. ECM provides vital signals to the CMs and permits anchorage and transfer of the contractile forces to the matrix to facilitate the muscular pump function of the heart [63–65].

The differentiation potential of iPSCs into CMs in an *in vitro* culture model was demonstrated by Mauritz *et al.* in 2008 using mouse iPSCs lines [63]. The iPSC-derived CMs were reported to show typical features of ESC-derived CMs, including spontaneous, rhythmic beating, expression of marker genes, expression of proteins typical of CMs, spontaneous rhythmic intracellular Ca^{2+} fluctuations, and the presence of the β-adrenergic and muscarinic signaling cascade. As a further step forward, Zhang *et al.* reported the cardiac differentiation of hiPSCs, showing that both hiPSCs and human-derived embryonic stem cells (hESCs) have a similar capacity for differentiation into nodal-, atrial-, and ventricular-like phenotypes [66]. Various studies have proven that under suitable conditions iPSCs can undergo long-term propagation in the undifferentiated state or can differentiate into many other cell types, including functional CMs [63, 66, 67].

iPSC-derived CMs mark an important achievement in the establishment of protocols for cardiac cell therapy. Many countries have prioritized stem-cell-based therapy for replacing lost myocardium. Stem cell transplantation is considered an effective treatment method for severe pathologic conditions. However, stem cell transplantation shows insufficient results because of poor survival and long-term engraftment of the transplanted cells [64]. Animal studies

of stem cell transplantation have revealed that stem cells could treat the damaged heart through paracrine mechanisms but not through direct differentiation [68]. Though stem cell transplantation shows initial positive results on myocardial function and perfusion, the disappearance of transplanted cells reduces the likelihood of sustained positive paracrine effects or sustained recovery of the function [68]. It is reported that more than 70% of the cells die during the first 48 h after needle injection due to the hypoxic, inflammatory, and/or fibrotic environment [69]. Hence, future research should focus on bioengineering strategies for improving the efficiency of stem-cell-based therapies for cardiac treatment [70]. The well-established strategy to improve the survival rate of the cells in a graft is by combining naturally derived biomaterials with stem cells to provide a favorable cellular microenvironment. As an effort to regenerate cardiac tissue with biomaterials, iPSCs were combined with biomaterials such as collagen I, fibrin, gelatin, or Matrigel [71]. These biomaterials provided favorable microenvironment and showed increased survival of the grafted cells as well as an improvement of the cardiac function. For instance, fibrin gels were reported to support the dedifferentiation and cardiac differentiation phases of the indirect reprogramming process [72]. In addition to natural-polymer-based biomaterials, decellularized scaffolds have also proven their potential for cardiac regeneration. For example, repopulation of a decellularized mouse whole heart with hiPSC-derived cardiovascular progenitor cells has shown promising results (Figure 4.4) [73]. Cell sheets derived from hiPSC-derived CMs have also been successfully used for CM transplantation. A combination of a cell sheet with an omentum flap, which is known to have a rich vasculature, resulted in increased survival rate of the transplanted hiPSC-derived CMs [74].

One the major challenges linked with the culture of iPSC-derived CMs on traditional cell culture polymer surfaces with negligible elasticity is their unsuitability for extended cultures. Embryonic CMs cultured on a series of flexible collagen-I coated polyacrylamide gel substrates showed that matrices that could mimic the elasticity of the myocardial microenvironment are the optimal systems for transmitting contractile forces to the matrix and promoting actomyosin striation along with beating at 1 Hz. On the contrary, hard matrices could cause overstrain for the cells and less striated myofibrils, and resulted in no beating [75]. Therefore, it emphasizes the fact that a matrix with heart-like elasticity is suitable for CM culture and regeneration. Polyacrylamide hydrogels with a matrix stiffness that matches the stiffness of the native cardiac tissue allow prolonged culture of functional iPSC-derived CMs [76]. Cell adhesion to the hydrogel matrix has been reported to be improved by using suitable cross-linkers such as 2,5-dioxopyrrolidin-1-yl-6-acrylamidohexanoate, which support the immobilization of the matrix protein (fibronectin) efficiently and facilitate long-term culture of iPSC-derived CMs on polyacrylamide hydrogels [76].

Furthermore, matrix properties and tractional forces have been reported to influence indirect cardiac reprogramming, which could further regulate the dedifferentiation and differentiation stages [72]. Other important determinants for cardiac reprogramming include cell proliferation, matrix mechanics, and matrix microstructure. Therefore, modulation of the extracellular microenvironment is necessary to enhance cardiac reprogramming [72]. Evidently, Ca^{2+}

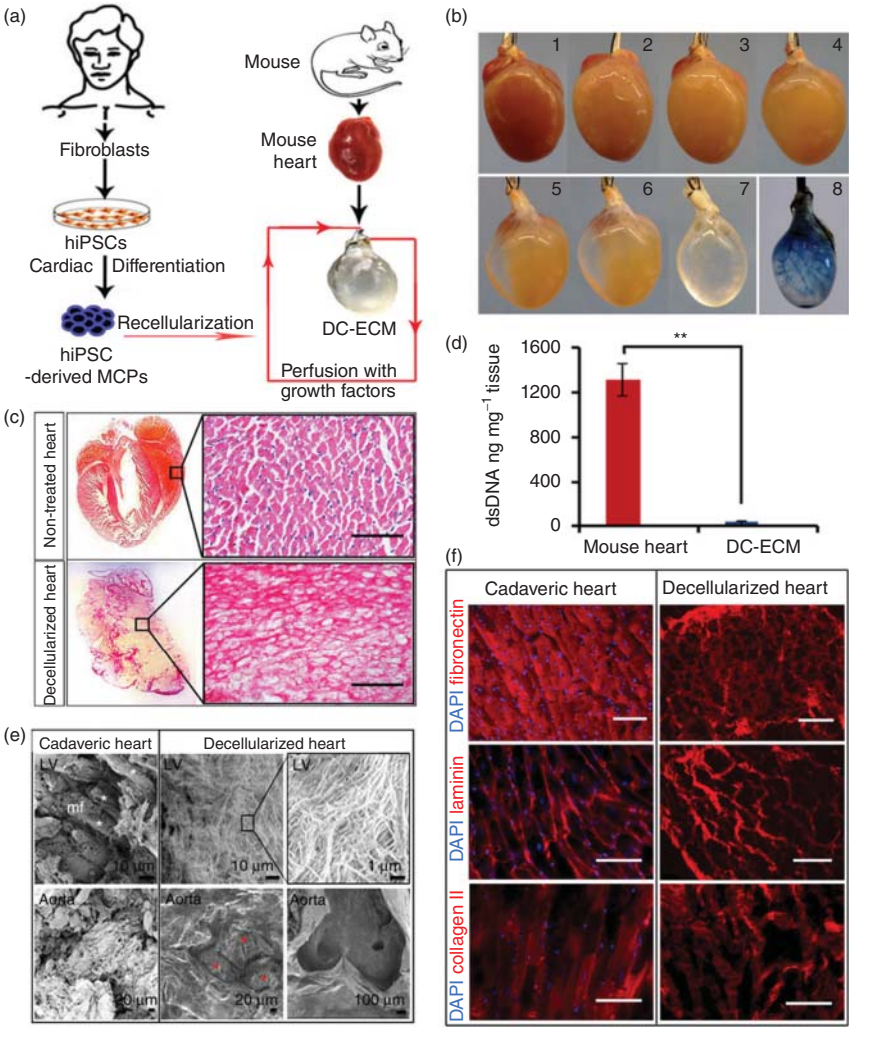

Figure 4.4 Decellularized mouse heart scaffolds repopulated with hiPSC-derived cardiomyocytes showing the growing potential for cardiac tissue engineering. (a) A scheme for the whole study. (b) Photographs showing each step of decellularization process: before decellularization (1); after deionized water perfusion (2); after PBS perfusion (3); after enzymatic perfusion (4); after 1% SDS solution perfusion (5); after 3% Triton X-100 solution perfusion (6); after acidic perfusion (7); perfusion of DC-ECMs with trypan blue solution to visualize the intact coronary vasculature (8). (c) H&E staining of sections from the cadaveric mouse heart (top) and DC-ECM (bottom). Scale bars, 10 µm. (d) DNA content quantification of cadaveric mouse hearts and DC-ECMs. Error bars show s.e.m of three independent experiments. **$P < 0.005$ ($n = 3$, unpaired Student's t-test). (e) SEM of cadaveric and decellularized hearts. Left ventricular (LV; top panel) and aorta (bottom panel). Myofibers (mf) were present in the cadaveric heart (white stars) but not in the DC-ECMs. Red stars indicate the aortic valve leaflets. (f) Immunostaining of cadaveric mouse hearts and DC-ECMs. Fibronectin (upper), Laminin (middle), and Collagen II (lower). No nuclear staining (DAPI) was observed in DC-ECMs. Scale bars, 50 µm. (Lu 2013 [73]. Reproduced with permission of Nature Publishing Group.)

cycling plays a critical role in the cellular phenotype of both inherited and acquired cardiomyopathies. Structured tissue culture substrates were proved to induce the regular alignment and anisotropy in the cells, improved Ca^{2+} cycling properties, and sarcomeric organization of neonatal rat ventricular myocytes [77–79]. In order to overcome this issue, several approaches have been employed, such as coating the substrate with micropatterned ECM components (such as fibronectin), micro-grooved load and nano-grooved culture substrates, and more sophisticated 3D constructs. The lack of mature Ca^{2+} cycling properties in iPSC-derived CMs appears to be an important obstacle in CM regeneration. Therefore, to improve Ca^{2+} cycling of iPSC-derived CMs, polydimethylsiloxane (PDMS) micro-grooved substrates coated with fibronectin

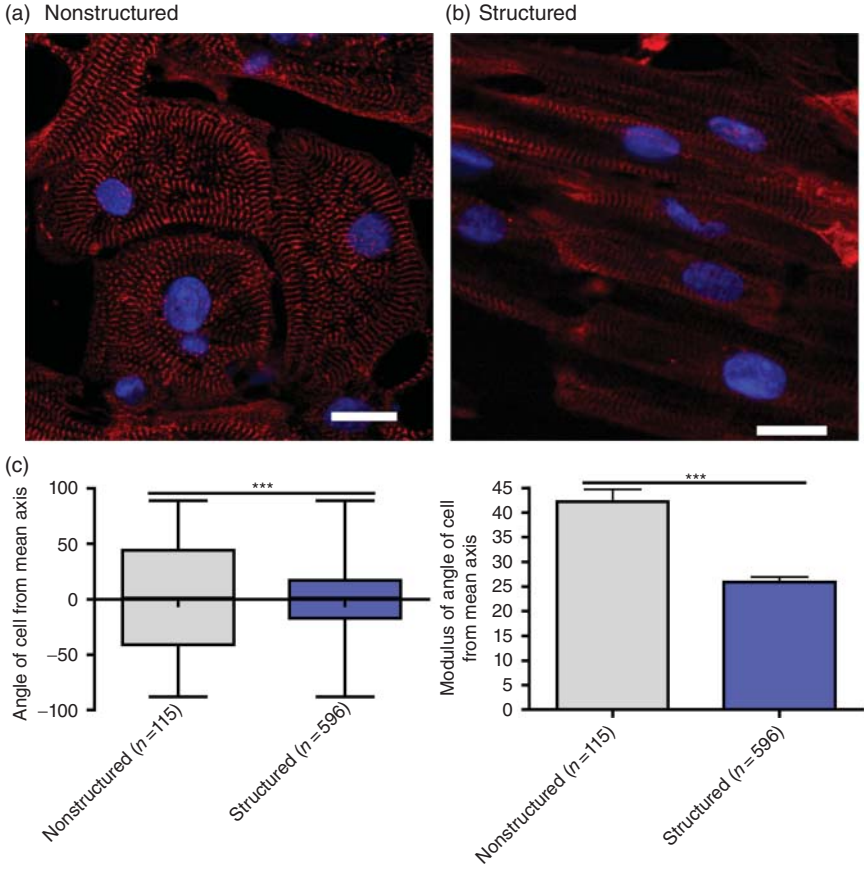

Figure 4.5 Representative immunofluorescence of iPSC-derived CMs cultured on unstructured PDMS (a) and micro-grooved PDMS (b); red – sarcomeric α-actin, blue – DAPI; scale bar 20 μm. Quantification of cell alignment of iPSC-derived CMs on structured and unstructured constructs (c). Microgrooved PDMS substrates significantly improved iPSC-CM alignment compared to the unstructured substrates (SD of Unstructured 50.11° n = 115, Structured 35.60° n = 596; F = 1.982, $p < 0.0001$). (Rao 2013 [80]. Reproduced with permission of Elsevier.)

were assessed using a photolithographic technique (Figure 4.5). The results of this study confirmed cellular alignment and a more organized sarcomere with PDMS micro-grooved culture substrates than with control [80]. The Ca^{2+} cycling properties of iPSC-derived CMs cultured on patterned PDMS substrates were significantly altered with a shorter time to peak amplitude ($p = 0.0002$ at 1 Hz) and a more organized sarcoplasmic reticulum. Ca^{2+} release was observed in response to caffeine ($p < 0.0001$) [80].

Natural biomaterial-based scaffolds, such as micromolded gelatin hydrogel, have also reported to tune the elastic modulus and extend the lifetime of the engineered cardiac scaffolds by matching the chemical and mechanical microenvironment with native cardiac tissues. hiPSC-derived CMs cultured on micromolded gelatin surfaces triggered the formation of physiologically functional tissue formation [81]. Contractile force, velocity, and power produced by hiPSC-derived CMs were assessed through seeding on elastomeric micropost arrays. Contractile function of the hiPSC-derived CMs could be traced by observing the deflection of a micropost beneath an individual hiPSC-derived CM using optical microscopy. In a comparative study, seeding of the hiPSC-derived CMs on laminin-coated elastomeric micropost demonstrated higher attachment, spread area, and contractile velocity than those seeded on microposts coated with fibronectin or collagen IV [82]. Under optimized conditions, hiPSC-derived CMs on laminin-coated elastomeric micropost were reported to spread up to an area of approximately 420 μm^2, with systolic forces of approximately 15 nN per cell, contraction and relaxation rates of 1.74 and 1.46 $\mu m\, s^{-1}$, respectively, and peak contraction power of 29 fW [83]. These studies discussed above, and other reported data, prove that iPSCs with engineered grafts could be a promising strategy for cardiac tissue regeneration and restoration of cardiac function.

4.5.4 Skin Tissue Engineering

Skin tissue engineering has progressed over recent years, but still many factors inhibit its development, most specifically the choice of seed cells. Theoretically, epidermal cells have been proven to be an ideal choice for skin tissue engineering, but the lack of surface antigens is the major drawback. An ideal seed cell should be pluripotent, highly proliferative, readily attainable, and easily expandable. iPSCs fulfills most of the ideal characteristics of a seed cell: for instance, it can differentiate into skin cells by reprogramming keratinocytes from hair follicle [84]. Bilousova *et al.* induced iPSCs *in vitro* to differentiate into a skin-like cell line and form multi-differentiated epidermis, hair follicles, and sebaceous glands [85]. Recently Yang *et al.* demonstrated the differentiation of hiPSCs into epithelial stem cells by controlling the actions of the epidermal growth factor, BMP signaling, and retinoic acid which is used for the reconstitution of epithelial cells of hair follicle and inter follicular epidermis [86]. Epidermal melanocytes play an essential role in protecting the skin from the harmful UV radiation and are also useful in treating a variety of skin diseases; hence methods of differentiating iPSCs into melanocytes have been developed using Wnt3a, stem cell factor (SCF), and endothelin-3 (ET-3) as culture supplements [87]. Furthermore,

Kawasaki *et al.* reported that neural differentiation of mouse ESC occurs via stromal cell-derived inducing activity (SDIA) culture method using PA6 feeder cells and that supplementation of BMP in SDIA culture promotes epidermal differentiation while suppressing neural differentiation [88]. In support of the previous studies, Yoshida *et al.* demonstrated the application BMP in the SDIA method for culturing fibroblast-derived mouse iPSCs and observed its differentiation into stratified epithelial cells [89]. The group further optimized the timing of adding BMP to produce a pure population of epithelial cells [89]. Recently, Kim *et al.* co-administered hiPSC-derived epithelial stem cells and SMCs for neovascularization and tissue repair in a murine dermal wound [90]. Such studies suggest the potential for iPSCs to generate functional, patient-specific skin equivalents that may be used in the treatment of both skin disorders and wound healing.

4.5.5 Neural Tissue Engineering

The nervous system plays a vital role in the human body. Regeneration of a nerve is an extremely complicated process: for example, small nerve injuries can exhibit regenerative potential, but for the larger nerve injuries surgical treatments are required. Neural tissue engineering is an encouraging technique for the regeneration of damaged nerves [91]. Studies have shown that ESCs and iPSCs can be used for neural regeneration for a range of diseases and disorders such as Parkinson's, Alzheimer's, Huntington's, spinal cord injury, and traumatic brain injury [92]. Recently, Wang *et al.* reported that iPSC-derived multipotent neural crest stem cells (NCSCs) seeded in nanofibrous tubular scaffold have the ability to differentiate into Schwann cells that can support the myelination of axons, thus encouraging nerve regeneration (Figure 4.6) [93]. PCL, poly(propylene glycol), and sodium acetate nanofibers were aligned longitudinally to impart mechanical strength, and the random fibers were placed on the longitudinal layer of the nanofibers to further improve the mechanical strength. Also, prior to the cell loading into the nanofibers, it was mixed with Matrigel and incubated in the NCSC maintenance medium. Interestingly, in a 1-year follow-up study of *in vivo* implantation of NCSC-seeded scaffolds, no teratoma formation was revealed. The formation of fully controlled microenvironment by the fabricated nerve conduit for the differentiation of iPSC-derived NCSC was observed, thus emphasizing on the potential of iPSCs as a cell source for nerve regeneration [93].

Scaffold properties can also modulate the behavior of seeded iPSCs [94], thereby emphasizing the manipulation of scaffold properties compatible with the seeded cells. For instance, Khayyatan *et al.* reported the impact of laminin-coated collagen scaffolds on the hiPSC-derived neural progenitor's behavior through integrins, which aids in cell–ECM adhesion and signaling [94]. The mechanical property of the collagen scaffold is dependent both on the polymer concentration and on the cross-linking. The maximum strength of the scaffold was found to be 1.8 MPa with 1% collagen and glutaraldehyde cross-linked. The laminin-coated scaffolds significantly increased the proliferation and infiltration of the seeded cells, and hence these scaffolds proved to be a potential contender for nerve tissue engineering [94]. Kuo and Chen aimed to use an inverted colloidal crystal (ICC) scaffold made up of alginate,

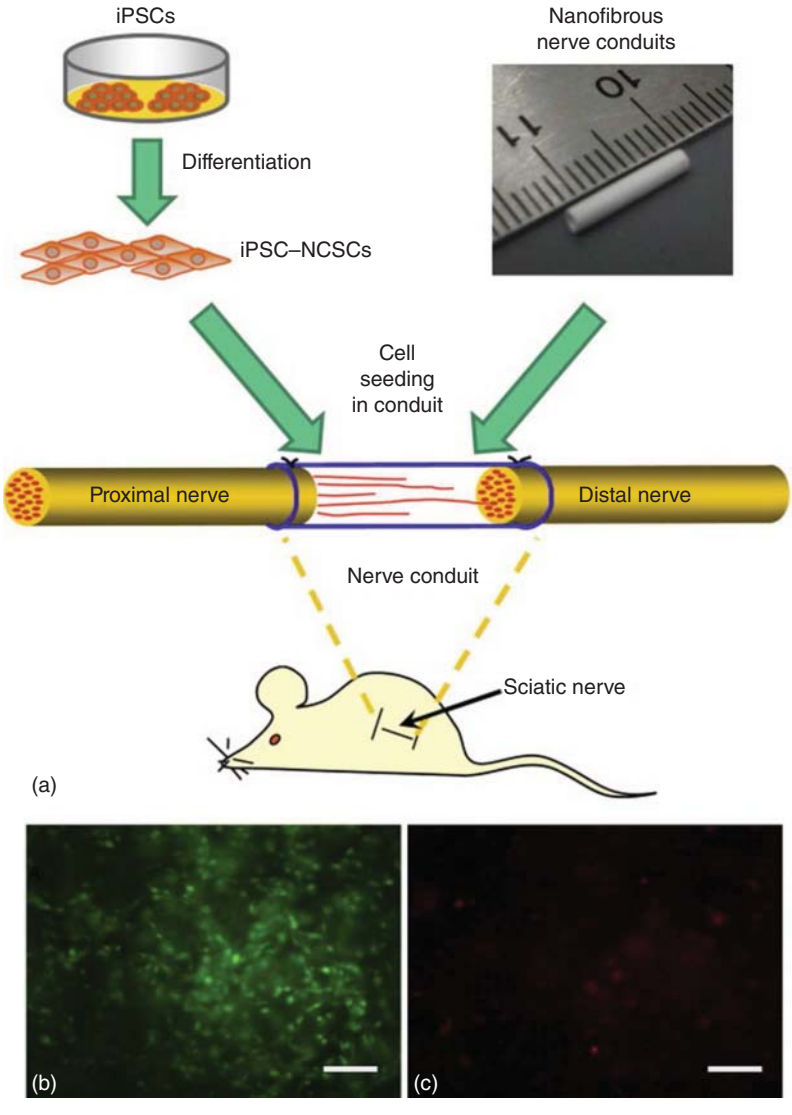

Figure 4.6 Tissue-engineered nanofibrous nerve conduits for peripheral nerve regeneration. (a) Schematic outline of the tissue engineering approach by combining NCSCs and a nanofibrous nerve conduit. (b,c) The NCSCs were mixed with Matrigel, injected in the nerve conduits, and cultured for 1 day *in vitro*. Scale bars are 100 μm. (Wang 2011 [93]. Reproduced with permission of Elsevier.)

poly(γ-glutamic acid), and a surface peptide (CSRARKQAASIKVAVSADR) for the differentiation of iPSCs into neuronal lineage [95]. They found that the use of a surface peptide in the ICC construct improved the differentiation of iPSCs into neuronal cell lineage, leading to better nerve tissue engineering [95]. Additionally, surface modifications of the scaffolds were evaluated to enhance iPSCs' differentiation potential. For instance, Kuo and Wan– demonstrated

the differentiation of iPSCs into neuronal lineage in heparinized PCL-PHB (poly(β-hydroxybutyrate)) scaffolds and found that the surface modification with heparin could direct the differentiation of iPSCs into neural cells [96]. All these studies together prove that the biomaterials loaded with iPSCs have great potential in the regeneration of nerve cells for the treatment of neuronal diseases.

4.6 Concluding Remarks

iPSCs are considered one of the prime cell sources for tissue engineering. Extensive research is being carried out in the field of iPSCs in order to explore and widen their applications in engineering physiologically functional tissues and organs, as seen from open-source publications. Right from the development of new mechanisms for cellular reprogramming that can activate a few transcription factors to change cellular fate and function to the development of interconverting procedures for mature cell types directly into each other such as bone, cartilage, cardiac, skin, and neural cell types is being studied. Cell–matrix interaction is one of the key factors for the success of engineering physiologically functional tissues and organs. Despite the numerous methods and technological advances in the cellular reprogramming of hiPSCs and their therapeutic applications, relatively little is known about their interaction with biomaterials, which is very essential to develop engineered tissues and organs. However, on the basis of the preliminary experimental data as discussed in this chapter and other reported studies, iPSCs and their derivatives have proven to be valuable cell sources for scaffold-based tissue engineering and regenerative medicine applications owing to their multi-lineage potential. Keeping these points in mind, future research may be focused to aim for converging biomaterial/scaffold engineering with stem cells technology in order to mimic the physiological complexity of the stem cell niche and ultimately provide the multitude of required cell types for clinical therapies in humans.

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5

Biosensors for Optimal Tissue Engineering: Recent Developments and Shaping the Future

Jihane Abouzeid, Ghinwa Darwish, and Pierre Karam

5.1 Introduction

In this chapter, we first explore the different biosensing techniques that have been applied or have shown great potential to be introduced to tissue engineering. We will then highlight the recent advances in sensing specific biomolecules related to tissue engineering for *in vitro* and *in vivo* systems. We will explore the challenges and hurdles that the field faces. We will also focus on implantable glucose sensors, as they constitute the most advanced example of biosensors interfaced *in vivo* measurements. We believe that the lessons learned from this example could be applied to developing future off-the-shelf implantable and regenerated tissues.

5.2 Fundamentals of Biosensors

The initial concept of the biosensor as it is known today dates back to the Clark microelectrode in 1956 [1]. It consisted of a thin layer of glucose oxidase (GOx) as part of a semipermeable dialysis membrane and functioned by measuring the amount of oxygen consumed in the glucose oxidation reaction [2]. Since then, the concept has developed to encompass a whole range of devices and techniques for sensing a variety of biological states and molecules [3].

New biosensing schemes and platforms are beginning to transform the ever-evolving frontier of preventive medicine, allowing the detection of multiple biomarkers to take place even on small platforms [4]. The definition of a biosensor can be just as varied as the analyte of interest. Biosensors can be broadly defined as systems that offer possibilities to transduce and amplify molecular interactions/sensing into detectable electrical, mechanical, or optical signals, which can be measured by readily available equipment (Figure 5.1) [5]. They typically consist of two main components: a bioreceptor and a transducer. The bioreceptor is a specific macromolecule that recognizes analytes of interest and is what distinguishes the biosensor from a typical chemical sensor; it could be an antibody, an enzyme, or a nucleic acid, for example, [6]. The transducer translates the analyte–bioreceptor interaction into a detectable signal.

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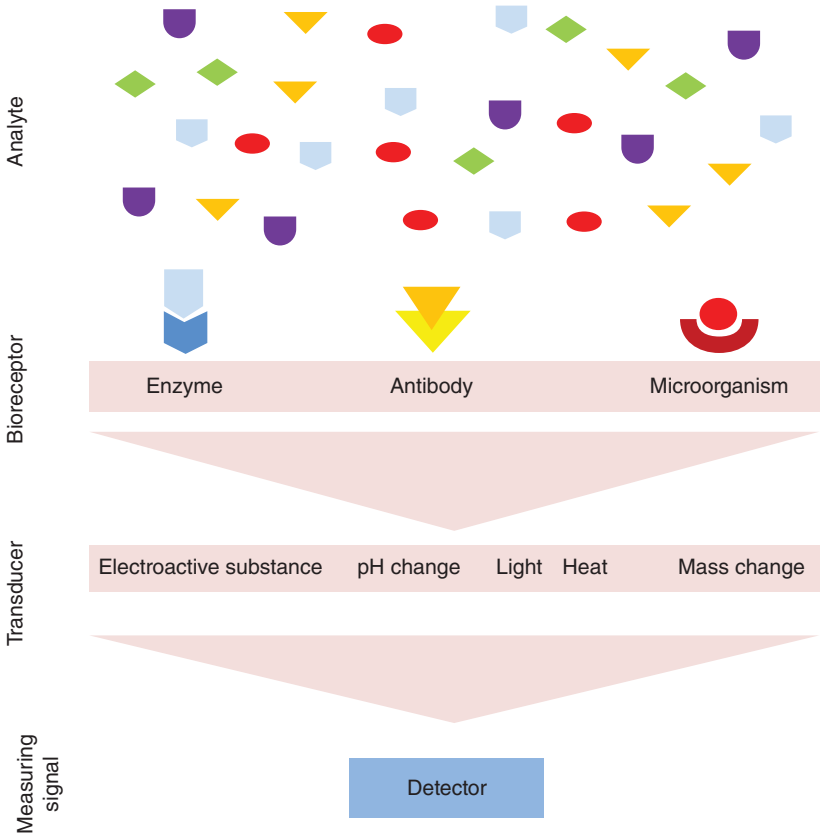

Figure 5.1 General scheme of a biosensor.

Biosensors have shaped the field of medicine by providing monitoring power at the diagnostic, treatment, and post-treatment stages [7]. More recently, biosensors have been rapidly expanding and impacting the field of tissue engineering by overcoming long-lasting hurdles via recent advances in the fields of nanotechnology and biomaterial science [8].

However, to provide an off-the-shelf engineered tissue, countless numbers of challenges still need to be overcome. All conditions must be optimized to create a healthy mass of cells expanding within an optimized scaffold in an environment that mimics the body. Cell fate is dictated by many elements and interactions that need to be strictly observed to allow efficient reconstruction of functional tissues [9]. Biosensors play a central role in this task.

One of these key factors is oxygen [10]. Blood vessel formation is a slow process at the beginning; a healthy tissue requires a well-vascularized mass where oxygen is efficiently distributed. As such, monitoring oxygen is considered an essential and vital component in tissue generation.

Other key factors are growth factors and cellular signaling; these instruct precise cellular responses in biological environments and may potentially allow control over tissue engineering, including stimulation or inhibition of cell growth,

survival, migration, and differentiation [11]. This in turn can dictate the final form of the tissue. Growth factors are often incorporated into the scaffolds to be slowly released, inducing tissue regeneration, and control over their concentrations is critical owing to their role in orchestrating tissue regeneration, specifically in inducing and controlling specific cellular functions [12]. Many efforts have been geared toward developing specific sensing platforms to continuously monitor their concentrations.

Information on how cells adhere to scaffolds or migrate is a critical step toward a successfully regenerated tissue [13]. It is only recently that we have begun to be able to visualize and understand this phenomenon at the cellular level, using sensitive probes that can decipher the interacting mechanisms and pressure distributions across the cell surface [14].

During tissue development, cells also undergo tremendous stress. This stress results in increased concentrations of reactive oxygen species (ROS), and other reactive species, which manifest in significant damage to cell structures and mediates apoptosis, leading to failure in tissue regeneration [15].

Currently, the majority of the methods for assessing the development of the cell numbers, growth, metabolism, and viability throughout the scaffold of regenerated organs require the destruction of the construct by means of biochemical or histological methods [9, 10b, 16]. Moreover, these methods are labor intensive. Many of the currently applied experiments are long, taking weeks or even months, to complete. Fast, simple, and readily applicable nondestructive methods for monitoring changes in tissue deposition and the aforementioned factors within the construct would be invaluable to the field of tissue engineering; such methods could detect adverse responses during the early stages of culture; thus, tissue failure or success depends heavily on advancing these methods.

Optimized conditions in tissue engineering differ between cell types and the end objective [17]. As such, there is a pressing need to develop biosensors that cater to the specific needs of the tissue engineering. Unlike other biosystems, biosensors applied to tissue regeneration need to fulfill the following criteria: they must (i) be biocompatible, thus inducing little to no foreign body reaction; (ii) be noninvasive while being able to probe the entire three-dimensional scaffold for specific markers; and (iii) provide continuous monitoring over long period (days to weeks).

As such, when designing a probe for tissue engineering, one is faced with stringent conditions, and only a handful of techniques can therefore be applied.

5.3 Biosensing Techniques

5.3.1 Spectroscopic Tools

5.3.1.1 Colorimetry Using Gold Nanoparticles

Colorimetric methods can be noninvasive and are naturally quite attractive as detection tools since the results can be easily detected by the naked eye. Gold nanoparticles exhibit special optical and electronic properties manifested by a pronounced absorbance band in the visible window [18]. This localized surface

plasmon resonance peak is sensitive to local changes in the refractive index and, most importantly, to interparticle distance changes, which typically results in a red shift in the maximum absorbance [19]. The useful properties displayed by gold nanoparticles coupled with the ease of colorimetric detection have led to various sensing applications, such as DNA and antigen sensing. [20] For example, detection of the prostate-specific antigen (PSA) has been reported at concentrations as low as 30 aM using gold nanoparticles [21]. DNA-functionalized gold nanoparticles have also been used for colorimetric detection of mercury ions (Hg^{2+}) and other heavy metals [22]. However, due to the limited transparency of most tissues, colorimetric techniques are limited to surface and thin tissue measurements, causing research thus far to be unable to fully implement colorimetry as a working technique for sensing applications in tissue engineering.

5.3.1.2 Fluorescence Spectroscopy

Fluorescence spectroscopy provides an easy tool to detect molecules with high sensitivity and low detection limit (down to a single molecule) [23]. Most importantly, it is noninvasive. By using recent fluorescent imaging tools (STORM, PALM, STED, etc.), we can reconstruct three-dimensional tissues with an impressive spatial resolution of a few nanometers [24, 25]. When a molecule is excited, the relaxation pathway greatly depends on its environment. A fluorescent molecule bound to an analyte will be either quenched or enhanced, as compared to its free form, or it can transfer its energy to an acceptor molecule.

One of the early seminal works of fluorescence techniques as applied to biochemical applications is the use of specific indicators to detect the physiological role of cytoplasmic Ca^{2+} and to quantify its concentration. Ca^{2+} ions are important intracellular messengers for controlling numerous biological processes. With the development of the Ca^{2+} specific probe, Grynkiewicz *et al*. have made it possible to study its role as an intracellular messenger in single cells, adherent cells layers, or bulk tissues [26]. Their system consisted of a highly fluorescent indicator, which upon binding with Ca^{2+} produced major changes in fluorescence wavelength and intensity in addition to high selectivity. Other specific biomarkers could be monitored similarly in real time during the development of tissues [27].

Recent progress in the development of sensitive fluorescent probes has offered an opportunity to detect, image, and therefore study the role of reactive oxygen and sulfur species in cellular signaling and regulation, which will be discussed later.

5.3.2 Electrochemical Methods

Electrochemical techniques have been widely applied in biosensor development because of their undisputable advantages [3, 28]. Contrary to fluorescence-based methods [29], electrochemical-based sensing methods are cheap, fast, and user-friendly, and can be readily developed into small, portable, point-of-care devices to perform online monitoring for a variety of small molecules of interest [30].

Glucose sensing is currently the most common and most established assay; however, many have built on the progress made by glucose sensing research to

develop electrochemical sensing platforms for quantifying a variety of biomarkers. A universal aptamer sensor has been developed based on the commercially available glucose sensors to detect a wide variety of small molecules (cocaine, adenosine, interferon, etc.) [31]. The sensor was developed by taking a standard personal glucose meter (PGM) and integrating invertase into the PGM in order to establish a direct relationship between the invertase concentration and target concentration; this was accomplished via target-induced release of invertase from a functional DNA–invertase conjugate or other conjugates using aptamer DNAzymes chosen according to the desired target.

Electrochemical blocking assays are another example of a sensitive, label-free detection method for probing a wide range of biomolecules including oligonucleotides, proteins, and specific cellular receptors [32]. Kelley *et al*. have reported the detection of the cancer biomarker CA-125 using electrochemical blocking assays with a low detection limit of $0.1\,\mathrm{U\,ml^{-1}}$, a level that is 150 times more sensitive than the current commercially available tests [32a]. Its simple working scheme relies on the steady diffusion of small electroactive molecules to a metal electrode, whereupon oxidation or reduction provides a detectable current. When the diffusion of the small molecules is hindered by the binding of the specific biomarker, the current dramatically decreases. Another electrochemical method that will be discussed in detail in this chapter involves nanowire field-effect transistors (FETs), which are sensitive tools for detecting an array of molecules.

5.4 Real-Time Sensing in Tissue Engineering and Cell Growth

In the following sections, we present specific examples where biosensors have been used to probe intra- and extracellular markers, both *in vitro* and *in vivo*. We are still far from a real-time monitoring device that can probe multiple specific markers and simultaneously reflect on the health of an implanted tissue, which would allow for adjustments to be made in order to give the tissue the best chances of success; nevertheless, much progress has been made in the past few years, giving us cutting-edge techniques that promise to revolutionize how we design and monitor tissue engineering.

In order to ensure healthy tissue growth, both the extracellular environment and certain key biological molecules need to be closely monitored. Critical parameters that need to be monitored to ensure optimal tissue growth *in vitro* are oxygen and nutrient uptake, pH levels, temperature, energy production, and excretion of waste. Biosensors have been widely developed for most of these key factors [1b], of which a select few will be discussed.

5.4.1 Metabolites

Monitoring the concentrations of metabolites can provide a good indicator as to whether the metabolism of a cell culture or tissue is functioning well. Glucose, the main source of energy for growing cells, is one example of such

an indicator. Another example is lactate, which is a major byproduct of actively growing cells and an important marker of tissue oxygenation. Its concentration rapidly increases when oxygen supply is compromised [33]. Vadgama *et al*. have developed needle-based, implantable sensors that allow continuous lactate monitoring [34]. The operational scheme of the sensor is based on lactate oxidase, which converts lactate to pyruvate, and hydrogen peroxide, which in turn is detected amperometrically on platinum. The sensor was biocompatible and performed steadily in *in vivo* tests performed on rats.

Another example is ammonia, which is produced as waste by cells during the consumption and breakdown of glutamine [35]. By observing the amounts of ammonia found in a sample, the growth behavior of the cells can be determined. The build-up of ammonia can inhibit cell growth; hence, measuring ammonia concentrations can provide a good indication of a successfully regenerated metabolic functional tissue, and monitoring and controlling its concentrations are vital for the continuous success of the regenerated organ.

Ammonia can be measured using an ion-selective electrode (ISE) technique, such as that developed by Radomska *et al*. [36]. Generally, ISEs were not considered effective instruments for biosensing because of their invasive nature, low biocompatibility (interaction with the biological surroundings), and short lifetime due to biofouling, which can, in turn, lead to inaccuracy. Various options to prevent biofouling and increase biocompatibility were investigated, thereby producing an ammonia-selective electrode that can be placed in direct contact with the cell medium (bioreactor) for continuous observation [36].

The polymer used for ISE membranes is usually poly(vinyl chloride) (PVC), which is not biocompatible. In order to overcome this problem and prevent biofouling of the membrane, the surface of the sensor could be modified with hydrophilic polymers such as alginic acid or polyethylene glycol (PEG), which would ensure that the polymer membrane can interact with proteins reversibly with insignificant conformational changes [36].

A second approach for minimizing protein biofouling of the ISE is charge modification of the polymer membrane. Negatively charging the membrane should prevent protein adsorption, by introducing carboxylic groups, for example [37].

In order to ascertain which ISE membrane composition is optimal, Radomska *et al*. created five different ammonia-sensitive electrodes with differing membrane compositions: carboxylated PVC, aminated PVC, and carboxylated PVC doped with PEG [36]. According to their studies, PEG-doped ISEs showed the greatest resistance to biofouling and were well suited for direct and continuous observation of ammonia *ex vivo*.

5.4.2 Oxygen Monitoring During Cell Growth

Constant oxygen supply is essential for successful tissue growth; thus it goes without saying that regular observation of oxygen gradients must be maintained. Tissues are generally supplemented with required metabolites, nutrients, and oxygen via specially engineered scaffolds in order to simulate a proper growth environment and increase waste removal efficiency. However, oxygen transport in engineered tissues can be problematic due to the fact that most systems only

provide transport at the surface level, with molecular diffusion being the only route for oxygen to reach the inner tissue [38]. In addition, the lack of adequate vascularization in tissues cultured *in vitro* results in oxygen being consumed before it can diffuse far enough (around 150 μm) to reach the inner cells [39]. This can result in hypoxia, which favors tumor progression, and a necrotic inner core of cells in the tissue [39].

Earlier methods for measuring oxygen concentration in tissues were mostly based on the use of invasive oxygen probes. Such probes depended on consuming oxygen in order to measure it, which is evidently counterproductive. Noninvasive, noncytotoxic, fluorescence-based methods that do not consume oxygen have been developed, from which the partial pressure gradients of oxygen in a tissue can be determined using the observed fluorescence intensities [40].

The main advantage fluorescence-based oxygen sensors provide is that they are noninvasive and noncytotoxic and do not consume oxygen [40, 41]. Sensing occurs by using fluorescence microscopy or spectroscopy to monitor the quenching of specially prepared fluorescent microparticles by oxygen.

Acosta *et al*. developed a method for sensing oxygen concentrations directly from the 3D scaffold itself using fluorescent oxygen-sensing microparticles bound to silica gel particles [40]. The system consists of oxygen-sensitive luminophores in addition to reference oxygen-insensitive fluorophores that are bound in poly(dimethylsiloxane) (PDMS)-encapsulated silica gel particles. The oxygen-sensitive and oxygen-insensitive fluorophores typically used are, respectively, $Ru_2(Ph_2phen_3)Cl_2$ and Nile Blue chloride.

Another useful application for noninvasive fluorescent oxygen-sensing is determining nondestructively the quantity of cells in a tissue construct from the real-time oxygen concentration, as demonstrated by Santoro *et al*. [41]. Their studies also show the potential for evaluating cell proliferation through studying the oxygen gradient in a tissue because of the fact that the oxygen gradient over time was shown to be proportional to cell growth.

5.4.3 Reactive Oxygen Species

As previously mentioned, hypoxic tissue environments can lead to necrotic cells and potentially favor tumor progression; similarly, hyperoxia can induce the formation of ROS, which in turn can also lead to cell death or apoptosis. [42] Moreover, regular stresses caused by tissue generation can also lead to ROS formation [15b].

ROS are a group of molecules of varying reactivity and functions that play a sensitive biological role. ROS such as superoxides, hydrogen peroxide, hydroxyl radicals, and HOCl perform physiological roles such as intracellular signaling in small, closely monitored concentrations that are kept in check by antioxidant defenses [43]; however, when concentrations exceed normal levels, these ROS can become pathogenic [15b, 44]. Detecting ROS is important for tissue generation in order to maintain the fine balance needed for healthy cell growth.

ROS can be detected using fluorescent microparticles, similar to oxygen [45]. One method of sensing involves using hydrocyanines, which have been reported to be easily synthesized from cyanine dyes by reduction with $NaBH_4$

[46]. Hydrocyanines are weakly fluorescent, but their fluorescence increases substantially upon oxidation by superoxides or hydroxyl radicals; additionally, they are stable with regard to autoxidation and can detect down to nanomolar concentrations of ROS [46].

5.4.4 Cell Adhesion

Cell attachment and migration is sensitized partially by external mechanical forces via specific adhesion molecules [47]. Cellular adhesion is crucial for maintaining a multicellular structure. Once in contact with the surface, cells can apply forces in the nano-newton (nN) scale. Quantifying the stresses exerted by cells in a three-dimensional scaffold is of great interest to the field of tissue engineering because this can reflect on cell health and vitality.

Using water-soluble CdSe/CdS semiconductor tetrapod nanocrystals, a two-dimensional *in vitro* stress assay was developed by Alivisatos *et al*. [14b, c]. These nanocrystals are robust and bright inorganic fluorophores whose emission depends on the external stresses. The tetrapod consists of CdSe as its core and CdS as four protruding arms. The arms play a critical role in the signal transduction to convert the applied stress to luminescent signals from the CdSe core. The system proved instrumental in measuring the forces applied by cells, but most importantly the nanoprobes were shown to be recyclable as they were able to endure multiple cycles of loading prior to failure. The range of applications is therefore limitless, since the tetrapod can be incorporated into many materials, allowing noninvasive stress measurement through fluorescence spectroscopy of the confined CdSe core states. The nanocrystals were easily prepared inside polymer fibers via electrospinning; as such, they could be potentially incorporated into three-dimensional structures for tissue engineering, specifically for spatial probing of cellular adhesion within a matrix (Figure 5.2). These nanomaterials are therefore uniquely positioned to quantify local stresses with high spatial resolution and can be incorporated in a variety of materials, notably tissue scaffolds.

However, as promising and exciting as they might seem, vigorous testing still needs to be undertaken to establish their cytotoxicity. CdSe, and CdS quantum dots are known to be toxic to multiple cell lines. A suggestion for circumventing potential cytotoxicity is incorporating a layer of silicon oxide, which could passivate and confine any leakages of the toxic cadmium ions.

5.4.5 Nanowire Field-Effect Transistors

5.4.5.1 Introduction to Field-Effect Transistors

FETs have been reported as sensitive tools to detect volatile molecules, small molecules, oligonucleotides, and proteins, to name a few [48]. An FET is a three-terminal device consisting of a semiconductor layer positioned between a source and a drain (Figure 5.3). The third terminal is a gate that modulates the charge carrier density in the semiconductor. Charge carriers electrostatically accumulate or deplete in the semiconductor at the semiconductor/gate dielectric interface, and any modulation in the microenvironment of the electric field affects the charge carrier density and leads to a change in measurable signals.

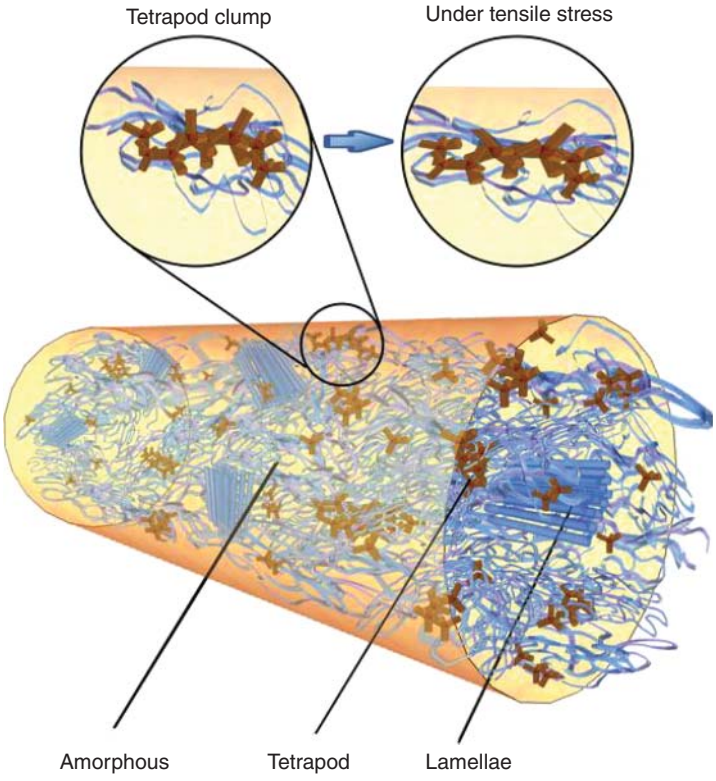

Figure 5.2 Illustration of embedded tetrapods in a polymeric fiber. (Raja 2013 [14b]. Reproduced with permission of the American Chemical Society.)

This effect can be transformed into a sensitive transducer for the detection of chemical and biological molecules; for instance, upon the analyte binding to a specific antibody immobilized at the interface, the change in the surface charge is similar to applying a new gate voltage across the interface. This results in changes in the charge carrier density and translates into a measurable signal [49].

5.4.5.2 Field-Effect Transistors for Intracellular Monitoring

Understanding and monitoring the intracellular environment of growing tissues has posed a number of challenges to tissue engineers. Cardiac tissue is one example of many where methods for quantifying electrical impulse propagation and mechanical function are of great importance, yet traditional techniques such as patch clamping, which was the go-to method for measuring intracellular electrophysiology and that of individual cells, are invasive and do not allow for continuous monitoring [50]. The large tips of the patch-clamp electrodes (1–2 μm) require breaking the cellular membrane in order to access the intracellular space; furthermore, over the course of the measurement, the intracellular content is replaced by the electrode fillings, and measurements are possible only for a maximum of 10 min before the cell is dialyzed. These major disadvantages prove this type of technique unsatisfactory for long-term monitoring in tissue engineering.

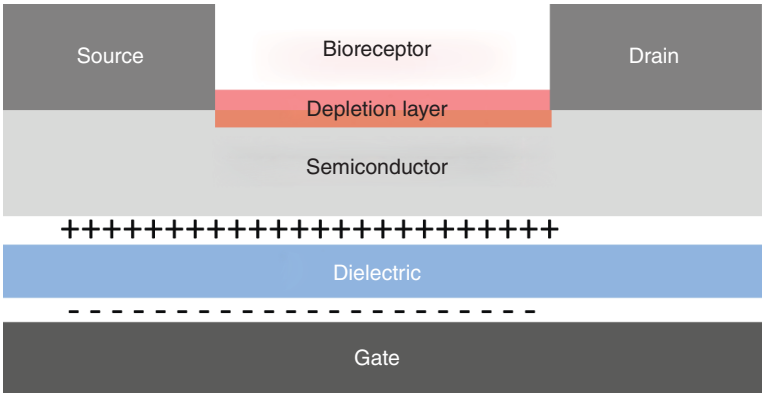

Figure 5.3 Schematic representation of a field-effect transistor.

To overcome these problems, sharp microelectrodes with narrow bores, which are able to preserve the intracellular milieu, have been developed; however, these microelectrodes display a relatively high resistivity, which compromises their sensitivity (30–100 mΩ) [51]. Metal-based electrodes or carbon-nanotube-based probes are limited by impedance as they decrease in size, which makes it difficult to formulate them into small electrodes that can probe intra- or extracellular activity. In addition, sampling can often be hindered by the limited multiplexing ability of the electrodes and by their rigid nature.

The ability to monitor intracellular activity has become a pressing need. Knowing that semiconductor nanowires, given their size and electronic properties, possess unique characteristics which make them ideal structures for establishing active interfaces with biological systems [52], it was realized that FETs could be formulated into nanoelectrodes, whose small sizes do not affect their limits of detection, contrary to metal-based microelectrodes. Nanoscale FETs thus emerged as promising models to address this issue [53].

Viventi *et al*. developed flexible silicon (Si) electrodes with multiplexing capabilities for measuring electrical signals in soft tissue [54]. The high mobility of single-crystalline silicon, relative to organic or other materials for flexible electronics, enables the amplifier to have a high bandwidth. The sensor consisted of 2016 silicon transistors with 288 measurement points and was able to measure the signal of a beating porcine heart *in vivo*. The system could generate a comprehensive map of the spread of ventricular depolarization in real time at high resolution. This was a huge step forward for online monitoring of cellular functionality.

Silicon nanowires were fabricated into flexible nanowire FETs and interfaced with embryonic chicken cardiomyocyte cells grown on a PDMS sheet. This system allowed, in return, multiplexed recording of signal propagation in cardiomyocytes to be determined with excellent spatial and temporal resolution [55]. Silicon nanowire FETs were also interfaced with a brain slice, enabling the visualization of a single cell and the assimilation of healthy neurons at upper and lower tissue surfaces [56].

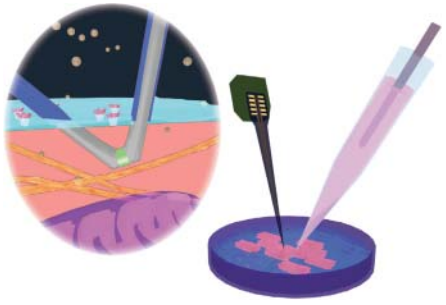

Figure 5.4 Schematic illustrating intracellular recording using free-standing nanowire probes. The probe is mounted on an *XYZ* micromanipulator (1). (2) Refers to a patch-clamp pipette. The probe (3) is coated with a lipid bilayer to facilitate cell membrane (4) entry into the cytoplasm (5). (Qing 2014 [57]. Reproduced with permission of Nature Publishing Group.)

While the FET devices presented thus far were able to provide a wealth of information by allowing multiplexed, accurate, continuous, and sensitive intra- and extracellular measurements, they still, however, required bringing the cells into direct contact with the stationary planar FET. Ideally, rather than forcing the cell to conform to the substrate, a movable nano-FET with the necessary source (S) and drain (D) electrical connections could be produced.

FETs were miniaturized to the nanoscale and transformed from planar electrodes into tip-like nanoelectrodes (about 50 nm) with an acute-angle kinked silicon nanowire, which allowed the probe to be brought into the cell and even probe within the cell membrane, leading to less tissue damage (Figure 5.4) [57]. This revolutionary design was made possible by controlling the cis versus trans Si nanowire crystal conformations between adjacent kinks [58]. Nano-tip FETs were induced through modulation doping. The probe showed exquisite sensitivity in aqueous solution, and when modified with a phospholipid bilayer, the nano-sized probe could enter a single cell without any induced destructive effect and allowed the measurement of the intracellular potentials.

Lieber *et al*. developed a three-dimensional macroporous nanowire nanoelectronic scaffold that could simultaneously monitor cell/tissue electrophysiology in addition to functioning as a standard scaffold for growing tissues [59]. The scaffold was prepared by deliberate incorporation of biomimetic and biological elements into a nanoelectronic network on a scale that ranged in size from nanometers to centimeters (Figure 5.5). Silicon nanowires were deposited, metalized, and epoxy-passivated, then individual nanowires FETs were lithographically patterned and integrated into a free-standing natural macroporous extracellular matrix to form a 3D scaffold. The scaffold was designed to mimic natural extracellular matrix structures and to have high porosity, flexibility, and biocompatibility, with its features in the nanometer to micrometer range. Lastly, specific cells were incorporated and grown. The prepared biomimetic structure allowed real-time monitoring of the local electrical activity of cardiomyocytes within the 3D constructs. Cellular response within the cardiac tissue model was studied upon exposure to specific drugs, showing changes in pH inside and outside the tubular vascular smooth muscle.

5.4.6 Microfluidics-Based Biosensors

Microfluidics is a field that involves the precise control of fluids in small spaces, utilizing the unique properties that fluids display at the micro or lower level, quite

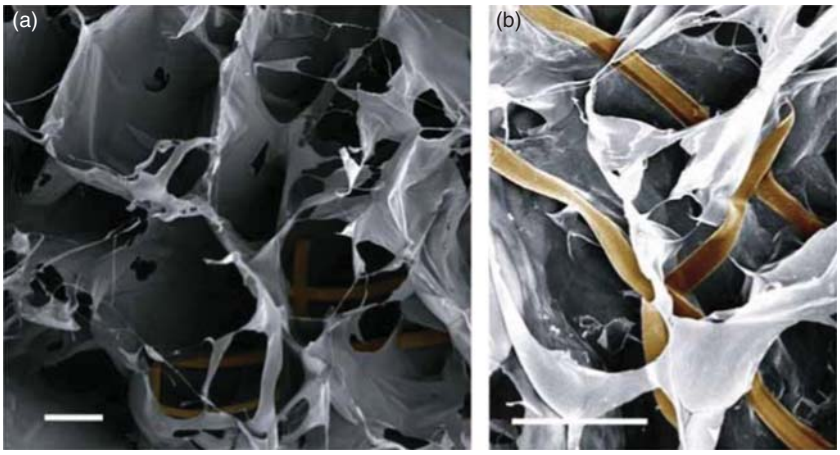

Figure 5.5 SEM images of the three-dimensional macroporous nanowire nanoelectronic scaffold. Scale bars, 200 μm (a) and 100 μm (b), respectively. (Tian 2012 [59]. Reproduced with permission of Nature Publishing Group.)

unlike those observed at the macro scale. Microfluidic technology has proved itself to be a tool with great potential for biosensing in tissue engineering applications, such as "organs on chips" and *in vitro* tissue models [60]. Some advantages that microfluidics provides include higher sensitivity, faster analysis times, and decreased reagent consumption, leading to lower costs. Microfluidic systems can also be of two types: continuous-flow or discrete (droplet-based), depending on what is needed [61]. Two main applications that could be utilized in the field of tissue engineering have emerged in the past decade: the microfluidic chip and microfluidic scaffolds [62].

5.4.6.1 Microfluidic Chips

Microfluidics technology has been integrated into the "lab-on-a-chip" concept, providing highly useful and versatile tools that could be used in biosensing in engineered tissues. For example, Weltin *et al*. developed a glass chip with fully integrated chemo- and biosensors combined with a cell cultivation chamber and microfluidics. The system was able to provide online monitoring of human cancer cell metabolism as well as pH and oxygen levels [63]. Other materials that can be used for fabricating such chips include polymers such as PDMS and PFPE (perfluoropolyether). PDMS, however, can sometimes swell and disintegrate in organic solvents, so it is preferable to use PFPE, which, like PDMS, displays high gas permeability, high flexibility, and low toxicity, in addition to increased resistance to swelling caused by organic solvents [62]. A third material that is very inexpensive and easy to use in microfluidics applications is paper. Paper-based microfluidics can be incorporated into devices with responsive hydrogels that perform as fluid reservoirs and respond to external stimuli to release fluid into the structured paper, as reported by Niedl and Beta [64]. Biosensors can be integrated into such systems and utilized in various tissue assay applications, such as that demonstrated by Derda *et al*. and explained in more detail in Section 5.4.6.2.

5.4.6.2 Microfluidics-Integrated Tissue Scaffold

The microfluidics-integrated scaffold is a recently developed analytical tool that can be used to replace the extracellular matrix for engineering 3D tissues, similar to the macroporous nanowire nanoelectronic scaffold discussed in Section 5.4.5.2. Introducing microfluidic systems within cell-seeded scaffolds allows further control of the distribution and flux of solutes and enables homogenization of the cultured tissue. In addition, such microfluidic systems can also be incorporated with microphotonics, which enables single-cell manipulation. These systems can help exploit and optimize the tissue microenvironment by facilitating chemo-mechanical manipulation through unique manifestations of physical properties in microfluidics such as shear force, surface tension, concentration, and so on.

Derda *et al*. have reported that paper-based microfluidic chips, mentioned in the previous paragraph, can be assembled into a 3D cell culture system by stacking layers of chromatography paper permeated with cell suspensions in extracellular matrix hydrogel, in which oxygen and nutrient gradients can be controlled and biochemical and genetic responses can be analyzed for bioassays [65].

However, despite noteworthy advancements in related biomedical fields [66], microfluidic applications for tissue engineering are still in their early stages due to various significant challenges that need to be overcome. Such challenges range from biofouling at the surface to difficulties in producing a microfluidic sensor with high enough sensitivity and accuracy to overcome the complex variety of molecules present in cell culture media [60].

5.5 *In Vivo* Implementations and the Challenges Faced

All the previously discussed examples reported on some fascinating advancements in the field of biosensing related to monitoring cell growth and viability. Most of these studies, however, were performed *in vitro*, and when tested *in vivo*, it was for the short duration of the experiment. The ultimate goal in the current field of tissue engineering is an off-the-shelf system in which organs could be regenerated with probes continuously monitoring the implant viability for the duration of the growth, without the need for patient intervention. One of the most studied and advanced systems is the implantable glucose sensor, for which much effort has been made and many technologies have been developed to overcome the major hurdles that one faces when a foreign structure is inserted into a human body. As a result, there is a wealth of knowledge to be drawn from on how the challenges faced with *in vivo* biosensing can be overcome in order to further progress toward off-the-shelf systems.

Plenty of reviews discussing the evolution of glucose sensing techniques are available [67]; therefore we will discuss only the innovative solutions for *in vivo* challenges that have been reported over the past few years. We believe the developments in this field could be easily translated to other types of sensors.

One of the greatest problems faced with biosensor applications is that poor biocompatibility can lead to foreign body response with symptoms such as tissue

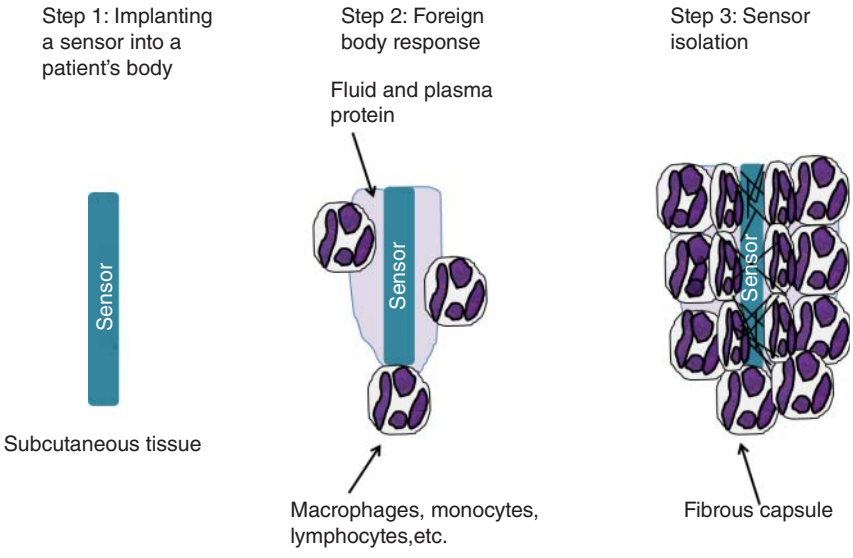

Figure 5.6 Schematic illustration of the foreign body response when a bioprobe is implanted in a human body.

inflammation that sets in within a few hours, and granulomatous formations around the sensors, leading to complete rejection [68].

In addition, biosensors inserted into the human body experience biofouling by proteins and cells. This can result in a thick (10–100 μm), fibrous capsule engulfing the implanted probe. This encapsulation leads to sluggish mass transfer of analytes, which prevents the sensor from interacting and probing its surroundings. It eventually leads to steadily diminishing sensitivity over long-term use (Figure 5.6).

5.5.1 Antifouling Coatings

In order to avoid the fouling problem, certain steps can be taken to ensure that the sensors are biocompatible. Sensors are not expected to be inert, but to be able to react with the surrounding tissue without adversely affecting or disturbing the homeostatic environment; surface modification of the sensors themselves is usually enough to prevent such adverse effects [69].

For example, Wang *et al*. studied the effects of coating dummy glucose sensors with a polymer compound consisting of poly(lactic-*co*-glycolic) acid microspheres dispersed in poly(vinyl alcohol) hydrogels [70], which were evaluated in both normal and diabetic rats over a 1-month period. According to the study, inflammation and fibrous encapsulation at the implantation sites were shown to be controlled via the slow release of tissue-modifying drugs by polymeric microspheres, with the hydrogel enabling swift analysis of diffusion.

Zwitterionic hydrogels have also proved to be effective for improving biocompatibility. Such hydrogels could resist collagen capsule formation for at least 3 months and have even be shown to promote angiogenesis, which could be particularly advantageous for tissue engineering applications [71].

However, these modifications did eventually induce an *in vivo* immune response, which led to the eventual formation of cellular capsules and the deterioration of the sensor performance [72].

5.5.2 Nitric Oxide

Nitric oxide (NO) is a free radical that is naturally produced in mammals with remarkable anti-platelet, anti-inflammatory, vasodilation, and wound-healing properties [73]. NO is produced by the endothelial cells within the vicinity of healthy blood vessels to prevent blood clot formation [74]. It presents an exciting direction for solving the fundamental biocompatibility problem of implanted sensors.

Schoenfisch *et al*. prepared silicon-based xerogels with NO-releasing capabilities via *N*-diazeniumdiolates. The newly prepared material was tested in a rat, and the effect of NO release to reduce cellular encapsulation was evaluated on the implant surrounding tissue at different time intervals (1, 3, or 6 weeks). The reported results were promising, where a 20–25% reduction in the capsule mass (at 3 and 6 weeks) was observed in addition to reduction in inflammatory cells and enhancement in vascular density near the implant (at 1 and 3 weeks) [75]. These results were a major step forward for developing stable implantable sensors.

This technology was subsequently tested on an implantable glucose sensor. Needle-type electrochemical glucose sensors coated with a polymeric membrane doped with (*Z*)-1-[*N*-methyl-*N*-[6-(*N*-butylammoniohexyl)amino]]-diazen-1-ium-1,2-diolate (DBHD/N_2O_2) was prepared and implanted in a rat model. [76] A slight improvement of 2–2.5% was reported in comparison to the control. Because of the stringent limitation of the outer membrane thickness (~10 μm) on the glucose sensor, the release time for NO was relatively short, about 16 h. As a result, small amounts of NO precursors could be packed into the membrane. Thicker membranes are not an option since they would drastically affect the sensitivity and response time of the probe.

An alternative approach of slow production of NO was made possible from endogenous *S*-nitrosothiols present in blood using a copper(II) complex [77]. Silica nanoparticles were used as the release vehicle for NO. The precursors *N*-diazeniumdiolate and *S*-nitrosothiol-modified silica nanoparticles were tested *in vivo*. [78] While the macromolecules doped with *N*-diazeniumdiolate showed a fast release (16 h), the *S*-nitrosothiol-modified silica nanoparticles released 99% of their content within the first 3 days. Nevertheless, NO was still detectable in small concentrations after 7 days. The two systems exhibited improved performance versus controls. The fast release of NO led to a positive improvement in clinical accuracy and glucose sensitivity. The slow release provided constant numerical accuracy for up to 10 days.

5.5.3 Templated Porous Scaffolds

Microorganisms and cells interfacing nano and macro surface structures in addition to porous materials have also been extensively studied [79]. When compared to smooth surfaces, these materials will heal in a less fibrotic and a more

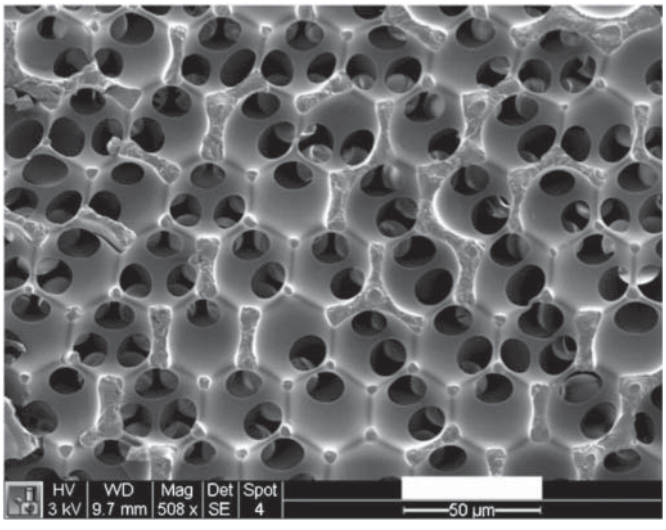

Figure 5.7 Porous templated scaffolds of 35 µm. Scale bar = 50 µm. (Bryers 2012 [82]. Reproduced with permission of John Wiley and Sons.)

vascularized manner. As such, those materials could help overcome the foreign body response in implantable devices.

Porous templated scaffolds are polymer structures in which every pore and pore interconnects are uniform in size [79c, 80]. Templated scaffolds were fabricated from many materials including poly(2-hydroxyethyl methacrylate)hydrogel, silicon elastomer, fibrin, alginate, and polyurethane, all in pore sizes ranging from 10 to 160 µm [81]. Ratner *et al*. used these porous structures to study the foreign body reaction in mice, rats, and rabbits. In a remarkable discovery, regardless of the polymer composition, scaffolds with 35 µm pore size showed the highest vascular density and excellent tissue recovery around the implant, making them a promising material for implantable sensors [81] (Figure 5.7).

5.6 Conclusion and Future Directions

Enormous achievements have been reported for continuous monitoring of specific analytes, in particular, markers that are critical for tissue engineering. Sensing of small molecules, forces, and electrical signaling has been made possible with exquisite sensitivity and spatial resolution. The major challenge is to translate those probes to the *in vivo* world.

Advancements in *in vivo* sensing have been hindered by the relative inaccessibility of tissues and difficulties in fundamental sensor design and application. There are many challenges that biosensors face for them to function optimally *in vivo*. These include biocompatibility, in which the sensor must be noncytotoxic, not perturb the homeostasis of the system, and ideally be noninvasive; preserving long-term stability, multiplexing, biofouling, and maintaining high spatial and

temporal resolution are the other challenges faced. Additionally, these difficulties vary in magnitude depending on the analyte in question.

Recent efforts have contributed to admirable results, especially with the continuously improving glucose sensing technologies; however, progress, particularly in other areas, is still gradual. More effort and resources need to be directed to what now seems to be the bottleneck for any potential translation of biosensors to *in vivo*. Specifically, efforts need to be channeled into developing efficient antifouling materials that would allow continuous monitoring of specific biomarkers. FETs present promising signal transducers but still come at a very high cost; thus, efforts ought to be directed toward creating less expensive FET devices. Alternative yet sensitive detectors are also necessary to design more affordable monitoring systems.

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6

Bioreactors in Tissue Engineering

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6.1 Introduction

Organs and tissues are highly complex systems. Hence, to utilize them for future applications it is important to know and understand these biological systems from molecular to multi-organ levels [1]. In the literature, we can find several applications of biological tissues, such as biomedical therapeutics and diagnostics. Tissue engineering (TE) is an interdisciplinary field of research that comprises stem cell research, regenerative medicine, biomaterials, and a set of microengineering technologies (e.g., microfabrication, microfluidics, and microelectronics), which are used together for the development of relevant *in vitro* tissue models [2]. Integration is a key aspect of this work. In recent years, micro- and nanotechnologies have been progressively integrated into this field to develop new scaffolds and bioreactors, which have become essential components of TE research [3].

As mentioned previously, TE is an interdisciplinary field of research that comprises knowledge on different cells and cultivation systems. One of the many key factors in the development of TE is the requirement to culture mammalian cells in large quantities and in an *in vivo* like environment. This requirement has resulted in the development of a wide variety of new bioreactor configurations and also the adaptation of different bioreactors based on traditional biochemical engineering designs. Bioreactors are laboratory cell culture devices in which different biological processes take place under controlled conditions. Design of bioreactors should be simple and efficient to minimize risk to the cells and the experimenter. The three main objectives of the bioreactors are (i) creating uniform cell distributions on three-dimensional (3D) scaffolds, (ii) controlling the level of gases and nutrients in the cell medium, and (iii) exposing the new tissue to stimuli, both physical and chemical. If these stimuli are inadequate or absent, cells cannot proliferate and differentiate; it can even lead to cell death. Bioreactors play also key role in the development of one of the most prominent areas of research that is spearheading TE research, namely the development of organs-on-a-chip. The idea is to integrate biosensor technology and nanotechnology with stem cell research and tissue engineering in a new concept of a lab-on-a-chip device. One of the main objectives for organs-on-a-chip researchers is to reproduce organs *in*

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vitro and obtain functional, engineered tissues or organs. In recent years, there has been great progress in this research field due to micro- and nanotechnologies. The achievements include the fabrication of micro- and nanoscaffolds with new functionalities [4, 5] and the development of new bioreactors [6]. Although numerous challenges remain for replicating *in vitro* tissues for clinical applications, the recent advances in microtechnology have given new opportunities for developing functional tissues in the near future. Of special interest is the application of organs-on-a-chip in the pharmaceutical industry [7]. These chips could be used in pharmaceutical assays, which would be a step toward the ultimate goal of producing *in vitro* drug testing systems crucial to the drugs and pharmaceutical industry. There is now substantial interest in developing minimal functional units that recapitulate tissue- and organ-level functions that mimic the *in vivo* system as closely as possible for the disease modeling and chemical testing. Most of the bioreactors used in this area of research are monoculture perfused microfluidic chambers. Microfluidic bioreactors are widely used to study physiological and biological phenomena, as they provide a better control over many system parameters than static cultures. These bioreactors and their applications in organ-on-a-chip devices are discussed in more detail in Section 6.2.5.

To date, researchers have developed a large variety of bioreactor systems for TE applications [8]. In the literature, we can find simple systems such as dishes, spinner flasks, and rotating vessels, where cells grow on fixed or floating scaffolds and the cell culture is exchanged at different intervals, or more complicated systems like perfusion chambers where cells grow on fixed scaffolds and the medium is circulated continuously. Of these, some systems are already commercially available [9]. In this chapter, we summarize and discuss the characteristics and applications of all bioreactors related to TE. This chapter does not simply present an exhaustive review of what has been reported in the literature, but also discusses the various aspects of the bioreactors used in the development of TE and attempts to incorporate the maximum amount of comprehensive information. The first part of this chapter briefly introduces the different bioreactor designs and their major characteristics. The second part discusses some of the TE research areas where these bioreactors have been used and have emerged as valuable technology. Detailed information has been classified regarding the different tissues and organs cultivated in the bioreactors, such as liver, musculoskeletal, neuronal, and cardiac tissues, and finally some compendium of tissues like cornea, bladder, and uterine.

6.2 Bioreactors

We can define a bioreactor as a device that uses mechanical forces to influence biological processes. Bioreactors are laboratory cell culture devices in which different biological processes take place under controlled conditions. In TE, bioreactors are widely used to stimulate cells, control their proliferation and differentiation, and stimulate cells to produce the extracellular matrix (ECM). A great variety of bioreactors exist, which can be classified by the means they

Table 6.1 Summary of characteristics of bioreactors used in TE.

Bioreactor type	Advantages	Disadvantages
Spinner-flask bioreactor	Easy system, cheap, high productivity, easy scale-up, homogeneous culture conditions	Poor aeration, turbulences (high shear), requires cleaning and sterilization, high space requirements in incubator
Rotating wall vessel bioreactor	Low shear stress, efficient gas transfer, 3D scaffold, homogeneous culture conditions	Complex system, increase sedimentation velocity, collision may induce cell damage
Wave bioreactor	Reduces contamination, easy scale-up, no need cleaning, and sterilization, low shear stress	Single use, high cost, low oxygen transfer
Perfusion bioreactor		
Parallel plates	Homogeneous culture conditions, high productivity, low shear stress, efficient gas transfer	Bioreactor geometry affect cell behavior, continuous removal of grow factors, difficult to harvest representative samples
Hollow fibers	High cell distribution, efficient gas transfer, low shear stress, mimic cell environment, 3D scaffold	Difficult to remove cells, pores can get clogged, difficult scale-up
Fixed and fluidized beds	Efficient gas transfer, 3D scaffold, cell/cell or cell/matrix interactions are possible, uniform cell distribution	Low sample volumes, shear stress in fluidized-bed systems, difficult scale-up

use to stimulate cells. Some of them are discussed in the following. Table 6.1 summarizes the advantages and disadvantages of bioreactors.

6.2.1 Spinner Flasks

Spinner flasks are simple bioreactor systems in which scaffolds are fixed at the end of needles or seeded onto microcarriers [10–15]. In this bioreactor, a magnetic stirrer or other mixing tools are maintained in a well-mixed environment, while gas exchange and medium oxygenation are carried out at the top of the vessel (headspace) (Figure 6.1a). This kind of bioreactor is easy to scale up because of its simple design. External mass transport is enhanced by using the convective forces generated by the stirring. However, turbulent eddies are also generated, which can adversely affect the development of tissues [16, 17]. To reduce this turbulent flow, Bueno *et al*. [18] modified the geometry of the spinner flask. They used a wavy-walled bioreactor (WWB) to culture the chondrocytes and compared the results with those from a typical spinner flask. They observed that the introduction of smooth waves enhanced chondrocyte aggregation and viability.

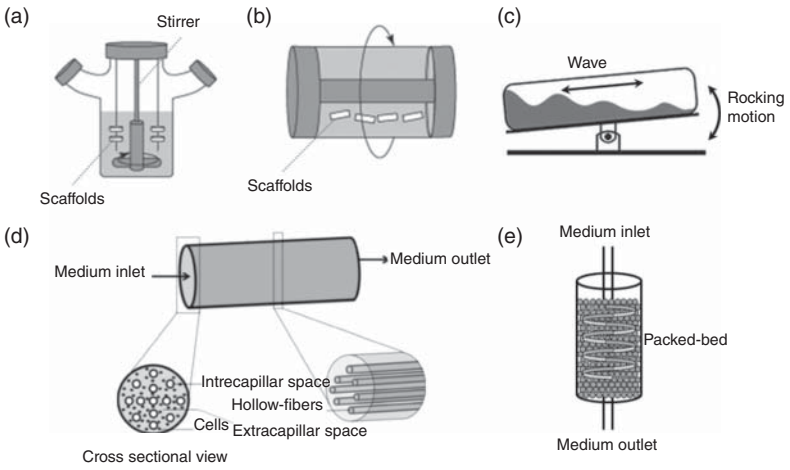


Figure 6.1 Schematic representation of several bioreactor configurations for TE applications: (a) Spinner-flask bioreactor; (b) rotating-wall vessel system; (c) wave bioreactor; (d) hollow-fiber bioreactor; and (e) fixed (packed)-bed bioreactor.

In the literature, we can find many examples using this kind of bioreactors for TE [18–21]. Alternatives to simple spinner flasks are the rotating-wall vessel (RWV) bioreactors with a dynamic culture and low shear stress, as discussed below.

6.2.2 Rotating-Wall Vessel Bioreactors

These bioreactors were fabricated by the National Aeronautics and Space Administration (NASA) and were primarily used to test simulated microgravity conditions [22]. In RWVs, the 3D cell constructs remain near a "free-fall" state and are subjected to a dynamic laminar flow. These systems are completely filled with the culture medium, and oxygen is supplied via a gas-permeable membrane (Figure 6.1b). These bioreactors provide a culture environment with a low shear stress; however, there is an inhomogeneous distribution of cells due to the low diffusion in the interior of the scaffold [23–26]. On the other hand, the size of the scaffold is an important factor, in order to avoid collisions with the reactor wall. Alternative configurations to minimize the internal diffusion and collisions have been developed, such as the rotating bed system (RBS) bioreactors [27–30], where the scaffolds are attached to the rotating vessel. These bioreactors provide alternate contact between the medium and the gas atmosphere, as they are half-filled with the medium, and allow long-term cultivation with low medium consumption. Suck *et al*. [27] used this bioreactor for osteoblastic differentiation of MC3T3-E1 cells. They observed that the proliferation and differentiation of the cells were enhanced as a result of the increased contact between cells, the medium, and oxygen (headspace). On the other hand, these bioreactors are limited to small scaffolds because the transport of nutrients cannot be guaranteed to the inner part. An alternative way to achieve a high mass transport within RWV bioreactors is to use a perfusion bioreactor described in the following.

6.2.3 Wave Bioreactors

Wave bioreactors are easy-to-use systems and include a single-use, flexible cell bag, which is pre-sterilized and half-filled with media and inoculated with cells into a fully monitored and closed system (Figure 6.1c). This system is based on a noninvasive rocking motion, which enables optimum mixing and oxygen transfer for cell culture. It is an easy-to-scale-up system, and there is no need for cleaning or sterilization although it entails a high cost of operation because the cell bags are expensive [31]. Some research groups have described the use of this system for the clinical-scale expansion of different cells [32–34]. For example, Timmins *et al*. [33] developed a closed and automated process for the isolation and expansion of human placental mesenchymal stem cells (hpMSC) using a wave bioreactor to facilitate its use in the clinic.

6.2.4 Perfusion Bioreactors

This system enhances mass transfer through the continuous exchange of media. Using this bioreactor, harmful metabolites can be eliminated, and growth factors and nutrients can be continually supplied. There are different configuration types of these bioreactors [35].

6.2.4.1 Parallel-Plate Bioreactors

Parallel-plate bioreactors consist of two primary compartments: a liquid compartment, which is filled with the culture medium and contains a tissue culture surface to facilitate cell adhesion; and a gas compartment filled with a mixture of gases, which is situated above the liquid compartment and separated from it by a gas-permeable, liquid-impermeable membrane [36]. In the literature, we can find several works using this bioreactor type [37–41]. Despite its widespread use, only very few studies have addressed effect of the bioreactor geometry on the behaviors of cells cultured in it. For example, Peng *et al*. [42] confirmed that the geometry of parallel-plate bioreactors affects cell growth and differentiation. They cultured parenchymal stem cells in four different geometries (slab, gondola, diamond, and radial) and observed that the best environment to grow the cells was provided by the radial configuration because of the absence of walls that create slow-flowing regions.

6.2.4.2 Hollow-Fiber Bioreactors

Hollow-fiber bioreactors (HFBs) provide a high surface area for cellular attachment and proliferation with a low level of shear stress [43]. This system mimics the capillary-type circulatory systems and offers an *in vivo* like environment to grow cells. An HFB comprises a group of thousands of hair-like hollow fibers within a tubular shell that has inlet and outlet ports (Figure 6.1d). Nutrients and metabolic products are pumped through the lumen, allowing the cells to grow. The cells are grown on the internal or external surfaces of the hollow fibers, which are permeable tubular membranes (around 200 μm in diameter) with pore sizes ranging from 10 kDa to 0.3 μm [44]. These membranes can be modified with ligands for better anchorage of cells [45]. In the literature, there are some examples of cell expansion and *in vitro* modeling of tissues using this bioreactor [43, 46–51].

At present, a number of commercial HFBs are available. However, there is no standard operating procedure for them, and only a handful of publications have used the commercial HFB systems [44, 52]. For example, Usuludin *et al*. used a commercial HFB as a tool for *in vitro* bone marrow modeling of leukemia [53]. They cocultured stromal and erythroleukemia cells and compared their results with a standard tissue culture polystyrene (TCP) culture. They observed that both systems promoted cell growth and supported the survival of erythroid and megakaryocytic cell lineages. However, only the HFB inhibited erythroid maturation. Furthermore, the cells proliferated better using HFB compared to the TCP dish.

6.2.4.3 Fixed (Packed) and Fluidized-Bed Bioreactors

Fixed or packed-bed bioreactors (PBRs) are compact systems providing high productivity. They typically are formed by a fixed scaffold (bed) arranged in a column, where the cells grow on or within carriers, and a tank that provides nutrients and oxygen through the bed (Figure 6.1e). The scaffold may be located either external to or within the tank of the medium. The column may consist of particles, either packed (fixed) or floating (fluidized). These systems have widely been used to culture mammalian cells using a fixed bed [54–57] or a fluidized bed [58–62] and to produce artificial tissues and organs [60, 63–67]. Another strategy to create this bioreactor bed is to encapsulate the cells in alginate microbeads [58, 66, 68].

6.2.5 Microfluidic Bioreactors

Microfluidic bioreactors are continuously perfused microdevices for culturing cells. These systems are widely used in cellular applications since they provide a large surface-area-to-volume ratio as well as many other biomimetic properties. They are manufactured using soft lithographic techniques [69], are cheap, and can be used to miniaturize assays; therefore, they can use reduced reagent volumes and cell numbers [45, 70]. Early experiments by Leclerc *et al*. demonstrated the utility of soft lithography for cell culture [71]. They fabricated a microreactor consisting of 10 stacked polydimethylsiloxane (PDMS) layers and 4 reservoirs for the cell culture; also it contained a reservoir for oxygen supply. When comparing their results with those from macroscale bioreactors, they observed that cell density was similar in both cases. Furthermore, the oxygen reservoir helped avoid cell detachment and allowed long periods of culture since it was not necessary to apply high flow rates of the medium. Toh *et al*. developed a PDMS microdevice for culturing MSCs and hepatocytes [72]. Vukasinovic *et al*. cultured tissue equivalents using a microfluidic platform in dynamically controlled environments [73]. Whitesides and coworkers combined microfluidic platforms with tissue engineering applications [74], and obtained an artificial "tissue construct" using a chip-based system [75]. At present, microreactors are considered powerful tools for the development of organs-on-chips. Such devices are 3D microfluidic chips that mimic the structure and functions of full-size organs but on a small scale. Microfluidic bioreactors provide better control over system parameters than static systems, thereby facilitating the study of physiological phenomena [76–78], and for this reason they are now widely used in the development of

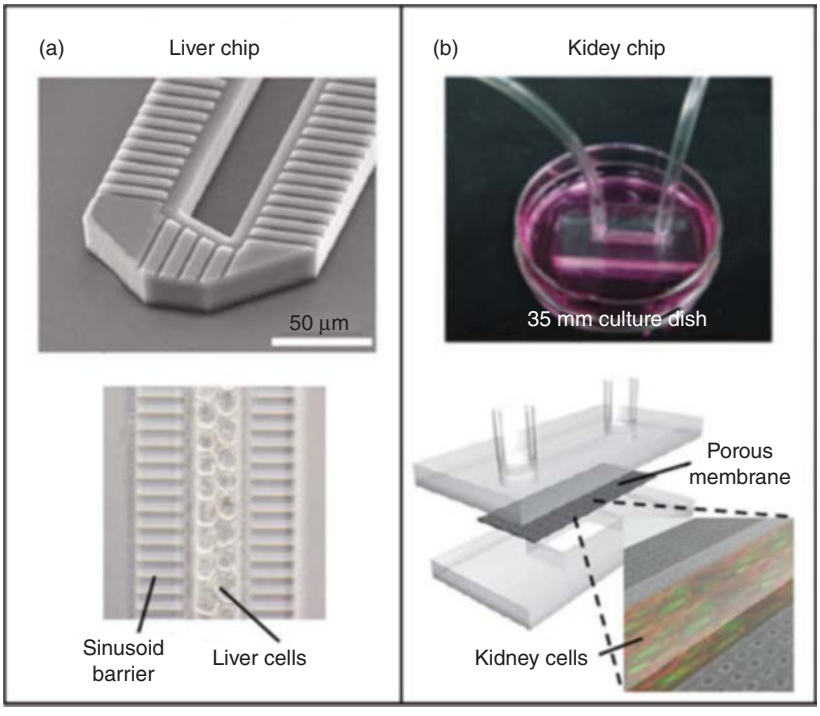


Figure 6.2 Mimicking organ-on-chip. (a) Liver-on-chip: image of a microfluidic liver chip with cell culture and flow chambers separated by a microfabricated baffle that mimics the endothelial–hepatocyte interface of the liver sinusoid. (Reprodued with permission from [81].) (b) Kidney-on-chip: image of a microfluidic kidney chip with a PDMS channel and a PDMS well bound to a semipermeable membrane where cells were cultured and subjected to fluid flow. This system mimics the interface between the epithelium and flowing urine. (Reproduction with permission from [83].)

organs-on-a-chip with different types of tissues or organs. In the literature, we can find several examples, such as liver-on-chip [79–81], kidney-on-chip [82, 83], lung-on-chip [84, 85], gut-on-chip [86, 87], muscle-on-chip [88], and many other devices [89–91] (Figure 6.2).

6.3 Applications of Bioreactors in Tissue Engineering

For the generation of 3D tissues, the development of new biological models and new techniques for culturing cells on a large scale cells are required. Bioreactors bring to TE research homogeneity, automation, and controlled environmental conditions. These features are necessary not only for basic clinical/lab research of 3D tissue development but also to decrease manufacturing costs, standardize the process and the produced tissues, facilitate their use in clinical trials, and their final translation to the industrial sector. Therefore, a comprehensive study for bioreactor culture is necessary for their application in different disciplines in

TE. In the following, we discuss the use of bioreactor culture for liver, neuronal, and cardiovascular tissue engineering among others.

6.3.1 Bioreactors for Liver Tissue Engineering

Liver is an important organ that is necessary for survival and has a wide range of functions, such as metabolization, detoxification, glucose metabolism, protein synthesis, and lipid metabolism. Liver is able to regenerate its lost tissue. Therefore, cell- and tissue-based therapy may offer an alternative to orthotopic liver transplantation. In the literature, we can find reports that demonstrate many types of stem cells that can be differentiated in hepatocytes, such as embryonic stem cells (ESCs), pluripotent adult stem cells, hematopoietic stem cells (HSCs), mesenchymal stem cells (MSCs), and induced pluripotent stem cells (iPSCs) [92–94].

Several groups have developed or adopted bioreactor configurations for liver culture and regeneration [13, 95–98]. For example, simple flat-plate bioreactors consisting of a monolayer culture of cells on a flat plate (such as collagen, laminin, or Matrigel) with a perfusion flow across the top of the monolayer were used for liver tissue culture and production. Pioneering work employing this system was done by Uchino *et al*. [99], who stacked monolayers of hepatocytes on the collagen plates and used them in the anhepatic dogs. More recent works have proved this system as a beneficial tool for the liver cell culture [100–103]. Overall, the results from the later studies were in 2D cultures. Therefore, it is required to develop more complex bioreactor configurations to closely mimic the *in vivo* environment of liver cells, maintain their hepatic functions, and finally make extracorporeal bioartificial liver (BAL) systems [13, 95, 96, 104, 105].

6.3.1.1 Spinner Flasks

Researchers have developed bioreactors using stirred systems to support stem cell culture and differentiation [14, 106–109]. For instance, Yin *et al*. developed a cultivation system to differentiate mouse embryoid bodies (EBs) into the hepatocytes using a spinner bioreactor [15]. They found that this system had high potential to be used as a large-scale hepatic differentiation system. However, the bioreactor mixing caused a wide distribution of sizes in aggregates of EBs. An alternative system uses microcarriers, which offer the potential of large-scale differentiation and more controlled conditions. Park *et al*. [110] expanded and differentiated rat multipotent adult progenitor cells (ratMAPCs) into the hepatocytes on dextran beads using a spinner reactor. They obtained results comparable to those using static cultures but with the advantage of large-scale production for future therapeutic and clinical applications.

6.3.1.2 Rotating-Wall Vessel Bioreactors

Primary hepatocytes and stem cells cultured on RWV reactors were also used to engineer BAL, since these systems may minimize turbulence and shear stress and maximize mass transfer [111–115]. For example, Ishikawa *et al*. found that the hepatocytes demonstrated a bile-duct-like or vessel-like structure after 10 days of culture using an RWV bioreactor system [113]. However, the

hanging-drop or spinner-flask method did not produce these structures. The tissues were unshaped or crumbled while using the hanging-drop approach, and most tissues were necrotic because of inadequate gas or nutrient supply while using the spinner-flask bioreactor. Wang *et al*. induced the differentiation of mouse ESCs into the hepatocyte-like cells using an RWV system [114]. They cultured ESCs on the biodegradable scaffolds of poly-L-lactic acid (PLLA) and polyglycolic acid (PGA) with exogenous growth factors and hormones. After 2 weeks of culture, they observed that the culture in the RWV reactor showed enhanced differentiation of stem cells compared to the static cell culture. Wang *et al*. further implanted the six cultured scaffolds into the peritoneal cavity of six immunodeficient mice. One or two weeks later, they examined the scaffolds and observed that the hepatocyte-like cells survived and maintained their hepatic function *in vivo*. The obtained results indicate that this system can be useful as a potential model for liver TE.

6.3.1.3 Perfusion Bioreactors

As mentioned previously, cell growth is not always uniform in RWV systems, and cells may be damaged as a result of collisions between the scaffolds and the reactor walls [116, 117]. Perfusion systems allow a more controlled environment, continuous introduction of gas and nutrients, and elimination of waste products. This configuration allows the perfusion of the medium or the patient's blood/plasma through a matrix filled with liver cells [64, 65, 118–122]. For example, Ishii *et al*. worked with a radial flow bioreactor and filled it with porous cellulose beads to obtain a high density of hepatocytes from the embryonic porcine liver (E35) cells with a higher concentration of hepatocyte growth factors [119]. After 1 week of culture, they observed the formation of small liver organoids floating in the medium with a palisading architecture and bile-duct-like structures. The authors concluded that this system was promising for use in transplantation medicine. Morsiani *et al*. developed one of the first radial flow bioreactors (RFB-BAL) for the clinical trials [123, 124]. In their studies, porcine hepatocytes were encapsulated within polyester microfibers and the patient's blood was perfused through the matrix. Their proposed system was tested in seven patients with acute hepatic failure, and the results showed a decrease in serum ammonia and transaminases in the patients. Although transition to the clinic is arduously slow, these results suggest that this configuration may be used as a temporary liver support system.

Fluidized-bed bioreactor systems have been employed for liver TE. In the later bioreactors, most of the cells were entrapped in alginate beads to create an immunological barrier to protect the implants from host rejection [59, 62, 67, 68, 125–127]. For example, Hwang *et al*. packed porcine hepatocytes entrapped within calcium alginate beads into a fluidized-bed bioreactor system to develop an extracorporeal bioartificial liver (EBAL) [127]. The authors demonstrated that the cells maintained their liver functions, such as ammonia removal and urea synthesis, *in vitro*. In addition, they used this system in *in vivo* conditions to treat acute liver failure in a porcine model. The results showed an increase in the serum ammonia compared to the control group. These results suggest that fluidized-bed bioreactor is a useful tool for liver TE.

Although different types of perfusion bioreactors have improved the liver cell function, the HFB system is the most commonly used configuration in TE applications [44]. There are several reports on the replication of liver tissue functions using HFB systems both *in vitro* and *in vivo* models [128–134]. In the case of drug and toxicology testing, these systems have widely been used because they are suitable for prolonged *in vitro* studies [47, 50, 135, 136]. Gerlach *et al*. developed a four-compartment hollow-fiber capillary membrane system to grow hepatocytes and liver endothelial cells [128]. This system was used to enhance the hepatic maturation in the fetal hepatocytes [137, 138] and to prolong their hepatic functions [139, 140]. More recently, Miki *et al*. used the same four-compartment hollow-fiber capillary system to increase the hepatic differentiation from human ESCs using growth factors [131]. The bioreactor had two bundles of hollow fibers to transport the medium as well as O_2 and CO_2 supplies, mimicking arteriovenous perfusion. They observed that this system induced more functional hepatocytes than a conventional 2D culture.

Zhang *et al*. developed a biocompatible polyethersulfone (PES) hollow fiber to promote the proliferation of HepG2 cells [141, 142]. They used a single layer of fiber instead of multiple layers because it does not possess an intermediate sponge layer and has higher flux and low fluid resistance. The authors demonstrated that the smooth inner surface of the fibers had good hemocompatibility and the rough/porous outer surface supported cell growth and proliferation. Two years later, Zhang *et al*. developed a hybrid artificial liver bioreactor (3DHB) incorporating PES hollow fibers into a porous polyurethane scaffold (hollow fiber/packed-bed bioreactor) to enhance the mass transfer in the 3D scaffold [49]. The fibers served as homogeneously distributed capillaries, avoiding the nonuniform nutrient and oxygen transport to the cells during the cell culture *in vitro*. After 7 days of culture, 3DHB showed enhanced the cell distribution, viability, and liver functions such as albumin secretion, urea production, and ammonia removal, compared to HFB. Furthermore, they evaluated the interaction between the 7-day-old hepatocytes and the plasma of patients with liver failure for potential clinical applications, and observed that the ammonia and unconjugated bilirubin concentrations decreased after 6 h of circulation in the bioreactor.

Several microfluidic bioreactors have been used for culturing liver tissues and testing hepatotoxicity [79]. Schuette *et al*. cocultured hepatocytes and endothelial cells in a microfluidic system to mimic an organ-like liver and for liver toxicity testing and liver sinusoids assembly [143]. Besides, Hattersley *et al*. studied the viability and functionality of rat explant liver tissue using a microfluidic approach. Their results demonstrated the advantages of using a microfluidic system to replicate pseudo *in vivo* and *in vitro* conditions, and the system was applied to the study of hepatotoxicity with ethanol [144].

Clinical trials of tissues and organs made in bioreactors are still enormously challenging because of the complexity of both the generated tissues and bioreactor systems. Even so, some configurations of BAL are under investigation for clinical applications [96, 104, 145], including hollow-fiber cartridges or chambers (i.e., ELAD [146], HepatAssist or HepaMate [147], MELS [148], TECA-BALSS [149], and HEBAL [150]), monolayer cultures [151], perfused matrices (BLSS [152] and

AMC-BAL [153]) using dedicated devices, and microencapsulation-based systems [126, 154, 155]. To date, HepatAssist and ELAD are the only BAL systems on which randomized and controlled trials have been performed. Their characteristics and the major clinical findings are listed in Table 6.2.

6.3.2 Bioreactors for Musculoskeletal Tissue Engineering

The musculoskeletal tissues include bone, skeletal muscle, ligament, cartilage, and tendon. In all these tissues, natural tissue regeneration is slow and needs medication or, in many instances, surgery. Nowadays, we can find different designs of bioreactors, such as spinner flask, RWV, and flow perfusion, for musculoskeletal tissue regeneration [156–159]. Several cell types have been used for regeneration, for example, the osteoblast cells for the bone regeneration [160], the primary chondrocytes and stem cells for the cartilage tissue repair [17, 161, 162], and the fibroblasts for the tendon and ligament repair [163, 164].

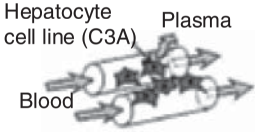
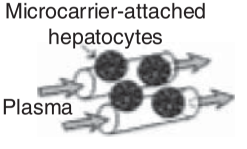
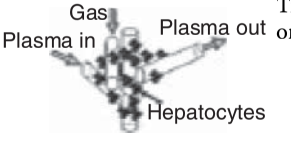
6.3.2.1 Rotating-Wall Vessel Bioreactors

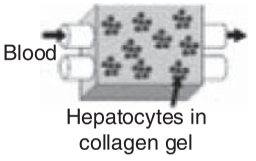
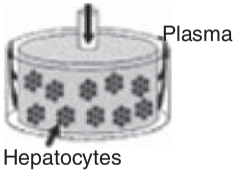
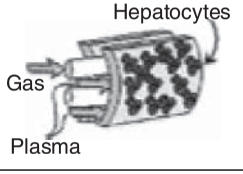
RWVs have already been used for the cultivation of bone and teeth tissues using microcarriers [165–167], porous scaffolds [12, 168–174], microspheres-based scaffolds [175–180], and cell aggregates [181–183]. Using traditional 2D and static culture methods, tooth germs present morphological changes, and their development would be limited [184, 185]. Recently, Sun *et al*. successfully used an RWV system to establish an *in vitro* tooth germ culture model [186]. They observed that tooth germs maintained their typical spatial shape both during and after the culture. After 6 days of culture, the thick layers of enamel and dentin grew on the cultured tooth. They reported that there was no obvious difference between the tooth germs grown in the bioreactor and *in vivo* conditions in a short period. These results show that the RWV system may be an efficient culture system for tooth development.

More recently, aerospace medicine has gradually been developed with the great interest shown in the outer space [187]. RWV may be an effective bioreactor for the study and analysis of cell behaviors in microgravity conditions. Jin *et al*. [188] and Tsai *et al*. [189] recently assessed the effect of microgravity on disk degeneration using bioreactors because astronauts experience back pain during and after space flights. Previous studies had shown that there were some histologic and biochemical changes in the rat intervertebral disks after space travel [190]. Jin *et al*. studied 12 lumbar disk tissues from 10-week-old Balb/C mice under dynamic and static conditions. They observed that the intervertebral disks suffered more degeneration as cultured in the RWV system. The latter result revealed, using reduced red Safranin-O staining within the annulus fibrosus, downregulated glycosaminoglycan (GAG) content and GAG/hydroxyproline ratio, increased matrix metalloproteinase-3 expression, and upregulated apoptosis.

In traditional RWV bioreactors, cell-laden scaffolds float in the medium while the vessel wall rotates. An alternative bioreactor is the rotating-bed system (RBS) [28, 29, 191]. In this system, the scaffolds are fixed on a shaft that rotates in a half-filled culture vessel. Anton *et al*. developed a new RBS bioreactor with a porous ceramic bed for MC3T3-E1 growth and osteogenic differentiation during

Table 6.2 Characteristics and clinical results of six BAL systems.

		Characteristics of six BAL systems					Clinical results			
	BAL system	Bioreactor Configuration	Shape of cells	Hepatocyte source	Immuno-logical barrier	Perfusion (plasma separation rate, ml min^{-1})	Reactor flow rate (ml min^{-1})	Neurological improve-ment	Ammonia removal	Bilirubin elimination
Hollow fiber systems	ELAD (Amphioxus Cell Tech-nologies)		Large aggregates	Human cell line (C3A) (400 g)	Yes (70–120 kD)	Blood (NA)	200	Probably	−8% (increased)	−20% (increased)
	HepatAassist (Circe Biomedical)		Microcarrier attached, irregular aggregates	Cryperseved porcine (5.7×10^9)	None	Plasma (50)	400	Yes	18%	18%
	LSS(MELS) (Charite, Humbolt Univ., Germany)		Tissue-like organoids	Porcine (600 g)	None (300 kD)	Plasma (31)	100–200	Yes	Not reported	Not reported

	BLSS (Excorp Medical, Inc.)		Collagen gel entrapped	Porcine (70–120 g)	Yes (100 kD)	Blood (NA)	100–250	No	33%	6%
Porous matrix systems	RFB-BAL (Univ. of Ferrara, Italy)		Aggregates	Porcine (200 g)	None	Plasma (22)	200–300	Yes	33%	11%
	AMC-BAL (Univ. of Amsterdam, Netherlands)		Small aggregates	Porcine (1.2×10^{10})	None	Plasma (40–50)	150	Yes	44%	31%

Source: Reproduced with permission from [104].

28 days of culture [28]. Two years later, the authors reported the same system with the primary osteoblasts and compared the results with static conditions. After 26 days of culture, the dynamic conditions enhanced osteoblast proliferation and matrix mineralization compared with static control conditions.

RWV bioreactors have been widely used for cartilage TE because they provide a favorable hydrodynamic environment for cartilage differentiation [24, 192–201] and skeletal muscle tissue regeneration [202–204]. A cell suspension of differentiated chondrocytes was seeded in an RWV without any scaffold support. After 90 days, the researchers obtained a 3D cartilage-like tissue encapsulated by fibrous tissue resembling the perichondrium [161]. In another study, Kobayashi *et al*. transplanted human cartilage progenitor cells (CPCs) into immunodeficient mice and obtained elastic cartilage reconstruction with the long-term morphological preservation [205, 206]. The same research group employed an RWV bioreactor to control the size and shape of a 3D elastic cartilage [207]. Human CPCs were seeded into a biodegradable, porous scaffold for 6 weeks. After the culture time, the cells differentiated into mature chondrocytes, and elastic fibers were present on the tissues. These results show that RWV bioreactors are useful tools to reconstruct articular tissues *in vitro*.

Marquette *et al*. developed a 3D skeletal muscle model from C2C12 myoblasts [202]. They cultured the myoblasts in a suspension without an exogenous matrix in an RWV system. The skeletal muscle myoblasts synthesized the ECM, an endogenous matrix that is more representative of *in vivo* conditions. Furthermore, the produced ECM induced the differentiation of C2C12 cells. After 12 h, they observed that the cells assembled into visible aggregates. Interestingly, the cell differentiation and fusion occurred without the use of a differentiation medium in the RWV system.

6.3.2.2 Spinner Flasks

Spinner-flask bioreactors have recently been used for TE and have shown promising results especially for bone TE applications. The cells can grow on fixed scaffolds, needles, or microcarriers [12, 208–215]. In general, the latter studies have shown that dynamic conditions had a positive effect on cell distribution and osteogenic differentiation. Recently, Teixeira *et al*. [216] used a multicompartment holder in spinner flasks for the osteogenic differentiation of human MSCs in 3D porous chitosan scaffolds. They observed that this novel and simple approach protected the cells from being removed by the turbulence generated by the stirring process. Furthermore, they observed that the dynamic conditions improved cell proliferation and increased the osteogenic differentiation and mineralization compared to static conditions after 14 days. Although these results are interesting, it would be more interesting to evaluate other scaffolds. In addition, the operating conditions need to be optimized.

For cartilage TE, spinner-flask bioreactors have been used to recreate the *in vivo* physicochemical conditions of cartilage development [16, 17, 217–226]. For example, Liu *et al*. [227] fabricated the trachea cartilage graft *in vitro* using rabbit MSCs and a poly(lactic-*co*-glycolic acid) (PLGA) scaffold in a spinner-flask bioreactor. The rabbit MSCs were prepared in a sheet form and wrapped around the PLGA scaffold and a glass rod to form the tubular structure. The scaffold was

cultured for 3 days under static conditions. After that, the glass rod was removed and the PLGA scaffold was cultured under dynamic conditions for 4 weeks. They observed that the PLGA scaffold completely degraded and the tubular graft contained mature and well-integrated cartilage tissue. However, the central area of the graft involved a fibrous tissue, showing the low mechanical properties of the graft.

As mentioned earlier, Bueno *et al*. modified the geometry of traditional spinner-flask bioreactors to reduce turbulent flow and developed the WWB [18, 19, 228, 229]. Recently, Yang *et al*. employed this kind of bioreactor to improve the mass transfer in the medium and provide mechanical stimuli for cartilage tissue generation [21]. The authors evaluated the effect of a solid fibrous capsule on the construct integration using a native articular cartilage. For this, they seeded the articular chondrocytes in the PGA scaffolds and cultured them with the growth factors IGF-1 or TGF-β1 for 28 days in a WWB system. The results showed that this system might encourage an early integration of the engineered and native cartilage tissues.

Monolayer expansion of tenocytes *in vitro* displays an altered phenotype compared to native tenocytes *in vivo*. In addition, differences in the gene expression profiles of monolayer tenocytes and native ones increase as the passage number increases [230]. For this reason, the use of spinner-flask bioreactors in tendon TE is an emergent approach to improve tendon repair because cells can continuously expand without passaging, and they provide a suitable basis for a continuous cell expansion without passaging the cells [231]. Stich *et al*. used a spinner-flask bioreactor to expand human hamstring tenocytes [14]. The authors seeded the tenocytes on Cytodex™ type 3 microcarriers and observed that the tenocytes adhered to the microcarriers, were alive, proliferated, and, after 2 weeks culture, showed higher glucose consumption and lactic acid formation. They demonstrated that it was possible to perform continuous tenocyte expansion without adding fresh microcarriers to the culture system.

Osteogenesis is higher when using spinner-flask bioreactors compared to when using RWVs. Rat MSCs were grown on biodegradable scaffolds under static and dynamic (spinner flask and RWV) conditions [11]. After 21 days of culture, the authors observed higher alkaline phosphatase (ALP) activity and osteocalcin secretion with the spinner-flask bioreactor compared to RWV. Furthermore, the cell proliferation rate and calcium content were enhanced. Recently, Wang *et al*. demonstrated a similar trend with human MSCs [12]. On the contrary, the RWV bioreactor promotes better cartilage growth and chondrocyte differentiation compared to other culturing techniques [232].

6.3.2.3 Perfusion Bioreactors

Perfusion bioreactors promote better *in vitro* osteogenesis differentiation compared to spinner-flask systems. For example, Goldstein *et al*. compared three different systems (perfusion bioreactor, spinner flask, and a rotary vessel) [160]. In their study, the osteoblastic marrow stromal cells were cultured in PLGA foam disks for 2 weeks in four different culture systems (static, spinner flask, RWV, and perfusion bioreactor). The researchers observed that cell seeding efficiencies were similar for all systems. However, the cell distributions in the RWV and

perfusion systems were more uniform compared to the other systems. The ALP activity was highest in the spinner flask and the perfusion bioreactor. Based on their results, the perfusion bioreactor seems an attractive culture system for bone TE. Osteoblastic differentiation is enhanced by the mechanical stimulation created by fluid shear forces within the perfusion system [233]. Also, numerous reports have demonstrated that fluid shear promotes *in vitro* osteogenesis [160, 234–241]. In addition, some studies have shown *in vivo* like bone formation of constructs cultivated in perfusion bioreactors [242–247].

HFBs were used for bone TE by Abdullah *et al*. [248, 249] and other researchers [53, 250–252]. For example, De Napoli *et al*. [251] studied the effect of Starling flow on sheep mesenchymal stem cells (shMSCs) that were grown on the extracapillar surface of the HFB. After 12 days culture, they observed that a low Starling flow did not promote cell proliferation and differentiation. Moreover, the cells formed thin layers that adhered to the outer surface of the fibers. Conversely, under high Starling flow, cell proliferation and differentiation were enhanced and the cells formed thick multilayer aggregates that filled the extracapillary space due to the higher transport of oxygen and nutrients to the cells.

Most studies on perfusion bioreactors have focused on the construction of small-sized, engineered bone tissues [243, 253–257]. Animal models were also developed to examine bone tissue repair [258–260] because the bone composition and physiology of pig, goat, sheep, and dog are similar to those of humans [261, 262]. For large-scale production of bone tissues, the commonly used bioreactors have some limitations; however, there are some perfusion systems that enable culturing large-size tissue constructs [263–268]. Recently, Gardel *et al*. [269] developed a bidirectional continuous perfusion bioreactor using starch poly(ϵ-caprolactone) (SPCL) fiber mesh scaffolds and then seeded goat marrow stromal cells in them. After 14 days of culture, the ALP activity was higher using the dynamic culture compared to static conditions. In addition, the cell distribution was more uniform. Scanning electron microscopy images showed that the cells proliferated into the core of the scaffolds in the dynamic culture, while they proliferated only on the surface of the scaffolds in the static cell culture.

Several research groups have assessed the influence of medium perfusion in cartilage and tendon TE both *in vitro* [270–278] and *in vivo* [279–281] conditions. The latter investigations demonstrated that flow perfusion enhances chondrocyte proliferation and ECM production for the production of large-size tissue constructs [271, 282–284]. However, several others have demonstrated that high shear stress altered the chondrocyte cell shape and alignment and even induced chondrocyte death via apoptosis [199, 285]. To protect cells from fluid flow and shear stresses in bioreactors, cell entrapment within hydrogel particles would be a practical solution. In addition, hydrogels provide a 3D environment mimicking *in vivo* conditions. Alginate is one the most frequently used biomaterials to prepare hydrogel scaffolds because it forms a gel that is biocompatible and biodegradable upon the cross-linking with calcium ions. The immobilized chondrocytes or stem cells in the alginate matrix were shown to retain their phenotype, proliferation, and chondrogenic differentiation [286, 287]. Several cell types have been successfully encapsulated in alginate sheets or microbeads [66, 224, 288–293]. For example, Osmokrović *et al*. cultivated murine bone MSCs in alginate microbeads

using a packed-bed bioreactor system [66]. After 5 weeks of cultivation, the authors observed that the cells remained viable and alginate microbeads retained their size and spherical shape. More recently, Gharravi *et al*. cultured chondrocytes in three different alginate scaffolds, namely beads, sheets, and a molded shape [288]. After 5 days in culture flasks, the scaffolds were transferred to the perfusion bioreactor for additional 3–5 days. The results showed that the cells on the sheet scaffolds presented a more homogeneous cell distribution than on the other scaffolds. However, there was no difference in cell viability between the alginate scaffolds. In addition, the histochemical and immunohistochemical staining methods showed that the alginate scaffolds secreted a new matrix that contained GAG and collagen, which enhanced chondrogenesis differentiation *in vitro*.

Recently, Dahlin *et al*. studied how the flow perfusion, stimulation with growth factors (TGF-β3), and coculture of MSCs affected the phenotype of bovine articular chondrocytes over 2 weeks of culture using electrospun poly(ε-caprolactone) (PCL) scaffolds [294]. They observed that the perfusion flow did not maintain the collagen II/I expression. However, when the TGF-β3 was added to the medium, the chondrogenesis was increased and the ECM obtained was more homogenous than that from static cultures.

In another study, Li *et al*. developed an acoustofluidic perfusion bioreactor for the cartilage TE [295]. This novel approach was used to fix chondral defects by combining ultrasonic cell trapping with a perfusion bioreactor. As mentioned earlier, in cartilage TE applications the perfusion bioreactor is frequently used because of their ability to create new cartilage tissue. A low-intensity pulsed ultrasound was shown to accelerate the repair of damaged cartilage in various studies [296–298]. After 21 days of culture in serum-free chondrogenic medium, the grafts were examined and it was observed that human articular chondrocytes maintained their viability. Histological examination and atomic force microscopy demonstrated that the new neocartilage grafts formed were similar to native hyaline cartilage. Then, the neocartilage grafts were implanted into the chondral defect and cocultured for 16 weeks *ex vivo*. The authors observed that the new hyaline cartilage-like tissues filled the defects, were totally connected to the host cartilage, and integrated with the surrounding native cartilage. These results show that this model can be a potential approach to engineer new cartilage grafts of human chondrocytes.

6.3.3 Bioreactors for Neural Tissue Engineering

Aging, injury, or the effects of disease of the nervous system can be combated by neural TE. RWV bioreactors have been widely used for the expansion, characterization, and neural differentiation of stem cells [299–307]. For example, Chen *et al*. demonstrated that simulated microgravity can enhance the differentiation of rat MSCs into neurons after 3 days of culture [308]. Lin *et al*. reported the proliferation and neural differentiation of stem cells on collagen-based carriers in a rotating wall bioreactor [25]. Ma *et al*. used collagen scaffolds to proliferate and differentiate neural stem and the progenitor cells into the neurons, astrocytes, and oligodendrocytes [309, 310].

Valmikinathan *et al*. [311] evaluated the impact of scaffold geometry (tubular, cylindrical, and spiral) on the rat Schwann cell behavior using an RW bioreactor. They observed that the new structure of the PCL-based spiral scaffolds, which is more open, enhanced the rate of cell proliferation and attachment after 4 days of culture, as compared to the static culture. In addition, when comparing cell proliferation under dynamic conditions between the different scaffolds, the authors observed that it was higher on spiral scaffolds because the open structure provided better influx of the medium into the scaffold. Based on these results, this new geometry seems to provide a short regeneration time for the grafts used in peripheral nerve regeneration.

Stirred bioreactors have also been employed to promote neural differentiation using microcarriers as scaffolds or in suspension culture (cell aggregates) [312–315]. For example, Liu *et al*. developed microcarriers of poly(ethylene terephthalate) (PET) to enhance the proliferation and neural differentiation of murine ESCs [316, 317]. The protocols for neural differentiation, including the formation a suspension of EBs and their subsequent replanting on ECM-coated surfaces, require highly skilled personnel and are difficult to scale up. Liu *et al*. [316] eliminated these steps by the addition of astrocyte-conditioned medium (ACM), thereby inducing directly neural differentiation. Moreover, they discovered that cells were protected from shear stress by the new microfibrous matrix, and high levels of nestin-positive cells, a neural stem marker, indicated the expansion and neural differentiation of ESC aggregates.

In another work, Baghbaderani *et al*. produced on a large scale human neural precursor cells (hNPCs) in suspension culture using a free growth medium within a spinner flask bioreactor (500 ml) [318]. The authors observed that the hNPCs formed aggregates after 10 days of culture and were positively stained for nestin and TUJ-1. In addition, they observed astrocytic and oligodendroglial phenotype differentiation.

Hollow fiber and perfusion systems have been widely used in the generation of *in vitro* models [73, 319, 320] and also in drug screening [136].The blood–brain barrier (BBB) acts as a defense barrier (both physical and metabolic) between systemic circulation and the central nervous system (CNS). HFBs have been developed to regenerate BBB *in vitro*. For example, Stanness *et al*. [321] developed an early model of BBB to culture endothelial cells (ECs) and glial cells from diverse sources (human, rat, and bovine) in an HFB coated with ProNectin. They observed consistent patterns of cell morphology and growth compared to those observed *in vivo*; however, the BBB model did not develop when EC and glial cells from different sources were used. In addition, in absence of glial cells, a low EC growth was observed within the fibers. The data obtained confirmed the use of HFBs to recreate BBBs for potential applications in pharmaceutical research. Neuhaus *et al*. utilized a commercial HFB system and seeded in the intraluminal space immortalized porcine brain microvascular endothelial cells (PBMECs/C1-2) and in the extracapillary space the glial cell line C6 cells [322]. The results showed that the flow exposure affected the growth and morphology of ECs, which led to longer cell survival within the HFB compared to the Transwell model.

A novel approach to treat Parkinson's disease is transplantation of dopamine neurons. However, one of the difficulties encountered in this approach is the collection of these neurons without damaging or losing the cells. To overcome these difficulties, Yamazoe *et al*. developed a new procedure where the undifferentiated ES cells were loaded into hollow fibers and grown in a PA6 conditioning medium and heparin to promote their differentiation into dopaminergic neurons [48]. The results showed that the cells grew and formed aggregates in the hollow fibers. In addition, supplementation with the PA6 conditioning medium increased the neural differentiation after 16 days in culture, but heparin addition showed no statistical significance. Using this model, dopaminergic neurons enclosed in the semipermeable hollow-fiber membranes were obtained without any loss of cells.

6.3.4 Bioreactors for Cardiovascular Tissue Engineering

Cardiac TE has emerged as a promising approach to repair damaged heart muscles and thereby cardiac regeneration. Three-dimensional culture of cells by seeding them into scaffolds in a bioreactor is a common approach in cardiac TE [323–326].

Spinner flask bioreactors are able to produce engineered cardiac tissues that are superior to those cultivated under static conditions [23, 117, 327–331]. However, these bioreactors may not be the optimal cultivation vessels for cardiac tissue because of the turbulence, which can be destructive for the seeded cardiac or stem cells. RWVs with slow rotation and gas exchange have also been used for the growth and differentiation of cardiac cells [332–336]. For example, Teo *et al*. encapsulated murine ESCs in an alginate hydrogel and cultured them in a novel perfused rotatory bioreactor for cardiac differentiation (Figure 6.3a) [335]. They observed that the cell proliferation rate and viability were higher in dynamic cultures (rotatory and perfused rotatory bioreactors) than in static cultures. In addition, the cells in the perfused rotatory bioreactor showed earlier gene expressions of cardiac troponin-T, α- and β-myosin heavy chains with higher percentages of cardiac troponin-I-positive cells, and more uniformity of sacromeric α-actinin expression compared to those the static reactors and rotary vessels (Figure 6.3b–d). Reichardt *et al*. used an RBS for the expansion of human umbilical cord artery cells (HUCACs) [30]. The measurement of glucose consumption rate showed that the cell proliferation was higher in RBS compared to that under static conditions. In addition, the HUCACs expressed more type I and III collagen, elastin, and fibronectin. It was demonstrated that the RBS was an appropriate device to achieve fast and automated expansion of HUCACs to engineer cardiovascular constructs.

Perfusion bioreactors have been widely used for *in vitro* cultivation of 3D cardiovascular tissues [337–344]. For example, Lu *et al*. constructed and validated a bioreactor system for simulating the cardiac niche. Their system was able to provide electromechanical stimulation and a continuous perfusion to culture human umbilical vein endothelial cells (HUVECs), adult rat ventricular myocytes, and human atrial fibroblasts [345]. The results from deformation analysis showed that the cell distribution across the surface was homogeneous. After 24 h of mechanical stimulation, fibroblasts were perpendicularly aligned. In

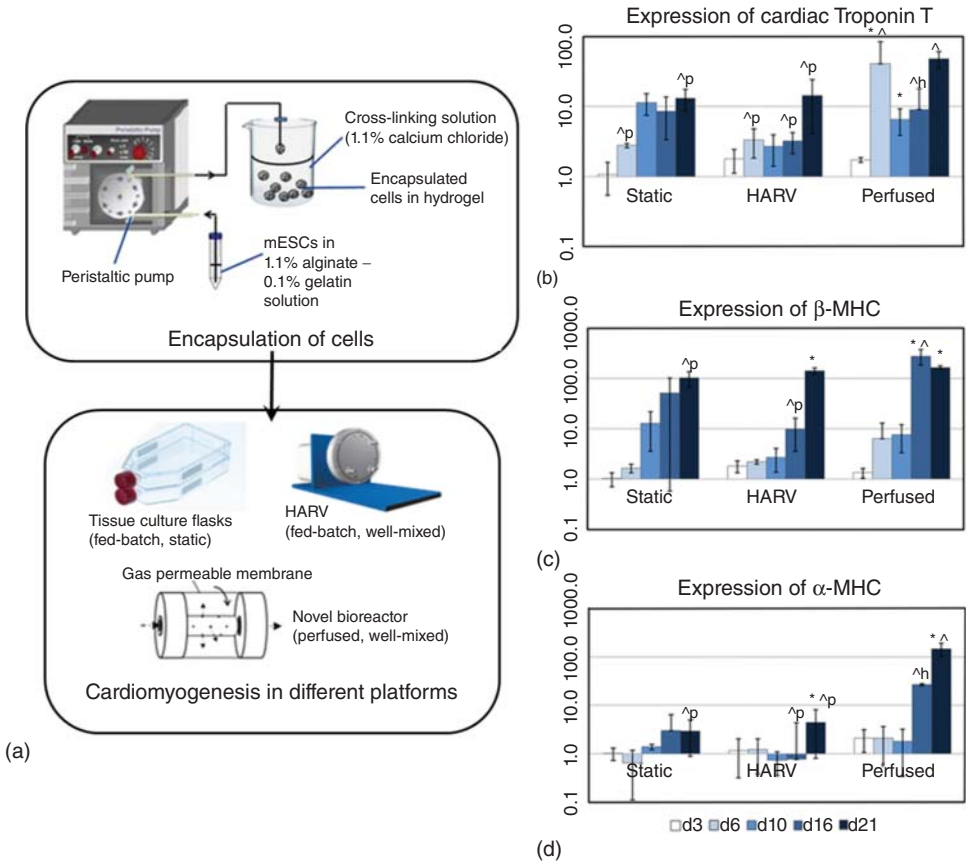


Figure 6.3 (a) Encapsulation process of murine ESCs in alginate hydrogel and their transfer to three different culture platforms for cardiogenesis: static culture (tissue culture flask), high-aspect rotating vessel (HARV), and perfused rotatory bioreactor. Fold changes in the expression of late cardiac markers (b) cardiac troponin-T (cTnT), (c) β-myosin heavy chains (β-MHC), and (d) α-myosin heavy chains (α-MHC) during the culture period ($N = 2$). Earlier and higher expressions of these cardiac markers imply an improvement of cardiomyogenesis in the encapsulated ESCs. (Wang 2009 [12]. Reproduced with permission of John Wiley & Sons.)

addition, electrical stimulation induced rhythmic contractions in the ventricular myocytes, with similar characteristics as when a standard field stimulation chamber is used.

Perfusion bioreactors were also developed for *in vitro* drug testing on cardiac constructs [346, 347]. For example, Xiao *et al*. fabricated a cardiac biowire bioreactor using both primary neonatal rat cardiomyocytes and human ESC-derived cardiomyocytes [348]. The biowires were made with a template of polytetrafluoroethylene (PTFE) tubing and the type I collagen. With this system, the authors generated different geometries for cardiac biowires. Besides, they integrated an electrical stimulator to enhance the phenotype of cardiomyocytes using carbon rod electrodes, which resulted in high Young's modulus and better electrical properties. After 3 and 4 days of post seeding, the neonatal cardiac biowires

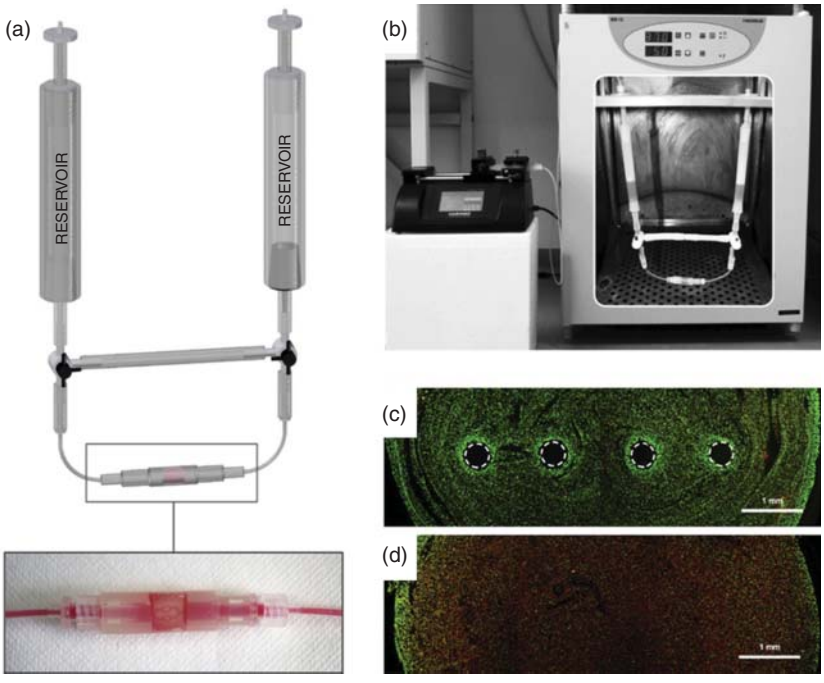


Figure 6.4 (a) Scheme and illustration of the perfusion bioreactor. (b) Image of the bioreactor system in the incubator connected to the syringe pump. The bioreactor was suspended vertically in the incubator to avoid cells from settling on the surface of the reservoir. Cross-sectional image montages of cell-laden gels with (c) and without fluidic channels (d). Live/dead fluorescence assay showed that viability was significantly higher in perfused microfluidic hydrogel (c) than in control (d). (Nold 2013 [250]. Reproduced with permission of Elsevier.)

spontaneously started to beat, and immunohistochemistry staining showed that the cells adhered to the surface of the template and expressed the cTnT protein.

One of the main problems faced is the lack of vascularization on the engineered constructs. Tocchio *et al*. worked on a novel method for controlled fabrication of robust vascular architecture in large and porous scaffolds using poly(vinyl alcohol) as a sacrificial template [349]. The novel bioreactor was inspired by the U-tube model and consisted of three parts: the input reservoir, the scaffold chamber, and the output reservoir (Figure 6.4). To demonstrate the effectiveness of the adopted approach in engineering vascularized thick tissues, the fabricated microfluidic scaffolds were successfully integrated with a 3D cell culture bioreactor and seeded with NIH-3T3 murine embryonic fibroblasts and HUVECs. They observed that this approach enabled the production of hierarchically branched endothelium and prevented the formation of necrotic tissue.

6.3.5 Bioreactors for Bladder, Uterine, and Cornea Tissue Engineering

Urethral defects resulting from urinary injury, tumor resection, infection, or malformation are painful urological diseases. TE is an interesting approach

for obtaining satisfactory urethral tissues that could be implanted in patients, without using the patient's own skin such as genital skin, bladder mucosa, or oral mucosa. Two studies have been described on the use of perfusion bioreactors to generate bladder tissue. In the work of Wallis *et al*. [350], the design and fabrication of a perfused bioreactor was to simulate the normal urinary bladder dynamics. The bioreactor was filled with an acellular porcine bladder matrix as the scaffold, and different mechanical properties were tested to simulate the urine flow in the system. Only porcine cells were used in this work. In a more recent approach, Wang *et al*. [351] used adipose-derived stem cells (ADSCs) from the dogs and polyglycolic acid as the scaffold for constructing muscular tubes of urethra in a perfused bioreactor. The use of stem cells and their differentiation in the bioreactor form an interesting approach toward obtaining human muscle tissues from pluripotent cells. However, the mechanical perfusion system is simple and lacks some interesting ideas of the previous work.

Malformations of the uterus are related to female infertility, and the uterus also an interesting organ target to study the proliferation of cancer. In this regard, TE can suggest solutions for both the generation of tissues and organs for therapeutic transplantation and investigation of cancer cells *in vitro*. As the first example of TE applications, Miyazaki *et al*. [352] used the decellularized uterine matrix from rat as the scaffold and seeded it with the rat uterine cells. The scaffold was then introduced into a bioreactor and was connected to the perfusion system. As a result, the uterine-like tissues were regenerated, which were maintained *in vitro* for up to 10 days. Furthermore, the uterine-like tissues were transplanted into the rats with a partially excised uterus. The regeneration of uterine tissues was almost complete, and achievement of pregnancy was almost comparable with that in rats with an intact uterus. As the second example of TE applications, Jaeger *et al*. [353] used a complex bioreactor to understand the effects of gas and nutrient transportation from the vessels to the ovarian cancer cells. They attempted to mimic the complex features of the *in vivo* tumor microenvironment in an *in vivo* system using porous silicones in a multilayered bioreactor. The bioreactor system was designed for mounting a micropatterned silicone hydrogel membrane between two sealed chambers and Matrigel as the scaffold for the cancer cells. The gas and the medium were introduced into the bioreactor through perfusion.

The next goal of tissue engineering comes from the need for vascularized organs and tissues *in vitro*. The thickness limit reached until now of engineered tissues is down to ~300 μm, which is the limit of diffusion of nutrients and waste products in a tissue without vascularization. There are some exceptions and some tissues do not need vascularization and therefore they can be studied and developed under static conditions. For such tissues, bioreactors with high mechanical features and complexity are not required. One of these tissues is the cornea. For example, the engineered cornea tissues were developed by Orwin *et al*. [354] in a static bioreactor, testing different materials as scaffold for the cells. In another work [355], equibiaxial and uniaxial strains and shear stress were incorporated to a static bioreactor. There have been a few attempts to engineer cornea tissues in perfused or stirred bioreactors. One of them is the work of Kang *et al*. [356], in which a perfused bioreactor was employed

to produce a biomimetic environment for limbal epithelial stem cells and to maintain their stemness *in vitro*.

6.4 Summary and Future Perspectives

The future of the TE research would involve the close collaboration and integration of different research fields such as physics, chemistry, biology, and medicine. The emerging groups of research in TE incorporate this knowledge to their own background. Fabrication and design of new bioreactors are the logical conclusion of this collaboration between experts in nanotechnology, biomaterials, microfluidics, and stem cells research. At present, TE research is being carried out with conventional bioreactor systems adapted to new cells lines and cocultures, to promote different differentiation pathways, to incorporate new scaffolds systems and biomaterials, and in general to produce tissues under *in vivo* like conditions. Much of the recent bioreactors research in TE field has been done for the production of reparative tissues for transplantation, such as vascular substitutes, skin, or cartilage. Moreover, bioreactors are also being investigated for use as short-term extracorporeal cell-based therapies such as the bioartificial liver or kidney. In the other hand, miniaturized bioreactors or microfluidic bioreactors have also been adapted for organ-on-a-chip research for either drug discovery or tissue functionality studies. Both lines of application introduce new research challenges; scalability of the production and standardization of the processes should be the next goal in the design and development of a new generation of bioreactors, especially because now is time to incorporate this promising technology into industry and bring TE approaches out from the lab bench to hospitals and pharmaceuticals and make them clinically accessible on a larger scale.

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Abbreviations

ACM	astrocyte-conditioned medium
BAL	bioartificial liver
BBB	blood–brain barrier
BMSC	bone marrow stromal cell
EBs	embryoid bodies
EBAL	extracorporeal bioartificial liver
EC	endothelial cell
ECM	extracellular matrix
ESC	embryonic stem cell

GAG	glycosaminoglycan
HFB	hollow-fiber bioreactor
hpMSC	human placental mesenchymal stem cell
HSC	hematopoietic stem cell
iPSC	induced pluripotent stem cell
MSC	mesenchymal stem cell
PBR	fixed or packed-bed bioreactor
PCL	poly(ϵ-caprolactone)
PES	polyethersulfone
PET	poly(ethylene terephthalate)
PGA	poly(glycolic acid)
PLGA	poly(lactic-*co*-glycolic acid)
PLLA	poly-L-lactic acid
ratMAPC	rat multipotent adult progenitor cell
RBS	rotating bed system
RWV	rotating wall vessel
TCP	tissue culture polystyrene
TE	tissue engineering
WWB	wavy-walled bioreactors

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Part II

Applications

7

Tissue-Engineered Human Skin Equivalents and Their Applications in Wound Healing

*Lara Yildirimer, Divia Hobson, Zhi Yuan (William) Lin, Wenguo Cui, and Xin Zhao**

7.1 Introduction

The skin is an essential organ as it protects internal organs, enables sensation, and allows absorption, excretion, and expression, as well as plays a critical role in maintaining body temperature and metabolism [1]. It is comprised of three layers, namely the epidermis, dermis, and hypodermis (Figure 7.1) [2–4]. The epidermis is the outermost layer and made of predominantly keratinocytes; other cell types are also found such as Langerhans cells and melanocytes [3, 5], which protect the body against infection and moisture loss. Immediately below the epidermis is the dermis, which contributes to the elastic nature and mechanical properties of skin. The dermis contains vascularized extracellular matrix (ECM) in which collagen, elastin, and glycosaminoglycans are abundant [3, 4, 6]. Cell types found in the dermis are fibroblasts, endothelial cells, smooth muscle cells, and mast cells [3, 6]. The hypodermis is situated beneath the dermis and acts as an energy source. The main components of the hypodermis are adipose tissue and collagen [2, 3, 5].

Any kind of failure in skin integrity can interfere with its ability to function, result in infection, and cause pain and discomfort. The healing of a large area of an adult skin wound is considered a highly complicated biological process that requires the synergistic functions of various cell types, ECM, as well as extracellular and intracellular signaling [7, 8]. Regeneration of perfect skin with full skin functions (protection, regulation, and sensation) is challenging and has recently become a major aim in wound healing [9].

Skin grafting is one of the most promising approaches to heal extensive wounds [10]. In skin transplantation, autografting represents the gold standard. However, autografts are not always available, particularly in burn patients. Cadaveric allografts are the next best option, even though these mostly serve only as temporary wound coverings to allow native wound healing. However, allografts are not commonly available due to the longer time periods needed for harvesting skin donations as compared to other organs, as well as concerns of lasting defect [11]. Xenografts from frog skin, bovine, and porcine sources have also been used

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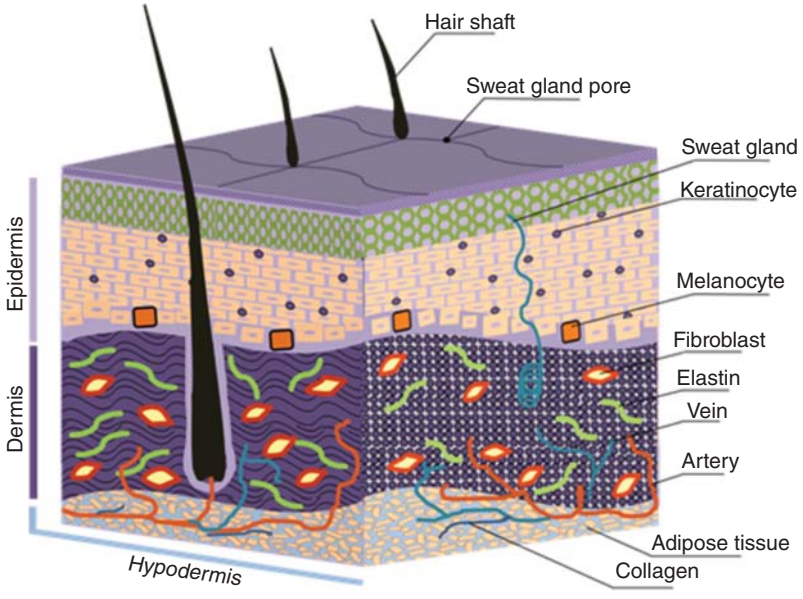

Figure 7.1 Skin structure.

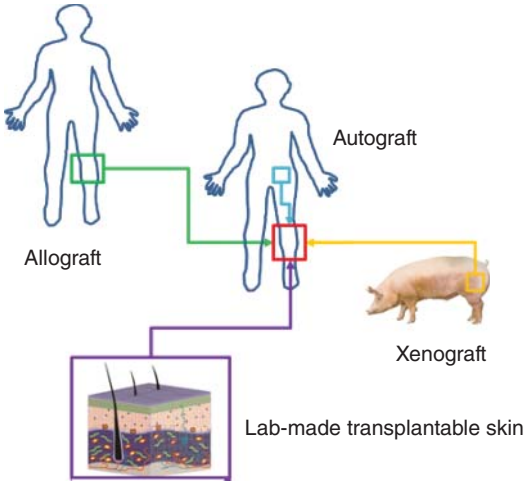

Figure 7.2 Types of different skin grafts.

clinically, but immunological issues and risks of pathogen transfer exist [12, 13]. Collectively, there is clearly an acute shortage of transplantable skin and overwhelming demand for alternative sources. Types of different skin grafts are summarized in Figure 7.2.

When tissue engineering was formally introduced in the late 1980s, possibilities of growing transplantable tissues in the laboratory [14] and developing off-the-shelf, tissue-engineered skin substitutes (TESs) became an attractive solution to treat acute and chronic cutaneous wounds. The common method in skin tissue engineering involves seeding a biodegradable matrix or scaffold with

cells, typically epidermal keratinocytes, dermal fibroblasts, or stem cells. The 3D matrix/scaffold provides a supportive environment for skin cell growth and is typically comprised of natural biomacromolecules including type I collagen, glycosaminoglycan, and chitosan, or synthetic polymers such as lactide-based aliphatic polyesters, glycolide, and caprolactone [15]. ECM is produced by cells as they proliferate and differentiate. As the matrix/scaffold degrades, it is gradually replaced by cells and ECM, which ultimately leads to the formation of the functional skin [14].

Successful isolation and cultivation of human epidermal keratinocytes were first reported in 1975 [16], and since then TES has evolved from simple epidermal substitutes to complex full-thickness skin with various appendages (Figure 7.3a) [5]. In a full-thickness skin, the upper thin epidermal keratinocyte layer provides protection by inhibiting infection and fluid loss. A much thicker dermal layer underneath the epidermal layer forms the basal body of TES to enhance mechanical properties and is comprised of dermal fibroblasts and ECM proteins. A pre-vascularized layer is positioned underneath the dermal layer in order to increase the chance of TES survival when applied into wounds. Skin appendages are implanted in order to mimic the appearance and functioning of normal skin. For example, melanocytes are implanted into the epidermal layer for pigmentation and hair follicles into the dermal layer for sensation.

In recent years, significant progress has been made in developing highly complex TESs that closely resemble natural skin by improving their durability,

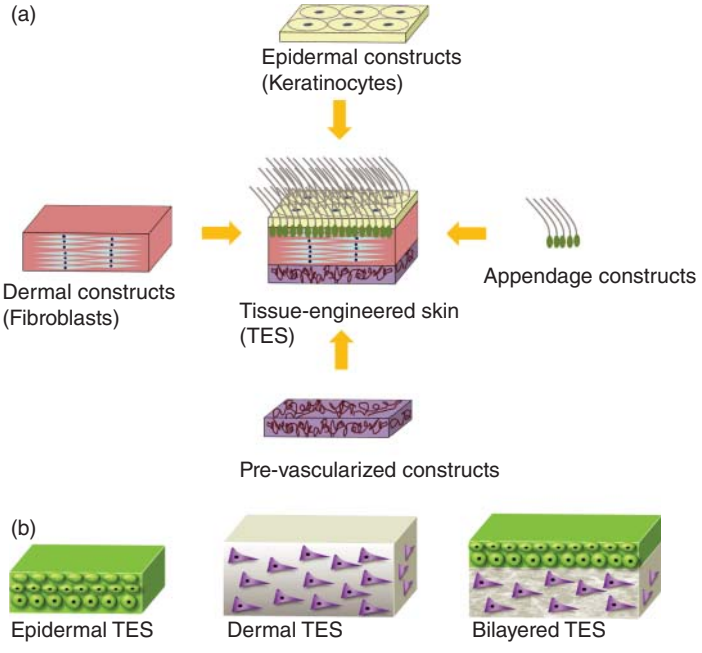

Figure 7.3 (a) Schematic illustration of full-thickness TES (Bottcher-Haberzeth 2010 [5]. Reproduced with permission of Elsevier.) . (b) Structure of epidermal, dermal, and bilayered TES.

elasticity, biocompatibility, functionality, and clinical efficacy. We introduce in this chapter the major milestones in TES development as well as their applications in wound healing.

7.2 Development of Tissue-Engineered Human Skin Equivalents

TESs can generally be categorized as (i) epidermal, (ii) dermal, or (iii) full-thickness models (Figure 3b). The technology for epidermal replacement (a confluent epithelial cell layer attached to a petroleum gauze carrier) was developed in the 1970s. Unfortunately, the rate of epidermal engraftment was less than ideal and even successful cases demonstrated poor durability and weak epithelium [17]. It was recently shown that the replacement of connective tissue could enhance the mechanical properties of healed skin [18, 19]. Consequently, dermal substitutes have been created to incorporate dermal fibroblasts into collagen scaffolds [20–22]. Since both the epidermis and dermis are essential for normal skin function and appearance, it is reasonable to replace both elements at the same time. Experience has demonstrated this to be true, as cellular interactions between dermal and epidermal elements improve epithelium maturation [23]. Bilayered TES (epidermal + dermal layer) are developed as a result, which permit the reconstruction of full-thickness skin, unlike either individual single-layered substitute. Advances in skin tissue engineering have further resulted in highly sophisticated TESs with very similar architecture and function as their natural counterparts, for example, full-thickness skin implemented with hair follicles [24, 25], a capillary network [26, 27], sensory innervation [28], adipose tissue [29], and pigment production [25, 30]. In the following sections, we give detailed explanations of each type of TESs, commercial or laboratory-engineered.

7.2.1 Epidermal Models

The epidermis is considered the outermost component of the skin and primarily comprised of keratinocytes, a specific type of epithelial cells. Application of an epidermal layer provides early reestablishment of a functional barrier, which is vital in the prevention of excessive transepidermal water loss and infection [28]. Moreover, re-epithelialization is crucial in the healing of cutaneous wounds, as it precedes repair in the dermis and accelerates the process of wound healing [29, 31]. In order to produce an epidermal skin replacement, the epidermis is usually separated from a skin biopsy, 2–5 cm^2 in size, and keratinocytes are subsequently cultured on fibroblasts [32]. Epidermal TESs are sold by a number of companies such as Genzyme's Epicel® (Cambridge, MA, USA). Epicel® is intended for grafts of burn wounds and consists of cultured epithelium using autologous epidermal cells. Laserskin® (Fidia Advanced Biopolymers Srl, Italy), is another epidermal TES model designated for the treatment of deep second-degree

burns and chronic ulcers. TES is comprised of a benzyl esterified hyaluronic acid derivative that forms a biodegradable matrix and is laser-perforated with microholes intended for autologous keratinocytes to grow inwards and proliferate [33].

7.2.2 Dermal Models

Dermal substitutes have also been created in addition to epidermal substitutes. Dermal skin substitutes provide higher mechanical stability and prevent wound contraction. They consist of collagen- and fibril-containing loose connective tissue that firmly secure the dermis to the epidermis [34]. Moreover, these substitutes are populated with fibroblasts and macrophages, which enable interactions between the dermis and epidermis and trigger synthesis of ECM components as well as keratinocyte cell growth and differentiation.

To date, there are many commercially available dermal wound products. For example, Transcyte® is a non-living wound dressing produced by Advanced Tissue Sciences, Inc. (La Jolla, CA, USA), which cryopreserves human dermal fibroblasts on a polymeric scaffold and has been established as a viable temporary wound dressing for excised burn wounds [35]. Dermagraft® is a derivative of Transcyte® made by Advanced Biohealing, and has demonstrated the potential for treating diabetic foot ulcers (DFU) [36]. It is manufactured by culturing fibroblasts isolated and expanded from human neonatal foreskin on a biodegradable polygalactin mesh. The produced dermal substitute is a 3D matrix containing human proteins created over a 3-week culturing period during which matrix proteins, such as human dermal collagen, and other soluble factors are secreted [32]. Integra® Dermal Regeneration Template (Intergra® DRT, Integra Life Sciences Corp., Plainsboro, NJ, USA) is a commercial composite skin graft consisting of a thin silicone film outer layer and an inner layer of complex cross-linked fiber matrix. After dermal layer regeneration, the silicone film can be replaced with an epidermal graft. Integra® DRT has shown promising results in burn wound treatment [37].

Nevertheless, the currently available single-layer skin substitutes (epidermal or dermal) all face various drawbacks since a skin layer made of only keratinocytes cannot fulfill functional requirements of fibroblasts, and vice versa. For instance, the fragile nature of Epicel® leads to instability without mechanical support from dermal substitute, whereas Intergra® is susceptible to infection due to the absence of stratum corneum from epidermal layer, which helps fend off foreign invasions [1]. In order to tackle these problems, bilayered skin substitutes with anatomical and functional resemblance to normal skin have been developed.

7.2.3 Bilayered Models

Epidermis and dermis are combined to form a bilayer structure in order to develop a full-thickness TES model, and keratinocytes and fibroblasts, either autologous or allogeneic, are employed in such processes [38]. This strategy,

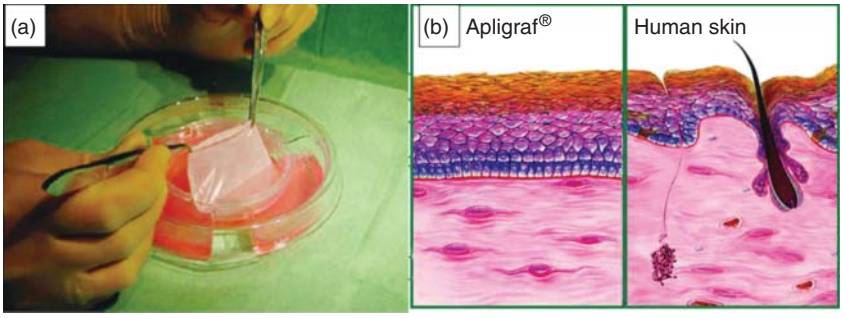

Figure 7.4 (a) Apligraf® being lifted with two smooth forceps, which will then be applied directly into a wound bed to help wound healing. (b) Comparison of Apligraf® and natural human skin. Apligraf® exhibits comparable structures to the natural human skin. (Zhong 2010 [42]. Reproduced with permission of John Wiley & Sons.)

apart from improving mechanical properties of the construct, is mainly intended for combining the roles of keratinocytes and fibroblasts in the wound-healing process, as they function in synergy to recruit cells that are necessary for complete wound closure and bestow unique properties that are vital for tissue homeostasis and wound healing [39]. Interactions between fibroblasts and keratinocytes have resulted in optimal keratinocyte proliferation and differentiation, where keratinocytes showed close resemblance to natural epidermis [40].

An early example of full-thickness TES was developed using a collagen lattice laced with fibroblasts and covered with epidermal cells [41]. Because of this breakthrough contribution, Organogenesis (Canton, MA, USA) became a pioneering company in the field of tissue engineering. Organogensis later established a living-tissue-engineered, bilayered skin model named Apligraf® by seeding keratinocytes in allogeneic skin fibroblasts, sourced from human foreskins, and soluble type I bovine collagen gel (see Apligraf® in Figure 7.4). Apligraf® has been implemented in the treatment of surgical wounds [43] and venous ulcers [44]. PermaDerm® (Regenicin, Inc.) is a promising new product with keratinocytes and fibroblasts seeded onto a collagen sponge that can indefinitely cover large skin burns and injuries [31]. In a more recent study, Zonari *et al.* fabricated a bilayered poly(hydroxybutyrate-*co*-hydroxyvalerate) (PHBV) construct with a 2D thin nanoporous membrane on top and a 3D porous scaffold at the bottom (Figure 7.5) [23]. The thin, nanoporous membrane mimicked the epidermis, while the scaffold simulated the dermis. Results showed minimal water loss, suitable mechanical properties, and desired susceptibility to enzymatic degradation. In addition, when cocultured with human fibroblasts, the seeded human keratinocytes differentiated and rearranged to form a multilayered structure as opposed to monolayer in the homotypic case, proving the construct to be a promising autologous skin graft.

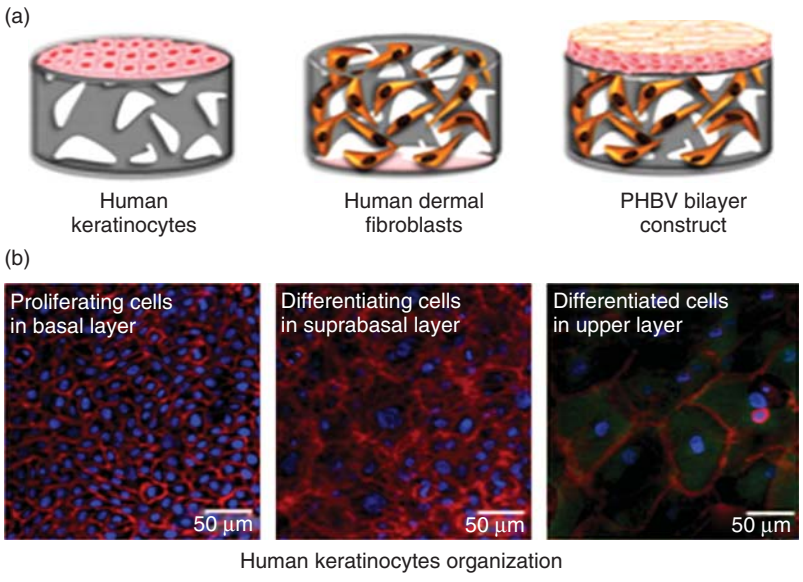

Figure 7.5 (a) Schematic of bilayered poly(hydroxybutyrate-*co*-hydroxyvalerate) skin construct seeded with human keratinocytes in the epidermal layer and fibroblasts in the dermal layer. (b) The keratinocytes grew and rearranged in a multilayer structure with proliferative cells in the basal layer and differentiated cells in the upper layer. (Zonari 2014 [23]. Reproduced with permission of John Wiley & Sons.)

7.2.4 Multifunctional Skin Models

Recent progress in skin biology has resulted in highly sophisticated and innovative approaches to reconstruct 3D skin equivalents with architecture and function very similar to those of their natural counterparts. The bilayered epidermal–dermal TESs have been implemented with various appendages, including hair follicles [24, 25], a capillary network [26, 27], sensory innervation [28], adipose tissue [29], and pigment production [25, 30].

For example, to develop a bilayered TES with hair follicles, Atac *et al.* fabricated a dynamically perfused chip-based bioreactor platform that was able to apply varying mechanical shear stress and significantly prolong culture periods [24]. This has resulted in ameliorated culture conditions that benefit numerous factors including biopsies of single hair follicular units. Overall, notable hair-fiber elongation from the epidermis was observed in the hair follicle cultures in the chip. These experimental findings at the miniature scale are promising indicators for greater skin emulation in engineered equivalents and hair follicle biology, as the hair follicle plays a role in skin metabolism and contains several stem cell lineages with regenerative capacity.

In addition, as is known, insufficient vascularization is a major threat for the clinical use of TES, as it can cause the TES to loosen, become susceptible to

infection, or experience partial necrosis. To circumvent this problem, Liu *et al.* fabricated a bilayered TES with capillary network by coculturing dermal fibroblasts with dermal microvascular endothelial cells [26]. Interactions of the fibroblasts with the endothelial cells formed a fibrous sheet, and after a 20-day period of coculture, capillary-like structures were observed. In order to build a bilayered tissue, epithelial cells were seeded on the fibrous sheet. Immunostaining demonstrated that epithelium promoted the formation of structures reminiscent of capillary. These structures were confirmed to be typical microblood vessels through transmission electron microscopy (TEM). The resultant blood vessels were believed to supply the cells in tissue graft center with nutrients and oxygen after implantation with improved clinical efficacy.

Moreover, the nerve component of the skin accountable for pain, temperature, and sensory perception can be destroyed by burns. Patients that receive standard bilayered TESs often suffer from reduced discriminative sensibility, hyperesthesia, and dysesthesia. To rescue this, Blais *et al.* integrated neurolemmocytes, which are also known as Schwann cells and are the principal glia of the peripheral nervous system, in reconstructed skin (RS) [28]. Specifically, Blais *et al.* demonstrated that Schwann cells stimulated an increase in the number of sensory neurites migrating in the 3D tissues by twofold as compared to the control with no Schwann cells. Moreover, through TEM, Schwann cells were found to colocalize along with neurites and to achieve *in vitro* myelin sheath formation. The effect of incorporating Schwann cells into *in vivo* nerve regeneration and function recovery was also studied through the transplantation of developed TES athymic mice. The researchers verified that the Schwann cells would survive the 25-day maturation period for the RS and that the addition of Schwann cells induced 1.81- and 1.71-fold increase in the amount of nerve fibers migrating in the graft at 60 and 90 days after the transplant, respectively. Such TES could be adopted as an effective strategy for improving nerve regeneration in wound healing [28].

Furthermore, absence of a subcutaneous fat layer in TESs may result in uncharacteristic mechanical and thermoregulatory properties as compared to normal skin. To address these issues, Monfort *et al.* fabricated a plasma-based trilayer TES, containing an epidermis, dermis, and hypodermis, that could generate a transplantable cell sheet [29]. Bone-marrow-derived mesenchymal stem cells (BM-MSCs) or adipose-tissue-derived stromal cells in a human plasma hydrogel were incorporated in the trilayer model and kept open to adipogenic clues for a 3-week period. Occasional feeding of fresh keratinocyte complete medium allowed continued viability of *in vitro* engineered adipocytes within the medium. The adipocytes were found to survive for a 2-year period and ~50% of the cells differentiated into mature adipocytes [29].

Another current challenge in skin grafts is the absence of pigmentation, which leads to noticeable cosmetic implications. Pigmented TESs may solve these problems through the incorporation of hair follicle melanocytes. Liu *et al.* isolated hair follicle melanocytes and keratinocytes from the human scalp and seeded them onto a chitosan–gelatin membrane to produce pigmented TESs [30]. The produced constructs were successively used to resurface skin defects in nude mice and found to be successfully restored after 4 weeks by observing a complete

epidermis structure and cuticular layer in the restored skin. This study demonstrates a successful example of using hair follicle cell sources to fabricate pigmented TES. The developed pigmented TES can find applications in the treatment of depigmentation diseases, skin deficiencies, and skin lacking pigmentation ability [30].

One more monumental achievement in TES development is the understanding of the role played by mesenchymal stem cells (MSCs) in skin regeneration. Besides their potential in morphogenesis, recent evidence suggests that BM-MSCs work in synergy with epidermal stem cells to accelerate re-epithelialization and have higher therapeutic potential in inducing formations of blood vessels and hair follicles than solely epidermal stem cells [45]. Similar to BM-MSCs, adipose-tissue-derived stem cells (ADSCs) can also differentiate into a number of skin cell types that can contribute to the wound-healing process [46]. One recent study in a bilayered TES system demonstrated that a mixture of dermal fibroblasts and ADSCs (1 : 1 ratio) had a better performance compared to that of each individual cell type in inducing keratinocyte proliferation and differentiation [47].

The aforementioned examples show promising progress in developing sophisticated TESs with architecture and function similar to those of natural skin with appropriate combinations of various cell types. Other factors associated with TESs to aid wound healing include delivery of beneficial growth factors and cytokines, which trigger proliferation of cells and synthesis of new matrix at the wound bed. The growth factors that have attracted significant attention include the epidermal growth factors (EGFs), transforming growth factors β (TGF-β), fibroblast growth factors (FGFs), vascular endothelial growth factor (VEGF), granulocyte macrophage colony-stimulating factors (GM-CSFs), platelet-derived growth factor (PDGF), and connective tissue growth factors (CTGFs) [48]. During the inflammatory phase of wound healing, upregulation of pro-inflammatory cytokines (e.g., interleukin (IL) IL-1 and IL-6, and tumor necrosis factors-alpha (TNF-α)) is observed [48]. For instance, IL-1 is secreted by monocytes, macrophages, and neutrophils. IL-1 is also produced by keratinocytes to aid wound healing. In addition to its paracrine effect, IL-1 also functions in an autocrine manner to facilitate keratinocyte migration and proliferation [48]. Barrientos *et al.* have made a summary of growth factors and cytokines involved in the wound-healing process [48]. *In vitro* cell culture bioreactor is another feasible tool to facilitate cell proliferation and differentiation in TES due to its ability to trigger biochemical and mechanical cues as well as to regulate tissue development [49]. There are three main components in bioreactors: (i) metabolically active cells that have different phenotypes and produce ECM; (ii) polymeric scaffolds that provide favorable 3D matrices for cell adhesion, tissue growth, nutrient/oxygen/waste exchange, as well as biological and mechanical stimuli; and (iii) an environment that simulates *in vivo* conditions in which cells and polymers form complexes which subsequently develops into natural skin-mimicking tissues [50].

With the evolving resemblance to native human skin, the developed TESs are expected to have improved durability, elasticity, biocompatibility, functionality, and clinical efficacy when employed to aid wound healing. In the following

sections, we will discuss the recent progress of clinical applications of developed TESs in wound healing and the uses of different TES models *in vivo* and *in vitro* to study the wound-healing process.

7.3 Application of TESs in Wound Healing

The development of RS models that bear greater resemblance to normal skin leads to success in burn and chronic wound treatments as higher graft take rates are achieved. This development also enables the creation of a variety of products including vascularized, pigmented, and adipose RSs, and promotes laboratory research and applications in the skin cosmetics and pharmaceutical industries.

The following paragraphs will explore how the developed TESs translate to facilitate cutaneous wound healing. The focus will be on clinical applications as well as *in vivo* animal models thereof. Toward the end, *in vitro* wound-healing models will be touched upon in an effort to illustrate how improving our fundamental understanding of the wound-healing cascade may guide more sophisticated and targeted approaches to wound repair and regeneration.

7.3.1 Clinical Wound-Healing Applications

7.3.1.1 Epidermal Skin Regeneration

Being a source of constantly renewing keratinocytes, the regenerative capacities of epithelial populations have fast garnered popularity as demonstrated by several commercialized epidermal substitutes including Epicel® and Laserskin®. Epidermis-derived keratinocytes cultured into monolayers have previously been shown to successfully regenerate an epidermis over full-thickness wounds [51–53]. Similar results have been found in clinical trials where the use of carrier dressings seeded with autologous keratinocytes improved the healing rates of DFU [54].

The commercialization of several epidermal skin grafts has provided realistic and clinically viable alternatives for extensive burn wound coverage. For example, Epicel® (Genzyme Biosurgery, Cambridge, MA, USA) consists of keratinocytes obtained from a single, small, full-thickness biopsy obtained from the wounded individual. Coculturing with irradiated murine fibroblast feeder layers achieves a stratified epidermis, which is transferred to the patient via a petrolatum gauze backing. Clinical feasibility was demonstrated in 22 extensively burned children; compared to conventional treatment consisting of meshed split-thickness autografts, cultured epithelial autografts (CEA) demonstrated an organized, fully differentiated epidermis with rete ridges and a vascularized neodermis 6–12 months after application. Average initial and final engraftment rates were acceptable at 79% and 84%, respectively [55]. A retrospective review of 30 patients with large-surface-area burns (mean of 78%) demonstrated an even higher initial survival rate of CEA at 90% [56]. This allowed a significantly smaller need for autologous skin harvesting, which is particularly important when healthy skin is scarce.

Laserskin® (Fidia Advanced Biopolymers Srl, Italy) is another commercially available CEA product, which is intended for either deep second-degree burns

or chronic ulcer treatments. A pilot noncontrolled study of 14 patients with nonhealing foot ulcers secondary to type 2 diabetes mellitus treated with CEA revealed complete healing of 11 lesions with an average healing time of 41 days [32]. Despite the lack of control, nonrandomization, and small sample numbers, these results suggest that CEA may be effective in accelerating the treatment of DFU. The main advantage of CEA is its immunological safety profile, as only autologous keratinocytes are transferred to the wound. On the other hand, high cost, short shelf-life, delicacy, and time spent on mandatory custom preparation are some of the many disadvantages. It is primarily intended for burn treatment with high body surface area because of the difficulty of obtaining CEA, but has yet to find extensive applications in chronic wound settings. More extensive controlled clinical studies are required to assess its efficacy as well as superiority over conventional treatment.

7.3.1.2 Dermal Substitutes

Besides the previously mentioned disadvantages of CEA, one major limitation remains, namely the lack of a dermal component that is frequently associated with wound contraction and poor graft vascularization, resulting in delayed or incomplete healing. Dermagraft® (Smith & Nephew, Inc., Largo, FL, USA) is a cryopreserved human fibroblast-derived dermal substitute approved for the treatment of chronic (>6 weeks) DFU. Fibroblasts isolated from human neonatal foreskin are cultured on a bioabsorbable polygalactin mesh, resulting in the formation of a 3D matrix suitable as a dermal replacement [57]. Earlier studies had indicated fast incorporation and vascularization into the wound bed [22, 58]. Interestingly, multiple applications of Dermagraft® onto nonhealing DFU over several weeks resulted in a cumulative effect and faster wound closure compared to conventional treatment with gauze [36]. Weekly treatment was found to be superior to 2-weekly or less frequent application, which is likely due to the continued constant levels of growth factors and cytokines necessary for graft vascularization [59]. Transcyte™ is another fibroblast-containing dermal substitute that acts as a substitute for cadaveric skin to temporarily cover burn wounds after surgical excision [60, 61]. A prospective, randomized comparison study on 14 patients with partial-thickness burns demonstrated faster wound healing with Transcyte™ compared to conventional treatments using topical antimicrobial agents in combination with repeated wound debridement and subsequent wound dressings [62]. Faster re-epithelialization of wounds treated with Transcyte™ was achieved because of continuous wound coverage (albeit temporary), which allowed undisturbed healing. This not only resulted in faster wound closure rates but further reduced hypertrophic scarring, which complicated the control group exposed to repeated debridement.

More often than not, however, clinical and laboratory-based *in vivo* skin regeneration models employ a bilayered structure composed of a top epidermal layer and a bottom dermal layer, which may naturally provide paracrine signaling factors, thus rendering external growth factor supplementation redundant. This, in combination with the emergence of genetically modified animals, is hoped to shed more light on the complexities of cutaneous wound healing.

7.3.1.3 Dermo-Epidermal Skin Substitutes

Akin to natural skin, most studies investigating skin regeneration focus on the development of cellular dermal–epidermal constructs. These constructs mimic the structure of natural skin in order to benefit from paracrine signaling, which reduces the risk of over- or under-dosing of exogenous signaling molecules. The dermal layer is of biological, synthetic, or combined origin and frequently encapsulates cells with or without additional growth factors.

Eaglstein *et al.*, for example, used a bilayered construct made of human fibroblast-seeded bovine collagen matrix with an overlying sheet of stratified human epithelium Apligraf® to cover excisional wounds in 15 patients [43]. Clinical evaluations were suggestive of reasonable graft take. However, the lack of controls and subjective, nonblinded assessments lessen the significance of these findings. A subsequent trial investigating the benefits of repeated applications of Apligraf® onto chronic rather than acute wounds was conducted in a larger cohort consisting of 233 patients [63]. Recalcitrant venous ulcers were successfully treated over 8 weeks with up to three separate applications of Apligraf® compared to simple compression therapy.

Larger wounds (>50% of total body surface area), particularly burn wounds, suffer from delayed and ineffective wound closure, partly due to a lack of autologous or allogeneic skin grafts. Boyce *et al.* applied a bilayered skin equivalent similar to Apligraf® but using the patients' own cells (PermaDerm™) onto 40 burn patients and compared wound-closure rates with those treated using split-thickness autografts [31]. Compared to the significant donor-site morbidity inflicted by skin harvesting, PermaDerm™ merely requires a punch biopsy, which accelerates recovery and is more acceptable among patients. Despite promising results using dermo-epidermal replacements, one major disadvantage prevails – the lack of important skin appendages such as hair follicles, capillary networks, sensory innervation, and pigmentation. More recent progress in skin biology has enabled the reconstruction of 3D skin equivalents which not only resemble natural skin in terms of architecture but also emulate skin's innate functions.

Delayed vascularization is a major contributor in skin engraftment failure, particularly in patients with extensive burns. Rapid vascularization and hence nutritional and oxygen provision to the graft are prerequisites for intermediate to long-term graft survival. In order to demonstrate the benefits of pre-vascularization strategies in terms of graft take rates, Tremblay *et al.* cultivated keratinocytes, fibroblasts, and endothelial cells in a collagen sponge to create a human endothelialized reconstructed skin (ERS) model, which was grafted onto excisional wounds in nude mice [64]. Macroscopic and immunohistochemical results demonstrated blood vessel formation and anastomosis between graft and host vasculatures, and this took place in 4 days compared to 14 days for non-vascularized RS (Figure 7.6).

In another example, Chan *et al.* demonstrated clinical feasibility with vascularized skin substitutes based on collagen–poly(ethylene glycol)–fibrin-based bilayer hydrogels seeded with autologous ADSCs from human burn tissue (Figure 7.7) [65]. Immunocytochemical analysis showed differentiated

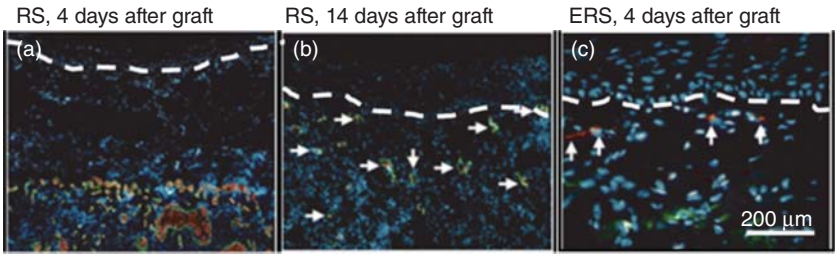

Figure 7.6 Immunohistochemical characterization of revascularization of the RS and ERS. Sections were immunostained with a marker of the cell nucleus (Hoechst, in blue), a marker of endothelial cells (in green), and with an antibody specific to mouse red blood cells (in red) (arrows). White dotted lines indicate the dermal–epidermal junction. In the RS, 4 days after graft, no endothelial cell tube containing red blood cells was observed under the epidermis, while some capillaries connected to the blood flow were detected in the bottom-half part of the graft thickness. Nevertheless, 14 days after graft, a homogeneous endothelial cell network filled with red blood cells was detected under the epidermis of the RS. Four days after graft of the ERS, human capillaries (arrows), which contained red blood cells, were observed close to the epidermis. (Tremblay 2005 [64]. Reproduced with permission of John Wiley & Sons.)

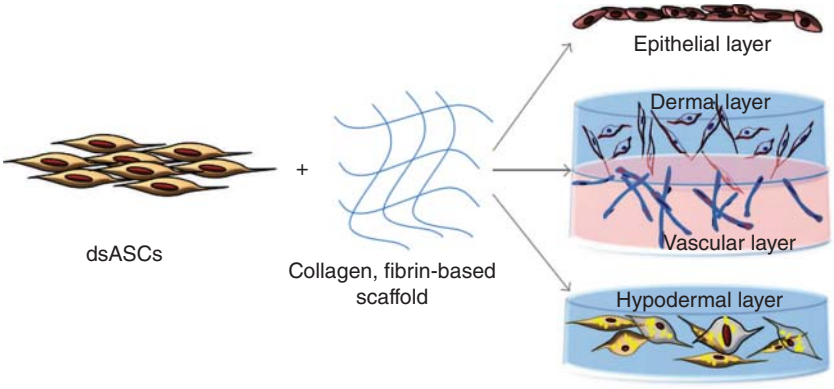

Figure 7.7 Schematic illustration of the development of different skin layers using surgically debrided adipose stem cells (dsASCs) and hydrogel scaffolds. (Chan, http://www.hindawi.com/journals/sci/2012/841203/. Used under CC-BY-3.0 http://creativecommons.org/licenses/by/3.0/.)

stromal, vascular, and epithelial cells, which indicated that debrided skin can become a source of autologous stem cells for the development of vascularized skin constructs with no need for culture expansion or exogenous growth factors.

Most skin equivalents only inadequately address the regeneration of skin appendages such as hair follicles or melanocytes. Numerous studies have demonstrated the successful regeneration of hair follicles in animal models [66–68]. However, *de novo* regeneration of human hair follicles has yet to be accomplished. It is hypothesized that this may be due to the loss of key inductive

properties of human dermal papilla cells upon culturing, which removes both contextual as well as positional cues from the surrounding epithelial cells. Aggregations of specialized mesenchymal cells located within the dermal papilla are known to control the cyclical growth activities of hair follicles [69], and dispersal of these cells results in a disruption of hair follicle development [70]. More recently, Higgins *et al.* succeeded in partially restoring such inductive capacities using an organotypic 3D spheroid culture system capable of growing human dermal papilla cells, thus paving the way for human hair follicle restoration [71].

Hypopigmentation, secondary to pigmentation disorders or after grafting of skin substitutes lacking melanocytes, is a common problem which can be particularly striking in darker skinned individuals. Apart from the obvious cosmetic implications, melanocytes are partly responsible for protection against UV irradiation, and therefore the absence of these cells within skin substitutes may have more serious implications especially where large areas of skin are affected [72]. A follow-up study of 132 patients suffering from leucoderma (an acquired condition with localized loss of pigmentation) demonstrated that treatment with autologous cultured melanocytes resulted in 100% re-pigmentation in select cases (stable leucoderma) [73]. Similar results were obtained in several other cases of post-burn leucoderma treated with melanocytes [74, 75].

Despite such promising results, there is no clinically available standard treatment for skin wound healing capable of perfectly regenerating skin including all its appendages. Thus, in an attempt to evaluate novel wound-healing therapies, scientists have created both *in vivo* and *in vitro* wound models that emulate human pathologies such as acute burn wounds [76, 77], chronic nonhealing wounds [78, 79], and skin conditions such as vitiligo [80, 81].

7.3.2 *In vivo* Wound-Healing Applications

Skin-humanized small animal models have emerged to mimic human skin healing as the model of choice for researchers because of multiple reasons including cost effectiveness, availability, and so on. Such models can be used to investigate various human wound conditions, for example, to investigate the mechanistic particulars of certain aspects of healing [82–84]. For example, Escamez *et al.* used transplanting dermo-epidermal equivalents containing fibroblasts and keratinocytes from immunodeficient mice as a base to develop a skin-humanized mouse model, which exhibited features of human wound healing [85]. Regeneration of human skin on the backs of nude mice was achieved by fabricating a fibroblast-encapsulating fibrin sponge, which served as a dermal equivalent, and culturing keratinocytes on top of it. Transplantation of this construct into mice and subsequent creation of full-thickness wounds enabled the study of human wound healing in an animal model. This is particularly useful, as ethical considerations and heterogeneous nature of the disease limit the advancement of the knowledge in diabetic wound healing. In another study, Martínez-Santamaría *et al.* demonstrated that the application of fibroblast-seeded, plasma-derived fibrin dermal scaffolds improved wound healing in diabetic skin-humanized mouse models when compared to acellular fibrin gels [86]. This was attributed to faster

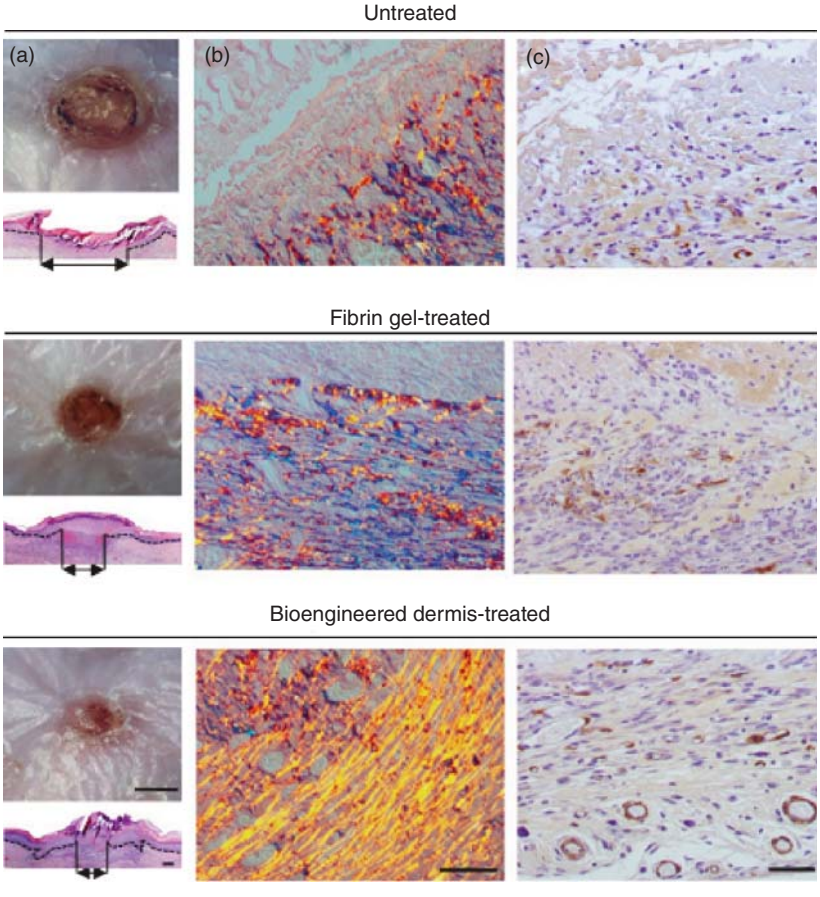

Figure 7.8 (a) Fibroblast-containing scaffolds enhance wound healing in a diabetic skin-humanized mouse model at 7 days post grafting. (b) Picrosirius red staining of a 7-day-old diabetic wound, demonstrating a collagen-rich granulation tissue in treated groups. (c) α-Smooth muscle actin (SMA) immunoperoxidase staining of a 7-day-old diabetic wound granulation tissue demonstrates a surge in vascular density. (Martínez-Santamaría 2013 [86]. Reproduced with permission of John Wiley & Sons.)

re-epithelialization, early granulation tissue maturation, and increased vascularization (Figure 7.8). The research findings using the preclinical wound-healing model can be further used to develop new therapeutic strategies for clinical uses.

7.3.3 *In vitro* Wound-Healing Models

TESs can also be applied as *in vitro* wound models to study the wound-healing process in detail. Such bench-based wound-healing models are particularly advantageous for preliminary investigations because of being easily manipulated and highly time and cost efficient compared to animal- or human-based studies and may eventually avoid animal testing [76]. Additionally, *in vitro* models enable the isolation and study of individual aspects of wound healing, which is

of fundamental importance in the elucidation of cellular and molecular cascades involved in the wound-healing process.

One of the simplest methods of assessing wound healing involves the creation of a 2D cell monolayer. For example, fibroblasts seeded into culture dishes and grown to confluence are scratched by running the sharp end of a razor blade or pipette tip along its surface, thereby removing a thin strip of the cell layer [87, 88]. This model is particularly well suited for relatively simple tasks involving the study of cellular migration and proliferation, as well as cytokine release profiles in response to injury [89]. Walter *et al.*, for example, utilized scratch assays to investigate the effects of a cell-conditioned medium on different rates of migration of dermal fibroblasts and keratinocytes [90]. Ojeh *et al.* demonstrated the inhibitory effects of caffeine on wound healing and epithelialization using a keratinocyte scratch assay [91]. The simplicity of scratch assays has enabled the isolated study of the effects of bioactive compounds on wound healing. For example, Ghazi *et al.* demonstrated that a scratched keratinocyte monolayer exposed to hyaluronic acid of different molecular weights healed quickest when exposed to medium molecular weight hyaluronic acid. These results may represent a facile topical therapeutic strategy to promote wound healing [88]. However, more sophisticated *in vitro* investigations must be performed prior to drawing conclusions regarding the molecular mechanisms of re-epithelialization and wound healing.

Bellas *et al.*, for example, created an *in vitro* trilayered skin equivalent based on silk and collagen seeded with ADSCs, endothelial cells, fibroblasts, and keratinocytes to study skin biology in a physiologically relevant and sustainable system (Figure 7.9) [92]. The combination of four different and spatially organized cell types is believed to create an optimal tissue microenvironment, which may serve as a human preclinical surrogate system to study the effect of different drugs on wound healing. By using the trilayered construct, rosiglitazone, an activator of the adipogenic program, was found to cause hyperproliferation (bracket) at the basal layer of the epidermis after 9 days of culture (Figure 7.9). This may be because the drug rosiglitazone slowed the inflammatory process, resulting in the hyperplasia of the epidermis [93].

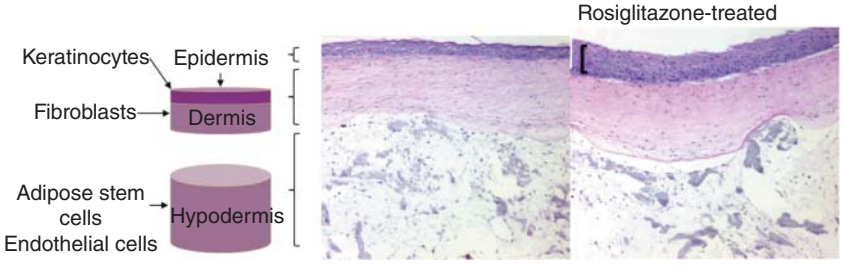

Figure 7.9 Schematic illustration of a trilayered, full-thickness skin model based on silk and collagen seeded with ADSCs, endothelial cells, fibroblasts, and keratinocytes. The trilayer constructs were found to be responsive to the adipogenesis stimulator drug rosiglitazone, which caused hyperproliferation (bracket) at the basal layer of the epidermis after 9 days of culture. (Bellas 2012 [92]. Reproduced with permission of John Wiley & Sons.)

Several other studies have subsequently used *in vitro* human skin models to study wound healing; a proof-of-concept study conducted by Maione *et al.* utilized human fibroblasts derived from DFU to recreate 3D chronic wounds which were characterized by stunted angiogenesis, delayed granulation tissue maturation, hyperkeratinization, reduced re-epithelialization, and impaired ECM deposition [94]. In addition to reflecting the clinical attributes of DFU, a correlation was established between the wound-healing potential of DFU fibroblasts and *in vivo* wound closure in mice models. Therefore, the reported 3D DFU models are biologically relevant tools for exploring cell–cell and cell–matrix interactions related to chronic wound pathogenesis and may further enhance clinical efficacy through translation of *in vitro* results.

Unfortunately, the currently developed *in vitro* models may suffer from general drawbacks including lengthy fabrication protocols, short-term viability, and significant variability in terms of study protocol standardization and performance evaluation, rendering systematic comparisons between studies difficult. While *in vitro* wound-healing models are preferred for the study of isolated actions of growth factors, cytokines, or genes on individual cell types, *in vivo* models are frequently deemed invaluable in elucidating the downstream effects of these factors on wound healing in models that closely mimic the physiology of human wounds. It is therefore vitally important to make continuous efforts to understand the complex biological processes, the interactions between various cell types, ECM, as well as extracellular and intracellular signaling during wound healing. Further technical advances may eventually lead to the production of new TESs resembling natural human skin.

7.4 Conclusions and Future Directions

The range of skin substitutes available and their successful application for *in vivo* and *in vitro* wound healing are a demonstration of advancement in the tissue engineering field. The terminal goal of skin tissue engineering is to regenerate a fully functional skin using TESs. The functional skin should possess all the skin appendages, including hair follicles, sweat glands, and sensory organs, as well as different skin layers including the epidermis, dermis, and fatty subcutus. In addition, rapid formations of vascular and nervous networks, as well as integration with the surrounding host tissues, are other key performance indicators of TESs. The regenerated skin should ideally have all the normal skin functions including barrier function, pigmentory protection against UV rays, temperature regulation, suitable mechanical properties, and esthetic appearance.

Existing TESs can already fulfill some of the aforementioned requirements. For instance, cultured keratinocytes applied on fibrin or matrix, either as cells or a cell layer, can quickly restore barrier function after skin burn or trauma. Another example is that keratinocytes seeded on a dermal substitute (e.g., collagen/fibronectin mixture with/without fibroblasts) are able to restore the mechanical properties partially. However, to date, issues of nonrapid availability and low commercial potential for greater scale have inhibited the creation of an

autologous bilayered skin substitute, that is, a substitute without the risk of host rejection. Heterologous cells such as poorly differentiated cells and stem cells do not have risks of graft rejection and their genetically stable nature inhibits tumor formation; these features are promising in terms of offering an off-the-shelf therapy that is commercially scalable. Applying the current knowledge of embryonic development and adult regeneration to develop a process of engineering such cells is critical for improving upon the existing TES. The in-depth understanding of differentiation mechanisms and means of cell manipulation require continued research. Elucidation of the fundamentals of stem cells could lead to the use of cellular therapy to accomplish local tissue repair.

In addition, TESs themselves are highly complex, for example, biomaterial scaffolds with controlled release of various signaling and differentiation molecules, as well as protein domains for promoting cell migration and adhesion. As advancement leads to the engineering of different tissues, a greater need for tissue-specific scaffolds will develop and require further research. Future scaffolds are anticipated to become more sophisticated and fulfill all the needs of various cells. Moreover, inclusion of intrinsic activity, mediated by cytokines or immobilized peptides, into scaffolds derived from biomaterials is another developing research area. In order to understand cytokine interactions on a cellular and molecular level, further fundamental research must be pursued. Similarly, the prevention of adverse host reactions at the immunological level is a subject that requires further investigation.

On the other hand, the substitutes also need to be simplistic for easy handling and to serve as carrier (e.g., hydrogel or electrospun fibers), which, upon noninflammatory degradation, allows cells to interact and to regenerate skin structures akin to natural skin. In this case, the cells are first undifferentiated prior to delivery, and then the substitutes rapidly disappear upon implantation. The creation of next-generation TES requires further understanding in the disciplines of embryonic development, stem cell biology, and biomaterial engineering.

Acknowledgments

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8

Articular Cartilage Tissue Engineering

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8.1 Introduction

Cartilage is an avascular tissue, which also lacks nervous and lymphatic systems. It plays a critical role both during embryonic development and in the adult. During the stage of embryonic development, cartilage is an extensively distributed tissue that provides templates for skeletal development through endochondral ossification. In adulthood, cartilage distribution is more restricted, but it still can be found at various sites throughout body with numerous functions, such as providing mechanical support, enhancing the strength of attached structures, and facilitating frictionless movements. According to extracellular matrix (ECM) composition, cartilage can be classified as hyaline cartilage (such as cartilage found in the joint, the nasal septum, tracheal rings and ribs), the elastic cartilages (such as cartilage found in the ear, epiglottis, and larynx), and fibrocartilages (such as cartilage found in the intervertebral disks, temporomandibular joint disk, and knee meniscus) [1]. Articular cartilage is a kind of hyaline cartilages that cover bony ends of synovial joints to constitute joint surfaces. Because of its unique ECM composition and highly organized structure, articular cartilage can thus provide a load-bearing surface with substantial durability to joints, as well as allow frictionless movement of joints as a result of its lubrication function.

8.1.1 Articular Cartilage Composition and Structure

Articular cartilage consists of chondrocytes and the ECM. Chondrocytes are cells of mesodermal origin and mainly responsible for the production and maintenance of the ECM. The main components of all cartilaginous ECM are collagen and proteoglycan. Type II collagen is the dominant collagen in articular cartilage, which is cross-linked by covalent bonds forming a collagen network within the cartilage ECM, making up 90% of the total collagen in the ECM of cartilage [2, 3]. However, in fibrocartilage, besides type II collagen, type I collagen also exists in the ECM [4]. Aggrecan is the predominant proteoglycan in cartilage ECM. It consists of one core protein backbone and many branched sulfated glycosaminoglycans (GAGs), mainly keratin sulfate (KS) and chondroitin sulfate (CS). Through a link protein, it is bound to a long, unbranched hyaluronic acid (HA) chain that is present throughout the cartilage ECM. The collagen network

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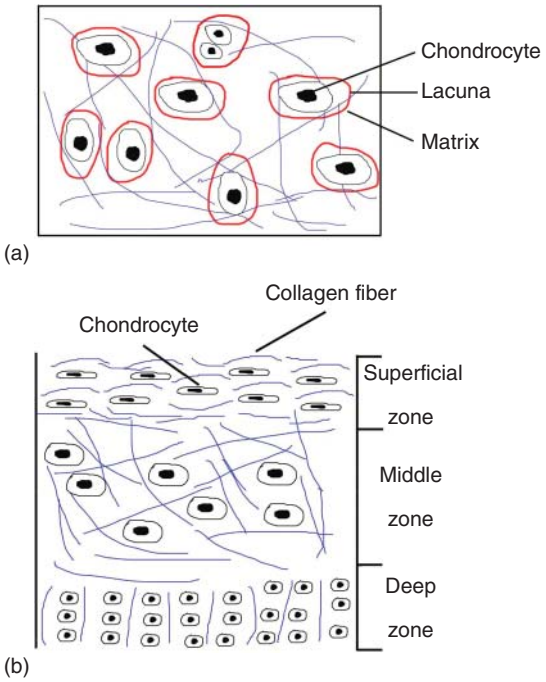

Figure 8.1 Structure of articular cartilage. (a) Cellular and ECM components of articular cartilage. (b) Zonal structure of articular cartilage.

that entraps HA chains bound with aggrecan molecules constitutes a highly organized and complicated structure of cartilage ECM [3]. Chondrocytes are scattered individually in this intricate network, with ECM directly surrounding a cell forming a cavity, which called the lacuna (Figure 8.1a) [5].

Besides complex ECM components, articular cartilage also contains three different zones, with each zone characterized by a distinct cellular phenotype and ECM structure (Figure 8.1b): the superficial zone, which contains the articulating surface of the joint; the middle zone; and the deep zone. In the superficial zone, cells are smaller than those of the two other zones, and type II collagen fibers are densely packed parallel to the articulating surface. Larger and more rounded chondrocytes can be found in the middle zone, with randomly oriented collagen fibers. The deep zone contains cells oval in shape, and the collagen fibers in this zone are oriented in direction perpendicular to the articulating surface [6].

8.1.2 Articular Cartilage Function

Articular cartilage, typically 2–5 mm in thickness covering the surface of articulating joints in the body, has the ability to withstand large amounts of repetitive mechanical loading for many years and provides smooth surfaces enabling frictionless movement between two articular surfaces in joints [3]. Proteoglycans and collagen networks are primarily responsible for the normal functioning of cartilage tissue [7]. Proteoglycans play an important role in resisting compressive loading. A large number of negative charges on densely packed GAGs of proteoglycans give a high osmotic pressure to this tissue. Negative charges attract cations, further increasing the osmolality in cartilage. A high osmotic pressure

finally results in cartilage being a tissue high in water content and compressive strength. Meanwhile, collagen networks provide tensile strength to cartilage. For example, strong type II collagen networks in ECM can prevent cartilage from being over-stretched [8]. In addition, a small amount of water will be pushed out from cartilage into the joint when cartilage resists load and deforms upon joint loading. In spite of the small volume, this liquid plays a critical role in reducing articular surface friction and the smooth motion of the joint [8].

8.2 Articular Cartilage Lesions and Repair

8.2.1 Articular Cartilage Lesions

Articular cartilage damage can be caused by arthritis or traumatic conditions. Arthritis occurs as a result of the degradation of cartilage, which finally leads to joint pain and loss of motion. Inflammatory rheumatoid arthritis (RA) and noninflammatory osteoarthritis (OA) are two major types of arthritis. RA is an autoimmune disease characterized by severe inflammation reaction surrounding the joint, while OA is a degenerative disease of the articular cartilage. In both these two diseases, the complex biochemistry and structure of the articular cartilage ECM become disrupted. Once the process of matrix degradation has begun, further degradation of the surrounding normal matrix will follow because of the release of degradative enzymes and cytokines by injured chondrocytes, finally leading to permanent loss of articular cartilage layer [9]. In addition, many traumatic conditions, such as intra-articular fracture, articular cartilage insult after a ligament injury, a single excessive load of great magnitude, or repetitive and prolonged overloading, can also cause damage of articular cartilage [10], with most significant injuries to articular cartilage eventually leading to the occurrence of OA [11].

Because cartilage is an avascular tissue low in cellularity, and chondrocytes are also low in proliferative capacity, cartilage has very limited repair capacity for articular cartilage damage. For cartilage damage that limits only to the cartilage layer, chondrocytes in the periphery of the injured zone will proliferate and increase ECM synthesis. However, the period of increased metabolic activity of the surviving chondrocytes is quite short, after which the synthesis rates fall back to normal. In addition, proliferating chondrocytes do not migrate into the defect, and therefore the edges of the defect do not fuse [12]. If the damage penetrates into the subchondral bone, it is called full-thickness defect [13]. Under this condition, although mesenchymal stem cells (MSCs) from the underlying bone marrow proliferate and undergo chondrogenic differentiation under the influence of local growth factors in the microenvironment, defects are repaired with tissues without the structure and function of normal articular cartilage [14]. Therefore, degeneration of the repaired tissues easily occurs with loss of hyaline cartilaginous matrix and death of the chondrocyte-like cells [15]. In the end, the remaining cells typically assume the appearance of fibroblasts, with the surrounding matrix composed primarily of densely packed type I collagen instead of type II collagen [16]. Fibrocartilage has inferior mechanical properties and durability

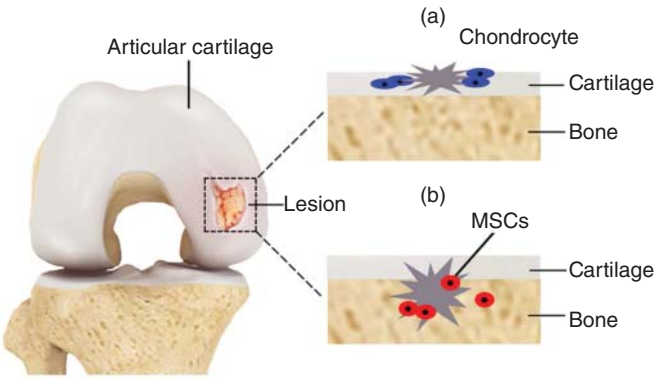

Figure 8.2 Damage and repair model in articular cartilage. (a) Cartilage damage limited to the cartilage layer; chondrocytes in the periphery of the injured zone proliferate to repair damage. (b) Cartilage damage penetrating into the subchondral bone; MSCs proliferate and undergo chondrogenic differentiation to repair damage.

compared to hyaline cartilage and will gradually degenerate with time, finally gets lost permanently, leaving areas of exposed bone [17]. Figure 8.2 illustrates two cartilage damage and repair models.

8.2.2 Current Treatments in Articular Cartilage Repair

Currently, a wide variety of approaches are available to restore the structure and functions of injured cartilage. Techniques used to regenerate cartilage in clinical settings can be divided into four categories: bone-marrow-simulation (BMS)-based techniques, osteochondral transplantation techniques, cell-based techniques, and tissue engineering-based techniques.

8.2.2.1 Bone-Marrow-Simulation-Based Techniques

Microfracture is the most commonly performed BMS-based procedure in the clinic. Microperforation of the subchondral plate is performed on defect site of cartilage, and bone marrow containing MSCs will fill up these microfractures and clot subsequently. Although MSCs have high chondrogenic differentiation potential, they are just able to repair cartilage defects with new tissues more like fibrocartilage, which are inferior in mechanical properties than hyaline cartilage [18]. This technique is a first-line procedure in acute knee injuries for athletes younger than 40 years of age, and favorable short-term outcomes can be observed if treatment is applied early after the injury and the defects are smaller than 2.5 cm [19]. However, inferior mechanical properties of repair tissues and imperfect integration of repair tissues with surrounding healthy cartilage are major drawbacks of microfracture, which may result in significant deterioration of fibrocartilage-like tissues in the long run [18].

8.2.2.2 Osteochondral Transplantation Techniques

According to the origin of the osteochondral graft, osteochondral transplantation can be divided into autologous osteochondral transplantation and allograft

osteochondral transplantation. Autologous osteochondral transplantation is a procedure that first harvests cartilage together with subchondral bone from nonload bearing sites of joint and subsequently transplants it to the damage area in the joint. This technique is used for small and medium size chondral or osteochondral defects and has shown good integration of the graft in the host bone and complete maintenance of the grafted cartilage in long-term follow-up [20]. However, drawbacks of this approach still exist. Donor site morbidity, mismatch in the surface shape of the treated joint versus the donor tissue, lack of integration of grafted tissue with host, and low weight-bearing capacity are the main issues associated with this approach [21].

When large osteochondral defects occur and autologous subchondral graft is insufficient to repair them, allograft osteochondral transplantation is indicated. In this approach, a fresh or fresh-frozen cartilage segment obtained from an organ donor is used to replace the injured cartilage. Fresh osteochondral allografts have been shown to reconstruct articular defects of the knee in the young active patient with high success percentage [22]. Limitations of this technique include limited graft availability, potential disease transmission, low chondrocyte viability in allograft, and nonstandardized allograft processing and storage procedure [23]. In addition, there is some controversy as to whether cartilage is immunoprivileged, and potential immune response is still the concern of many people [22, 24].

8.2.2.3 Cell-Based Techniques

The first cell-based regenerative technique, called autologous chondrocyte implantation (ACI), was reported in 1994 [25]. In this procedure, chondrocytes are harvested and isolated from a less load-bearing area in the joint and then expanded in a monolayer culture *in vitro* before transplantation of proliferated cells back into the damaged area in the joint. ACI is used for inconsistent medium to large sized defects [26] and has been successfully used to treat full-thickness cartilage lesions in the knee with long-term durability of functional improvement for more than 10 years [27], with good integration of the newly formed repair tissue with the surrounding cartilage [28]. However, ACI still has many disadvantages, including donor site morbidity, requirement for two surgeries, implanted chondrocyte leakage from the recipient site, uneven distribution of the cells in the defect, and long recovery time after operation [29]. In addition, a critical issue associated with this approach is the dedifferentiation of chondrocytes during *in vitro* monolayer culture for expansion, with a shift from type II collagen-rich hyaline cartilage to primarily type I collagen-containing fibrocartilage-like tissues [30], which results in defects treated with ACI to be primarily filled with fibrocartilage in the end [31]. For this reason, selection and expansion of a subgroup of chondrocytes characteristic of expression of specific gene markers and phenotype was performed in order to regenerate cartilage with more hyaline-like tissue [32]. Nonetheless, fibrous tissue repair is still highly likely to happen.

8.2.2.4 Tissue Engineering

Though partial success has been achieved by the current clinical techniques, each method has its own intrinsic limitations that prevent further advancement to

be made in cartilage regeneration therapy. There is no current clinically mature approach capable of regenerating cartilage with ECM composition and physiological function similar to those of native cartilage [33].

Tissue engineering is a relatively new field that has seen dramatic developments during recent decades, with the purpose of creating tissue-like constructs via using organ-specific cells, biomaterials, bio-reagents, and mechanical stimulation. Implantation of these artificial tissues into the body to repair a structure-compromised organ or replace function of a failed organ is the final aim of tissue engineering [34]. Cartilage tissue engineering involves the culture of isolated autologous cells in scaffold with appropriate biochemical and mechanical stimulations to create a relatively mature cartilage *in vitro* before its implantation. An ideal modality for cartilage tissue engineering involves several key procedures. First, an autologous cell population is obtained from the patients. These cells can be either derived from chondrocytes in the donor site or stem or progenitor cells within the body. The focus of this step is to identify qualitatively and quantitatively reliable cell sources for cartilage. Next, these cells are cultured at *in vitro* environments that can give cells appropriate biological and mechanical signals to proliferate, differentiate, and function as they do *in vivo*. Biomaterials that can mimic some critical aspects of natural environments and biological and mechanical stimuli that can promote cell differentiation and tissue maturations play significant roles during this procedure. Finally, after the optimal *in vitro* culture period, the artificial cartilage tissue generated is implanted back into the defect site of the body [35].

For an ideal engineered cartilage tissue, issues of current regenerative treatments encountered can be overcome. Because of the presence of the matrix around the cells, donor cell retention at the repair site can be enhanced. In addition, the ECM around cells can protect cells from disastrous inflammatory environment in the damaged area of the joint. Most importantly, it is easier to handle the engineered cartilage, which is capable of bearing mechanical loading upon implantation into the defect sites in the joint [36].

8.3 Basics of Articular Cartilage Tissue Engineering

8.3.1 Cells

In cartilage tissue engineering, quantitatively sufficient and phenotypically stable cell sources are critical for successful artificial cartilage graft construction. Cells should be easily accessible, high in proliferative capacity, abundant in quantity, stable in phenotype, and capable of cartilage-specific ECM production and accumulation, as well as nonimmunogenic and nontumorigenic [37]. Chondrocytes are one of most widely used cell sources in cartilage tissue engineering because they are resident cells of cartilage and can be obtained from nondamaged areas of articular cartilage in patients [38]. MSCs, derived from bone marrow, synovium, adipose, and so on, are also extensively explored because of their high proliferative capacity and the multilineage differentiation potential [39]. In addition, recent advances in pluripotent stem cells, such as induced pluripotent stem cells

(iPSCs), have allowed these cell types to be used in cartilage tissue engineering as feasible and promising cell sources [40, 41].

8.3.1.1 Chondrocytes

Chondrocytes, which are the resident cells of cartilage and able to maintain cartilage-specific ECM, represent one cell type that is commonly used for engineering articular [42]. The primary cell source for articular cartilage tissue engineering is chondrocytes originating from articular cartilage. Generally, expansion of autologous cells following cell harvest *in vitro* via monolayer culture is necessary in order to obtain sufficient number of cells for engineering artificial cartilage constructs. However, dedifferentiation always occurs during this monolayer proliferation process. Chondrocytes will become flattened, losing their rounded morphology, and synthesize collagen type I instead of collagen type II [43]. But this process of dedifferentiation is reversible. Once implanted back into a 3D environment, the dedifferentiated cells have the ability to redifferentiate, that is, chondrocytes will recover their differentiated phenotype [44]. Even though the 3D environment can help redifferentiate chondrocytes to some degree after their monolayer expansion *in vitro*, the native phenotype of chondrocytes can never be fully restored without the natural biochemical and biomechanical environment, resulting in fibrocartilage formation finally in most cases [24, 45]. Growth factors that can stimulate redifferentiation of chondrocytes, such as transforming growth factor-beta (TGF-β)-1, bone morphogenetic protein (BMP)-2, and fibroblast growth factor (FGF)-2, and biomolecules such as insulin and triiodothyronine, have been used for enhanced cartilage-forming capacity during chondrocyte monolayer expansion [46] or 3D culture of engineered constructs [47, 48]. In addition, there is large variability in the chondrogenic capacity of chondrocytes from different individuals, which consequently leads to quantitatively variable cartilage construct formation by those chondrocytes [46]. Therefore, studies to find optimal *in vitro* culture conditions for tissue-engineered cartilage, such as appropriate growth factor supplements and biomechanical stimuli application, and to identify chondrocytes with enhanced chondrogenic potentials are greatly needed in future.

Moreover, chondrocytes can also be harvested from other sources, such as nasal septum and ribs, both of which belong to hyaline cartilage. Compared to articular chondrocytes, nasal and costal chondrocytes have been shown to have a higher capacity to generate cartilage-specific ECM [49, 50]. Even though nasal chondrocyte derives from neuroectoderm and articular chondrocyte originates from mesoderm, nasal chondrocyte can reprogram to a gene profile similar to that of articular chondrocyte upon implantation into cartilage defect [51].

8.3.1.2 Mesenchymal Stem Cells

8.3.1.2.1 Bone-Marrow-Derived Stem Cells

The first type of identified MSCs is bone-marrow-derived stem cells (BMSCs), which are still commonly used in preclinical and clinical studies [52]. BMSCs have the ability to proliferate more rapidly than chondrocytes and differentiate into multilineage cells, including chondrocytes, which was first reported in 1998

[53, 54]. In addition, transplantation of autologous BMSCs has been shown to be able to facilitate the repair of large focal articular cartilage defects in a case report [55] and then has proven to be as effective as chondrocytes for articular cartilage repair in a cohort study [56]. Despite similar clinical outcomes, BMSCs provide additional advantages over chondrocytes. BMSCs can be harvested from body through bone marrow biopsy, which is less invasive than knee arthroscopy. Moreover, donor site morbidity can be avoided, since normal articular cartilage is not extracted as cell source [56].

However, collection of BMSCs involves still an invasive procedure, and their percentage in the bone marrow is quite low (0.001–0.01%). Furthermore, their differentiating potential decreases with age. In elderly patients, a significant decrease in the number of BMSCs and their ability to proliferate and differentiate was observed [57]. In addition, chodrogenic differentiation of BMSCs often result in the chondrocyte hypertrophy, which is characterized by the expression of type X collagen and matrix metalloproteinase (MMP)-13, and finally lead to vascular invasion and ossification of neocartilage [58]. Finally, the differentiation potential of BMSCs will decrease significantly with the increase in the number of cell passages. Excessive expansion of BMSCs is therefore not favorable for cells to maintain their differentiation capacity [59].

8.3.1.2.2 Synovium-Derived Stem Cells

Synovium-derived stem cells (SDSCs), originating from the synovial membrane surrounding joints, were first isolated in 2001, with superior chondrogenic potential both *in vitro* and *in vivo* [60]. Many studies have demonstrated that SDSCs have greater chondrogenic ability but less osteogenic tendency than stem cells derived from other tissues *in vitro* [61]. Once implanted into in a cartilage microenvironment, they can differentiate into chondrocyte-like cells simultaneously and maintain stable hyaline cartilage ECM without indications of fibroblastic or hypertrophic phenotype change [62]. Thus, SDSCs are a promising source of stem cells for cartilage tissue engineering.

Synovium can be obtained through arthroscopy. From a small synovium punch biopsy, a clinically sufficient amount of SDSCs can be isolated and expanded for cartilage defect repair. This technique is still effective for patients over 50 years old, possibly for the reason that the differentiation and proliferation potential of SDSCs is age-unrelated [63]. Moreover, less donor-site morbidity will be caused by synovium harvest, since synovium has been shown to have the capacity to regenerate after surgical removal [63]. However, invasiveness and complications to patients are still unavoidable, even though the procedure is minimally invasive and leads to much less donor site morbidity compared to autologous chondrocytes harvest.

8.3.1.2.3 Adipose-Derived Stem Cells

Adipose-derived stem cells (ASC) are another kind of multipotent adult stem cells residing in adipose tissues in the human body with cell surface marker profiles and differentiation characteristics similar to those of BMSCs. The chondrogenic potential of ASCs was first discovered in 2001 [64]. ASCs can also be differentiated into multilineage cells, including chondrocytes, osteoblasts,

adipocytes, neuronal cells, and muscle cells, under controlled culture conditions [65]. ASCs provide many advantages as a cell source for tissue engineering. First, ASCs are readily accessible. Since ASCs can be isolated in large quantities from adipose tissues that are routinely available from liposuction surgeries and expanded to larger numbers quickly *in vitro*, sufficient quantity of cells can be obtained from such adipose tissues [66, 67]. In addition, ASCs are abundant, because they can be found in adipose tissues that abundantly exist in the human body. Moreover, repeated access to the subcutaneous adipose tissue to obtain ASCs is feasible because of the rapid recovery capacity of adipose tissue [68]. For cartilage tissue engineering, ASCs isolated from the infrapatellar fat pad of the knee is an attractive type of ASCs with additional advantage, since ASCs in this adipose tissue contain a high proportion of highly chondrogenic cells [69–71]. However, compared to BMSCs, ASCs have been shown to have inferior potential for chondrogenesis [72, 73].

8.3.1.2.4 Other Mesenchymal Stem Cells

Although MSCs from other tissues are not so extensively explored as the aforementioned stem cells, some kinds of MSCs also show the potential to be a candidate cell source for cartilage tissue engineering. Stem cell from the Wharton's jelly of the human umbilical cord is another type of potent MSCs that can be differentiated into chondrogenic lineages *in vitro* and *in vivo*, with additional advantages like noninvasive collection [74–77]. Muscle-derived stem cells (MDSCs) were also reported to have the capacity of multilineage differentiation, including condrogenesis potential, which has been demonstrated both *in vivo* and *in vitro*, and thus is a potential new cell source for cartilage tissue engineering [78]. Amniotic fluid stem cells (AFSCs), isolated from the amniotic fluid, show a higher expansion and differentiation capacity and lower accumulated somatic mutations compared to adult stem cells, as well as lower tumorogenicity compared to embryonic stem cells (ESCs) [79, 80].

8.3.1.3 Pluripotent Stem Cells

8.3.1.3.1 Embryonic Stem Cells

ESCs, which originate from the inner cell mass of blastocyst embryos, have the capacity to proliferate in an undifferentiated state for quite a long time in culture, as well as the potential to differentiate to any kind of cells from three embryonic germ layers [81]. These cells can proliferate rapidly and unlimitedly *in vitro* to provide large quantities of cells [82] and maintain expression of primitive ESC markers along with doubling all the time [80]. Additionally, ESCs can undergo chondrogenesis, which can be demonstrated by the formation of teratoma, a disorganized tissue with components from all three germ layers including cartilage [83]. Differentiation of ESCs into chodrogenic lineage by various means has been reported, and the potential of ESC-derived chondrogenic cells for cartilage tissue engineering and regeneration with no evidence of tumorigenicity has also been demonstrated both *in vivo* and *in vitro* recently [82, 84].

In addition to ethical controversies involved in ESC studies, and since undifferentiated ESCs are tumorigenic with high risk of teratoma formation

in vivo, there are also safety concerns regarding the use of ESCs in cartilage tissue engineering, and hence it is essential to develop a stable and efficient culture system for differentiation of ESCs into a defined chondrogenic lineage [84]. Furthermore, in order to ensure that tissue-engineered constructs consist of homogeneous and thoroughly differentiated ESC-derived chondrocytes, production of nonuniform cell populations post differentiation is another issue that needs to be solved [85]. A nonuniform cell population not only increases the risk of teratoma formation by undifferentiated cells contained in the population but also limits the effectiveness of cell-based therapy [86]. Thus, purification of chondrogenic, differentiated ESCs and the preservation of their desired function as chondrocytes are critical [81].

8.3.1.3.2 Induced Pluripotent Stem Cells

iPSCs were inducted by retroviral transduction of somatic cells with four transcription factors (Oct4, Sox2, Klf4, and c-Myc) by Yamanaka for the first time [41]. iPSCs are similar to ESCs in many aspects, such as gene expression profile, morphology, *in vitro* and *in vivo* differentiation and proliferation potential, *in vivo* teratoma formation capacity, and even the ability to give rise to viable embryos [87]. The main advantage of iPSCs over ESCs is that iPSCs can be used to generate autologous and patient-specific cell types for clinical use, with no need to generate and destroy human embryos, thus avoiding ethical concerns of using ESCs for therapy.

As to iPSCs' use in cartilage tissue engineering, great progress has been achieved recently. First, readily available cell sources, such as the adult skin, can be used to generate patient-matched iPSCs because they are derived from the patient's own cells [88]. These transduced somatic cells have significant expansion potential as well as multilineage differentiation capacity. As a result, large numbers of patient-matched cells with chondrogenic potential can be obtained for cartilage tissue engineering [89, 90]. Second, both chondrogenic differentiation of normal somatic cell-derived iPSCs to form functional cartilaginous tissue [91] and chondrogenesis of osteoarthritic chondrocyte-derived iPSCs [92] have been shown to be possible *in vitro*. Besides, the potential of using iPSCs for cartilage defect repair *in vivo* has also been proved recently [93].

However, challenges still remain. For example, the origin of the somatic cell type influences the molecular and functional properties of iPSCs generated subsequently [94]. Additionally, reprogrammed cells may have the tendency to differentiate along cell lineages related to their original somatic tissue, which restricts alternative cell fates [95]. Therefore, in order to obtain iPSCs that allow highly reproducible differentiation of themselves into desired cell lineages, it still remains to be determined what type of initial somatic cells is to be transduced and whether the reprogramming methods are optimal for iPSC generation. Moreover, clinical use of iPSCs is still restricted by safety concerns due to the possibility of iPSCs forming tumors due to genetic mutation as well as genomic methylation, an epigenetic modification of the DNA that influences gene expression, that occurring during the process of reprogramming [96]. Consequently, to ensure cell safety, genetic screening iPSCs before clinical application is to be performed. Finally, as with ESCs, the difficulties of uniform

Table 8.1 Cell types used in cartilage tissue engineering.

Cell types		Advantages	Disadvantages	References
Resident cells	Chondrocytes	• Autologus cell type • Maintenance of cartilage specific ECM	• Limited source • Dedifferentiation	[42–51]
MSCs	BMSCs	• Rapid differentiation • Multilinage differentiation • Less invasiveness • Avoidable donor site morbidity	• Low percentage in bone marrow • Unstable in chondrogenically differentiated phenotype	[52–59]
	SDSCs	• Superior in chondrogenic and proliferation potential	• Invasiveness • Occurrence of complications associated with procedures	[60–63]
	ASCs	• Easily accessible • Abundant in sources	• Inferior potential for chondrogenesis	[64–73]
Pluripotent stem cells	ESCs	• Self-renewing ability • Rapid and unlimited proliferation • High differentiation potential	• Ethical controversies • Risk of teratoma formation	[80–86]
	iPSCs	• Self-renewing ability • High differentiation and proliferation potential • Generation of autologous and patient-specific cell types • Circumvention of ethical concerns	• Risk of tumor and teratoma formation	[87–97]

differentiation of iPSCs into the cell type of interest also limit the use of iPSCs for therapeutic applications. Undifferentiated cells contained in post-differentiated cell population not only affect the effectiveness of engineered constructs but also have a high possibility to lead to teratoma formation [97]. Table 8.1 lists the commonly used cell sources in cartilage tissue engineering.

8.3.2 Scaffold

8.3.2.1 Desired Properties for Scaffolds

During monolayer culture *in vitro*, chondrocytes will lose their differentiated phenotype, being spindle shaped and having increased type I collagen expression [98]. Scaffolds are thus used to provide the 3D environment to chondrocytes to mimic *in vivo* conditions where they are surrounded by an abundant ECM and able to keep their round shape and type II collagen-producing capacity [99].

The optimal scaffold for cartilage tissue engineering should possess following properties:

1) *Biocompatibility*. The scaffold should provide a favorable microenvironment for the chondrocyte to maintain round morphology and phenotype, proliferate, differentiate, and produce cartilage-specific ECM [100]. Host immune reactions in response to the scaffold material should also be minimized following *in vivo* scaffold implantation.
2) *Porous structures*. A porous environment is necessary for adequate diffusion of nutrients and waste, as well as some soluble bioactive molecules [40].
3) *Biodegradability*. The scaffold should provide room for newly formed tissue via proper degradation rate that can match tissue growth [24].
4) *Mechanical properties similar to those of native cartilage tissue* [101]. Because engineered tissue constructs would have to withstand high mechanical loading immediately upon implantation into cartilage defects, sufficient mechanical properties of scaffold, such as tensile and compressive strength, are crucial for the cartilage tissue construct to function.

Currently, scaffolds made from natural proteins and synthetic materials are fabricated into hydrogels, sponges, or fibrous meshes for cartilage tissue engineering. Of all these different forms, hydrogel is a promising scaffold structure, since it is a network with high water content similar to cartilage ECM and therefore can provide a biomimetic 3D environment to the cells [102]. It can also serve as a permeable and viscoelastic platform to allow efficient nutrient and waste transport, diffusion of soluble bioactive factors [103], and efficient mechanical load transfer [104]. Furthermore, hydrogel has the ability to effectively and uniformly encapsulate cells for cell delivery vehicles [105, 106]. However, hydrogel has poor mechanical properties, which restricts its wideer application in cartilage tissue engineering.

8.3.2.2 Classification

A variety of materials are being extensively explored as scaffolds in cartilage tissue engineering, which can be classified into natural and synthetic materials. Recently, newly emerging scaffolds, decellularized ECM scaffolds [107], and platelet-rich plasma (PRP)-derived scaffolds [108] are also receiving increasing attention.

8.3.2.2.1 Natural Materials

Natural materials can be categorized into protein-based materials and polysaccharide-based materials [102]. Protein-based materials include collagen, gelatin, and fibrin, and polysaccharide-based materials include agarose, alginate, HA, and chitosan.

Collagen Collagen is the main ECM component of cartilage, with bioactivity, hydrophilicity, and cell-adhesion sites [100]. Scaffolds composed of collagen thus can provide a more biomimetic environment for cultured chondrocytes to survive, proliferate, and generate cartilage-specific ECM [109]. In addition, collagen has excellent biocompatibility and low antigenicity, which makes it a commonly used biomaterial in cartilage tissue engineering [110]. Besides, collagen can be

used as a carrier for efficient delivery of growth factors, such as BMP-2 [47], and bioactive molecules, such as insulin [111]. Although type I collagen used in cartilage tissue engineering has demonstrated usefulness to some degree in cartilage defect repair [55, 75], type II collagen promotes more efficient chondrogenic differentiation of embedded MSCs [104] and supports more homogeneous cell distribution throughout the matrix compared to type I collagen [112].

However, the main drawbacks of collagen, such as poor mechanical properties and rapid degradation *in vivo*, should be overcome before its wide application in tissue engineering. Considering that collagen architecture, including fiber density, diameter, orientation, and degree of cross-linking, plays a critical role in determining the compressive properties and tensile strength of cartilage, current studies primarily focus on collagen content, alignment, and cross-linking degree to improve the biomechanical properties of engineered articular cartilage graft [113]. Besides, creating a hybrid scaffold combining collagen and other materials with good mechanical properties is an alternative means to increase collagen's mechanical strength [110, 114]. Meanwhile, in order to slow down collagen degradation *in vivo*, increase in collagen cross-linking via chemical [115] or biological method [110] is also an efficient approach. Coating biomaterial scaffolds with the collagen-mimetic peptide GFOGER may be an alternative way, which can maintain the mechanical properties of the biomaterial as well as the bioactivity of collagen [116].

Fibrin Fibrin gel is a kind of natural material derived from fibrinogen via thrombin-mediated cleavage of fibrinogen followed by conformational changes and exposure of the polymerization sites [117]. Fibrin can provide an adhesion substrate and hydrophilic environment to cells, where cells can attach via adhesion molecules on fibrins [118] and generate cartilage-specific ECM [119]. In addition, it is biocompatible, especially when it is harvested and isolated from autologous blood. Furthermore, it has been demonstrated that fibrin gel can maintain the phenotype and function of chondrocytes and facilitate chondrogenic differentiation of BMSCs [120]. Moreover, fibrin can also be used as a carrier for growth factor delivery [121].

Similar to collagen, fibrin suffers from fast degradation and low mechanical strength, which limit the wide application of fibrin in tissue engineering. Since the degradation of the fibrin network occurs *in vivo* through the function of plasmin in a process called fibrinolysis, fibrin degradation can be controlled by using agents that can affect fibrinolysis [122]. Meanwhile, increase in the cross-linking density of fibrin can improve fibrin's mechanical property, similar to collagen.

Gelatin Gelatin, which is a partially denatured hydrolysis derivative of collagen, possesses good biocompatibility and bioactivity. Compared to collagen, gelatin is more water-soluble, cheaper, and nonimmunogenic. Since gelatin still contains some signaling sequences of collagen, such as the Arg-Gly-Asp (RGD) sequence, it still has the ability to promote cell adhesion and migration [123], proliferation, and differentiation [124]. Because of its lower mechanical strength, pure gelatin is rarely used as a scaffold for cartilage tissue engineering [125]. Composite scaffolds composed of gelatin and other materials with more excellent mechanical

properties are therefore fabricated for improving the mechanical strength of gelatin [126]. On the other hand, gelatin is always made into microspheres as carriers, which are widely used in cartilage tissue engineering. Gelatin microspheres are then embedded into other materials for delivery and controlled release of growth factors to enhance chondrocyte attachment and proliferation or to better facilitate chondrogenic differentiation of MSCs [127].

Alginate Alginate is a polysaccharide isolated from brown algae, and is composed of 1,4-linked β-D-mannuronic acid (M-block) and α-L-guluronic acid (G-block) units. Aqueous solutions of alginates can gel upon the addition of multivalent cations via ionic interactions between the acid groups on G blocks and the gelating ions [128]. Such ionic-association-based alginate hydrogel is resistant to compression and can support *in vitro* culture of chondrocytes for several weeks. Besides, alginate is biodegradable and biocompatible, and is a Food and Drug Administration (FDA)-approved polymer [129]. When implanted *in vivo*, alginate induced only a very mild inflammatory reaction [130]. Therefore, alginate has become one of the most important biomaterials for diverse applications in regeneration medicine [129, 131]. In addition, the degradation rate and mechanical properties of alginate-based biomaterials are influenced by the molecular weight of alginate. Higher molecular weight decreases the number of reactive sites available for hydrolytic degradation, which further facilitates a slower degradation rate. Meanwhile, degradation also inherently influences the mechanical properties because of structural changes both at the molecular and macroscopic levels [129]. Moreover, since alginate is inert, hydrophilic, and resistant to protein and cell adhesion, it has the remarkable ability to maintain the phenotype of chondrocyte and support ECM accumulation [132–134]. Finally, because of the high encapsulation efficiency and mild fabrication conditions, alginate is frequently used as a vehicle for maintaining the bioactivity of growth factors and their controlled release [135]. When alginate is modified with sulfate, higher growth factor loading capacity and more sustained release could be achieved, as demonstrated by both *in vitro* and *in vivo* studies [136]. However, this kind of hydrogel has a much lower mechanical strength compared to that of native cartilage, which is the major challenge for extensive alginate application in cartilage tissue engineering [126]. Because of the gelating ions' elution into surrounding environment, alginate hydrogels will further degrade and lose their mechanical properties [137]. In addition, for anchorage-dependent cells, such as MSCs, the capacity of alginate to allow cell attachment is insufficient, which, however, can be increased by covalently modifying alginate with adhesive molecules such as RGD [138]. Alginate immobilized with the RGD peptide has been shown to promote chondrogenic differentiation of human MSCs [132].

Agarose Agarose is a polysaccharide derived from marine algae and consists of thr basic repeating units 1,4-linked 3, 6-anhydro-α-L-galactose and 1,3-linked β-D-galactose. When agarose is heated up to 65 °C and above, it becomes soluble in water. When temperature is lowered down to 17–40 °C, spontaneous gelation will occur due to hydrogen-bonding interaction between agarose polymers. Agarose has been shown to have the ability to maintain the phenotype of

chondrocytes and support functional ECM production, thereby increasing the mechanical properties of cell-embedded constructs to the same degree as native cartilage [139]. For this reason, agarose also has been widely used as a model system to study the effects of dynamic mechanical loading on the differentiation and behavior of encapsulated cells during construct culture [59, 140, 141]. In addition, agarose is biocompatible, as no immune reaction could be observed following agarose implantation *in vivo* [128, 142]. However, agarose is nondegradable because no enzyme for agarose degradation exists in the human body. Exogenous enzymes, such as agarase, can be introduced to degrade agarose before implantation, thereby increasing the collagen content and mechanical properties of engineered constructs to some degree [143]. Like alginate, agarose also lacks bioactive signals, which can be overcome by modification of agarose with bioactive molecules, such as a short adhesive peptide sequence [126].

Hyaluronic Acid HA, which found primarily in cartilage ECM [84], is a linear polysaccharide composed of alternating D-glucuronic acid and *N*-acetyl-D-glucosamine, serving as a core molecule for the binding of aggrecans to form aggregates with the function of resisting compressive load in joints [144]. HA is a natural polymer with excellent bioactivity. It is involved in many biological processes, such as cell proliferation, adhesion, migration, differentiation, morphogenesis, inflammation, and wound repair [67, 145], and can interact with cell surface receptors of HA, such as CD44 and CD168 [146]. HA–CD44 interaction is probably the main reason for HA-induced chondrogenesis in MSCs [147]. In addition, HA hydrogel is able to support chondrocyte phenotype retention and ECM production [148], as well as promote MSC chondrogenesis [135, 144].

Despite its capability of promoting cartilage regeneration, HA hydrogel has some limitations as scaffold for cartilage tissue engineering. First, the mechanical strength of HA hydrogel is not sufficient to withstand the mechanical load in joints after *in vivo* implantation. Mechanical properties, however, can be enhanced with increased cross-linking densities in the network [126]. In addition, because of the slow degradation of HA *in vivo* via hyaluronidases, cartilage-specific ECM accumulation and neocartilage formation are very limited in HA scaffolds. Incorporation of degradable components into HA scaffolds can increase the HA degradation rate for better ECM distribution and new tissue formation [149]. Such components include hydrolytically degradable units [149] and MMP cleavable peptide for cell-mediated degradation [150]. Furthermore, degradation products of HA have the tendency to induce chondrocytic chondrolysis, that is, enhanced chondrocyte catabolism, which can result in extensive loss of cartilage proteoglycan [151].

Chitosan Chitosan, derived from partially deacetylated chitin found in the exoskeleton of crustaceans, is a polysaccharide consisting of glucosamine and *N*-acetyl glucosamine [152]. Chitosan is biocompatible and biodegradable. Its degradation products are nontoxic [153] and elicit only negligible immune reaction *in vivo* [123, 152]. In addition, because chitin is widespread in nature,

chitosan is cheap and can be available on a large scale with exceptional antimicrobial property [123]. Furthermore, chitosan shares some structural characteristics with GAGs and HA in cartilage ECM, making it an ideal scaffolding material in articular cartilage engineering [154]. GAGs and HA play a critical role in modulating chondrocyte morphology, differentiation, and function [155]. Chitosan has been shown to support chondrogenic differentiation of MSCs [156] and cartilage ECM protein expression by chondrocytes [157]. As a result, it is widely used as a scaffold in tissue engineering. Finally, upon addition of glycerophosphate, chitosan can form a novel thermosensitive hydrogel, transition from a viscous liquid state at room temperatures to a semisolid-like gel at body temperature, which makes it promising as an injectable hydrogel [158, 159].

However, chitosan does not favor cell adhesion [153]. Combining chitosan with other bioactive materials, such as gelatin, is a method to create suitable biomimetic microenvironments for cells [110, 159–161]. Besides, depending on the source of chitosan and the preparation method, its molecular weight may range from 10 to 1000 kDa and its deacetylation degree can oscillate from 50% to 95%, which will greatly affect the scaffold structure of the obtained chitosan and subsequent biological responses of cells seeded on it. Thus, the choice of chitosan with appropriate molecular weight and deacetylation degree is necessary before its application in tissue engineering [162]. Furthermore, the brittleness of chitosan scaffolds in its wet state is another drawback for its application in cartilage tissue engineering [157]. When combined with negatively charged materials, such as poly(L-lactic-*co*-glycolic acid) (PLGA) and gelatin, the positively charged chitosan can form polyelectrolyte complexes due to ionic interaction between the two molecules, resulting in further gelation and improved mechanical properties [68, 163].

8.3.2.2.2 Synthetic Materials

Compared to natural materials, synthetic materials have unique advantages in that their porosity, hydrophilicity, degradation time, and mechanical properties are well defined and can be controlled accurately. In addition, they can be manufactured in large quantities with a high degree of reproducibility [164].

Polyester Polyester is one kind of synthetic material, which includes poly(glycolic acid) (PGA), poly(L-lactic acid) (PLLA), and poly(L-ε-caprolactone) (PCL), and their copolymers such as PLGA and poly(L-lactide-*co*-ε-caprolactone) (PLCL). It has many advantages as scaffolds in cartilage tissue engineering. First, these materials are degradable, which means initially they can provide the necessary mechanical support to cells and then gradually degrade to make room for the newly formed tissues. Depending on the molecular weight, polymer morphology, crystallinity, and composition, the degradation time of these materials can vary extremely, from days to years [165]. Second, because these materials are synthesized under definite conditions, they are highly reproducible. Third, as synthetic materials, their composition, architecture, and physical properties are adjustable [166]. For example, copolymers have attracted special attention, as they have the unique advantage that their physical and chemical properties can be adjusted by varying the ratio of each monomer [167]. Polymer blends, such as

PLLA/PCL, are also an alternative strategy to achieve adjustable microstructures as well as physical and chemical properties [168]. Finally, these materials are biocompatible and have been approved by the FDA for clinical use [169]. However, because of the fast degradation of some of these materials *in vivo*, acidic degradation products easily accumulate locally, leading to inflammation reaction in the area of construct implantation [170]. In addition, because of rapid degradation, these materials cannot main their architectural integrity and mechanical properties during the long-term *in vitro* culture before implantation [165, 171]. Meanwhile, these materials also are also hydrophobic, that is, without a hydrophilic surface that supports cell proliferation and ECM synthesis [170]. Finally, these materials are brittle and lack the necessary flexibility to mimic cartilage tissue [168]. PCL is synthetic polymer of this kind with much slower degradation; it will take up to 3 years for complete removal from body [172]. Moreover, PCL has been shown to have elastic properties close to those of native cartilage [165]. PLGA is a degradable synthetic polymer with wide application in tissue engineering because of its excellent biocompatibility, biodegradability, and mechanical strength [173], as well as low cytotoxicity and immunogenicity, compared with protein-based polymers [174]. PLCL is able to degrade much more slowly than PLLA and thus can avoid abrupt pH drops like those reported in a PLLA scaffold [175]. In order to overcome the shortcomings of this kind of synthetic material, a combination of this with a natural material is a commonly used strategy to enhance its wettability and bioactivity. For example, PCL can be more beneficial for chondrocytes to grow on it when it is combined with the natural material chitosan, which can enhance the hydrophilicity of PCL [157].

Other Synthetic Polymers Poly(*N*-isopropylacrylamide) (pNIPAM) is a synthetic polymer with the ability to switch from a low-viscosity solution to a high-viscosity solution at body temperature, because it undergoes a thermally induced phase transition at 32 °C from a swollen hydrated state to a shrunken dehydrated state. Thus, this hydrogel is extensively investigated as a noninvasive, injectable hydrogel for clinical use. However, its inability to degrade *in vivo* hinders its use in body [166].

Polyurethane (PU) is a kind of polycarbonate with proper mechanical strength, favorable elasticity, suitable degradation time *in vivo*, nontoxic degradation products, and high biocompatibility. Also, it can support both cell attachment and ECM accumulation. As a result, these properties make it a candidate material in cartilage tissue engineering [176]. However, because of its surface hydrophobicity, cell distribution and ECM protein accumulation on this material is poor. Moreover, chondrocytes seeded on PU will dedifferentiate upon long-term culture [164].

Poly(ethylene glycol) (PEG) is a hydrophilic, relatively inert, and biocompatible material. It has been proven to support chondrocyte proliferation and ECM accumulation [177–179]. However, due to the absence of bioactive moieties, PEG does not favor cell growth and function to the same degree as some natural materials [103]. Table 8.2 summarizes the materials used in cartilage tissue engineering.

Table 8.2 Materials used in cartilage tissue engineering.

Type	Material	Advantages	Disadvantages	References
Protein-based	Collagen	• Promotion of MSC chondrogenesis • Hydrophilicity	• Low in mechanical properties • Fast degradation *in vivo*	[100, 104, 109–116]
	Fibrin	• Promotion of MSC chondrogenesis • Hydrophilicity • Low in antigenicity	• Low in mechanical properties • Fast degradation *in vivo*	[117–122]
	Gelatin	• Promotion of MSC chondrogenesis • Hydrophilicity • Low in antigenicity • Low cost	• Low in mechanical properties	[123–127]
Polysaccharide-based	Alginate	• Maintenance of chondrocyte phenotype • Biodegradability • Biocompatibility • Hydrophilicity • Mild fabrication conditions	• Low in mechanical properties • Low in bioactivity	[126, 128–138]
	Agarose	• Maintenance of chondrocyte phenotype • Support of ECM accumulation • Biocompatibility	• Nondegradability • Low in bioactivity	[139–143]
	Hyaluronic acid	• Support of chondrocyte phenotype retention and ECM production	• Low in mechanical properties • Slow degradation *in vivo* • Risk of chondrolysis induction via degradation products	[84, 144–151]
	Chitosan	• Maintenance of chondrocyte phenotype • Biodegradability • Biocompatibility	• Low in mechanical properties • Low in bioactivity • Variations in quality	[152–163]

Synthetic	PLLA	• Biocompatibility	• Hydrophobicity • Fast degradation *in vivo* • Low in bioactivity	[165–168]
	PCL	• Elasticity • Biodegradability • Biocompatibility	• Hydrophobicity • Low in bioactivity	[165, 172]
	PLGA	• High in mechanical strength • Biocompatibility • Biodegradability	• Hydrophobicity • Low in bioactivity	[173, 174]
	PLCL	• Biodegradability • Biocompatibility	• Hydrophobicity • Low in bioactivity	[175]
	pNIPAM	• Used as noninvasive injectable hydrogel due to phase transition property	• Non-degradability	[166]
	PU	• High in mechanical strength • Elasticity • Biocompatibility • Biodegradability	• Hydrophobicity	[164, 176]
	PEG	• Hydrophilicity • Biocompatible	• Low in bioactivity • Non-degradability	[103, 177–179]

8.3.3 Biochemical Stimuli

8.3.3.1 Growth Factors

Chondrogenic differentiation and phenotypic expression of stem cells are primarily affected by TGF-β and BMP. Other growth factors, such as FGFs, insulin-like growth factor (IGF), and connective tissue growth factor (CTGF), are also involved in the chondrogenesis of stem cells. These biomolecules have a wide range of activities, including stimulating cell proliferation, enhancing the synthesis and accumulation of cartilage-specific ECM, and facilitating chondrogenesis [57].

8.3.3.1.1 Transforming Growth Factor-Beta

TGF-β is the most commonly used growth factor for stimulating chondrogenesis. It has the capacity to maintain the phenotype of chondrocytes, attenuating their dedifferentiation during monolayer expansion. The addition of TGF-β, alone or in combination with other factors, into the culture during the *in vitro* expansion phase of chondrocytes can contribute to a better maintenance of the cartilage phenotype compared to control cultures without TGF-β [180]. In addition, TGF-β can induce full chondrogenesis of stem cells, which manifests as the expression and synthesis of type II collagen and aggrecan by these stem cells. Upon addition to cell cultures, it initially induces cell proliferation and then enables cell condensation into clusters for subsequent differentiation into chondrocytes [132]. Besides, TGF-β has been demonstrated to have the ability to upregulate the superficial zone protein expressed by the chondrocytes in the superficial zone of articular cartilage [181–184]. TGF-β has three subtypes: TGF-β1, TGF-β2, and TGF-β3. However, TGF-β1 [120, 132, 136, 185, 186] and TGF-β3 [70, 135, 187, 188] are more commonly used than TGF-β2 [189] because of their capacity to induce higher levels of aggrecan and type II expression by chondrogenically induced cells [43].

Although TGF-β can induce stem cells to form cartilage-like tissue *in vitro*, these differentiated cells will easily proceed to a hypertrophic state especially after implantation into an uncontrolled *in vivo* environment, which commonly results in extensive ECM calcification and vascular invasion [190]. Additional biosignals, such as signals provided by parathyroid hormone-related protein (PTHrP), are needed to prevent the chondrogenically induced MSCs from further differentiating into a hypertrophic phenotype [135].

8.3.3.1.2 Bone Morphogenetic Protein

BMP, belonging to TGF-β superfamily, has similar effects on chondrocyte phenotype maintenance and chondrogenic differentiation of stem cells as TGF-β. First, several subtypes of BMP have been demonstrated to enhance the synthesis of type II collagen and aggrecan by chondrocytes. For example, BMP-2 has the potential to maintain chondrocyte phenotype and facilitate dedifferentiated chondrocytes to redifferentiate, thereby enabling them to produce cartilage-specific ECM again [47]. Also, high expression of BMP-2 resulting from by the delivery of the vector encoding the BMP-2 gene in dedifferentiated chondrocytes could help reverse compromised chondrogenic properties and improve the quality of newly formed

cartilage by chondrocytes [191]. Finally, BMP-2 enhanced the expression of type II collagen and aggrecan of chondrocytes when they were cultured in an appropriate 3D environment [192]. Second, different BMP members have been shown to stimulate chondrogenic differentiation of stem cells. BMP-2, BMP-6, and BMP-7 have been used to induce chondrogenesis of stem cells [156, 186], and BMP-2 and BMP-7 show much higher chondrogenic potentials than TGF-β [193]. In addition, several members of BMP, such as BMP-2, BMP-4, and BMP-6, also have been proved to enhance TGF-β3-induced stem cell chondrogenesis because of their ability to induce the expression of the TGF-β receptor [194]. Among them, BMP-2 has been shown to be more effective than BMP-4 and BMP-6 in stimulating the chondrogenesis induced by TGF-β3 [195]. BMP also has the capacity to facilitate the healing response of full-thickness defects of cartilage. For example, BMP-7 has been shown to promote cartilage defect repair *in vivo* [196]. Furthermore, BMPs, such as BMP-7, can increase accumulation of superficial zone protein in articular cartilage [182]. The disadvantage of BMP is that a higher amount of BMP has high risk of causing ectopic bone formation *in vivo* due to its narrow spectrum of tissue regenerative dose.

8.3.3.1.3 Other Growth Factors

Other growth factors also have been demonstrated to affect cell chondrogenic differentiation and phenotypic expression. FGF-2, a chondrocyte mitogen that exists in normal cartilage, can stimulate chondrocytes to synthesize cartilaginous ECM [197]. FGF-2 has been demonstrated to accelerate cartilage repairs on the articular defects *in vivo* with improved biomechanical properties [198, 199]. IGF-1 can not only enhance cartilage formation in tissue-engineered cartilage constructs [200] but also promote cell-based repairs of articular cartilage defects *in vivo* [201], because IGF-1 is important in regulating cartilage growth. CTGF is strongly expressed in growing cartilage and enhances the articular chondrocyte differentiation, growth, synthesis of matrix, and inhibition of articular chondrocytes mineralization [202]. In addition, it has been shown to accelerate the regeneration of articular cartilage in rat knee joints [203]. However, CTGF is limited in its application because of its rapid diffusivity, denaturation, and degradation when used directly *in vivo* [204].

8.3.3.2 Other Bioactive Reagents

In addition to growth factors, other bioactive reagents are also extensively explored to promote cartilage regeneration, and already have shown encouraging results. PTHrP, expressed by periarticular perichondrium cells in response to signals from prehypertrophic chondrocytes, has the ability to inhibit chondrocyte maturation and hypertrophy [205]. PTHrP is also expressed in chondrocytes of articular cartilage to maintain their phenotype and prevent their terminal differentiation [206–208]. In addition, PTHrP has been demonstrated to improve the chondrogenesis of MSCs and simultaneously suppress their hypertrophic differentiation to obtain stable chondrocyte phenotype [209, 210]. Moreover, PTHrP has been shown to improve cartilage repair and regeneration in a rabbit model as a result of suppressed terminal differentiation and enhanced chondrogenesis of repaired cartilage tissues [211].

Small molecules, with selective gene transcription modulation capacity, are also useful tools in stimulating chondrocyte progenitor cell differentiation. For example, the heterocyclic drug-like small molecule kartogenin has been demonstrated to promote MSC chondrogenic differentiation via specific modulation of transcription factors associated with chondrogenesis [212].

PRP, an autologous concentration of platelets in a small volume of plasma, contains various enriched growth factors and bioactive proteins [213]. It has been reported that PRP can promote articular cartilage regeneration because of its capacity to stimulate chondrocyte proliferation and maintain chondrocyte phenotype both *in vitro* and *in vivo* [214], as well as enhance the chondrogenic differentiation of MSCs from different sources [78, 215]. Also, PRP has been shown to have the potential to improve osteochondral healing in a rabbit model [216] and facilitate cartilage regeneration in the inflammatory microenvironment of OA [217].

8.3.4 Mechanical Stimuli

Healthy articular cartilage in a joint is subjected to mechanical loading on a daily basis, and it is widely known that mechanical loading plays an important role in the maintenance of the normal structure and function of cartilage by influencing metabolism of chondrocytes within the tissue. Therefore, in addition to biochemical stimulations, mechanical stimulation is also used in cartilage tissue engineering to obtain cartilage tissues with adequate structural and mechanical properties.

Different forms of mechanical loading, such as dynamic and static, have been adopted for improved mechanical properties of the engineered cartilage. It has been found that dynamic loading is more favorable than static loading in promoting cartilage ECM synthesis. For example, a study on agarose-based cartilage constructs has proved that a static load decreased proteoglycan and protein synthesis, whereas dynamic compression at physical frequencies and amplitudes stimulated ECM synthesis [218]. Although short-term continuous dynamic loading at physiological levels has been shown to enhance cartilage ECM deposition in engineered cartilage to some degree [219, 220], intermittent dynamic loading is more mimicking of *in vivo* joint mechanical environment and can support chondrocyte proliferation and ECM accumulation within scaffolds more significantly [141, 221]. For example, with 4 weeks of intermittent dynamic loading (1 h loading on and 1 h loading off, three times per day, 5 days per week), a 21-fold increase in stiffness of engineered cartilage grafts was achieved [141]. Moreover, the timing of the mechanical loading imposed on engineered cartilage dramatically influences the cartilage tissue maturation process. In order to obtain mechanically competent artificial cartilage, mechanical loading is supposed to be applied when chondrocytes produce sufficient ECM to help them sense mechanical stimuli [220]. A study revealed that both the beneficial effect of dynamic loading and adverse influences of static loading on agarose-based cartilage graft were more pronounced in the late stage of culture, indicating that the ECM produced by chondrocytes has great importance on transduction of the mechanical signals to chondrocytes embedded within it [222]. Also, when PEG-based constructs were

subjected to dynamic loading soon after chondrocyte encapsulation, chondrocyte proliferation and ECM synthesis were inhibited since there was not sufficient ECM deposition within hydrogel, enabling efficient signal transduction from mechanical stimuli to biosignal that could be perceived by cells [221, 223, 224].

Mechanical stimulation can not only improve the structure and mechanical properties of chondrocyte-based engineered cartilage but also lead to enhanced chondrogenic differentiation of stem cells and their synthesis of cartilage-specific ECM [225, 226]. For example, chondrogenesis of BMSCs in a 3D environment could be improved by periodic dynamic compression, with the formation of ECM of a higher quality both *in vitro* and *in vivo* [227]. Chondrogenic differentiation of stem cells from other donor sites, such as ASCs, could also be enhanced by dynamic compression [228]. Puetzer *et al.* demonstrated that cyclic hydrostatic pressure alone could initiate ASC chondrogenic differentiation even under culture conditions without soluble chondrogenic factors [59]. In addition, the frequency and amplitude of mechanical loading can also affect the chondrogenesis of stem cells. It has been reported that higher load frequency and higher compression amplitude resulted in enhanced chondrogenesis of MSCs, with higher GAG synthesis, chondrogenic gene expression, as well as TGF-β1and TGF-β3 gene expression [229]. Thus, loading regimes for each specific stem cell should be modified for optimized chondrogenic differentiation.

Furthermore, because of kinematically complex joint movements, the application of a single stimulus cannot precisely reflect the complex *in vivo* situation. A bioreactor that is a capable of mimicking the kinematics of the joint was recently developed. Using this bioreactor system, several different stimuli, such as compression, shear, and fluid flow, can be applied to engineered cartilage simultaneously. Compared to single loading, complex loading not only upregulates chondrogenic gene expression in chondrocytes [230] but also results in improved chondrogenic differentiation of stem cells [176, 231]. For example, it has been reported that compression or shear force alone was insufficient for the chondrogenic differentiation of MSCs when no exogenous growth factors were supplemented, while the application of shear and dynamic compression simultaneously significantly increased chondrogenic gene expression [232].

8.4 Strategies in Current Cartilage Tissue Engineering

8.4.1 Controlled Delivery of Biochemical Factors

In order for the growth factors to be uniformly accessible to the cells in the engineered constructs, including those located in the middle part of engineered constructs, over a prolonged period, they are always incorporated into scaffolds so as to stimulate the proliferation, chondrogenic differentiation, and phenotype maintenance of cells encapsulated into scaffold in a spatially homogenous manner [47, 120, 196, 233]. However, such directly loaded growth factors will quickly denature and lose their biological activity shortly. In addition, the initial burst release and abrupt fluctuation of the growth factor level is also not desirable for their application in tissue engineering. Finally, since growth factors are potent

biosignals that can raise a wide range of effects *in vivo* even when there is only a slight change in their level, their delivery and release must be localized.

Therefore, approaches for sustained and local release of growth factors in scaffold are explored. One of the methods is to load growth factors into delivery vehicles to achieve controlled release. Various delivery vehicles capable of sustained and local release of growth factors have already been developed. For example, Ahearne *et al.* utilized gelatin microspheres to load TGF-β1 and then incorporated these microspheres into fibrin hydrogels. Results showed that TGF-β1 was released from the gelatin microspheres in a controlled manner and promoted chondrogenesis of stem cells [71]. By adjusting the cross-linking levels of gelatin microspheres, the rate of polymer degradation can be controlled and thus the release rate of growth factors that are loaded into these microspheres can also be tailored [234]. Bian *et al.* developed an alginate microsphere-based carrier for TGF-β3, which retained TGF-β3 bioactivity in the scaffold and promoted chondrogenic differentiation of MSCs [135]. Spiller *et al.* used degradable PLGA microparticles to load IGF-1, observing sustained release of IGF-1over 6 weeks *in vitro* [200]. Other polymeric microspheres are also studied for the possibility of sustained and controlled release of growth factors [234]. However, the possible effect of the microspheres themselves on differentiation of MSCs has not yet been testified. In addition, heparin, which can bind growth factors and form a stable complex, has also been incorporated into the scaffold to maintain the biological activity of growth factors and retard their release. For instance, Park *et al.* used heparin to bind TGF-β3, and such heparin-bound TGF-β3 enhanced chondrogenic differentiation of rabbit MSCs [235]. Nonetheless, besides binding with growth factors, heparin also has several other important biological functions, such as anticoagulation, some of which may result in reduced effectiveness of the growth factors [236]. Further studies are needed to clarify its influence when used to carry growth factors. Moreover, presentation of growth factors via their affinity interactions with the scaffold material, which mimics natural presentation of growth factors by ECM *in vivo*, has also attracted great attention. For example, Re'em *et al.* employed alginate sulfate to bind TGF-β1 via affinity interactions, which led to increased TGF-β1 loading and decreased initial release as well as more sustained release of TGF-β1, compared to TGF-β1 carried in non-sulfated alginate without affinity binding. Accordingly, chondrogenic differentiation of MSCs was greatly enhanced by such affinity-bound TGF-β1 [136]. Table 8.3 summarizes the strategies for controlled release of growth factors that are commonly used in cartilage tissue engineering for enhanced chodrogenesis.

8.4.2 Combination Tissue Engineering with Gene Therapy

Gene therapy, which is used in tissue engineering for increased expression of some proteins by transfected cells, is extensively explored to enhance cell proliferation capacity, differentiation potential, or ability to maintain phenotype [188, 191, 228, 237, 238]. Compared to exogenous growth factor supplementation, gene therapy is able to provide more sustained expression of endogenous chodrogenic factors [204, 239]. For example, Lu *et al.* showed that genetically engineered ASCs could persistently express TGF-β3/BMP-6, which improved the

Table 8.3 Strategies for controlled release of growth factors used in cartilage tissue engineering.

Growth factors	Delivery vehicle	Study type	Main effects	References
BMP-2	Collagen hydrogel	*In vitro*	Increased viability of chondrocytes and ECM synthesis	[73]
TGF-β1	Fibrin gel	Both *in vitro* and *in vivo*	Facilitated chodrogensis of BMSCs and enhanced repair of full-thickness cartilage defects in rabbits	[120]
BMP-7	PLGA matrix	*In vivo*	Facilitated cartilage regeneration on osteochondral defects in rabbits	[196]
TGF-β1	Gelatin microspheres	*In vitro*	Promoted chondrogenesis of MSCs with increased GAG and type II collagen synthesis	[71, 234]
TGF-β3	Alginate microspheres	*In vitro*	Increased mechanical properties and cartilage ECM content of MSCs seeding tissue constructs	[135]
IGF-1	PLGA microparticles	*In vitro*	Enhanced cartilage formation with increased mechanical properties and cartilage ECM content	[200]
TGF-β3	Heparin-bound hydrogel	*In vitro*	Promoted cartilage tissue formation with higher proliferation rate and cartilage specific ECM production by MSCs	[235]
TGF-β1	Sulfated alginate hydrogel	Both *in vitro* and *in vivo*	Induced chondrogenic differentiation of MSCs both *in vitro* and *in vivo* with deposited type II collagen and aggrecan found in tissue constructs	[136]

production of cartilaginous constructs *in vitro* and cartilage regeneration *in vivo* [194]. Generally, there are two approaches for gene therapy to be applied in tissue engineering. In the first, cells are first cultured in monolayers and then transfected by gene/vector complexes, followed by subsequent encapsulation of cells into scaffold to form a tissue construct [237]. For instance, Lee and Im transduced ASCs with SOX-5, SOX-6, and SOX-9 genes during their monolayer culture, followed by their encapsulation in a fibrin gel to treat osteochondral defects in a rat model, which resulted in enhanced chondrogenic differentiation of ASCs and accelerated cartilage healing [106]. However, this method has limited application *in vivo* because of the migration and apoptosis of the transplanted cells.

In the other method, gene/vector complexes are first loaded into the scaffold and then cells are incorporated into this scaffold for *in situ* transfection. In this case, transfection of embedded cells will occur both before and after implantation [185]. For example, Wang *et al.* loaded plasmid DNA encoding TGF-β1 into the scaffold to transfect encapsulated MSCs. Such tissue constructs were then used to repair full-thickness cartilage defects in rabbits. Results showed that cartilage defects were successfully regenerated with the formation of hyaline cartilage-like tissues abundant in type II collagen. Also, better chondrogenesis of MSCs was achieved, as evidenced by upregulated cartilage-specific genes expression in the repaired tissues [53]. *In situ* transfection of seeded cells may be more promising, since gene transfection is localized and can be carried out under good control, thus ensuring the safety of gene therapy to some degree.

8.4.3 Biomimetic Hierarchical Cartilage Tissue Engineering

Mimicking the native architecture and function of a tissue as closely as possible is the general paradigm of tissue engineering. Based on this principle, in order to promote osteochondral defect regeneration, bilayered scaffolds that mimick natural cartilage and bone interface have been designed, with one layer of the scaffold stimulating of chondrogenesis and the other layer facilitating osteogenesis simultaneously [240]. The material and mechanical properties in each layer are usually different, thus enabling one layer more conducive to articular cartilage regeneration and the other layer more favorable for bone regeneration [173, 241–243]. For example, Ding *et al.* designed a biphasic scaffold, which consisted of a PGA/PLA layer for cartilage repair and a PCL/hydroxyapatite layer for bone repair [244]. Galperin *et al.* fabricated a bilayered scaffold consisting of one layer that had a single defined, monodispersed pore size of 38 μm with surfaces coated with hydroxyapatite particles to promote regrowth of subchondral bone, and the other layer that had 200 μm pores with surfaces decorated with hyaluronan for articular cartilage regeneration [245]. Zhang *et al.* developed a bilayered collagen–silk scaffold, with a collagen layer for cartilage repair and a hydroxyapatite–silk layer for subchondral bone repair [211]. Biological methods are also used to promote cartilage and bone regeneration. For example, Chen *et al.* designed a bilayered scaffold consisting of one layer containing TGF-β1 plasmid for chondrogenesis and the other containing BMP-2 plasmid for osteogenesis. Upon seeded on this scaffold, MSCs in different layers underwent chondrogenic differentiation and osteogenic differentiation, respectively, *in vitro*. Additionally, such bilayered scaffolds incorporated with MSCs facilitated the regeneration of both articular cartilage and subchondral bone in a rabbit knee ostochondral defect model [161].

In addition, native articular cartilage also has a complex zonal organization as evidenced by different cell morphologies, cell arrangements, biochemical compositions, and mechanical properties [246]. For example, the gene expression profiles in the surface, middle, and deep zones of hyaline articular cartilage are distinct; especially the gene expression patterns between the superficial and middle zones have obvious differences, and the functions of different zones are also different [247]. Since the normal physiologic role of articular cartilage is determined by zonal organization, it is important to reproduce such fine architecture

of the native cartilage when attempting to engineer the truly usable cartilage tissue substitute. Numerous studies have been performed to mimic the zonal structure of articular cartilage. The superficial zone of articular cartilage is important because chondrocytes in the superficial zone can synthesize the superficial zone protein, which is a key mediator of boundary lubrication of articular cartilage in joints. Yamane and Reddi used BMP-7 and TGF-β1 to prime stem cells from synovium toward chondrocytes in the superficial zone of articular cartilage, which can produce the superficial zone protein. As a result, the surface layer of the cartilage was engineered [248]. For zonal structural engineering, Kim *et al.* regenerated the zonal organization of articular cartilage by encapsulating chondrocytes from different zones of the articular cartilage into multilayered hydrogels, with each zone of the multilayered constructs showing similar histological findings to that of native articular cartilage after 21 days of *in vitro* culture [249]. Besides using zone-specific chondrocytes isolated from cartilage, development of multilayered hydrogel constructs with different compositions capable of directing specific differentiation of a single stem cell population is also an approach to mimic the spatial organization of cartilage. For example, Nguyen *et al.* demonstrated that a native-like articular cartilage with spatially varying mechanical and biochemical properties could be created by a three-layer PEG-based hydrogel with CS and MMP-sensitive peptides incorporated into the top layer, CS incorporated into the middle layer, and HA incorporated in the bottom layer [250]. However, whether such zonal cartilage constructs can still maintain zonal organization and regenerate cartilage defects with repair tissue properties similar to the native cartilage *in vivo* needs to be further investigated.

8.4.4 Application of Cartilage-ECM-Derived Scaffolds

Since cartilage ECM components, such as GAGs and collagen, can stimulate the chondrogenic differentiation of stem cells, scaffolds directly derived from devitalized cartilage also have the ability to induce chondrogenic differentiation of such cells [251]. Such scaffolds have been developed recently and demonstrated to highly promote chonodrogenic differentiation of stem cells, thus showing great promise in cartilage tissue engineering [252]. These scaffolds can be created by chemical or physical processing. Physical methods, including thermal shock, freeze–thaw cycles, and mechanical crushing, will disrupt the cellular membranes and nuclei but will not remove cell components and DNA, leading to devitalization scaffold formation [253]. Chemical methods include the use of detergents or other chemicals to break down cellular and nuclear membranes, followed by several washing steps to totally remove cell components and DNA. Chemical methods combined with physical methods are effective in obtaining fully decellularized scaffolds [254]. The devitalization process is easy to perform, but the cellular components easily give rise to immune reactions after implantation *in vivo*. Although the decellularization process removes all cells and cellular antigens, the bioactive cues that reside in the ECM are hard to be preserved. Whether absolute decellularization is necessary is still under discussion, and further studies addressing this issue are needed in the future. In addition, according to the source of the cartilage ECM, there are two kinds

of ECM from which scaffolds can be made: native cartilage-derived ECM, and cell-derived ECM. Native cartilage-derived ECM is ECM from native cartilage, which can be obtained from human cadavers or xenogeneic sources, whereas cell-derived ECM is ECM secreted by cells cultured *in vitro* [107]. Native cartilage-derived ECM scaffolds suffer from problems of host responses, while cell-derived ECM scaffolds can be prepared from autologous cells expanded *in vitro* after isolation from patients, thus circumventing the undesired host responses that may be induced by allogeneic or xenogenic materials [255]. Thus, cell-derived ECM scaffolds show greater promise.

Many studies have used cartilage ECM-derived scaffolds for cartilage defect repair both *in vitro* and *in vivo* [243, 251]. For example, Yang *et al.* obtained a native cartilage-derived ECM scaffold through a decellularization process, including physical shattering of cartilage, decellularizing cartilage with the use of hypotonic buffer and nuclease solution forming an ECM suspension, cross-linking, and finally freeze-drying the ECM suspension to obtain ECM-derived scaffold. Such generated native cartilage ECM-derived scaffold supported both *in vitro* MSCs adhesion, proliferation and differentiation, and *in vivo* cartilage-like tissue formation [256]. In another example, Cheng *et al.* seeded ASCs on to porous scaffolds derived from adult porcine articular cartilage and cultured the cells without any exogenous growth factor supplement. Results indicated significant ECM synthesis and accumulation within the scaffold, as well as enhanced mechanical properties of the construct similar to those of the native cartilage [257]. In addition, cartilage-ECM-derived scaffolds have the potential to be used as carriers for controlled and sustained delivery of growth factors. For example, TGF-β3, which was loaded onto the scaffold, was released in a controlled manner over the first 10 days of culture. Chodrogenic differentiation of stem cells on such TGF-β3-loaded constructs was comparable to that of stem cells on the scaffolds with exogenous TGF-β3 added to the medium [70].

8.4.5 Scaffold-Free Cartilage Tissue Engineering

Although various scaffolds have been developed to support cells proliferation, differentiation, and function in a 3D environment in cartilage tissue engineering, there are still some concerns regarding scaffold degradation rates, toxicity of scaffold degradation products, scaffold retention, biocompatibility, and mechanical properties that can match those of native cartilage. In addition, during embryogenesis, cells organize into differentiated tissues simultaneously via biochemical signals and cell-to-cell interaction rather than exogenous scaffold. Therefore, techniques used to generate cartilage constructs without a scaffold have been recently developed. For example, Kaneshiro *et al.* developed layered, scaffold-free chondrocyte sheets using temperature-responsive culture dishes and successfully repaired a partial cartilage defect in a rabbit model [258]. The temperature-responsive culture dish is a dish with its surface coated with a polymer that can become hydrophilic or hydrophobic in a reversible manner depending on the temperature [259]. When the temperature is 37 °C or higher, the surface of the temperature-responsive culture dish is weakly hydrophobic

and thus can be used to culture cells in a conventional manner. However, when temperature decreases to 32 °C or below, the dish surface becomes hydrophilic and dissolves in culture medium and the confluent sheet of cultured cells will be released from the dish's surface. A single cell sheet can be harvested using this approach, and several cell sheets can be layered to form 3D cartilage constructs for cartilage defect repair [260–262].

In addition, a membrane-based method was also a recently developed to generate scaffold-free neocartilage *in vitro*. For example, Mayer-Wagner *et al.* placed MSCs on top of a cellulose acetate membrane filter and cultured them at the medium–gas interface, successfully producing a scaffold-free stem-cell-derived cartilage tissue construct [263]. Similarly, Murdoch *et al.* cultured MSCs on a permeable membrane of transwell, which also resulted in a scaffold-free cartilage-like tissue formation [264]. Moreover, self-assembly is also used for engineering cartilage constructs [265–267]. Self-assembly involves seeding chondrocytes at high density into prefabricated, nonadherent, cylindrical molds. Cells condense into disk-shaped constructs and, over time, synthesize ECM rich in collagen and sulfated GAGs.

Using the scaffold as a transit support to cells during the initial cell seeding, and removing the scaffold subsequently when cells produce sufficient ECM to self-support, is a new strategy to generate scaffold-free cartilage tissue. For example, Su *et al.* created a cartilage graft comprised of pure chondrocytes and the ECM secreted by them using alginate as an interim scaffold. In this system, chondrocytes and gelatin microspheres were encapsulated into an alginate-based hydrogel. When chondrocytes proliferated and secreted an extensive interpenetrating network of ECM, making the construct self-supporting, the alginate hydrogel was removed under a mild condition by exposing it to sodium citrate, leaving a scaffold-free cartilage graft [268]. Non-cross-linked gelatin microspheres incorporated in the alginate hydrogel quickly dissolved when cultured in 37 °C and were used to create microcavities throughout the alginate hydrogel to provide enough space for neocartilage formation and efficient nutrient diffusion. *In vivo* studies using this scaffold-free cartilage graft in a rabbit model for cartilage defects repair showed full-thickness repair with good graft–host integration (Figure 8.3) [268].

Other methods explored by researchers include fusion culture. Lehmann *et al.* established fusion culture by several steps. First, a suspension of chondrocytes was added to agar-coated dishes without using any scaffold material, and then stable chondrocyte aggregates could form spontaneously after 2 days of culture. Three-dimensional cartilage-like constructs were formed via fusion of several aggregates [269].

8.4.6 Homing Endogenous Cells for Cartilage Regeneration

Cartilage regeneration by recruitment of endogenous cells, such as stem or progenitor cells, through biological cues spatially embedded in cartilage defects is a new strategy in cartilage tissue engineering. The cell source is always the main obstacle in cartilage tissue engineering. Mature autologous chondrocytes are limited by their low availability and loss of their phenotype during *in vitro* expansion,

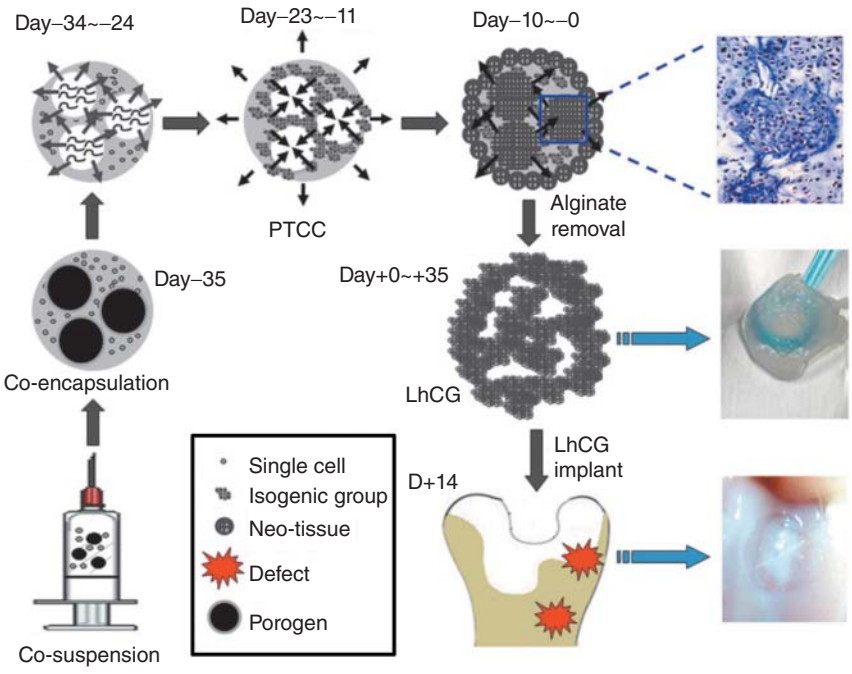

Figure 8.3 Schematic illustration of scaffold-free cartilage graft fabrication process. (Clockwise from left bottom) Porcine chondrocytes are coencapsulated with gelatin microspheres (porogen) in alginate hydrogel. After the gelation of alginate, the dissolution of gelatin microspheres creates cavities within the gel bulk. Chondrocytes proliferate and produce ECM, forming micro tissue nodules within the alginate hydrogel scaffold. The micro tissues interact and secrete ECM extensively, forming an intricate interpenetrating network of ECM and hydrogel. Upon removal of the alginate scaffold, the structural integrity of the construct remains intact and a pure, scaffold-free cartilage graft is thus formed. (Su 2012 [268]. Reproduced with permission of John Wiley & Sons.)

while stem cells are restricted in their chondrogenic differentiation and are difficult to be put under complete control, especially under the complex *in vivo* environment. Recruitment of chondrogenic stem cells *in vivo* to cartilage defects thus can circumvent this problem, and recent studies have already given promising results for this idea. Lee *et al.* employed a TGF-β3-adsorbed acellular scaffold to repair cartilage defects in rabbit. Four months later, cartilage defects that were treated with TGF-β3-adsorbed scaffold fully regenerated with hyaline cartilage in the articular surface, and 130% more cells were recruited in the regenerated cartilage by TGF-β3 delivery than by spontaneous repair response without TGF-β3 (Figure 8.4) [270]. Similar results could also be observed when scaffolds mixed with FGF-2 were used to repair large osteochondral defects of rabbit [199]. Treatment of cartilage defects with a combination of scaffold and biochemical signals that can induce homing of endogenous cells can avoid potential problems associated with cell-based tissue constructs, such as immune rejection, pathogen transmission, and tumorigenesis caused by stem cells. Therefore, this approach shows a higher tendency to be acceptable and widely used in clinical settings. In

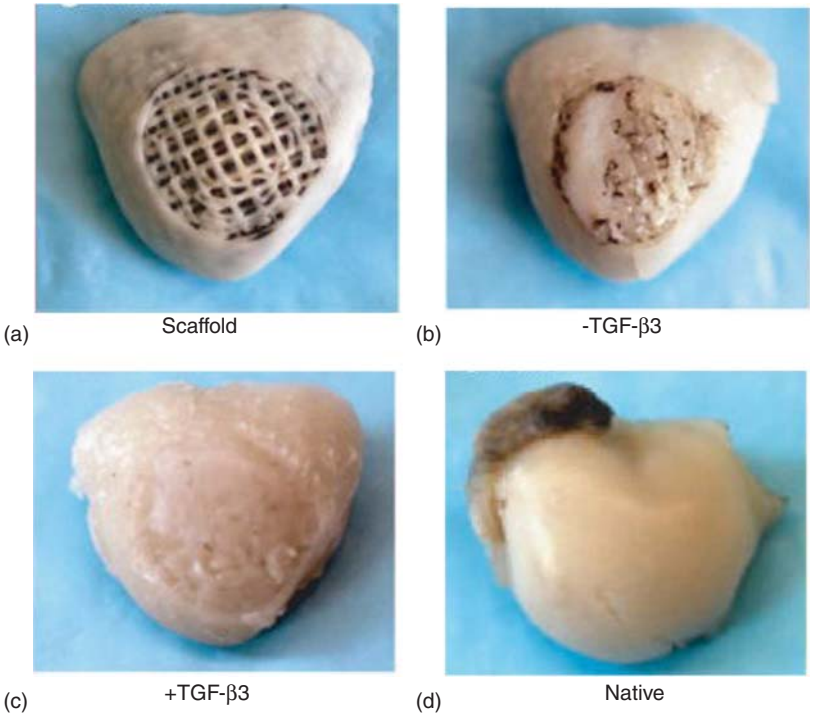

Figure 8.4 Effects of TGF-β3-adsorbed acellular scaffold on cartilage defect repairs in a rabbit model. (a) Unimplanted scaffold, (b) TGF-β3-free scaffold, (c) TGF-β3-adsorbed scaffold, and (d) native cartilage. (Lee 2010 [270]. Reproduced with permission of Elsevier.)

addition, homing of endogenous cells can also be modified by providing biochemical signals from autologous cells, which is more mimicking the regeneration process *in vivo*. For example, Chang *et al.* used a PLGA scaffold seeded with autologous endothelial progenitor cells (EPCs) to repair a full-thickness osteochondral defect in rabbits. Seeded EPCs created a favorable microenvironment for regeneration via secretion of various biomolecules, which triggered the mobilization, proliferation, and differentiation of endogenous progenitor cells and thus promoted both chondrogenesis and osteogenesis [271].

8.5 Conclusions and Future Directions

Although great advancements have been made over the past few decades and many novel approaches have been developed in the field of cartilage tissue engineering, engineered cartilage with properties identical to those of native cartilage is currently unavailable. Cell sources ensuring optimal and stable chondrogenic phenotype of cells, scaffold design mimicking zonal organization of cartilage, scaffold-free cartilage tissue engineering, and re-creation of cartilage substitutes with the structure and properties of native cartilage are major challenges of the future.

Chondrocytes are native resident cells in cartilage and can readily be used during *in vitro* expansion. However, they easily dedifferentiate during *in vivo* culture. In addition, the chondrogenic properties of chondrocytes are not consistent in isolated population. Purification of chondrocytes using cell markers associated with high chondrogenic potential before cell seeding can enhance the hyaline quality of the generated cartilage from expanded chondrocytes [272]. MSCs are promising cell source for cartilage regeneration since they can be isolated from different tissues in the human body in large quantities and also they have ability to proliferate rapidly *in vitro*. But chondrogenic differentiated MSCs easily proceed to the hypertrophic stage rather than maintain stable chondrocyte phenotype, which will result in extensively calcified ECM formation. Because of the MSC-induced chondrocyte proliferation and chondrocyte-enhanced MSC chondrogenesis, cells from cocultures of primary chondrocytes and MSCs seem to be a new promising cell source for cartilage tissue engineering, avoiding hypertrophic differentiation of MSCs [273]. Recruitment of endogenous stem cells that can enhance cartilage regeneration into cartilage defects is also an innovative strategy that can circumvent conventional stem cell isolation and differentiation procedures [270]. However, further research is still needed to verify its validity. Genetically modified cells have shown great potential for enhanced chondrogenic differentiation as well as long-term chondrocytic phenotype maintenance. Although these gene-manipulated cells are excellent candidates for model studies in laboratories, safety issues regarding gene therapy is still the main concern before genetically modified cells are used in clinical practice.

Fabrication of scaffolds with properties closest to the structure and function of native cartilage is the future direction for scaffold design. Scaffolds that can directly bear mechanical loading in the joint have already been developed [274]. Scaffolds with biphasic design showed improved bone and cartilage integration [241]. Engineering cartilage with the zonal structure has already received much attention in order to generate cartilage substitutes with similar structure and function as those of native cartilage [250]. Multiple layered scaffolds mimicking different cartilage zones have high possibility to demonstrate excellent mechanical properties and host tissue integration. Depth-dependent variations in scaffold design (pore size, porosity, mechanical properties, and addition of growth factors) and the origin of the seeded cells (superficial zone, middle zone, or deep zone) can be used as tools in designing zonal scaffolds [249]. Scaffold-free approaches that can allow the formation of constructs totally free from scaffold material with a strategy that takes scaffold in a transit role during cartilage tissue formation are showing great promise [268]. However, scaffold-free cartilage constructs are weak in mechanical properties because of the lack in mechanical support from the scaffold. Scaffold-free cartilage constructs that can bear load immediately upon implantation is desirable in the future.

Stimulation during neocartilage formation using biochemical stimuli, mechanical stimuli, or a combination of both, is employed in cartilage tissue engineering [228]. Biochemical stimuli are commonly used to promote chondrogenesis in traditional culture systems. However, the complex mechanism involved in cartilage regeneration is still not fully understood, which makes it difficult to establish precise differentiation methods for chondrogenesis. Small molecules that promote

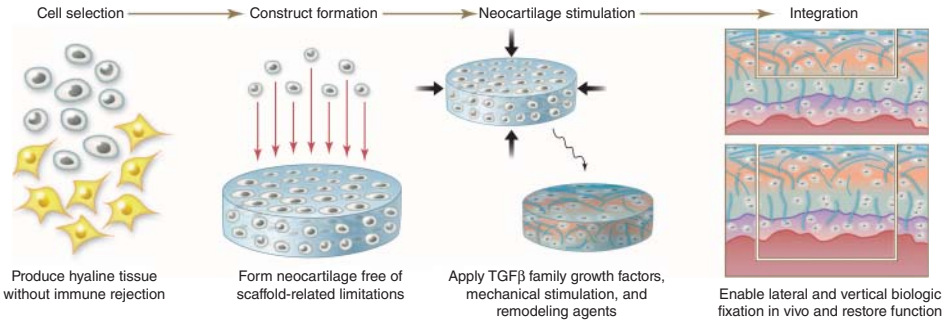

Figure 8.5 Scaffold-free cartilage tissue engineering through various stimulations to fabricate cartilage grafts with the ability to integrate with host articular cartilage and normal articular cartilage function. (From [24]. Reproduced with permission from AAAS.)

the chondrogenic differentiation of MSCs into chondrocytes may finally lead to a controlled differentiation system for MSCs *in vitro* [212]. Various studies have already shown that mechanical stimulation can increase the production of ECM, cell proliferation, and mechanical properties of cartilage constructs [59]. But the optimal combination of biochemical stimuli and mechanical stimuli to cartilage formation is yet to be established. Understanding how these factors synergistically promote chondrogenesis is essential for successful cartilage engineering strategies in the future.

Articular cartilage constructs in the future should be engineered free from biomaterials so as to avoid issues associated with scaffolds derived from these materials. At the same time, various stimuli, such as biological and mechanical ones, should be applied to newly formed cartilage tissues in order to fabricate mature cartilage tissues with mechanical properties similar to those of native cartilages. Essential biological molecules involved in chondrogenesis are yet to be found, and efficient mechanical stimulation regimens for enhanced ECM production also need to be explored. Also, these mature cartilage substitutes should quickly integrate with host articular cartilage upon *in vivo* implantation. Such tissue-engineered cartilage grafts are the desired products for clinical use, which can help restore the function of damaged articular cartilages as soon as possible (Figure 8.5).

List of Abbreviations

3D	three-dimensional
ACI	autologous chondrocyte implantation
AFSC	amniotic fluid stem cell
ASC	adipose-derived stem cell
BMP	bone morphogenetic protein
BMSC	bone-marrow-derived stem cell
CS	chondroitin sulfate

CTGF	connective tissue growth factor
ECM	extracellular matrix
EPC	endothelial progenitor cells
ESC	embryonic stem cell
FDA	Food and Drug Administration
FGF	fibroblast growth factor
GAG	glycosaminoglycan
HA	hyaluronic acid
IGF	insulin-like growth factor
iPSC	induced pluripotent stem cell
KS	keratin sulfate
MDSC	muscle-derived stem cell
MMP-13	matrix metalloproteinase
MSC	mesenchymal stem cell
OA	osteoarthritis
PCL	poly(L-ε-caprolactone)
PEG	poly(ethylene glycol)
PGA	poly(glycolic acid)
PLCL	poly(L-lactide-*co*-ε-caprolactone)
PLGA	poly(L-lactic-*co*-glycolic acid)
PLLA	poly(L-lactic acid)
pNIPAM	poly(*N*-isopropylacrylamide)
PRP	platelet-rich plasma
PTHrP	parathyroid hormone-related protein
PU	polyurethane
RA	rheumatoid arthritis
RGD	Arg–Gly–Asp (peptide)
SDSC	synovium-derived stem cell
TGF-β	transforming growth factor-beta

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9

Liver Tissue Engineering

Jessica L. Sparks

9.1 Introduction

Orthotopic liver transplantation remains the only definitive treatment for end-stage liver disease [1]. Because of the critical shortage of donor organs, much research effort has been invested to develop alternatives to liver transplantation, such as extracorporeal bioartifical liver (BAL) devices, cell transplantation, and tissue-engineered constructs [1–3]. To date, BALs have not yielded significant improvements in survival rates when tested in clinical trials [4, 5]. While cell transplantation has been used in certain liver-based metabolic disorders, cell engraftment rates are low and the engrafted cells may be lost over time [1]; thus, this technique currently cannot substitute liver transplantation. Liver tissue engineering strategies, particularly whole-organ decellularization/recellularization methods, are promising techniques because they have the potential to generate transplantable whole organs [6–8]. Scaffolds made from a decellularized liver retain much of the organ's native extracellular matrix (ECM) architecture and intrinsic network of vascular channels [6]. Thus, these scaffolds have the potential to provide appropriate microenvironmental cues and hemodynamic conditions to support cell attachment, proliferation, differentiation, and function [9].

Some of the key challenges for liver tissue engineering include identifying appropriate cell sources and developing optimal seeding and conditioning strategies to promote tissue organization and function in perfused three-dimensional (3D) scaffold systems. Significant numbers of cells are required for scaffold seeding. Approximately 10^7–10^8 cells have been used to seed decellularized rat liver scaffolds [9], and it is estimated that a minimum of $2.5–7.5 \times 10^9$ cells would be required for a clinical product for human use [1]. Stem cells are among the most promising cell sources for liver tissue engineering because of their higher proliferative capacity and their potential to differentiate into multiple cell types. Stem cells from multiple sources have been used for liver tissue engineering applications, including embryonic, adult, mesenchymal, and induced pluripotent stem cells. The advantages and disadvantages of various cell sources for liver tissue engineering have been reviewed thoroughly elsewhere [1, 9]. With regard to developing optimal seeding and conditioning strategies for the growth and

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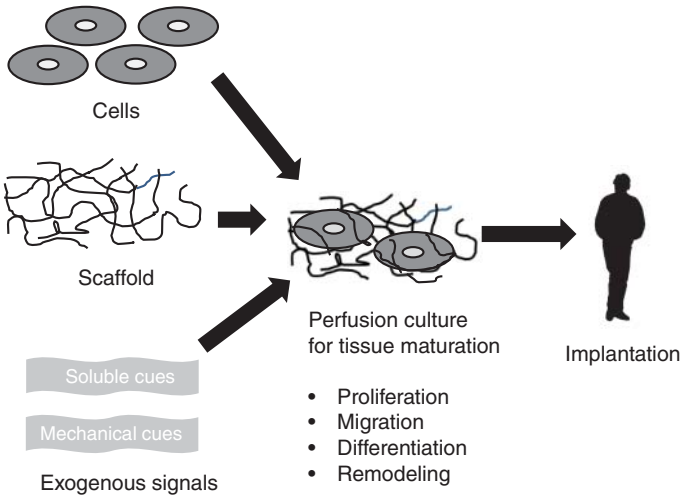

Figure 9.1 Schematic representation of liver lobule.

maintenance of perfused 3D scaffold constructs, several studies have addressed these questions in recent years [6, 7, 10–11]. Various aspects of scaffold seeding and maintenance have been considered, such as flow rate, media composition, and gas exchange, as well as the mechanism and route of cell delivery into the scaffold.

While it is well recognized that soluble biochemical cues provided to seeded cells are of vital importance, it is becoming increasingly clear that the *biomechanical* cues that exist in perfused liver scaffolds also must be considered (Figure 9.1). There is growing evidence that most, if not all, liver cell types exhibit mechanosensitivity to biomechanical cues such as shear stress, hydrostatic pressure, and ECM stiffness [11–14]. Mechanical stimuli have been shown to play a role in key cellular processes such as motility, differentiation, morphological changes, and response to injury [12, 15–16]. In the liver, biomechanical factors are involved in regulating hepatic stem and progenitor cell fate [12, 14] and directing stellate cell, portal fibroblast, and hepatocyte activity in liver fibrosis and cirrhosis [13, 17–18].

Mechanical forces are known to impact multiple liver cell behaviors relevant for tissue engineering applications. For example, Schmelzer *et al.* have reported increased hepatic differentiation of hepatic progenitor cells (HPCs) cultured under constant perfusion, compared to static controls [12]. Others have reported that 3D perfusion culture enhances functional maturation of hepatic differentiated human embryonic stem (EC) cells [19, 20]. Finally, the work of Baptista *et al.* suggests that hemodynamic stresses in vascularized, bioengineered liver tissue can influence the self-organization of different cell types to their respective niches within the native liver architecture in decellularized liver scaffolds [21]. In sum, biomechanical cues are key regulators of liver cell biology and are expected to play an important role in the development of clinically relevant bioengineered liver constructs.

The goal of this chapter is to provide an introduction to the mechanical forces that exist in liver tissue and to explore the significance of biomechanical cues for liver tissue engineering. The following sections will (i) review liver structure; (ii) provide an overview of liver biomechanics, emphasizing organ-, and tissue-scale characterizations of liver's mechanical properties and hemodynamics; (iii) summarize current knowledge of liver mechanobiology, highlighting cellular-scale mechanical forces in liver, cellular mechanotransduction mechanisms, and literature evidence of the mechanosensitivity of numerous liver cell types; and (iv) discuss the dynamic biophysical stimuli that are present in current perfusion-based liver tissue engineering strategies.

9.2 Liver Biology

9.2.1 Organ-Scale Anatomy

The liver is the largest gland in the body, weighing roughly 1.0–1.5 kg in human adults [22]. This complex organ plays an important role in carbohydrate, protein, and lipid metabolism, as well as the synthesis and secretion of blood and bile components and the detoxification of blood [23]. It is located beneath the diaphragm, primarily in the upper right quadrant of the abdomen. It is surrounded by a smooth capsule of connective tissue termed Glisson's capsule.

The liver is highly vascularized. It receives a blood flow of about 1.5–2.0 l min^{-1} in adults, which represents ~25% of the resting cardiac output [24]. About 25% of the total liver blood flow comes from the hepatic artery, while the remaining 75% derives from the portal vein, which drains the splanchnic viscera (stomach, intestines, and spleen) [24]. Blood drains from the liver via the hepatic veins, which open into the inferior vena cava [25]. The parenchyma of the liver can be characterized as a soft, sponge-like mass penetrated by tunnels containing interdigitating networks of vessels [24, 26]. Structural support for this extremely compliant tissue derives from the blood vessels and their investments of connective tissue. The connective tissues surrounding the branching afferent vessels of the liver form portal tracts, which are continuous with the mesenchymal components of Glisson's capsule, forming the liver's "skeleton" [24]. The ducts of the biliary system are also found within the portal tracts. These ducts form progressively larger branches until they emerge at the liver hilum as the right and left hepatic ducts. Functionally, the liver is divided into two major lobes, right and left, each with its own blood supply and its own venous and biliary drainage [25]. The primary branches of the portal vein and hepatic artery can be used to further divide a lobe into segments, with the hepatic veins located between segments and draining adjacent ones [25].

The liver produces a large volume of lymph. The body's lymphatic system processes up to 8 l of lymph per day [27, 28], and as much as half of this lymph comes from the liver [24, 29]. Lymphatic drainage of the liver is accomplished through superficial lymphatic vessels found mainly in Glisson's capsule and the deep lymphatic vessels found in the portal tracts or along the hepatic veins [29].

The smallest lymph vessels end blindly in the portal tracts and are not found in the parenchyma outside of the portal tracts [24, 29, 30]. It has been argued that the perisinusoidal space of Disse can be regarded as a pre-lymphatic space from which hepatic lymph could originate [29], and tracer studies suggest that lymph originates in the parenchyma [24, 31].

9.2.2 Histological Structure

The classical structural unit of the liver is the hepatic lobule [22]. In a 2D cross-sectional view, the hepatic lobule is often represented in schematic form as a hexagon (Figure 9.2), with portal tracts found at the corners and the central vein located at the center of the hexagon. This classical framework is idealized, but it is a useful scheme for understanding liver's architecture and the patterns of microvascular blood flow. The portal tracts contain a trio of structures known as the portal triad: a branch of the hepatic artery, a bile duct branch, and a pre-terminal (or penultimate) branch of the portal vein. Connective tissue, lymphatics, and nerves are also found in the portal tracts, as described previously. The pre-terminal portal veins give rise to terminal portal vein branches, which are oriented approximately 120° apart and travel along the boundaries of the hexagon (Figure 9.2). In some mammals, such as swine, the terminal portal veins are associated with a noticeable connective tissue sheath that clearly demarcates the lobular "boundaries" on the histological section. In humans and many other mammals, the terminal portal vein branches lack a conspicuous connective

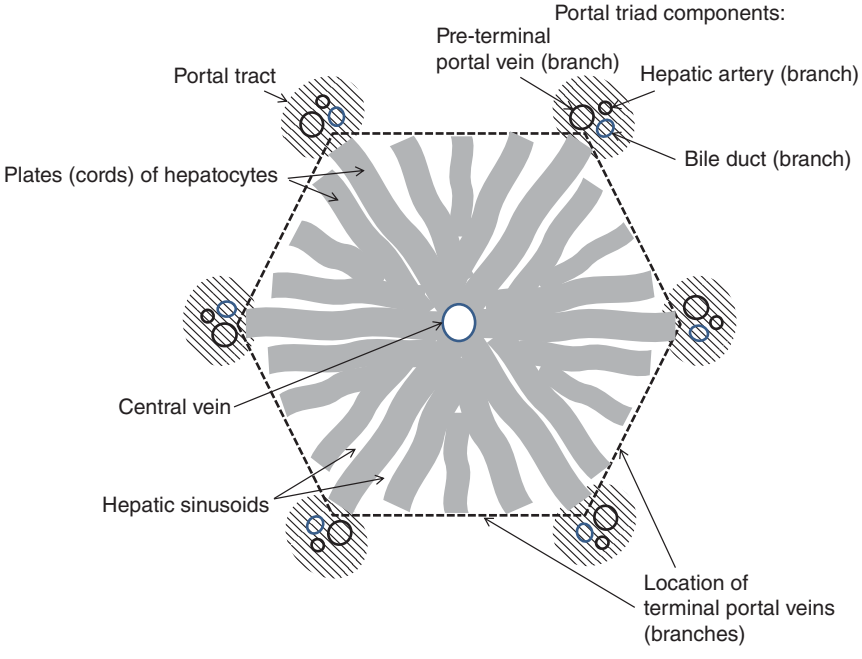

Figure 9.2 Liver tissue engineering aims to recreate the organ *in vitro* for subsequent implantation, to enhance or replace patient liver function [1].

tissue sheath [22, 24]. Regardless of species, Grisham has argued convincingly that the boundaries between adjacent liver lobules are best understood not as structural boundaries but rather as watershed or hemodynamic barriers which distinguish the flow of blood between adjacent microvascular segments formed by the local distribution of blood flow sources and sinks [24].

Hepatic sinusoids, the specialized "capillaries" of the liver, are oriented radially and carry blood from the perimeter of the lobule toward the central vein (Figure 9.2). The central veins, oriented along the axis of the lobule, ultimately drain into the hepatic veins which empty into the inferior vena cava [32]. The hepatic sinusoids receive blood from both hepatic arterial and portal venous inflow sources. During their course through the parenchyma, the terminal portal veins break up completely and give rise directly to the sinusoids [24, 33]; however, the path from the hepatic arterioles to the sinusoids is more complex and is reported to be species-dependent [33]. Within the portal tracts, hepatic arterioles give rise to the peri-biliary plexus, a capillary network around bile ducts. Branches from the peri-biliary plexus drain into the sinusoids and into adjacent portal veins [24, 33–35]. For a review of hepatic circulation, see [36]. The structure of the hepatic sinusoids is unlike that of capillaries elsewhere in the body. Hepatic sinusoids lack a basal lamina (basement membrane) and are lined by a discontinuous layer of fenestrated sinusoidal endothelial cells [22, 24]. The fenestrations are ~100 nm in diameter, lack a diaphragm, and are grouped in clusters [22, 37]. Collectively, the gaps between sinusoidal endothelial cells, the fenestrations within the cells, and the lack of a basal lamina combine to make hepatic sinusoids extremely permeable vascular structures. A sub-endothelial space called the "space of Disse" exists between the sinusoidal endothelial cells and the hepatocytes. The high permeability of the sinusoidal endothelium enables free flow of plasma and macromolecules, but not cellular elements, from the sinusoidal lumen into the space of Disse [22]. This exchange gives hepatocytes access to large molecules for catabolism and also facilitates hepatocyte secretion of macromolecules (e.g., albumin, lipoproteins, fibrinogen) into the blood [22]. While the porous ECM of the space of Disse permits ready passage of fluid and solutes, some solutes can bind to matrix molecules located in the space of Disse, limiting their availability at the hepatocyte surfaces [24, 38].

Functionally, the hepatic lobule can be divided into three distinct zones: the peri-portal region (zone 1) at the lobule periphery, the mid-lobular region (zone 2), and the peri-central region (zone 3) surrounding the central vein. The zones are numbered in accordance with the direction of blood flow through the lobule, entering via the vessels in the peri-portal region and exiting via the central vein. Accordingly, various gradients exist along the portal–central axis including gradients in oxygen tension, solute concentration, and matrix chemistry, as well as hepatocyte ploidy, functional capabilities, susceptibility to damage or injury, cell maturation, and gene expression [23, 24, 39]. Sinusoids are narrower and more tortuous in zone 1, endothelial cell fenestrations are smaller and less numerous in this zone, and the plates or cords of hepatocytes merge and branch more frequently in this region [24]. Gene expression patterns of other liver cell types, such as stellate cells, also vary along the portal–central axis [24, 40].

The stem/progenitor cell niche in adult liver is located in the canals of Hering (CoH) [41–49]. The CoH are the anatomical link between the hepatocyte canalicular system and the biliary tree [41, 49]. The niche is composed of hepatic stem and progenitor cells situated on a basal lamina, with intimate contact between the stem/progenitor cells and supportive cells, which include stellate cells, myofibroblasts, and Kupffer cells [50]. Studies of the lineage stages of human liver stem cells have described the following maturational sequence: hepatic stem cells (HSCs), the self-renewing multipotent stem cells residing in the CoH, give rise to HPCs, which are diploid bipotent cells that give rise to committed hepatocytic progenitors and committed cholangiocytic progenitors [41]. The bipotent HPCs make up <0.01% of the parenchymal cells in adult livers, but they are known to expand during regenerative processes associated with diseases such as cirrhosis and hepatitis B virus (HBV) infection [41, 51].

9.2.3 Cell Types of the Liver

The major cell types of the liver include hepatocytes, cholangiocytes, liver sinusoidal endothelial cells (LSECs), stellate cells, Kupffer cells, and immunocytes (lymphocytes and dendritic cells). The cells of the liver are often divided into parenchymal cells (hepatocytes) and nonparenchymal cells (other cell types); however, this distinction is somewhat artificial in that hepatocytes do not perform the functions of the liver parenchyma in isolation but rather function in close coordination with nonparenchymal cells. A brief overview of liver cell types is given here. Detailed reviews are available in [23] and [52].

Hepatocytes are highly differentiated epithelial cells that comprise 60% of the total cell population of the liver [23, 53]. These large polygonal cells, roughly 25–30 μm in diameter, are responsible for many of the liver's metabolic and synthetic functions. They possess one or more nuclei and display variable polyploidy (4*N*, 8*N*) that increases from zone 1 to 3. Hepatocytes are highly polarized with distinct cell membrane domains that are specialized for the sinusoidal surface and the bile canalicular surface of the cell. Bile canaliculi, tubular spaces 1–2 μm in diameter, are the smallest segments of the biliary tree and connect with bile ducts near the portal tracts via the CoH. Adjacent hepatocytes are bound tightly together through intercellular adhesion complexes (tight junctions, intermediate junctions, desmosomes) that form a permeability barrier between the space of Disse and the bile canaliculi [24]. Gap junctions are also found frequently between adjacent hepatocytes and are important sites of intercellular communication [22].

The non-parenchymal cell fraction represents 40% of the total liver cell population [23]. Major non-parenchymal cells types include cholangiocytes, LSECs, stellate cells, Kupffer cells, and immunocytes (lymphocytes and dendritic cells). Cholangiocytes are cuboidal to columnar epithelial cells that line the bile ducts. The luminal surface of the cholangiocyte cell membrane contains microvilli as well as a primary cilium, which relays information regarding bile flow and content to the cell cytoplasm [24]. Functionally, cholangiocytes contribute to bile secretion and are also involved in the reabsorption of biliary constituents [23]. LSECs are flattened, elongated cells that line the walls of the hepatic sinusoids. They are

actively pinocytic cells. As described previously, LSECs possess greater intercellular permeability than a typical endothelium, as well as fenestrations organized into sieve plates. They also lack a prominent basal lamina between LSECs and the neighboring hepatocytes. These features greatly enhance hepatocyte exposure to soluble components in blood and improve passive transport of endogenous and xenobiotic substances. In addition to their role as a selective sieve between blood and hepatoctyes, LSECs function as a scavenger system to clear the blood of macromolecular waste products. They play a role in hepatic immunity, and they are involved in the clearance and bioactivation of drugs and other xenobiotic substances [23]. Stellate cells, or "pericytes," are found in the space of Disse partly encircling the sinusoids [24]. Stellate cells are involved in the metabolism and storage of vitamin A, the production and degradation of ECM, and the regulation of sinusoid contractility [23]. In response to liver injury, stellate cells can become actively migratory and acquire a myofibroblastic phenotype characterized by expression of α-smooth muscle actin [24]. In their activated state, stellate cells play a major role in the development of the inflammatory fibrotic response of the liver. Kupffer cells are the resident macrophages of the liver and are found in the lumens of sinusoids, most prominently in the portal regions [24]. They possess long cytoplasmic extensions that make direct contact with hepatocytes [23]. Kupffer cells are actively phagocytic, and they function together with the LSECs to remove worn out cells and proteins from the circulating blood [24]. They play a key role in immune surveillance, and when activated they produce cytokines that modulate hepatocyte metabolic activity and induce expression of acute phase proteins [23]. Finally, the immune cells of the liver include lymphocytes of different phenotypes as well as dendritic cells. The liver's immune system represents a major fraction of the body's innate (native) immune capacity [24]. Liver immune cells are involved in the clearance of foreign antigens and play a role in regulating liver repair after injury [24].

9.2.4 Liver Extracellular Matrix

In healthy adult liver tissue, the molecular composition of the ECM is known throughout the hepatic lobule, including in the region of the liver stem cell niche (the CoH) [41, 54]. In most tissues of the body, epithelial cells are understood to reside on a basal lamina, typically composed of type IV collagen, laminin, nidogen (also known as entactin), and proteoglycans [55]. Adult liver ECM is unique in that hepatocytes, the terminally differentiated epithelial cells comprising the liver parenchyma, do not rest on a traditional basal lamina ECM. Rather, the ECM scaffolding for hepatocytes is formed of net-like, porous 3D lattices composed of collagen I, III, IV, V, and VI, with abundant fibronectin and heparan sulfate proteoglycans [24]. Moreover, there is a gradient of ECM components along the axis from the portal triad to the central vein. This gradient has been reported to mirror a maturational lineage system in healthy adult liver, with stem/progenitor cells beginning peri-portally (zone 1) and progressing in maturation toward the peri-central region (zone 3) [41, 54]. In contrast to the lack of basal lamina ECM in the hepatic parenchyma, a traditional basal lamina ECM does exist around vascular structures and bile duct epithelium found in the portal tracts, and around

the central veins [32]. Importantly, the ECM of the hepatic stem and progenitor cell niche has been characterized as a basal lamina: it is reported to contain collagen IV, laminin, and nidogen [50] and is restricted to the peri-portal region in a healthy liver. It is also known to contain hyaluronan, collagen III, and chondroitin sulfate proteoglycans [54].

9.3 Liver Biomechanics

9.3.1 Liver Biomechanical Properties

Liver tissue is extremely compliant. From an engineering perspective, it has been described as a very soft, fluid-like viscoelastic solid [56]. Like any biological tissue, the biomechanical properties of liver are complex and depend on numerous factors including test modality (tension, compression, shear), strain rate, species of origin, postmortem time, sample storage conditions, sample hydration, the presence/absence of active tissue perfusion, inclusion/exclusion of Glisson's capsule or large vessels within the sample, and the presence of fibrosis.

Numerous studies have been conducted to quantify the macroscopic biomechanical properties of liver [56–68] using specimens with dimensions on the order of millimeters to whole organs (see Table 9.1). The motivation for these biomechanical studies comes from several sources. One driving force for liver biomechanics research is the need to develop and validate advanced finite element models that are capable of predicting traumatic liver injury risk due to motor vehicle crashes or ballistic impacts. In order to be effective, such models require detailed liver tissue mechanical characterization studies with particular emphasis on tissue failure properties and response to high rate loading conditions [57, 59]. A second driving force is the diagnosis and management of liver diseases. For example, measuring liver mechanical properties has been investigated as a means to detect liver fibrosis [66], and tissue biomechanical properties are being used to create virtual surgical simulations with haptic feedback for physician training purposes [62, 64]. A third driving force for liver mechanical characterization is the increasing importance of biomechanical factors in the development of functional tissue-engineered constructs and scaffolds [60, 67].

Table 9.1 illustrates a range of liver biomechanical properties that have been reported under various experimental conditions. The elastic (Young's) modulus E and shear modulus G are measures of a material's ability to resist deformation under an applied load. Typical liver shear moduli range from 200 Pa to 5 kPa, and typical elastic moduli range from ~1 to 20 kPa (Table 9.1). If the liver is assumed to be an incompressible material (i.e., to maintain a constant volume under mechanical loading), which is a common assumption in many biomechanics studies, then to a first approximation the elastic modulus is roughly equivalent to 3 times the shear modulus ($E \approx 3G$).

Many studies have assumed that the liver parenchyma is an isotropic material, meaning that its mechanical properties are the same when measured in any direction (for samples that do not include portions of Glisson's capsule or

Table 9.1 Selected macroscopic liver biomechanical properties reported in the literature for millimeter-scale tissue specimens to whole organs.

Liver biomechanical property	Reported value	Liver tissue origin	Test conditions	References
Failure stress in tension	69–77 kPa (true stress)	Human	Uniaxial tension, for loading rates of 0.008 and 0.089 s^{-1}	[57]
	139–184 kPa (true stress)	Porcine	Uniaxial tension, for loading rates of 0.008 and 0.089 s^{-1}	[58], cited in [57]
Failure stress in compression	−38.9 to −145.9 kPa (true stress)	Human	Uniaxial compression, for loading rates from 0.012 to 10.708 s^{-1}	[59]
Failure strain in tension	1.29–1.28 (nominal strain)	Human	Uniaxial tension, for loading rates of 0.008 and 0.089 s^{-1}	[57]
	1.28–1.27 (nominal strain)	Porcine	Uniaxial tension, for loading rates of 0.008 and 0.089 s^{-1}	[58], cited in [57]
Failure strain in compression	−0.48 to −1.15 (true strain)	Human	Uniaxial compression, for loading rates from 0.012 to 10.708 s^{-1}	[59]
Shear modulus (G)	200 Pa (initial shear modulus of solid matrix, from porohyperviscoelastic model)	Bovine	Uniaxial unconfined compression tests on perfused *ex vivo* liver samples	[60]
	300 Pa (initial shear modulus, from two-term Mooney–Rivlin model)	Porcine	Combined compression and elongation experiments on cylindrical samples	[62], cited in [61]
	795 Pa (initial shear modulus of solid matrix, from porohyperviscoelastic model)	Porcine	Uniaxial unconfined compression tests on nonperfused *ex vivo* liver samples	[63]
	1400 Pa (initial shear relaxation modulus)	Bovine	Shear stress relaxation tests on cylindrical samples	[56]
	1823 Pa (initial shear modulus, from two-term Ogden model)	Porcine	Hemispherical indentation tests on *in vivo* porcine liver	[64]

(Continued)

Table 9.1 (Continued)

Liver biomechanical property	Reported value	Liver tissue origin	Test conditions	References
	~5–40 kPa (complex shear modulus, evaluated over 1.3–13 kHz)	Porcine	Dynamic torsional resonator device, porcine liver without capsule	[65]
Elastic (Young's) modulus (E)	0.64– 2.0 kPa (for 5–15% pre-strain)	Human (fibrosis score = 0)	Cyclic compression–relaxation experiments on *ex vivo* samples	[66]
	1.65–19.98 kPa (for 5–15% pre-strain)	Human (fibrosis score = 5)	Cyclic compression–relaxation experiments on *ex vivo* samples	[66]
	10.5 kPa (long-term modulus, from poro-viscoelastic model)	Ferret	Macroscopic spherical indentation of perfused native liver (whole organ with capsule)	[67]
	20 (long-term modulus) and 60 kPa (instantaneous modulus)	Human	Aspiration experiments on *in vivo* liver (whole organ with capsule) during open surgery	[68]

obvious vascular structures). Chui *et al.* [69] have reported findings that suggest porcine liver is transversely isotropic, but the microstructural implications are difficult to interpret since the orientation of the stiffer material axis could not be related directly to connective tissue fiber orientations or hepatic lobule orientations within the samples. Based on the liver's relatively uniform histology throughout the organ, the assumption of liver isotropy is considered to be reasonable by other authors, especially if the length scale of the mechanical test specimen is on the order of 10–200 mm [70].

Because of the liver's complex architecture and equally complex mechanical behavior, a variety of constitutive models (mathematical laws) have been used to quantify its biomechanical properties. A complete review of these constitutive laws is outside the scope of this chapter, but a brief overview will be provided. The major categories of constitutive laws that have been applied to liver include viscoelasticity, hyperelasticity, and poroelasticity. Viscoelasticity refers to materials that exhibit time-dependent features in their mechanical response, such as creep, stress relaxation, or strain rate dependence. The liver is known to be linearly viscoelastic in shear up to shear strains of 0.2% [56]. Beyond this limit, it exhibits nonlinearly viscoelastic behavior [71], with a shear relaxation modulus that is dependent on strain. Like many biological tissues, the liver exhibits nonlinear stress–strain behavior under large deformations. For this reason, liver is often

modeled as a hyperelastic material. A plethora of strain energy functions have been used in hyperelastic models of liver tissue [62, 64]; however some of the more commonly used forms include the Ogden, Mooney–Rivlin, neo-Hookean, polynomial, and reduced polynomial models. Hyperelastic models can accurately describe the large deformation behavior of liver tissue, but by themselves they are unable to capture viscoelastic time-dependent effects in the material. Finally, since liver tissue is highly vascularized and saturated with fluid, several investigators [60, 63, 64] have used constitutive models based on poro-elasticity theory [72] to characterize liver biomechanics. These models regard the liver as a fluid-saturated, sponge-like material. Poro-elastic (PE) models are valuable because they can describe not only the solid "matrix" behavior but also the flow and pressure conditions for fluid within the tissue "pores." This is noteworthy, since perfused liver tissue is known to differ significantly from nonperfused liver in its mechanical behavior in tests conducted using both macroscopic and microscopic indentation methods [67, 73]. The simplest PE models assume that the solid matrix is a linear elastic material. In some studies, such an assumption has been found to be inadequate to capture liver behavior [63]. Therefore, more complex poro-viscoelastic (PVE) and poro-hyper-viscoelastic (PHVE) models have been employed [60, 63, 64, 67], which capture the viscoelastic and hyperelastic features of the solid matrix while still accounting for the mechanical contribution of the pore fluid.

9.3.2 Liver Hemodynamics

The liver vasculature is a high-capacity, high-compliance, and low-resistance system [24]. It has been estimated that, in adult humans, ~100–110 ml of blood passes through 100 g of liver tissue per minute [74]. While the portal vein at the liver hilum carries roughly 75% of the blood that enters the liver, pressures in this vessel are low at roughly 9 mmHg [75]. The remaining 25% of afferent hepatic blood flow derives from the hepatic artery at prevailing arterial pressure and oxygenation [24]. While arterial pressures exhibit a time-varying cyclic pulsatile pattern, as a rough approximation the average pressure in the common hepatic artery has been estimated as 95 mmHg [75].

Pressures in the hepatic microvasculature are of particular interest for understanding the fluid biomechanical environment within the hepatic lobule. The following microcirculatory hemodynamic data are taken from a detailed review by Oda *et al.* [75]. The hepatic arteriole, the branch of the hepatic artery located in the portal tracts at the corners of the hepatic lobule (Figure 9.2), was estimated to have a pressure of 30–35 mmHg. According to the authors' observations, hepatic arterioles give rise to terminal hepatic arterioles, which can form the peribiliary plexus, open directly into sinusoids or empty into the terminal portal veins. The pressure in the terminal hepatic arterioles was estimated as 22–29 mmHg. The pressure in the terminal portal veins was reported as 3.7-4.4 mmHg, and the terminal hepatic venule (also known as the central vein) pressure was reported as 0.7 mmHg. Therefore, the sinusoidal pressure was estimated to fall between these values, with the authors' stated range between 0.7 and 1.5 mmHg [75]. Other authors have reported a pressure gradient ranging from 4.4 mmHg in the terminal

portal vein to 1.8 mmHg in the central vein, placing the sinusoidal pressure gradient between these limiting values [24].

The mechanisms regulating blood flow within the sinusoids are controversial [24]. It has been argued that the large pressure drop from terminal hepatic arteriole to sinusoid may be regulated in part by a pre-capillary sphincter (see reviews in [76] or [77]). However, other studies have been unable to detect these sphincters [24, 78]. It has also been hypothesized that numerous large-diameter fenestrations in the peri-portal sinusoidal endothelium facilitate rapid transport of plasma into the space of Disse and help dissipate the high-pressure influx of flow from the hepatic arterioles [76, 79]. In addition, it is known that sinusoidal flow is strongly affected by post-sinusoidal resistance [24, 80].

The sinusoids are more tortuous, the sinusoidal diameters are smaller, and the blood flow velocity is slower in zone 1 (periportal) compared to zone 3 (pericentral) [75]. Sinusoid diameter can range from 7 μm in the periportal region to 15 μm pericentrally [23, 81]. Oda *et al.* [75] estimated the blood flow velocity in the sinusoids as 407–451 $\mu m\ s^{-1}$. This velocity is much smaller than that observed in typical capillaries in the body, which has been reported as 500–1000 $\mu m\ s^{-1}$ [75, 76] or as high as several millimeters per second depending on surrounding conditions [82].

9.4 Liver Mechanobiology

Mechanobiology is the study of how "tissues are produced, maintained, and adapted by cells as an active response to biophysical stimuli in their environment" [83]. The mechanobiology of mechanically stressed tissues, such as muscle, bone, cartilage, and blood vessels, has inspired a significant and growing body of literature [84, 85]. However, it is well recognized that all cells are mechanosensitive [84], including those of the liver and other organs not traditionally associated with mechanical loading. As a field, liver mechanobiology is in its early stages of development. Continued growth of this field is critically important to the future success of liver tissue engineering. Furthermore, it has great potential to shed light on fundamental processes of liver development, pathology, and regeneration.

9.4.1 Cellular-Scale Mechanical Forces

The *in vivo* mechanical environment of liver cells includes forces generated by flowing fluids, such as blood, bile, and interstitial fluid. The cellular mechanical environment also includes forces transmitted between adjacent cells or between cells and the ECM. Cells can detect two distinct types of fluid-mediated mechanical stimuli: fluid shear stress and hydrostatic pressure. Fluid shear stress is produced when fluid flows across a cell surface, acting parallel to the direction of flow. Hydrostatic pressure is produced in a fluid at rest because of gravity. For a simple scenario such as fluid flowing through a horizontal tube, gravity can be neglected and the hydrostatic pressure may be regarded as the lateral (static) pressure acting perpendicular to the direction of flow through the tube [82]. In addition to

Table 9.2 Selected reports quantifying mechanical cues in liver cellular environment.

Mechanical stimulus	Reported value	Conditions	References
Parenchymal (interstitial) fluid pressure in normal liver	2.86 ± 1.04 mmHg	Measured in rat livers *in situ*	[87]
	5.8 mmHg	N/A	[29]
	0–5 mmHg	N/A	[88]
Parenchymal (interstitial) fluid pressure in cirrhotic liver	24.6 ± 6.2 mmHg	Measured in 10 patients with cirrhosis	[89]
Fluid shear stress on the walls of hepatic sinusoids	0–1 Pa (0–10 dyn cm^{-2}) for majority of sinusoidal channels Local maximum of 3.80–6.62 Pa in narrowest channels	Computational fluid dynamic analysis of hepatic microcirculation	[90]
Fluid shear stress in biliary system	~0.014 Pa (~0.14 dyn cm^{-2})	Estimated from observations and calculations based on previous animal model studies	[91]
Young's (elastic) modulus of liver extracellular matrix	0.9 kPa	Microscale indentation experiments on decellularized ferret livers, combined with finite element analysis	[67]
Bulk compressive modulus of liver extracellular matrix	1.25–1.31 kPa	Unconfined compression experiments on decellularized porcine liver tissue disks	[92]

detecting fluid-mediated mechanical stresses, cells can also probe their physical environments through cell–cell adhesions and cell–ECM adhesions [84]. These adhesions are mechanically coupled to the cytoskeleton and allow transmission of forces from the extracellular to intracellular space. For example, cells can "feel" changes in substrate stiffness through integrins and focal adhesion proteins that link the cell's actin cytoskeleton to the ECM [86]. Selected values reported for various mechanical stimuli in liver are summarized in Table 9.2.

9.4.2 Cellular Mechanotransduction Mechanisms

Several biological components have been proposed as mediators of cellular mechanotransduction events [86, 93]. These include the ECM, cell–ECM adhesions, cell–cell adhesions, the cytoskeleton, nucleus, glycocalyx, primary cilium, and stretch-activated ion channels, among others. Various signal transduction mechanisms have been proposed. For instance, tensile forces in the ECM

can trigger tension-induced changes in protein conformation that can initiate signaling events [94] such as exposure of binding sites. In epithelia, as well as some other tissue types, strong cell–cell adhesions are mediated by transmembrane proteins, such as cadherins, that are anchored on the intracellular side to the cell's cytoskeleton [95]. Both integrin-mediated and cadherin-mediated adhesions are known to enlarge and strengthen in response to tension [86, 96]. The nucleus is mechanically connected to the rest of the cell through LINC (linker of nucleoskeleton and cytoskeleton) complexes, and these complexes enable cytoskeletal and external forces to produce nuclear deformations [97]. In endothelial cells, the glycocalyx covers the apical cell surface and has been proposed to mediate mechanotransduction of fluid shear stress [84, 94, 98]. The primary cilium is a single nonmotile extension that projects from the surface of almost every cell type of the body (hepatocytes are a notable exception) [99]. It functions as a mechanosensor of fluid flow and can also sense changes in the cell's chemical environment [99]. Lastly, stretch-activated ion channels open in response to membrane strain, such as during osmotic swelling, and permit the influx of calcium and other ions [84, 86]. For detailed reviews of cellular mechanotransduction, see [84, 93, 94].

9.4.3 Mechanosensitivity of Liver Cell Types

There is increasing evidence in the literature that numerous liver cell types exhibit mechanosensitivity to external cues such as mechanical stretch, ECM stiffness, or fluid shear stress. A brief overview is provided here, organized by cell type.

Mechanical factors have been shown to play a role in the myofibroblastic transdifferentiation of hepatic stellate cells (HSCs) and portal fibroblasts, with important implications for liver fibrosis [17, 18, 100, 101]. Gaca *et al.* cultured primary HSCs on polyacrylamide substrates with defined stiffness values (elastic moduli) ranging from 100 Pa to 12 kPa, and found that HSC transdifferentiation toward the myofibroblastic phenotype increased as substrate elastic modulus increased [17]. Similarly, Li *et al.* reported that both tumor growth factor (TGF)-β and substrate stiffness are critical factors in regulating portal fibroblast activation [18]. Using a rat model of liver fibrosis, Georges *et al.* found that liver stiffness increased prior to fibrosis, suggesting that increased liver stiffness may be involved in initiating the early stages of fibrosis [100]. Finally, Sakata *et al.* found that for a human HSC cell line (LI90), cyclic mechanical stretch increased TGF-β expression on both mRNA and protein levels [101].

Cholangiocytes and hepatocytes have also been shown to be responsive to mechanical cues in their environment. Using micro-perfused, isolated intrahepatic bile duct units (IBDUs) from rats, Masyuk *et al.* found an increase in cholangiocyte intracellular calcium concentration in response to fluid flow as well as flow-induced changes in cAMP (cyclic adenosine monophosphate) signaling [102]. The primary cilium was suggested as the putative mechanosensor mediating these effects, since ciliary ablation and downregulation of the ciliary proteins polycystin-1, polycystin-2, and adenylyl cyclase isoform 6 significantly reduced or abolished the flow-induced changes in intracellular calcium and

cAMP levels [102]. Woo *et al.* reported that for cholangiocyte cell monolayers, exposure to fluid shear stress produced a dose-dependent increase in adenosine triphosphate (ATP) release for shear stresses ranging from 0.08 to 0.64 dyn cm^{-2} [91]. Fluid shear stress was also associated with increased intracellular calcium and increased membrane chloride permeability in this study [91]. Nakatsuka *et al.* found that fluid shear stress induced hepatocyte PAI-1 (plasminogen activator inhibitor-1) expression at mRNA and protein levels, in experiments on cultured primary rat hepatocytes exposed to shear stress levels up to 40 dyn cm^{-2} [103]. Using partial hepatectomy and portal vein branch ligation models in rats, Schoen *et al.* reported that increased hemodynamic variables (portal venous pressure, fluid shear stress) were associated with increased hepatic c-fos mRNA expression and increased proliferative factor (PF) activity in the blood, two markers of the initiation of the liver regeneration cascade [104]. These effects were inhibited by administration of a nitric oxide synthase antagonist, suggesting that mechanical stress-induced NO release plays a role in initiating the liver's regenerative response [104]. Lastly, a study by Semler *et al.* reported that for primary rat hepatocytes cultured on substrates of low versus high elastic moduli, the effects of hepatocyte stimulation by EGF and HGF (epidermal and hepatocyte growth factors) varied based on the substrate mechanical compliance [105].

Endothelial cells are well known to sense and respond to fluid shear stress produced by blood flowing across their surface [106–109]. This overview will focus on mechanosensitivity findings related to LSECs, the specialized population of endothelial cells that line the hepatic sinusoids. Shah *et al.* reported that for *in vitro* and *in vivo* conditions, LSECs express endothelial NO synthase (eNOS), produce NO basally, and increase their production of NO in response to fluid flow [110]. Braet *et al.* noted a significant upregulation of vascular endothelial growth factor receptor (VEGFR)-1, VEGFR-2, and neuropilin-1 mRNA following LSEC exposure to shear stress *in vitro* [111]. LSECs can undergo a dedifferentiation process, termed "capillarization," when cultured over time *in vitro* or in response to certain experimental interventions [37, 112]. Capillarization involves loss of LSEC fenestrations and the formation of an organized basement membrane, both of which would signal that the altered LSEC phenotype now more closely resembles that of "typical" vascular endothelium found outside the liver. DeLeve *et al.* used cell surface expression of CD31, the platelet endothelial cell adhesion molecule, as a marker of dedifferentiated, non-fenestrated LSECs [112]. They found that LSEC surface expression of CD31 could be prevented in culture through paracrine VEGF signaling from hepatocytes or stellate cells, under coculture conditions for up to 3 days. The same effect was produced for LSECs cultured alone in the presence of VEGF [112]. The authors also noted that blocking autocrine production of NO by the LSECs prevented the ability of VEGF to preserve the LSEC phenotype [112]. This is important in the context of mechanosensitivity since NO production in LSECs, as well as LSEC VEGFR-1 and VEGFR-2 expression, is regulated in part by mechanical stimuli (shear stress) [110, 111]. Finally, Hwa *et al.* investigated the maintenance of LSEC phenotype using a 3D coculture system with microperfusion capabilities [113]. They reported that adult

rat liver LSECs maintained SE-1 expression (a marker of differentiated LSEC phenotype in rats) for 13 days in culture, in the absence of external VEGF and serum, when cocultured with primary adult rat hepatocytes in the 3D perfusion system [113]. These results were dependent on the 3-D microperfused coculture format, since the SE-1 positive cells did not survive more than a few days when cultured alone in the 3-D perfusion system or in 2D static coculture with hepatocytes [113].

Mechanical cues have been implicated in guiding the hepatic lineage specification and phenotype of ES cells [114], mesenchymal stem cells (MSCs) [115], and hepatic stem and progenitor cells [14]. Studies have examined cellular responses to substrate or ECM stiffness changes as well as changes in fluid dynamic conditions. Using murine ES cells that were induced to differentiate toward hepatocyte-like cells via exposure to sodium butyrate, Li *et al.* examined whether altering the substrate stiffness could improve or stabilize the hepatocytic phenotype of these cells [114]. They reported that, overall, hepatic functions such as intracellular albumin content and albumin secretion were sustained and enhanced on softer substrates (5 kPa) compared to stiffer substrates (47 or 230 kPa). Gordon-Walker *et al.* cultured cells from a murine HPC line as well as primary murine HPCs on laminin-coated polyacrylamide supports with stiffness values ranging from 1 to 12 kPa [116]. They found that increased stiffness was associated with increased cell spreading, increased proliferation, and increased expression of the HPC/biliary marker CK19, while lower stiffness was associated with reduced cell proliferation and increased expression of hepatocytic markers albumin and CYP7A1. Lozoya *et al.* cultured hepatic stem cells in 3D microenvironments composed of hyaluronan hydrogels of varying stiffness and found that the mechanical properties of the microenvironment could regulate hepatic stem cell differentiation [14]. In addition to substrate stiffness, fluid dynamic conditions have also been noted to influence hepatic stem/progenitor cell behavior. Miyoshi *et al.* examined the effect of medium flow rate on the cell growth and hepatic functions of murine and porcine fetal liver cells cultured in a perfused 3D packed-bed reactor system [117]. They found that the fetal liver cells were highly sensitive to the flow rate and that low shear stresses were necessary to maintain cell function. Ji *et al.* cultured murine MSCs in a biomatrix scaffold, obtained from decellularized liver, coupled with dynamic perfusion [115]. They found that the dynamic cultured scaffold system stimulated the MSCs to express endodermal and hepatocytic genes and proteins, and that when the optimal flow rate was used, the dynamic cultured scaffold system promoted better cell proliferation compared to the biomatrix scaffold under static (no flow) conditions or the monolayer (control) static culture system [115]. The authors noted that for static culture and for dynamic culture with very low flow rates, cell proliferation was inhibited as a result of severely limited oxygen and nutrient diffusion. On the other hand, very high flow rates induced massive cell apoptosis due to high shear stress exposure [115]. The optimal flow rate may have achieved a balance by enhancing mass transport and removal of waste products while minimizing shear stress to limit cell apoptosis. It is worth noting that neither of the previous two studies directly controlled or quantified the fluid shear stress applied to the cells within their perfused 3D culture platforms; however, Ji *et al.*

commented that the shear level within the system should be optimized for cell growth and differentiation [115].

9.5 Biophysical Stimuli in Liver Tissue Engineering Scaffolds

There is increasing interest in using *in silico* experiments, based on accurate computational models, to optimize experimental conditions in tissue engineering studies and help speed up the development of functional tissue engineering constructs ready for clinical use [8, 118]. Models have been developed to predict, for example, the structural properties of scaffolds generated through rapid prototyping methods [119], with simple microstructures and well-defined geometries. Alternative modeling approaches based on poly-lines [120] have been developed for predicting the mechanical properties of more complex scaffold architectures produced via electrospinning. Models of scaffold perfusion have been developed using computational fluid dynamics (CFD) approaches based on Navier–Stokes equations [121], as well as continuum-level models based on Brinkman's [122] or Darcy's [123] equations for flow through porous media. Some investigators have also coupled scaffold perfusion models to models of cell population dynamics [124] or nutrient transport [125]. Any modeling strategy has strengths and limitations. CFD models can provide detailed predictions of fluid velocity profiles and the resulting shear stress distributions on fluid channel walls or cell surfaces; however, these models require detailed knowledge of microstructure and pore/fiber geometry and have high costs in terms of computational time and resources, and thus can be used only in limited cases or for small spatial domains [122]. While continuum models based on porous media provide only approximations of shear stress, rather than full Navier–Stokes solutions, continuum models are computationally efficient and do not require precise spatial and geometric information about every fiber or cell in a tissue [123, 126]; hence their use in perfusion models of porous materials.

A recent review by Bijonowski *et al.* [127] provides an excellent overview of the development of bioreactor designs for perfusion-based tissue engineering of highly vascularized 3D organs, as well as the design challenges yet to be addressed. From a computational modeling perspective, native liver perfusion has been modeled at various length scales ranging from whole-organ hemodynamics to models of the hepatic microcirculation [60, 128–130]. In contrast, computational modeling of decellularized organ perfusion is a relatively new field of investigation. Evans *et al.* modeled the biomechanics of perfused decellularized liver, based on indentation experiments across several flow rates and at multiple length scales [67]. Moran *et al.* measured parenchymal fluid pressure (PFP) in native and decellularized liver tissue as a function of perfusion flow rate, demonstrating that PFP in the decellularized scaffolds could be controlled by varying the flow rate [87]. These experimental results were then used by Nishii *et al.* to develop a computational model of decellularized organ perfusion [131]. In sum, perfused 3D culture systems such as the seeded decellularized

scaffolds and packed-bed reactors described above are promising tools for liver organ engineering. Advanced computational tools are required to optimize these complex dynamic environments.

9.6 Conclusion and Future Directions

In conclusion, the *in vivo* environment of liver cells contains subtle but detectable mechanical stimuli including variations in ECM stiffness as well as hydrostatic pressures and shear stresses associated with the flow of blood, bile, and interstitial fluid. Increasing evidence is becoming available to indicate that hepatocytes, cholangiocytes, HSCs, sinusoidal endothelial cells, and hepatic stem and progenitor cells can sense and respond to mechanical cues in their environment. Providing dynamic perfusion to cells in 3D culture systems, including vascularized decellularized scaffolds, aids in nutrient transport and removal of cellular wastes but can also impart mechanical stresses that can lead to cellular injury or apoptosis.

Looking to the future, it is likely that computational modeling will play an increasingly important role in optimizing the complex dynamic environment inside perfused 3D culture systems for liver tissue engineering. For complex multiscale experimental systems, such as decellularized/recellularized whole-organ scaffolds, multiscale modeling strategies are required to quantify local forces at the cellular scale as a function of macroscopic input conditions such as inlet flow rates or pressure gradients. Advanced models can simulate cell-level conditions during the initial seeding process as well as throughout the process of tissue development and maturation. In addition, computational modeling can aid in scaling up optimized seeding and maintenance protocols from small animal models to larger and more clinically relevant sized organs. Standards for computational model validation will become even more important to ensure the accuracy of the models for a given range of experimental conditions, so that they can serve as valuable tools to advance liver tissue engineering research.

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10

Development of Tissue-Engineered Blood Vessels

Haiyan Li

10.1 Introduction

To regenerate damaged or lost tissue/organs, tissue engineering (TE) is a promising technology. The basic principle of TE is culturing cells within a biomaterial scaffold *in vitro* in order to reconstruct new tissues or organs [1]. Although there have been tremendous advances in TE, there are still several key challenges that limit the applications of TE products. Recently, as one of the key challenges in TE, angiogenesis has attracted much attention [2, 3]. It is well known that existing blood vessels can grow into the new tissues spontaneously when they are induced by *in vivo* signals. However, the in-growth speed of blood vessel is very slow, which results in inadequate nutrients for the cells within the scaffold [4]. In addition, the engineered tissue cannot rely on the diffusion of oxygen and nutrients from the surroundings into the engineered construct because they will be consumed if the distance is longer than a few hundred micrometers [5]. Thus, the thickness of an engineered construct cannot be more than 150–200 μm if there is no capillary network inside the engineered construct. To promote more tissue-engineered organs for clinical applications, development of organs/tissue with a microvasculature is critical. Since engineered skin and cartilage do not need much nutrients and oxygen, they have found some successful applications in clinics [6]. However, for regenerating large, whole organs with thick and complex constructs, such as the liver, kidney, or heart, TE technology has encountered difficulties because of the regeneration of blood vessels within these organs. Therefore, strategies for promoting vascularization/angiogenesis are necessary in order to provide the cells within the large tissue-engineered constructs with oxygen and nutrients.

In addition, in the Western countries, cardiovascular disease is now the major cause of death, as almost 1 million people die of cardiovascular problem every year in the United States [7]. To solve cardiovascular problems, currently, implantation of a vascular graft surgically is a clinical choice. To revascularize myocardial and limb, coronary artery bypass and peripheral bypass are applied, respectively. To facilitate dialysis, fistulae arteriovenous (AV) are applied. All these procedures involve small-diameter (<6 mm) vascular grafts [8]. Although autologous vessels can be used to alleviate cardiovascular diseases, their availability is limited. Some synthetic vascular grafts, however, still show inadequate

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performance [9]. Therefore, development of small-diameter vascular grafts with proper functionalities for clinical applications has become very urgent. Among all strategies, reconstruction of vascular grafts by TE technology holds great potential [9, 10].

In this chapter, the recent advancements in techniques for fabricating *in vitro* blood vessel models and vascularized networks in 3D engineered tissue constructs are reviewed. The natural biological character of blood vessels, in terms of different types of vascular structures, and natural processes of blood vessel formation *in vivo* are introduced. Then, different techniques for fabricating vascular structures, including engineering microvascular networks as well as vascular replacement grafts, are discussed in detail.

10.2 Biology of Blood Vessels

10.2.1 Structure and Component of Native Blood Vessels

Native blood vessels have complex structures. Different types of vessels have different thicknesses, diameters, and lengths. The inner diameter of the smallest capillaries can be 5 μm, while that of largest artery, for example, the aorta, can be 25 mm. In addition, there is a three-layer structure in mature blood vessels, which contains the tunica intima, the tunica media, and the tunica adventitia [11]. Within the different layers of the blood vessels, the type of cells and the matrix that compose each layer are different. Figure 10.1a shows the configuration, architecture, and cellular components of blood vessels. In the tunica intima, endothelial cells (ECs) adhere to each other to form an endothelium with a thickness of ~2 μm supported by a basal lamina of collagen with a thickness of ~1 μm. Smooth muscle cells (SMCs) are densely populated and concentrically aligned. SMCs, combined with elastic lamina and trace quantities of collagen, play an important role in the tunica media. Fibroblasts are predominantly located in the tunica adventitia of the collagenous extracellular matrix (ECM). Special structures can be found during the development of blood vessels. For example, capillaries have only a single layer of ECs, the basement membrane, and pericytes, while arterioles and venules are mainly composed of SMCs [8, 14–16].

10.2.2 Functions of Native Blood Vessels

Understanding blood vessels, especially the functions of blood vessels, is critical for regenerating vascularized tissue or fabricating vascular grafts to replace damaged blood vessels. Blood vessels, as the name partially suggests, transport blood throughout the body. With blood circulating through the whole body, necessary oxygen and nutrients are supplied and waste products are removed [17]. Besides, it has been reported that blood vessels play important roles in maintaining proper solute to water balance in the blood and tissues, protecting the parenchymal cells from interstitial fluid shear, as well as providing appropriate physical and chemical signals to the surrounding tissues [13].

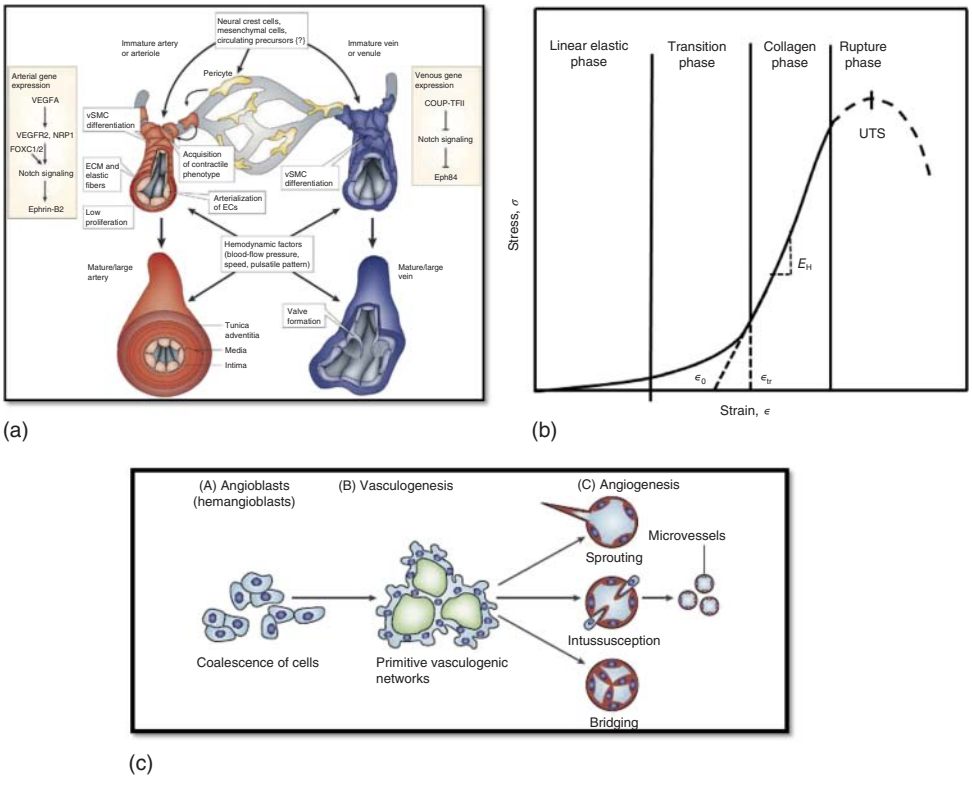


Figure 10.1 (a) Schematic diagrams of structure of blood vessels: Cross-section of arteries with their major components and maturation steps are shown. (b) A typical uniaxial tensile stress–strain curve for soft biological tissues such as human aortic and pulmonary heart valve leaflets. (Images adapted from [12] with copyright permission from Elsevier.) (c) Vasculogenesis and angiogenesis: two predominant mechanisms of blood vessel formation. (Images adapted from [13] with permission from Elsevier.)

The composition, organization, and structure of blood vessel's ECM determine the mechanical properties of the vessels, including tensile stiffness, elasticity, compressibility, and viscoelasticity, which ultimately decide their deformations and stress fluctuations [8, 18, 19]. Collagen, proteoglycans, and elastin are the three main components of the ECM of blood vessels. Each of them has a unique function [20, 21]. Collagens type III and type I are the most abundant types in blood vessels and provide the tensile stiffness to the blood vessels. In addition, collagens self-assemble into fibrils with a particular orientation to endow tissues with anisotropic mechanical properties. The presence and activities of proteoglycans provide the vessels with viscoelastic properties, which enable the blood vessels to go through significant deformations. Elastin, a highly elastic protein, can self-assemble into three-dimensional (3D) networks. Once it senses stress, the elastin 3D network can get oriented and store potential energy, which accounts for the elasticity and compressibility of blood vessels. Through the

combined function of collagen and elastin, blood vessels also show viscoelastic properties [22].

As blood vessels exist in a special environment where continuous stress is induced by the blood flow, there is always stress–strain deformation in blood vessels. The response of the blood vessels to stress can be divided into three typical phases: the linear phase, the nonlinear phase, and the stiffening phase [11, 12, 23]. In the linear phase, the elastin fiber stretches with the stress, and an isotropic response can be observed. This is manifested through a linear stress–strain curve, which can be observed in this phase. After that, collagen fibers in the ECM start to straighten in order to resist the stress, which causes the stress–strain curve to become nonlinear. With the increase in stress, the collagen fibers are finally completely elongated and the vessel become stiffer in this third phase than that in previous phases [17, 24] (Figure 10.1b).

Besides the functions of the ECM components in blood vessels, the cells in each layer of the blood vessels also have specific functionalities. The first function of ECs is the formation of a continuous endothelium monolayer in the inner layer of blood vessels, which acts as a barrier between the blood and subendothelial tissues through their wide range of strong cell-to-cell junctions [25, 26]. ECs have a distinguishing property: they can adjust their cell–cell junctions and morphology in response to shear stress induced by blood flow in order to maintain the intactness and permeability of blood vessel [27, 28]. In addition, an intact and quiescent endothelium can inhibit thrombosis by producing the anticoagulant protein S, nitric oxide (NO), prostacyclin, and tissue plasminogen activator, and synthesizing heparin sulfate proteoglycans [29]. Furthermore, ECs can direct the behavior of SMCs and white blood cells and regulate homeostasis by secreting various signaling molecules, including NO [30]. Therefore, a confluent, anti-thrombogenic endothelial layer is essential for vascular graft success.

SMCs form the tunica media. As this layer is responsible for vascular tone/diameter, highly differentiated SMCs play important roles in the dilatation and constriction of vessels [31]. SMCs are of two phenotypes: a "synthetic" phenotype and a "contractile" phenotype. The former is proliferative, while the latter is biomechanically active but quiescent. The differences between the contractile and synthetic phenotypes of SMCs have been widely studied, and several markers have been reported for confirming the contractile phenotype of SMCs [32]. SMCs can easily switch their phenotypes between contractile and synthetic under normal conditions and injured conditions because of their phenotypic plasticity, which facilitates the initiations of cell proliferation, enzymatic degradation of ECM, and vessel wall remodeling to finally form new blood vessels [33]. SMCs can sense strain through the interaction of their integrin-mediated connections with the ECM. Controlled strain can orient SMCs in an organized manner.

Fibroblasts in the adventitia are mainly responsible for the secretion of highly collagenous ECM [34, 35]. In addition to being regarded as supporting components for blood vessels, fibroblasts in blood vessels have been reported to be able to interact with SMCs and ECs to accelerate regeneration and repair of blood vessels [36, 37]. Besides, fibroblasts can convert into myofibroblasts in response to triggers such as injury [38].

10.2.3 Vasculogenesis and Angiogenesis

There are two routes of formation for new blood vessels: vasculogenesis and angiogenesis. Figure 10.1c shows the major characteristics of vasculogenesis. The molecular mechanism for vascular plexus formation from the mesoderm by differentiation of angioblasts and generation of primitive blood vessels has been termed vasculogenesis. During this process, the mesodermal stem cells first differentiate into endothelial progenitor cells (EPCs), also known as angioblasts [3–5, 39–41]. Then, the angioblasts migrate over long distances, forming a vascular plexus at a region different from where they originated. Afterward, the angioblasts differentiate into ECs. Finally, the ECs migrate and organize themselves into nascent endothelial tubes and form capillaries. Several growth factors (GFs), proteases, and cell–cell junction molecules are involved in this process [26, 42–47]. In particular, the vascular endothelial growth factor (VEGF) plays a very critical role in every stage of this process [45]. Besides, interactions between pericytes, SMCs, fibroblasts, and ECs are critical for turning endothelial tubes into more mature and larger blood vessels such as arterioles, arteries, venioles, and veins [37, 48–51].

Angiogenesis refers to the molecular mechanism of new blood vessels growth from an existing blood vessel through sprouting of ECs [5, 41, 52–54]. Angiogenesis is an important step not only in the growth and development of the embryo but also in the regeneration of many organs and wound healing. In this process, once the ECs sense the signals that new blood vessels are needed, they will first produce molecules to degrade the ECM. In addition, the molecules will induce changes in the cell–cell junctions between ECs and in the adhesive properties of ECs to the ECM. These changes will promote EC release and migration. Several extracellular signals, including secreted paracrine factors and ECM components, along with various cell–cell and cell–matrix interactions, are involved in protecting ECs from apoptosis and stimulating the migration. Finally, ECs differentiate into new capillaries with the stabilization by pericytes.

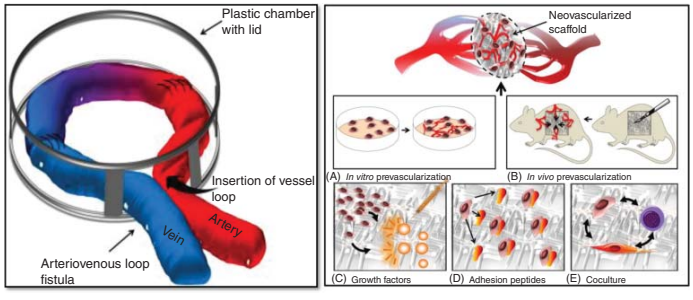
10.3 Tissue Engineering of Blood Vessels

10.3.1 Tissue Engineering of Microvascular Networks

Various strategies for engineering microvascular networks have been developed, including prevascularization-based techniques as well as vasculogenesis- and angiogenesis-based techniques. These strategies are illustrated in Figure 10.2.

10.3.1.1 Prevascularization-Based Techniques

Prevascularization is a method of forming blood vessels within constructs before the latter are implanted in the defect sites. The preexistence of blood vessels in the constructs can ensure that the seeded cells are supplied with oxygen and nutrients and the wastes are removed from the beginning. This can contribute to the proliferation, growth, and differentiation of the seeded cells and finally stimulate the regeneration of tissues or organs. Therefore, prevascularization of engineered tissue or organ constructs has attracted much attention [54]. There are two main



(a) (b)

Figure 10.2 (a) *In vivo* prevascularization with an artery (A) and a vein (V) (AV) loop surgical procedure. (Images adapted from [4] with permission from Elsevier.) (b) Five different strategies to induce vasculogenesis and angiogenesis. (Images reprinted from [3] with permission from Elsevier.)

techniques for prevascularization: *in vivo* prevascularization and *in vitro* prevascularization.

10.3.1.1.1 In vivo Prevascularization

In vivo prevascularization is a method that first incubates the constructs in vascularized tissue to allow the in-growth of blood vessels from the surrounding environment into the engineered constructs. Afterward, the prevascularized construct is harvested and implanted into defect sites [4, 55, 56]. In this method, to have a transplantable macrovessel in or around the construct, an artery is normally implanted into the engineered construct or an axially vascularized tissue is wrapped around the engineered constructs once the constructs are implanted into a vascularized tissue. At this stage, the functions of the transplantable macrovessel are inducing microvascular network formation within the construct and supplying the microvascular network with blood. Then, this prevascularized-tissue-engineered construct is harvested and reimplanted at the defect site. Since there is a vascular axis or an artery with the construct, the vascular axis or artery can be connected to the host vasculature through microsurgery. After that, the entire construct can be perfused with blood through the vascular axis and the microvascular network within the construct. As mentioned previously, this technique can ensure the immediate perfusion of the whole construct at the beginning by surgical anastomosis, which can benefit the survival, proliferation, and differentiation of seeded cells and the regeneration of tissues. Fontaine first reported the infiltration of an unseeded macroporous scaffold [57]. Since then, studies have demonstrated that the in-growth of host cells into a suitable 3D engineered constructs can form a perfusable vascular network [58–60].

Cell sheet engineering and AV loop techniques have been used to generate prevascularized constructs *in vivo* [61–63]. In cell sheet engineering techniques, cells are cultured *in vitro* to form to a confluent monolayer and then harvested from cell culture dishes. As the cell culture dishes are temperature-responsive, changing the temperature can easily detach the whole cell sheet. The cell monolayer can be stacked layer by layer to obtain a three-dimensional (3D) cell-dense sheet before they are transplanted into the defect sites. This method can successfully provide newly formed vessels and increased blood perfusion in different approaches. AV loops have been used for *in vivo* anastomosis to generate vascularized tissue. In this approach, an AV loop chamber is placed in a scaffold, and the scaffold is first cultured *in vitro* until a new vascularized connective tissue is achieved (Figure 10.2a). The scaffolds with the AV loop are then microsurgically transplanted to the defect sites [55, 64–71]. In order to generate an autologous tissue flap, Tanaka *et al.* placed an AV shunt loop under the inguinal skin. The AV loop was wrapped with a sheet of artificial skin dermis between the right femoral artery and vein [71]. Kneser *et al.* first revascularized solid, porous matrices by means of an AV loop. After the AV loop was implanted in a processed cancellous bovine bone matrix for 8 weeks, an axial vascularization was observed [55].

However, this technique first needs to implant the cell-free scaffold, then remove the prevascularized scaffold, and finally implant the prevascularized

scaffold again, which is very complicated. In the first surgery, a macrovessel, such as a vascular axis or an artery, has to be removed together with the constructs for late anastomosis, which is relatively complicated and time consuming. In addition, during the first prevascularization stage, the delivery of nutrient and oxygen supplements to the seeded cells within the construct relies on the spontaneous in-growth of blood vessels from the surrounding tissues, which takes a relatively long time and may result in limitation of nutrient and oxygen to the seeded cells, and finally damage the viability of the cells [69].

10.3.1.1.2 In vitro Prevascularization

In contrast to *in vivo* prevascularization, *in vitro* prevascularization refers to the process through which microvascular networks are formed by self-assembly of preseeded ECs within engineered constructs. The cells are then cultured under optimal conditions. Afterward, the prevascularized construct is implanted into the defect site [60, 72–75]. In contrast to microsurgical vascular anastomosis, *in vitro* prevascularization techniques allow the spontaneous anastomosis of the host's in-growing vasculature after implantation. This allows for the construct to be supplied with nutrients. In this approach, the microvascular network within the construct is formed by the self-assembly and differentiation of ECs. As a result, the in-growth of host blood vessels into constructs is not necessary. After implantation, the host vasculature only needs to grow to reach the surface of the prevascularized constructs. The shorter growth distance needs less time compared to the time needed for complete vascularization within the whole construct.

Compared to the *in vivo* prevascularized construct, the *in vitro* prevascularized construct has much slower blood perfusion, as its microvascular network is not microsurgically but spontaneously connected after implantation. Time is needed for this spontaneous connection between the host vasculature and microvascular networks. A combination of *in vivo* and *in vitro* prevascularization may be able to solve this problem by creating a vascular axis within an *in vitro* prevascularized construct. This vascular axis can then be surgically connected to the host vasculature during the implantation process [76–80]. To direct ECs formation of the microvascular networks *in vitro*, a single channel or channel networks within the constructs are necessary for ECs adhesion. So far, three methods have been applied to create the channel or channel networks: (i) subtractive methods, (ii) additive methods, and (iii) the bioprinting method which has been reviewed by Hasan *et al.* [13].

10.3.1.2 Vasculogenesis- and Angiogenesis-Based Techniques

As mentioned previously, there are two ways to generate blood vessels: through vasculogenesis and angiogenesis, which represent the blood vessels generated from angioblasts and blood vessels branched from existing blood vessels, respectively. Currently, there are four laboratory techniques to regenerate blood vessels through these two ways: (i) micropattern-based techniques; (ii) GF-based techniques; (iii) cell-based techniques; (iv) scaffold and material-based techniques, which are shown in Figure 10.2b.

10.3.1.2.1 Micropattern-Based Techniques

Patterning ECs through microfabrication techniques has been used to promote the formation of vascular networks in engineered tissue constructs. Various microfabrication techniques, including photolithography, laser photolithography, micromolding, microcontact printing, nanoprinting and UV light-based chemistry, have been used to pattern ECs [13, 81–89]. Among these methods, techniques based on photolithography have been widely used. In the photolithographic technique, a photosensitive polymer solution and a photomask are normally used. The part of the polymer solution not covered by the mask is exposed to light of a certain wavelength, which induces cross-linking. As the rest of the polymer solution is not cross-linked because of the shielding by the photomask, it can be removed by washing. Finally, a 3D construct with patterned structures can be obtained, and ECs can be immobilized within this microengineered hydrogels. Using standard photolithographic technique, Dickinson *et al.* patterned fibronectin (Fn) molecules onto silicon masters with stripes of micrometer-sized widths [85]. They found that the patterned structures with Fn modification guided the orderly adhesion of human EPCs, supported their elongation, and subsequently assembled them into chains and tubular structures (Figure 10.3a,b).

Laser photolithography was developed from photolithography by replacing the photomask with laser beams that can selectively photopolymerize biomolecules to form 3D hydrogel structures [91]. The hydrogels can be a mixed solution with multiple types of cells, biomolecules, and GFs aimed at inducing vasculogenesis of the ECs. For example, Lee *et al.* micropatterned the cell adhesive ligand (Arg-Gly-Asp-Ser-Lys, RGDS) onto a collagenase-sensitive poly(ethylene glycol-*co*-peptide) diacrylate hydrogel using two-photon laser scanning (TPLS) photolithographic technique. The spatial presentation and concentration of RGDS within the hydrogel scaffolds were controlled by the TPLS technique. Then, human dermal fibroblasts (HDFs) were encapsulated within the micropatterned collagenase-sensitive hydrogels. As RGDS is helpful in cell adhesion and inducing vasculogenesis, HDFs migrated only into the RGDS-patterned regions of the hydrogels (Figure 10.3c,d) [90].

10.3.1.2.2 Growth-Factor-Based Techniques

Angiogenic GFs have been widely applied to promote vasculogenesis and angiogenesis based on the mechanism that GF concentration gradients can induce the migration of ECs and EPCs from regions of low GF concentration toward regions of high GF concentration of in 3D TE scaffold or in a microfluidic channel [92, 93]. The main GFs used to induce vascularization include VEGF, the basic fibroblast growth factor (bFGF), hepatocyte growth factor (HGF), angiopoietins (Angs), transforming growth factors (TGFs), and platelet-derived growth factors (PDGFs) [44, 52, 94–99].

Application of these GFs generally results in vasculogenesis and angiogenesis. However, the blood vessels induced by GFs are often immature, with disorganized and unstable structures, which results in leaky and hemorrhagic properties [96, 100, 101]. SMCs or pericytes are necessary for forming blood vessels, as they can synthesize ECM for stabilizing those blood vessels [52, 92, 102, 103]. Among the

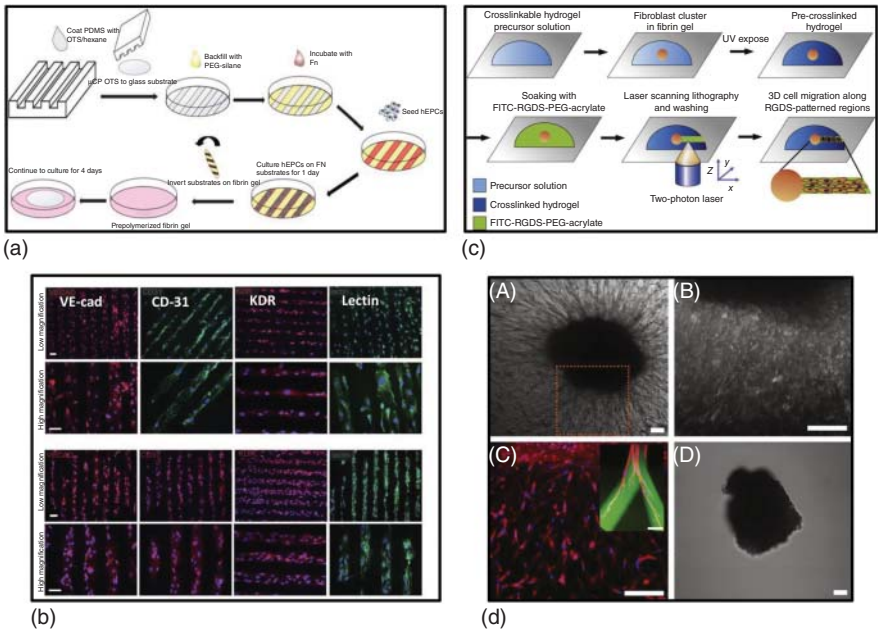


Figure 10.3 (a) Schematic describing the development of Fn functional surfaces, followed by hEPC seeding and culture. (b) Characterization of hEPCs cultured on Fn patterns. (Panels (a) and (b) adapted from [85] with permission from the Royal Society of Chemistry.) (c) The overall methodology for three-dimensional RGDS patterning by two-photon laser scanning (TPLS) photolithography. (d) DIC and confocal fluorescence images of HDFs undergoing 3D migration within degradable PEG hydrogel with homogenously distributed RGDS; scale bar = 100 μm. (Images adapted from [90] with permission from Elsevier.)

above-mentioned GFs, VEGFs and bFGFs have been reported to be able to induce the formation of blood vessels. PDGF, TGFs, and Angs are important for stabilizing new vessels since PDGF can recruit SMCs and pericytes. TGF-β is critical for the deposition of ECM and the communication between ECs and mural cells. In addition to applying a single type of GF, which can induce and subsequently stabilize new blood vessels, applying a combination of GFs has been demonstrated to induce the creation of functional vascular networks [43, 44, 104]. Synergistic effects between VEGF and FGF2 have also been observed with the combined application of these two GFs in a rabbit ischemic hind limb, while application of either VEGF or FGF2 alone did not show a similar effect [46, 104].

In addition to directly applying the above GFs for stimulating blood vessel formation or maturation, there are indirect approaches. Sonic hedgehog homolog (SHH) [105], hypoxia-inducible factor 1 (HIF-1) [106], and bone morphogenetic proteins (BMPs) [107–109] have been demonstrated to be able to stimulate cells neighboring the vascularization site to produce GFs of angiogenesis. This indirect approach is advantageous than the aforementioned direct approach. The advantages include its ability in enabling the cells to secrete multiple types of GFs simultaneously, regulating and ensuring the concentration of GFs from cells

to be proper, and responding to the requirements of vessel formation in different stages, resulting in proper GF gradients that are important for both vessel formation and maturation.

As an overdose of the angiogenic factors may result in risks such as severe vascular leakage, destabilization, and hypotension [110, 111], direct injection of GFs is not a good way to apply GFs *in vivo*. Therefore, an area-restricted and controlled delivery strategy is necessary. Several strategies have been developed to protect GFs from denaturation and degradation, as well as deliver GFs in a controllable and effective manner. These strategies include encapsulation, immobilization of GFs protein or genes within a biocompatible biomaterial, and the use of transfected cells genetically engineered to overexpress specific factors [44, 100, 112–117]. Various biomaterials have been designed to carry GFs and release them in a controlled manner [96, 112, 118–126]. Poly(lactic-*co*-glycolic acid) (PLGA) or poly-L-lysine (PLL) has been used to deliver those GFs in an effective, long-term way by taking advantage of the degradation properties of these biomaterials [118–120, 124]. However, during encapsulation or immobilization, proteins may get denatured because of the harsh conditions due to high temperatures or organic solvents [127]. In addition, as the *in vivo* environment is complicated, the manner of delivery of GFs from biomaterial matrices *in vivo* is difficult to control or tune well [111]. All these concerns indicate that the delivery of GF through biomaterials or scaffolds is of limited application.

Another approach is to supply GFs by using genetically transfected cells. These cells can overexpress angiogenic factors and provide a sustained GF release once they are seeded in the scaffolds [111, 128]. Yang *et al.* transfected the eukaryotic expression vector pcDNA 3.1-hVEGF$_{165}$ into MSCs and injected the transfected MSCs at the heart infarct zone of inbred Wistar rats. Results showed that the transfected MSCs could improve myocardial perfusion and restore heart function better than with either cellular or gene therapy alone [128]. However, this method requires complicated transfection technologies, which also limit its application.

10.3.1.2.3 Cell Coculture-Based Techniques

In addition to applying a single type of cells, such as ECs or EPCs, to regenerate blood vessels, coculturing of different types of cells has been developed to induce spontaneous alignment of the vascular cells and generate vascular networks based on the fact that there are many types of cells involved in the angiogenic process [37, 48, 49, 51, 62, 77, 129–131]. SMCs, fibroblasts, keratinocytes, adipocytes, osteoblasts, and bone marrow stromal cells have been selected to be cocultured with ECs or EPCs [48, 49, 132–135]. A fibroblast and EC coculture model has been widely applied for studying the angiogenesis mechanism. This coculture system could significantly enhance angiogenesis because of the favorable ECM supplied by fibroblasts and the intimate communication that occurred between fibroblasts and ECs (Figure 10.4a,b) [37, 136]. In addition, mesenchymal stromal cells have been cocultured with ECs to induce, stimulate, and stabilize vascularization of ECs through the molecular machinery contained in stem cells [77, 132, 137–139]. Coculture of HUVECs and human BMSCs also significantly improved the formation of capillary-like networks through

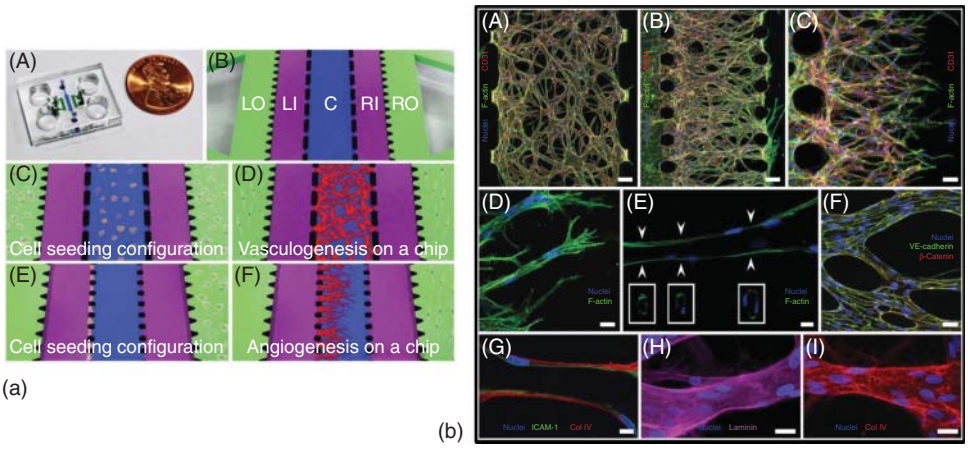


Figure 10.4 (a) Microfluidic chip design and cell-seeding configurations for microvascular network and angiogenic sprout formation. (b) Engineered 3D microvessels characterized by immunofluorescence. (A,B) Confocal micrographs showing the overall architectures of vascular networks established by (A) vasculogenic and (B) angiogenic processes at day 4. Scale bar, 100 μm. (C) Angiogenic sprouts grown for 2 days. Scale bar, 50 μm. (D) Higher magnification image shows the F-actin-rich filopodia extensions of angiogenic tip cells. Scale bar, 20 μm. (E) Cross-sectional images of a blood vessel showing a hollow lumen enclosed by ECs. Scale bar, 10 μm. (F) Microvascular network immunostained against adherens junction proteins, VE-cadherin (green) and β-catenin (red). Scale bar, 50 μm. (G) Longitudinal cross-section of a TNF-α-stimulated blood vessel stained for ICAM-1 (green) and collagen IV (red). Scale bar, 10 μm. (H,I) Confocal micrographs of vessels stained for the major components of basement membrane, laminin (purple), or collagen IV (red). Scale bar, 20 μm. (All images adapted from [136] with permission from the Royal Society of Chemistry.)

paracrine effects, and angiogenic factors were reported to play an important role in these paracrine effects [132, 138, 139]. Boyd *et al.* cocultured HUVECs with multipotent MSCs derived from human embryonic stem cells in 3D collagen I–Fn scaffolds. Results showed that the coculture could increase the branching of EC networks and maintain the network integrity for up to 6 days. In contrast, the networks formed by HUVECs alone regressed very fast. This phenomenon is reflected by the high average lengths and large number of branch points per network formed by the coculture [140].

10.3.1.2.4 Scaffold and Material-Based Techniques

Scaffold-Structure Design and Fabrication Techniques It has been widely reported that the structure of a scaffold strongly affects the vascularization of the scaffold after it is implanted. In particular, the pore size and interconnectivity of the scaffold determines the extent of blood vessel in-growth [141–144]. Faster blood vessel in-growth has been observed in scaffolds with pores exceeding 250 μm than in scaffolds with smaller pores [143–145]. Moreover, the interconnectivity of the pores is important since the migration of cells for vasculogenesis or angiogenesis relies on the interconnectivity of the pores. Vasculogenesis or angiogenesis will be reduced if the pores in scaffolds are unconnected even when the porosity

of the scaffold is high. Therefore, developing scaffolds with large pores and high interconnectivity is crucial for inducing vascular genesis as well as angiogenesis.

There are many conventional methods to fabricate 3D scaffolds, such as phase separation, freeze-drying, particular leaching, and gas foaming [146]. Although these methods are able to create scaffolds with porous structures, interconnectivity cannot be guaranteed. To achieve the properties mentioned above, recently several scaffold fabrication techniques have been developed, mainly including (i) electrospinning techniques, (ii) rapid prototyping (RP) and solid free-form techniques, and (iii) microfluidic techniques.

Electrospinning has been used for fabricating TE scaffolds or drug delivery system with interconnected pores for many years [147–152]. However, the conventional electrospinning method is not good enough for fabricating cell scaffolds, as it can easily get the fibers tightly packed into a sheet-like structure, which actually inhibits cell penetration into the scaffolds [147, 153–155]. The fiber sacrificing method involves co-electrospinning two different kinds of fibers with different chemical or physical properties. During postprocessing, one type of fiber is sacrificed by leaching out in order to form voids within the whole scaffold. This is achieved by exploiting the different physiochemical properties of the two fibers. Scaffolds prepared by this method have large pore size and high porosity, which are beneficial for cell infiltration. Baker *et al.* obtained a highly porous polycaprolactone (PCL) scaffold by removing poly(ethylene oxide) (PEO) from a PCL/PEO electrospun scaffold [156]. This can be seen from Figure 10.5a. Mixing nanofibers with microfibers is an alternative method for producing porous electrospun scaffolds (Figure 10.5b) [157–159]. Santos *et al.* fabricated a PCL-based scaffold containing a nano- and microfibrous network. This topography not only guided the 3D distribution of ECs but also allowed ECs to stretch between single microfibers and form capillary-like networks. After implantation, it was discovered that these scaffolds enhanced *in vivo* blood vessel formation (Figure 10.5b) [157].

In addition to increasing the pore size and pore interconnectivity in electrospun scaffolds, aligning electrospun nanofibers uniaxially or into various patterns to promote blood vessel growth has been a subject of much attention in the field of TE [160–163]. Many publications have reported that alignment of nanofibers can direct the adhesion, elongation, alignment, migration, and differentiation of blood vessel cells [160, 164, 165]. As mentioned before, in order to withstand the high blood pressure in vessels, SMCs and collagen fibrils in the tunica media are aligned in a re-marked circumferential orientation to endow the whole blood vessel with enough mechanical strength. Therefore, the alignment of blood vessel cells has been widely studied with the aim of improving vascular TE [164, 165]. SMCs, ECs and myofibroblasts are sensitive to nanofiber alignment in electrospun matrices. For example, parallelly aligned poly(L-lactide-*co*-ε-caprolactone) (P(LLA-CL)) nanofibers have been reported to affect almost all behaviors of human coronary SMCs [165]. Although electrospinning techniques have shown many advantages in fabricating scaffolds for directing ECs growth, differentiation, and vascularization, they cannot fabricate scaffolds with complex 3D structures and shapes [146].

RP and solid free-form fabrication (SFF) systems have attracted much attention because these can fabricate scaffolds with complex architecture and desired

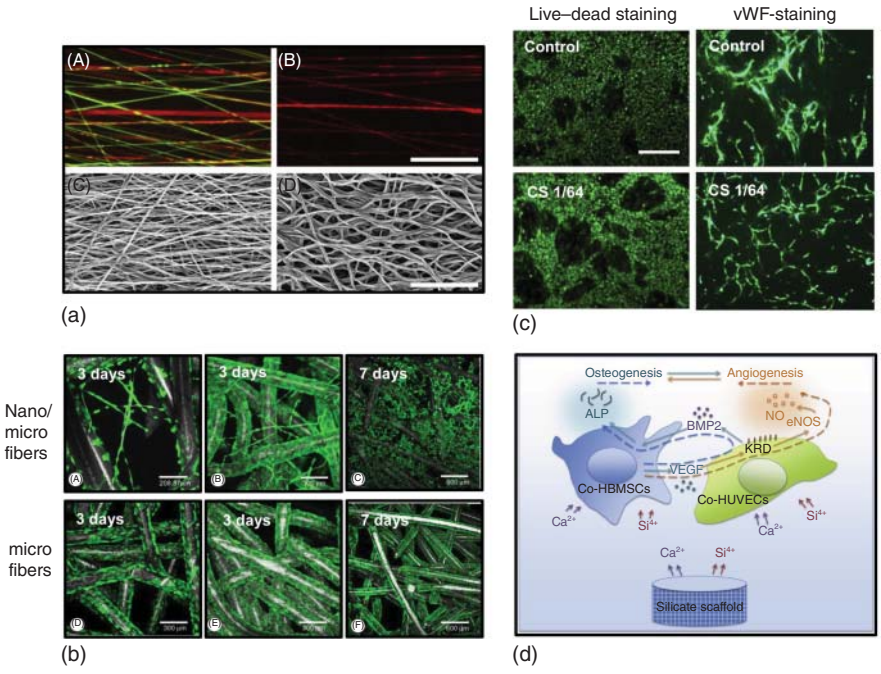


Figure 10.5 (a) Composite fibrous scaffolds can be formed with individual fibers of distinct polymer compositions. Removal of one sacrificial fiber population increases scaffold porosity. (A) Fluorescently labeled PCL (red) and PEO (green) fibers showed pronounced alignment and interspersion. (B) Submersion of scaffolds in an aqueous solution removed the PEO component but left the PCL fibers intact. SEM images of as-spun (C) and post submersion (D) composite scaffolds revealed increases in pore size with the removal of sacrificial PEO fibers. Scale bar: 50 mm. (Images reprinted from [156] with permission from Elsevier.) (b) Confocal fluorescent micrographs of viable HDMECs (A, B, D, E) and HUVECs (C, F) growing on fibronectin-coated nano/micro-fiber-combined scaffolds (left column) and on micro-fiber scaffold (right column) after 3 (A, D, C, F) and 7 days (B, E). (Images reprinted from [157] with permission from Elsevier.) (c) The angiogenic responses of cocultured cells in different culture media. Cells were stained with live–dead and vWF. Scale bar = 200 μm. (d) The proposed mechanism through which the CS stimulated the interactions between HUVECs and HBMSCs in cocultures, which finally enhanced the osteogenesis and angiogenesis/vascularization. (Panels (c) and (d) adapted from [135] with permission from Elsevier.)

pore structure [166–169]. There are many techniques belong to RP or SFF, including the aforementioned laser-based systems, multiphoton polymerization, 3D inkjet and wax printing systems, or nozzle-based procedures [3, 166, 170]. With computer-aided design (CAD) technology, the obtained constructs possess desired pore morphology and porosity, aspired interconnectivity, and proper mechanical strength, all of which facilitate the desired cell behaviors and finally vascularization. For example, fiber deposition technology has been applied to fabricate TE scaffolds. With this technique, various types of biomaterials, including molten polymers, hydrogels, or biomaterial pastes, can be processed. These materials are extruded in the form of fibers, which are subsequently

deposited layer by layer into 3D constructs designed by CAD technology [169, 171–173].

Microfluidic techniques can fabricate devices with microchannels using procedures adapted from soft lithography [174–178]. As there are many channels with small dimensions, microfluidic devices can create laminar flow, which is able to mimic the *in vivo* vascular microenvironment [13, 179, 180]. After the vascular cells are seeded within the microfluidic channels, angiogenesis or growth of microvascular networks in biomaterials can be induced by applying GFs or fluid pressure with certain well-defined gradients. However, so far this technique could not be used to produce 3D scaffolds with complicated structures since it is limited by geometric and surface chemical considerations [181].

Bioactive-Material-Based Techniques Recent evidence indicates that some bioactive materials possess pro-angiogenic potential. This has attracted much attention because of its simplicity and high efficiency in inducing vascularization. So far, biomaterials that have been reported to be capable of inducing vascularization are primarily silicate biomaterials and ion-substituted bioceramics, including but not limited to 45S5 Bioglass® (BG), mesoporous BG, akermanite (Aker), calcium silicate (CS), strontium-substituted calcium silicate (Sr-CS), and copper-doped calcium silicate (Cu-CS) or copper-Bioglass® (Cu-BG) [135, 182–192]. Several studies have demonstrated that 45S5 BG could stimulate fibroblasts to secret GFs into medium. A medium of fibroblasts could significantly increase the proliferation of human dermal microvascular ECs and stimulate the tubule network formation. This is because the presence of BG causes the fibroblasts to secrete large quantities of VEGF and bFGF into the culture medium. Leu *et al.* incorporated BG into collagen and reported that the BG/collagen sponges had strong stimulatory effects on EC proliferation, tubule formation, and VEGF production. The presence of BG *in vivo* could enhance blood vessel and bone formation in critical-sized calvarial defects [184, 185].

In addition to BG, silicate bioceramics also have the ability to enhance vascularization. Zhai *et al.* demonstrated that a Ca-Mg-Si bioceramic upregulated the expression of VEGF and bFGF receptors, nitric oxide synthase (eNOS), and synthesis of NO in human aortic endothelial cells (HAECs), which resulted in vasculogenesis of HAECs [192]. Li *et al.* demonstrated that CS, a simple silicate bioceramic composed of only calcium and silicon, could not only stimulate HUVECs to perform vascularization but also enhance the interactions between HUVECs with fibroblasts or HBMSCs and improve the vascularization in an *in vitro* HUVEC–HBMSC coculture system (Figure 10.5c) [135]. The proposed mechanism is that the ionic products released from CS enhance the paracrine effects between HUVECs and HBMSCs *in vitro* and *in vivo*, thus promoting cocultured HBMSCs to express VEGF and initiate the vascularization pathway (Figure 10.5d).

Some elements, such as copper, strontium, and cobalt, have been doped to bioceramics or bioglass to improve the angiogenic ability of biomaterials. For example, strontium has been incorporated into CS, and the *in vitro* results showed that the ionic products of Sr-CS could stimulate the proliferation,

differentiation, and angiogenesis of HUVECs and *in vivo* promote the angiogenic activity of ECs [187]. Since copper can stimulate angiogenesis, Kong *et al.* synthesized a Cu-CS product and found that it could further accelerate vascularization compared to CS alone due to the combined effects of Cu and Si ions [183]. Biomaterials with pro-angiogenic ability have also been incorporated into hydrogels to engineer hydrogel systems with angiogenic properties [193, 194].

10.3.2 Strategies for Engineering Vascular Replacement Grafts

Cardiovascular diseases involving small and medium-sized blood vessels are the main cause of death in the Western society [9, 16, 195, 196]. For treating large diseased arteries with inner diameter >6 mm, artificial vascular grafts, such as expanded polytetrafluoroethylene (ePTFE) or polyethylene terephthalate (PET), have been successfully developed and used [197, 198]. However, much less success has been reported in treating small-diameter blood vessels (inner diameter <6 mm) with synthetic vascular grafts. Therefore, clinical needs of vascular grafts for replacing diseased arteries are urgent. The emergence of TE technology, undoubtedly, brings great potential to replace diseased and damaged native blood vessels with engineered vascular grafts. An ideal engineered functional vascular graft should meet the following requirements: (i) proper mechanical properties; (ii) matching the compliance of the adjacent host vessel; and (iii) favorable for cell attachment and phenotype keep of critical vessel cells [198, 199]. There are many technologies that have been developed for engineered vascular grafts with small diameter that meet the above-mentioned requirements, which mainly include biomaterials as well as scaffold-based and cell sheet-based techniques [38, 199–204]. The cell sheet-based technique was first established by L'Heureux and colleagues, and has been reviewed by McBane *et al.* [38, 195, 200, 201]. This method obtained a cell sheet by rolling sheets of human vascular SMCs and dermal fibroblasts around a mandrel in concentric layers. It is an innovative, implantable, biological-based, tissue-engineered vascular graft that does not use synthetic biomaterials. This method has been reviewed in many publications. It has been reported that the cell-sheet technique is a promising means to reconstruct vascular grafts for the replacement of small and medium-caliber vessels, but the high cost and long production time required for autologous blood vessels limit the clinical applications of this technique [38]. Therefore, here the material selection and scaffold fabrication points of biomaterial- and scaffold-based techniques rather than cell sheet-based techniques will be focused.

10.3.2.1 Material Selection

Because of the elastic properties of blood vessels, materials used for constructing a vessel substitute are normally polymers. Various materials have been applied to fabricate tubular scaffolds for blood vessel engineering. These materials include type I collagen, styrenated-gelatin, polyethylene oxide, elastin, segmented polyurethane, ePTFE, PET, PCL, PLGA, and so on [195].

Natural proteins, including collagen, fibrin, and gelatin, are biomaterials that first attracted researchers in the blood vessel TE field due to their excellent

biological compatibilities and functions [21, 205–210]. Collagen was the first biomaterial used for engineering a vascular graft. In 1986, Weinberg and Bell first constructed a bioengineered vascular conduit with a collagen scaffold and vascular cells. Huynh *et al.* coated the submucosa of a pig small intestine with fibrillar type I bovine collagen [211].

Rich cell signaling components found within the matrices recently made the decellularized biological scaffolds attractive for use as TE scaffolds. This is because those biological clues present are essential for all cell behaviors [212–215]. As the host cellular antigens have been removed during the decellularizaiton process, the remaining ECM will not result in inflammatory response and immune rejection [204, 212]. Meanwhile, the decellularized ECM not only remains in a tubular arrangement but also contains the main EMC components, such as collagen, elastin, and glycosaminoglycans [10, 216], which can endow the decellularized ECM with certain mechanical properties that are similar to those of native blood vessels. All these advantages indicate that decellularized tubular scaffolds have great potential in blood vessel TE. Various decellularized matrices have been used to engineer blood vessels [204, 217, 218]. Zhou *et al.* modified a decellularized canine carotid artery with heparin immobilization and VEGF coating. They found the modified scaffolds showing significantly higher patency than nonmodified scaffolds after the scaffold was implanted as a bilateral carotid allogeneic graft for 6 months in a canine [219]. To avoid the risk of transmitting animal pathogens to humans, vessels from humans have been applied to engineer blood vessels [204, 220]. For example, the human umbilical vein (HUV) has been decellularized to obtain a scaffold for blood vessel TE [220]. The obtained scaffolds contain biological proteins for cell integration and proper mechanical properties. As a result, HUV scaffolds are highly applicable to vascular engineering.

Recent investigations in blood vessel TE focus more on bioresorbable grafts than nonbiodegradable substitutes. Polyglycolic acid (PGA), PCL, and synthetic polymers modified from PGA and PCL are the most acceptable materials for bioresorbable grafts [77, 221–225]. Modified synthetic polymers can improve the biological compatibility of synthetic polymers and adjust their degradation rate since they lack the appropriate cell signaling cues for inducing cell proliferation, maturation, differentiation, and ECM deposition. Moreover, some of the polymers degrade fast, which results in an acidic environment and leads to inflammation. [8, 16, 195, 197].

On comparing the natural proteins, decellularized biological tubular scaffolds, synthetic bioresorbable grafts, and nonbiodegradable polymers, each material has its advantages and disadvantages. Natural proteins are similar to the components of ECM and there are no non-autologous cells, which indicates that they are relatively nonimmunogenic and biocompatible. However, vascular grafts made from natural proteins normally possess poor initial mechanical properties [210]. Decellularized biological tubular scaffolds do not easily cause immunogenic response of tissue because no cells are left in the matrix. However, it is difficult to form an EC layer in the decellularized scaffolds [195]. Besides, the procedures of decellularizaiton are quite complicated and hard to control. Synthetic materials possess the advantages of being relative cheap and easy to obtain,

having consistent and stable components, and easy processing and fabrication. As synthetic materials, nondegradable polymers have been first used clinically for grafts with large diameters. However, their mechanical properties are not favorable for soft-tissue regeneration [9]. The mechanical properties of degradable polymers can be tailored, and most degradable polymers are compatible with cells. However, their acidic degradation products and poor mechanical properties still need improvement [226, 227]. Taken together, to date, no single material can meet all the requirements of a functional vascular graft. It has been proposed that scaffold grafts made of two or more types of biomaterials are advantageous over grafts composed of a single type of biomaterial [228].

10.3.2.2 Tubular Scaffold Fabrication

In order to mimic the shape of blood vessels, the above biomaterials need to be fabricated into tubes. Many methods have been developed, including wrapping biomaterials around a mandrel or a rod and subsequently removing the mandrel or rod, and electrospinning techniques [150, 198, 229, 230]. Roh *et al.* constructed a biodegradable tubular scaffold with an internal diameter of <1 mm by using a cylinder chamber and a polyester sealant [230]. Recently, electrospinning techniques have been widely used for fabricating blood vessel TE scaffolds because of its many advantages. In particular, its capacity to form fibrous scaffolds with nano and micro fibers can strongly affect cell behavior and subsequent blood vessel regeneration. Recently, Hasan *et al.* have reviewed electrospun vascular grafts [198].

Therefore, here the application of scaffolds with controlled structures fabricated by electrospinning techniques in vascular engineering is reviewed. It is well known that component, morphology, alignment of the nanofibers and the 3D shape of electrospun scaffolds are able to strongly affect the properties of engineered vascular tissue [151, 161, 198, 231]. Chen *et al.* fabricated a tubular scaffold with two layers: the core layer is composed of thermoplastic polyurethane (TPU) nanofibers, while the outer layer is composed of collagen (TPU/collagen) [160]. They demonstrated that TPU could endow the scaffolds with better mechanical strength, while collagen could provide better biocompatibility and hence better promote cellular proliferation when compared to pure collagen or TPU scaffolds. In addition, to mimic the alignment of collagen fibrils in the natural blood vessel and obtain a scaffold with improved mechanical properties to withstand the high pressure in the circulation system, electrospun scaffolds with aligned fibers have been fabricated. Zhang *et al.* used a template to prepare a tubular scaffold with designed pattered nanofibers, which is shown in Figure 10.6a [232]. He *et al.* fabricated a bi-layered elastomeric poly (ester-urethane) urea scaffolds (Figure 10.6b). Then, pericytes were seeded into the scaffolds and the pericytes-seeded scaffolds were implanted into Lewis rats as aortic interposition grafts. After 8 weeks, extensive tissue remodeling with collagen and elastin present could be found in the implantation (Figure 10.6c). Xu and coworkers prepared P(LLA-CL) scaffolds with parallelly aligned nanofibers and found that the behaviors of human coronary SMCs cultured on the scaffolds were strongly affected [165]. Wang *et al.* used electrospinning to fabricate a poly(L-lactide) (PLLA) scaffold with aligned

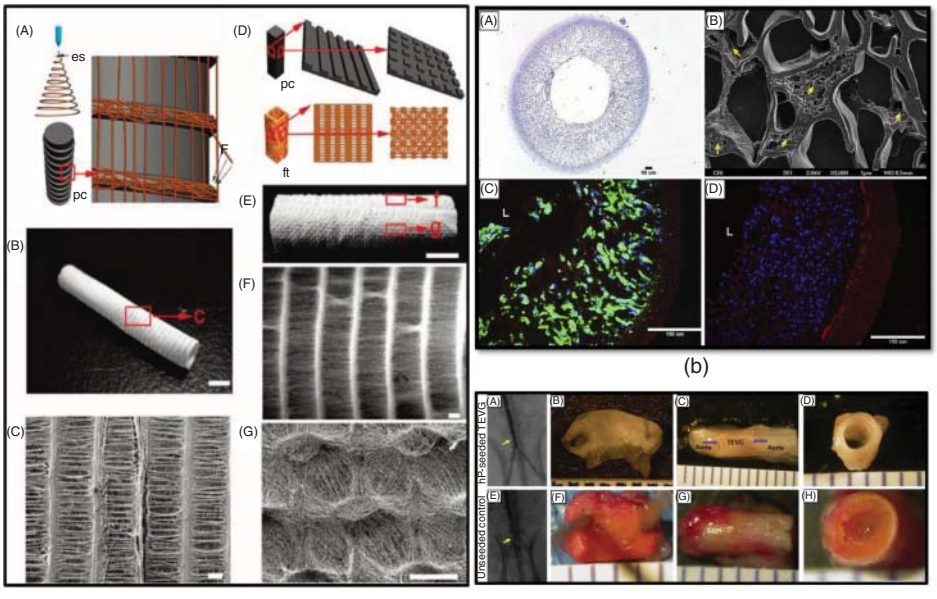


Figure 10.6 (a) (A) Schematic illustration of collecting process using a cylindrical collector with equally spaced circular protrusions (es, electrospinning process; pc, patterned collector). (B) A fibrous tube with patterned architectures (scale bar = 5 mm). (C) Magnified panel of (B) (scale bar = 200 μm). (D) Schematic illustration of collectors with two different patterns and relevant fibrous tube (pc, patterned collector; ft, fibrous tube). (E) A fibrous tube with two different patterns (scale bar = 5 mm). (F,G) Magnified images of two different patterns of (E) (scale bar = 200 μm). (Images adapted from [232] with permission from the American Chemical Society.) (b) Characterizations of the pre-implanted hP-seeded ES-TIPS PEUU scaffolds after dynamic culture. (A) H&E staining; (B) SEM (arrows = cells); (C) F-actin staining (red = scaffold; blue = nuclei; green = F-actin), and (D) nuclear staining (red = scaffold; blue = nuclei). "L" indicates lumen. (c) Representative angiographic and gross pathology panels of the (A–D) hP-seeded TEVG and (E–H) unseeded control. (A and E) Angiographic images (arrow ¼ implant); (B and F) macroscopic images of the lumen, (C and G) entire length, and (D and H) whole cross-section. Arrows in (C) indicate anastomosis marked by suture lines. (Images adapted from [236] with permission from Elsevier.)

nanofibers in the inner surface of the tubular scaffolds and found that these aligned nanofibers could induce SMC orientation and differentiation in a contractile phenotype [164]. Furthermore, the tubular shape of electrospun scaffolds could mimic the shape and mechanical properties of native blood vessels [233–235]. As tubular-structured scaffolds with aligned nanofibers can provide not only proper mechanical properties but also good biological properties, Wang *et al.* prepared an electrospun PLLA/polydimethylsiloxane (PDMS) tube with circumferentially aligned PLLA fibers in the inner surface of the tube and PDMS on the outer surface of tube, which was responsive for inducing SMC orientation and differentiation and providing better compressive property, respectively [164].

10.4 Conclusion and Perspective

Vascularization is one of the biggest challenges for engineering implantable constructs with complex structures and large size, or for repairing and regenerating living vascularized tissues. So far, several strategies for engineering blood vessels, including TE of microvascular networks, and strategies for engineering vascular replacement grafts have been studied and developed. For engineering microvascular networks, prevascularization techniques as well as vasculogenesis- and angiogenesis-based techniques have been introduced in this chapter. Microfabrication-based techniques, GF-based techniques, cell-based techniques, and scaffold- and material-based techniques for vasculogenesis and angiogenesis have been reviewed in detail.

Although various strategies have been developed for improving vascularization, no single vascularization method can fabricate functional vascularized tissue or organs. Available channels allowing immediate perfusion of growth media or blood can be achieved with prevascularization techniques, which are perhaps suitable for the fabrication of larger blood vessels. However, these techniques are not suitable for fabricating microcapillary beds with fine bifurcations down to few micrometers in size. Regarding vasculogenesis- and angiogenesis-based approaches, it usually takes days to weeks before cells can organize and give rise to perfusable lumens and, in this process, the temporal and spatial factors cannot be controlled, which is not suitable for the formation of vascular structures in the size ranges suitable for suturing and anastomosis with the host vasculatures [13]. In addition, it is hard to control the integration between the implanted vascularized tissue constructs and the host tissue. In addition, for practical use in the clinic, cost, vascularization speed, and functionality of the newly formed blood vessels are to be taken into account. Therefore, so far there is no single strategy that is able to sustainably vascularize constructs with a critical size after implantation. In the future, the combined application of several strategies is obviously a promising way to overcome the current problems and obtain a functional vasculature.

Large-diameter vascular grafts are available for clinical applications, but small-diameter vascular grafts are still under study. For engineering small-diameter vascular grafts, recent discoveries on cell-seeding methods, scaffold structures, material design, and cell sources have contributed much. However, techniques for *in vitro* expansion of cells still face major problems, as current techniques cannot meet the requirements for improving both the quality and amount of cells for blood vessel TE. Therefore, ideally, no seeded cells should be used in a tissue-engineered, small-diameter vascular graft in order to eliminate the expense and time and facilitate the manufacture of an "off-the-shelf" graft. Recently, *in situ* cellularization has attracted much attention and shown great promise. This strategy takes advantage of the bioactivity of biomaterials or some pharmaceuticals to increase circulating progenitor cells and improve homing capabilities. To meet this requirement, smart biomaterials and advanced scaffold fabrication techniques should be explored to endow the scaffolds with inherent bioactivities from component and structure, which will attract the host cells and direct the behaviors of the cells to regenerate blood vessels. To obtain grafts

with inherent bioactivity, electrospinning holds great promise since various bioactive components can be added into the materials before electrospinning. Besides, electrospinning can fabricate tubular grafts with various microstructures, which might improve the mechanical and biological properties of the grafts.

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Foreword

With the rapid advances that are seen nowadays in the area of tissue engineering, and the lack of manuscripts dealing with the state of the art of the field, this book presents a comprehensive review of tissue engineering in its various applications. Although a relatively new field, tissue engineering has seen huge progress in the past few decades, and scientists from various backgrounds are jointly working on making it a reliable method not only for organ replacement but more recently for *in vitro* disease models as well.

The book presents a collection of basic topics related to tissue engineering, which are organized in a progressive manner. Starting with a brief historical background on the development of tissue engineering as a substitute for organ transplantation, the current state of the art and the future prospects are also pointed to. The different pillars of the field are then discussed: biomaterials, stem cells, biosensors, biomanufacturing, and bioreactors are all necessary topics to consider when trying to engineer tissue constructs for organ replacement. For a new researcher entering the field, this first part gives an idea of the multidisciplinary aspect of tissue engineering. In fact, a combination of efforts is needed from experts in engineering, cell biology, materials, chemistry, and even microfluidics to be able to construct a scaffold for the engineered tissue, seed it with cells, and ensure proper attachment and functioning of the cells inside it. In the second part of the book, specific organs are treated in more detail, and different tissues are considered, from simple skin substitutes to the more complex organs such as lungs, and even to the nervous system with neural tissue engineering. Examples are cartilage, bone, musculoskeletal, cardiac, and heart vessel tissue engineering. Other organs are also treated, such as the liver, kidney, and pancreas. In a more recent advance, the final chapter of the book deals with the use of bionics in tissue engineering, which is an area with great future prospects.

The structure of the book should make it easy for the reader to follow the progression of tissue engineering from its basic elements (materials, cells, etc.) to

the full reconstruction of organs. In the second part, a whole chapter is dedicated to each type of tissue reconstruction in order to discuss all of its details and challenges.

In summary, the book offers a detailed and clear perspective of tissue engineering in its recent forms and applications. Given the variety of topics discussed in the context of recent advances, it could serve both as an introductory step into the field for new researchers in tissue engineering and as a reference for more advanced readers interested in learning about a particular topic.

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Preface

Tissue engineering is a new interdisciplinary field that aims at developing or regenerating artificial tissues and organs by using a combination of cells, biomaterials, engineering techniques, and suitable physicomechanical and biochemical stimuli to improve or replace biological functions. It is a relatively young field, as it emerged as a properly defined academic field only in the 1980s. However, it has progressed rapidly in terms of striking advances in the use of cells, biomaterials, and mechano-biochemical stimuli for the purpose of artificial organ generation.

The motivation for tissue engineering research emerges from the fact that millions of people worldwide go through various biomedical implant surgeries every year and the total cost for organ replacement surgeries is in billions of dollars annually. The currently available options for these transplants are autologous grafts, allografts (donor/cadaveric), xenografts, and artificial prostheses. All of these currently available options and technologies for organ replacement have severe limitations. The use of autografts and allografts, for example, are limited because of the lack of tissue donors, previous harvesting, or anatomical variability, as well as the concerns about their long-term functionality. Xenografts suffer from their relatively short life span, poor control over their physical and mechanical properties, inflammation, and calcification. Synthetic prosthetics often get rejected within a few months to a few years by the immune system of the body.

Tissue engineering has emerged to resolve these issues by generating implantable artificial tissues and organs. The ability to repair or regenerate damaged human tissue has already been a tremendous leap forward in the field of medicine. The goals and aims of tissue engineering have expanded rapidly from growing engineered skins for replacing the skin of burn victims and repairing cartilage joints of arthritis victims to re-creating tissues of more complex human organs such as the liver, kidneys, and heart, as well as regenerating lost neurons in patients with Alzheimer's disease and growing engineered blood vessels for cardiac bypass grafts. The targeted applications of engineered tissues have also expanded from implantable organs to *in vitro* models of tissues for the development of various *in vitro* disease models that could revolutionize the new therapeutics for numerous diseases. Furthermore, the progress of patient-specific smart diagnostics and personalized medicine will greatly benefit from the development of functional healthy or diseased models of engineered

tissues on microfluidic platforms. As a result, billions of dollars have been poured into bioengineering research over the last two decades, particularly focusing on organ regeneration. This has been resulting in exciting advancements and progress in the field.

However, the number of textbooks on tissue engineering for artificial organs have remained very limited, most of which are also not up to date. This book is intended to fill in a large part of this gap, reporting the major recent advances in tissue engineering and regenerative medicine. Tissue engineering for these applications requires the understanding of various fields of science and engineering. Biology, chemistry, microfluidics, as well as structural and material engineering are just some of the fields that help in understanding the concept of replacing and regenerating body tissues. Various biomaterials, their biocompatibility with *in vivo* conditions, and understanding the chemical makeup and engineering structure of the engineered tissue to fulfill its task are also important. Therefore, this book is divided into two parts. The first part aims to give an introduction to tissue engineering in general. The history of tissue engineering, the recent breakthroughs, and the future perspectives are discussed in Chapter 1. The major topics associated with tissue engineering, namely biomaterials, stem cell sources, induced pluripotent cells, biosensors, and bioreactors are presented in Chapters 2–6, respectively.

The second part of this book delves deeper into a more detailed discussion of various tissues and organs of the body. From skin, cartilage, bone, and musculoskeletal tissue to blood vessel, cardiac, heart valve, lungs and tracheal tissue as well as pancreas, kidney, liver, neural tissue, and bionics, the book presents the challenges that are faced in engineering each type of tissue. Also, it shows how to successfully engineer a tissue that does the function of its natural and biological counterpart.

This book has a slightly different focus from other available books on tissue engineering in that here the focus is on the application of tissue engineering and regenerative medicine to smart diagnostics and personalized medicine in addition to the generation of implantable tissues and organs.

Publication of this book would not have been possible without the tireless efforts of all the contributors. I would therefore like to extend my thanks and sincere gratitude to all the authors from across the globe for the successful completion of this work.

March 2017

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11

Engineering Trachea and Larynx

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11.1 Introduction

Diseases related to the respiratory system and cancers are two of the most common causes of death worldwide. Patients may face dramatic airway resection due to the cancer-associated problems, which involves the loss of associated functions. Even though there are several well-established methods to overcome disease and cancer-related tissue loss for larynx and trachea (such as anastomosis of the tracheal sections following partial resection), there is still an unmet need for functional replacements for ensuring a high quality of life for the patients. Speech loss, permanent separation of the respiratory and digestive systems, and complications related to the presence of a tracheostoma (a surgically implemented opening connecting trachea directly with the ambient air to replace normal respiratory pathway) all underline the need to develop a functional replacement without any ensuing problems.

In order to overcome organ donor shortage, and the need of immunosuppressant drugs, one possible solution is the development of tools and methods for personalized, engineered substitute tissues, which has become a well-developed research area. Personalized medicine is not only limited to the pharmacological applications and to the advancement of using different dosages to specific patients but also include medical treatments and diagnostic systems that consider the genetic, anatomical, and physiological characteristics of each patient. Lately, within the context of tissue engineering and regenerative medicine, personalized medicine is extended to the construction of a specific solution to each patient by using advanced technologies such as rapid prototyping. In the near future, it will be possible to develop an organ substitute that will match the size and the exact tissue composition of the target organ that is completely adapted to the patient's regenerative capacity. Tracheal replacement is one of the areas where tissue engineering has become a clinical reality, with several successful implantations already reported [1, 2]. However, larynx is not aimed much because of its complex structure, but for a few transplantation attempts. In the following sections, we describe these two organs in detail, explain the medical conditions that necessitate their partial resection or removal, and elaborate on the methods

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for their replacement with a special focus on the clinical larynx and tracheal replacement trials.

11.2 Basic Anatomy and Histology of the Larynx and Trachea

In this section, the basics of the laryngeal and tracheal anatomy and histology will be described briefly. The goal is to provide the anatomical background in order to understand better the associated clinical problems that would require larynx and trachea replacement.

11.2.1 The Larynx

The larynx is a multifunctional organ located in the anterior and medial part of the neck at the level of C5–C6 cervical vertebrae. It establishes the limit between the upper and lower airways. It is followed by the cervical part of the trachea in the basement of the neck, which plays a role in breathing, swallowing, and phonation. The larynx has a cartilaginous part with nine components connected each to other, which provides the necessary support for its function. This cartilaginous part of the larynx includes the thyroid cartilage, the cricoid cartilage, corniculate cartilages, the epiglottis, cuneiform, and the arytenoid cartilages.

The *thyroid cartilage* is considered the largest of the cartilages. It is composed of two laminae that fuse at their anterior parts to form the laryngeal pro-eminence. This pro-eminence is larger in adult males than females. The *cricoid cartilage* is a complete ring-shaped cartilage lying under the thyroid cartilage and connected to it with the cricothyroid membrane in the anterior part and the cricothyroid joints in the posterior side. The cervical trachea is connected to the inferior part of the cricoid cartilage. The *epiglottis* is attached to the internal side of the anterior part of thyroid cartilage. It is leaf-shaped and situated behind the base of the tongue. The *arytenoid cartilages* are paired and triangular and rest on the cricoid ring on the cricoarytenoid joints located at the posterior and upper side. They regulate the level of tension and position of the vocal cords. The *corniculate* and *cuneiform cartilages* are both paired and located at the apex of arytenoids.

All these structures are maintained together with ligaments and supported by several muscle insertions. The extrinsic muscles are important for positioning the larynx in the central part of the neck and its mobilization during its different functions. They suspend the larynx to the hyoid bone. In addition, the intrinsic muscles play a role in vocal cords mobilization, as they are the ones responsible for allowing the opening and closing of the vocal cords and the regulation of the laryngeal field during breathing, phonation, and swallowing. The vocal cord region is also called *glottis*. At this level, the pharyngeal tract is divided into the respiratory tract (trachea) and the digestive tract (esophagus).

The larynx's sensory and motor innervation is provided by the vagus nerve, with the superior and the recurrent laryngeal nerves. The internal side of the larynx is covered with the malpighian epithelium. The functions of glottis and epiglottis can be compared to a multifunctional valve.

During breathing, the vocal cords are maintained open and the epiglottis is in the raised position. This allows air to flow from the upper aerodigestive tract to the lower airway, namely the trachea. Similarly, during phonation, the epiglottis stays in the same raised position, and the vocals cords' opening and tension are controlled to permit their vibration with expired air, resulting in sound production. On the other hand, during swallowing, the larynx plays a crucial role in the lower airway protection, which is achieved by a highly regulated sequence of actions. The swallowing process starts with a controlled, voluntary phase (mastication), which is followed by an involuntary phase (deglutition). The deglutition process is comprised of a series of events. First, the laryngeal ascension takes place, and then the recession of the root of the tongue occurs. Then, the vocal cords move to the closed position, and the epiglottis moves down to a horizontal position covering the vocal cords. Afterward, the alimentary bolus is directed to the esophagus by the contraction of pharyngeal muscles. All these steps are controlled by the sensory and motor innervation and are crucial for a good swallowing process. This means that if any of these parts are missing or impaired, the normal functions associated with them will be compromised.

11.2.2 The Trachea

The trachea, also called the windpipe, begins in the inferior side of the cricoid cartilage at cervical basement, more precisely at the C6 level, and runs in the mediastinum. It is classically described as two parts: cervical trachea from the cricoid ring to level T2, where it enters the rib cage. Its inner diameter changes in males and females. It is larger in the male adults (~1.8 cm). It bifurcates at level T5 into two primary bronchi. The trachea, the initial part of the lower airway, conducts air to the lungs during inspiration and expiration for gas exchange.

The trachea, which is about 15 cm long, is constituted by 16–20 C-shaped, incomplete cartilage rings, stacked against each other. These rings are maintained together by the presence of fibrous tissue in the anterior and lateral parts of the annular ligament of the trachea. The posterior part of the trachea is a fibromuscular tissue that is attached to the anterior side of the esophagus. It runs in the mediastinum just behind the breast bone. This cartilaginous scaffold prevents the trachea from collapsing during inspiration and expiration. The cervical part of the trachea supports the thyroid gland on its anterior face, which is crucial for regulating metabolism, growth, development, and the temperature. At the rib cage level, the trachea is surrounded by important anatomical structures such as the aorta, the superior vena cava, and the pulmonary artery. The inner lumen of the trachea is covered with the respiratory epithelium. This epithelium is pseudo-stratified, columnar, and ciliated. The respiratory epithelium of the inner lumen of the trachea is composed of the following cells that are lined on the lamina propria, which is basically a fibroelastic connective tissue: (i) *ciliated columnar cells*, which are elongated cells that are ciliated on the apical side. They are responsible for moving the mucus on the epithelium; (ii) *goblet cells*, which are exocrine, mucus-producing cells, individually disseminated in the epithelium; (iii) *basal cells*, which are round cells located at the basal surface of the epithelium, which basically are a reserve of stem cells for replacing ciliated and goblet

cells; (iv) *brush cells*, which are columnar cells with apical microvilli with a sensory receptor role as they synapse with the dendritic endings of sensory nerve fibers; and (v) the *dense core granule cells*, which are endocrine cells that appear to be related to the amine precursor uptake and decarboxylation (APUD) system and may release vasoactive substances. From the description and explanations presented here, it can be appreciated that the trachea is more than a simple tube but is a complex organ with different tissues and cells. All these structures have specific functions. The pseudo-stratified, columnar, ciliated epithelium plays a role in heating the inspired air and also moistening it. The ciliated cells clear the airways by moving the produced mucus to the upper airways. In this way, the entrapped dust particles and exogenous microbial elements that are inhaled are eliminated.

Thus, in any laryngotracheal replacement, all these multiple functions need to be replaced and ensured. The biomaterial to be used needs to be compatible with the trachea's environment. The multiple functions of larynx (protection of the airway from food aspiration while providing the route for breathing) and the actions of the pseudo-stratified, ciliated epithelium in the trachea (first line of defense against insults, removal of particle and bacteria, etc.) should be, at least minimally, achieved in an engineered tissue or implant for an optimal replacement.

11.3 Indications for Tracheal Resection

In this section, the conditions under which tracheal or laryngeal resections are necessary are described in detail.

For tumors, or tracheal lesions due to trauma involving more than 50% of the trachea in adult patients or one-third in children, direct anastomosis cannot be performed even with maximum tracheal mobilization. Extensive tracheal resection remains a persistent clinical problem in terms of surgical reconstruction. The indications of tracheal resection include laryngotracheal stenosis, benign tracheal tumors, and malignant tracheal tumors either primary or secondary.

11.3.1 Laryngotracheal Stenosis

Laryngotracheal stenosis is the decrease of the caliber of the respiratory tree at the level of the larynx or trachea. Diagnosis and staging are based primarily on laryngotracheal endoscopy under general anesthesia. The severity of the stenosis is evaluated based on the degree of reduction of the cross-section of the stenotic segment (classified from grade I, which corresponds to reduction up to 50%, to grade IV, which corresponds to a total stenosis).

Simple monitoring is sufficient if the degree of reduction of the cross-section is less than 50%. Otherwise, the stenotic segment should be addressed surgically. Depending on the characteristics of the stenosis, treatment can be performed either endoscopically by a laser (carbon dioxide (CO_2) or potassium titanyl phosphate (KTP)) or by using other external approaches. While addressing such lesions, two principles are adopted depending on anatomical considerations: widening of the stenosed segment, in which case laryngotracheoplasty using

mostly costal cartilage; or excision of the pathological segment completely by cricotracheal resection anastomosis or pure tracheal resection anastomosis.

Historically, acquired stenosis was mainly secondary to infectious diseases, trauma, hamartomas, or amyloidosis. Currently, the leading cause of acquired laryngotracheal stenosis is iatrogenic, that is, prolonged intubation or too high a tracheostomy (performed close to the cricoid cartilage). The estimated risk of laryngotracheal stenosis after prolonged intubation varies from 6% to 21% of cases and after a tracheostomy between 0.6% and 21% of cases [3]. The use of low-pressure, high-volume cuffs in endotracheal intubation resulted in decreased risk [4].

11.3.2 Benign Tumors of the Trachea

The treatment of these tumors is generally endoscopic, with the necessity, in some advanced cases, for partial or circumferential tracheal resection. In pediatric population, benign tumors represent ~90% of tracheal tumors [5]. Up to 20% of them are inflammatory pseudotumors. Hamartomas, including chondroid hamartomas, are the second most common ones [6]. Diffuse papillomatosis is rare (about 2%) and is seen in children ranging from 18 months to 3 years of age.

In adults, benign tumors of the trachea and bronchi represent only 4.7% of tracheobronchial tumors. They can be of various origins: epithelial, mesenchymal, nervous, or mixed. Mixed tumors (inflammatory tumors or granulomas) are among the most common benign tumors. They are usually associated with chronic irritation (foreign body, bronchopulmonary infection), but can also be specific (due to tuberculosis or syphilis).

Chondroid hamartomas are due to an original embryonic injury. The average age of diagnosis is between 60 and 70 years, with a male predominance. Malignant transformation is rare. Epithelial tumors include papilloma in adults (usually solitary), which is more likely to originate in the larynx or bronchi than the trachea. Adenoma is also exceptional. Among the mesenchymal tumors is the granular cell tumors (Abrikossoff tumor) of neural origin developed from Schwann cells. There is also neurofibroma, schwannoma, fibroma, leiomyoma, fibroleiomyoma, and chondroma. They can be also benign and localized in trachea through infiltration in diseases such as amyloidosis, sarcoidosis, Wegener's granulomatosis, and tracheopathia osteochondroplastica.

11.3.3 Primary Malignant Tumors of the Trachea

In these kinds of tumors, the main surgical technique is tracheal resection–anastomosis. In the absence of a tracheal substitute and if an extended tracheal resection is needed, treatment is palliative. For trachea, primary malignant tumors are rare and represent ~2% of malignant tumors of the respiratory tree. In 90% of cases, it is either squamous cell carcinoma or adenoid cystic carcinoma [7, 8].

Squamous cell carcinomas are the most common primary tumors in adults with a male predominance and a peak incidence between 60 and 80 years. Smoking is the main risk factor.

Adenoid cystic carcinomas (ACCs) or cylindromas represent 0.1% of cancers of the respiratory tract. They are the second most common primary malignant tumor of the trachea, of which the most common origins are the major and accessory salivary glands. These tumors are observed more frequently in young patients than other malignancies, with no gender predominance. They are not associated with smoking [9]. Other primary malignancies of the trachea include, in the order of decreasing frequency, carcinoid tumors (typical and atypical), adenocarcinomas, anaplastic carcinomas, small-cell carcinomas, mucoepidermoid carcinomas, and melanomas.

11.3.4 Secondary Malignant Tumors of the Trachea

The secondary tumors of the trachea result either from a locoregional invasion or by distant metastasis. Locoregional invasions can be linked to laryngeal, bronchogenic, thyroid, or esophageal cancers, which typically occur through a tracheoesophageal fistula. Distant metastases are frequently hematogenous. Their sites of origin include breast cancer, kidney, colon, hepatocellular carcinoma, and melanoma.

11.3.5 Indications of Total Laryngectomy

Total laryngectomy is the complete excision of the larynx, from the cricoid cartilage to the hyoid bone. Once the pharynx is closed by several mucosal and muscular planes, the digestive tract becomes completely separated from the respiratory tract. The proximal end of the trachea is sutured to the skin to form a permanent tracheostome. The International Association of Laryngectomees reports that 57 000 laryngectomees are performed in the United States (http://www.theial.com). At the moment, a definitive laryngeal replacement without considerable decrease in the quality of life of the patient is not available [10]. Total laryngectomy is indicated for larynx or hypopharynx cancers, in advanced cases of larynx benign tumors, and in nonfunctioning larynx either post-traumatic or due to neurological diseases.

11.3.5.1 Cancer of Larynx or Hypopharynx

Total laryngectomy is performed in more than 98% of cases in the treatment of advanced cases of laryngeal or hypopharyngeal cancer that is classified T3 or T4. In over 95% of cases, it is squamous cell carcinoma. When the local extension of the primary tumor contraindicates the performing of functional partial laryngectomy, and when organ preservation protocol (chemoradiotherapy) is impossible or has failed, total laryngectomy becomes the treatment of choice. The 5-year survival of laryngectomees, all stages, is over 43% [11]. Although this surgery gives a satisfactory life expectancy, it causes major difficulties in the daily lives of patients.

11.3.5.2 Benign Tumors of the Larynx

The treatment of benign tumors tends to be more conservative: that is, endoscopic excision or partial laryngectomy. Total laryngectomy can be considered only in progressive tumors that are very large and which cause complete stenosis

of the larynx, or when it is impossible to deal with them with a more conservative approach. Its indication is thus exceptional. It has been reported in the literature in cases of chondroma [12], rhabdomyoma, and Abrikossoff tumor [13].

11.3.5.3 Nonfunctioning Larynx

Surgeries are resorted to when there is major swallowing disorders with aspiration (mainly in neurological diseases) in order to protect the lungs by separating the respiratory and digestive tracts. The options are closing the laryngotracheal tract, tracheal diversion, or total laryngectomy. Total laryngectomy, although effective in controlling aspiration, implies a mutilating surgery with an irreversible effect on the voice quality. Currently, it is replaced by other equally effective procedures such as laryngeal prosthesis or closed tracheal diversion [14, 15].

Very rare cases of severe external trauma to the larynx (gunshot or highway accidents) may require total laryngectomy. It is the same for severe necrosis of larynx after radiotherapy for a laryngeal or hypopharyngeal cancer [16].

11.4 Available Remedies Following Total Laryngectomy

Currently, there are four ways for restoring the laryngeal functions after total laryngectomy: (i) laryngeal transplantation, (ii) grafts, (iii) biomaterial-based solutions (implants), and (iv) tissue engineering.

11.4.1 Laryngeal Transplantation

There are only a few examples of laryngeal transplantation in humans. Transplants have been mostly carried out on animals, particularly on rats [17], dogs, and pigs [18]. A percentage of animals survived in these trials, but nerve sutures were either not performed or nonfunctional. Thus the main objective of the transplantation cannot be attained: that is, obviating the need for a tracheostomy. Also, animals are not the best models for conducting such research because of the voluntary control of swallowing in humans on one hand and anatomical differences on the other. Strome *et al*. [19] managed to transplant a human larynx. The graft was viable and resulted in good voice quality. The tracheostomy tube was, however, left in place because of incomplete motor function of the transplant. Their experience confirms the two obstacles associated with this surgical procedure: the need to maintain immunosuppressant therapy, and satisfactory re-innervation of the larynx or stimulation of nerve branches.

11.4.2 Grafts

11.4.2.1 Implantation of Nonviable Tissues

Bio-prostheses consisting of lyophilized tissue [20], frozen or chemically fixed, have been tested. Tracheal allografts treated with glutaraldehyde in rats and pigs, lyophilized aortic grafts in dogs, and trachea fixed with formaldehyde in humans have been implemented without success. This kind of treatment can avoid rejection, but these nonviable tissues are not revascularized and eventually become necrotic. Regrowth of the epithelium from both tracheal ends leading to formation of a granulation bed is also another associated problem.

11.4.2.2 Tracheal Transplantation and Autografts (Viable Tissues)

Nonvascularized grafts, either in the form of autografts or allografts, of tracheas have led generally to failures either by stenosis or necrosis of the graft. On the contrary, the use of a tracheal autograft, for example, the resection of some tracheal rings followed by their grafting, has demonstrated the viability of such grafts provided that the graft has a vascular support. But tracheal transplantation with its vascular pedicle is particularly difficult because of the caliber of anastomotic vessels. All trials encountered such problems of vascular anastomoses except in cases where tracheal transplantation was associated with pulmonary and bronchial transplantation, leading inevitably to the use of immunosuppressive therapy.

The trachea does not have an extensive vascular supply. It is connected with the vasculature by a network of vessels, the origins of which are the bronchial and the right inferior thyroid arteries. Thus, the provision of vascular support, particularly in the case of allogeneic replacements, is highly challenging [21, 22]. Additional constraints to successful tracheal tissue engineering applications should be resolved to make it a reality, such as the absence of a fully functional respiratory epithelium; foreign body response or other immunologic complications; local infection; biomaterial failure; and repeated operations, including tracheostomy [23]. Moreover, unlike the liver, heart or kidney, tracheal transplantation is not possible because of the number and size of its vascular supply. It can only be transplanted together with the adjacent organs such as the esophagus or thyroid, which have a common blood supply [22] due to their adjacent location.

11.4.2.2.1 Allografts

Despite all these problems, Rose *et al*. reported in 1979 the first case of allogeneic transplant of the trachea. First, a tracheal graft from a cadaver was transplanted into the sternocleidomastoid muscle in order to achieve revascularization (3 weeks). Afterward, the vascularized tracheal graft was implanted. A second case was described in 1993 by Levashov *et al*. In order to achieve vascularization indirectly, they performed omentopexy in a single-stage transplantation. Though this case seemed promising at the time, the allograft viability and functionality were not reported subsequently. Ten years later, Kleteptko *et al*. reported heterotypic vascularization in the momentum of an allogeneic graft. The functional and structural integrity of the graft was maintained. However, all allogeneic transplantations require lifelong immunosuppression, which has considerable side effects. It also presents a significant risk for patients with malignancies. However, immunosuppression is inevitable because the tracheal epithelial cells and the chondrocytes can trigger acute inflammation, which can lead to chronic inflammation/rejection [21].

Cryopreservation of the grafts can decrease their antigenicity, as demonstrated by Murakawa *et al*., who compared allografts (fresh, frozen, and cryopreserved) in a primate. Although cryopreservation resulted in amelioration, there was still infiltration by mononuclear cells after 1 year, which points out that, in the long term, immunosuppression is still necessary [24].

11.4.2.2.2 Autografts

Another possibility is to use non-tracheal autologous tissues. These are tissue grafts from the same individual. This kind of transplantation can take place either with or without a vascular pedicle. Autologous tissues without a vascular pedicle are used primarily to repair tracheal defects. The most widely used tissues are fascia, cartilage, and periosteum, which ultimately are expected to turn into cartilage [25]. The precarious nature of the vascularity of tissues in contact with the flap often causes necrosis of the implanted tissue, leading to anastomotic dehiscence, infection, and formation of fibrosis.

As it is difficult to consider using an aortic autograft in humans, allografts of fresh aortas were taken to be implanted to replace trachea in sheep [26, 27]. The results were similar to those reported in autologous reconstruction, and a gradual transformation of the aortic graft was noted between the third and the sixth month. After extensive inflammation of the tissue, the vessel was re-colonized by a differentiated ciliated epithelium, and cartilage islands appeared in the connective tissue. No immunosuppression was required because of the disappearance of the vascular tissue [28, 29]. These results are quite encouraging. This strategy opens a new avenue for tracheal or laryngeal reconstruction but needs further studies and confirmation.

Transplantation of autologous tissue with vascular pedicle allows larger reconstructions with a decreased risk of necrosis. They may also be associated with biomaterials. These techniques take into account the quality and the long-term functional outcomes; rejection is not possible as all tissues are autografts. Nevertheless, these techniques require a long surgery time, they are tedious, and they are not easy to repeat.

11.4.3 Biomaterials

What kind of biomaterials can be used for the restoration of a rigid structure [30]? A few teams are currently working on the design of an artificial laryngeal prosthesis. The benefits of such a prosthesis are as follows: (i) the prosthesis can be placed during same operation, just after the total laryngectomy; (ii) proper biomaterials that are not considered as a contraindication for adjuvant radiotherapy or magnetic resonance imaging (MRI) can be used; and/or (iii) there would be no need for immunosuppressants.

However, there are many steps to consider for an artificial larynx system: first, the need to validate the acceptance of a long-term implanted material in a potentially septic environment; second, the design and manufacture of a triple sphincteric valve that can replace the functions of phonation, swallowing, and respiration; and finally, the surgical restoration of the pharynx. Despite these challenges, an implantable laryngeal prosthesis has been gradually developed by our team [31, 32] by combining a biocompatible titanium immovable prosthesis of alternating smooth and porous parts for the anastomoses, which could replace laryngeal cartilage and/or the tracheal tube, with a removable functional structure (valves synchronizing the functions of respiration and swallowing), intended to be fixed on the immovable prosthesis. The valves have been developed to be replaced under laryngoscopy suspension. Many materials have been used

primarily for tracheal reconstruction in animal studies (dogs, sheep, goats, pigs, rabbits, rats) and in humans. As there exists no animal model for a complete laryngeal replacement [33], we have conducted experiments based on tracheal replacements to judge the tissue acceptance.

Surface treatment processes have become of major importance for many industrial processes, particularly in the biomedical field. Thus, the great challenge now lies in the development of new surface structures whose reactivity can be controlled and modulated to induce specific and appropriate cellular responses (adhesion, proliferation, differentiation, etc.). Currently, nanoscience and nanotechnology present a unique opportunity to achieve such properties and to modify the surfaces of biomaterials in use. In our system, they have been especially considered for the porous titanium structures. *In vivo* acceptance of such prosthesis, supporting active valves as described above, may be potentiated by nanostructuration of the surface of any type of porous structure of a polymeric product, associated with biological factors. It seems possible, in the light of the work in both small and big animals (i) to improve the sealing of the porous titanium prosthesis during the first days/weeks after implantation to limit the deposition of mucus and bacterial colonization from the tracheal lumen, and (ii) to accelerate cell colonization by stimulating the colonization of porous titanium beads. Thus, it is possible to improve the material response by combining surface treatments with the use of degradable and functional components.

Even if laryngeal transplantation problems, such as the need for immunosuppressants and nerve anastomoses, are solved in the future, the limited number of donors still remains a limiting factor to solve this medical problem. In fact, one of the limiting causes is actually the shortage of donor organs for this kind of applications. To recreate a larynx, the trachea should be elongated by an integrated hollow structure, which is vascularized by the surrounding tissues. Porous titanium seems to fit to the demands of such an implant, allowing our team to perform the first implantations with an artificial larynx (ENTegral™, Figure 11.1a–e). ENTegral™ is composed of two parts: (i) a permanent tracheal prosthesis containing a porous titanium connector which is in contact with trachea, and (ii) a removable valve-based system that provides the route for inspiration and expiration. The implantation of the valve part is done endoscopically. Recently, we have reported the implantation of this system in a 65-year-old patient who had the artificial larynx system for 8 months [10] (Figure 11.1). Our main objective is to implant the artificial larynx just after total laryngectomy in order to avoid delays in postoperative radiotherapy. However, further research is necessary for the regular implantation of the artificial larynx in the clinic. We hope that the artificial larynx design can be incorporated in the tissue-engineered trachea development, which is covered in the next sections.

11.5 Regenerative Medicine Strategies and Tissue Engineering Tools for Tracheal and Larynx Replacement

Replacement of the respiratory tissue is needed after a tumor resection, trauma, stenosis, or in tracheal defects (such as tracheomalacia or tracheal strictures),

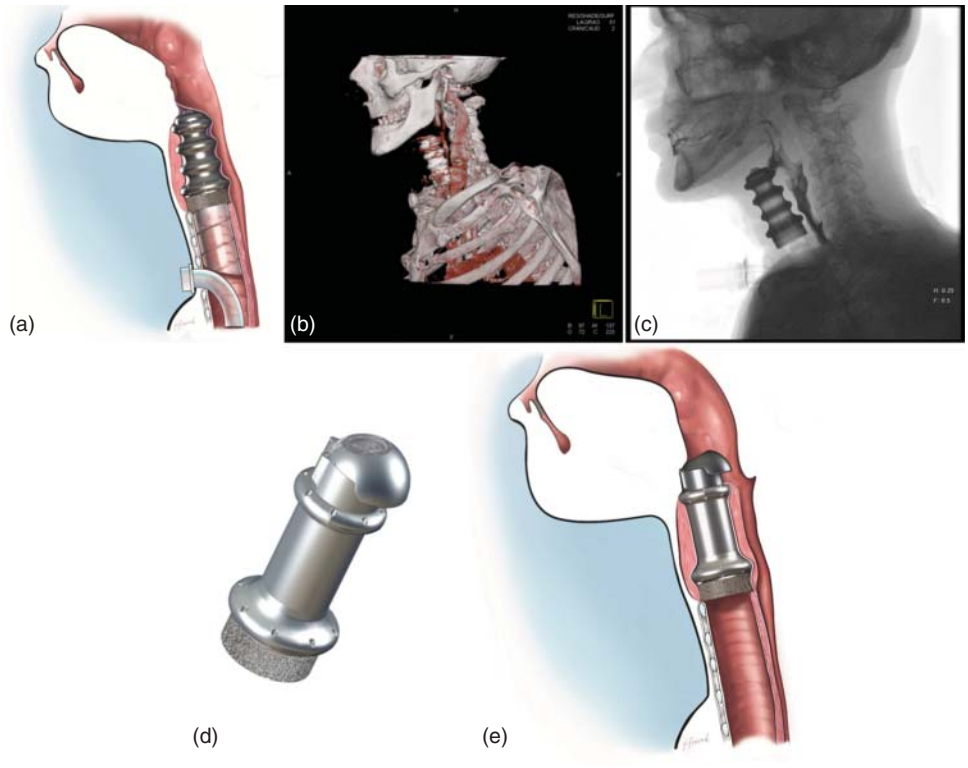

Figure 11.1 ENTegral™ Artificial Larynx system. (a) ENTegral after the first surgical step (implantation of the permanent part). (b) 3D CT scan shows the positioning of the implant. (c) Barium swallow test confirms the absence of fistulas. (Reprinted with permission from [10], Head and Neck.) (d) The overall structure of the implant with a removable valve system to restore the breathing and swallowing functions and a permanent part with a porous tracheal connector to ensure the proper integration of the implant with the airway. (e) The full implant in its orthogonal position. (Courtesy of Protip Medical.)

which causes high levels of morbidity [21, 23, 34]. Around 150 000 patients have complications due to mechanical ventilation or endotracheal intubation every year in the United States. In the case of brain or spinal cord injuries, the complications are more common, as long-term ventilator support and intubation is often necessary. Chronic airway problems results in high mortality rates (11–24%), which poses an important clinical challenge due to the limitations of surgical resection, as will be explained below [35]. Currently, the only curative treatment for primary cancers of trachea is resection [21].

Tracheal replacement still is a major challenge in thoracic surgery and also in regenerative medicine. Over the past 60 years, Grillo and several surgeons worldwide have solved the different problems associated with tracheal surgery in order to provide standardized approaches for airway diseases [36]. Thus, the majority of tracheal lesions requiring surgery can be now treated by resection followed by primary end-to-end anastomosis. Surgical techniques have been also developed for cases with laryngeal or cardinal extension. However, the resection of

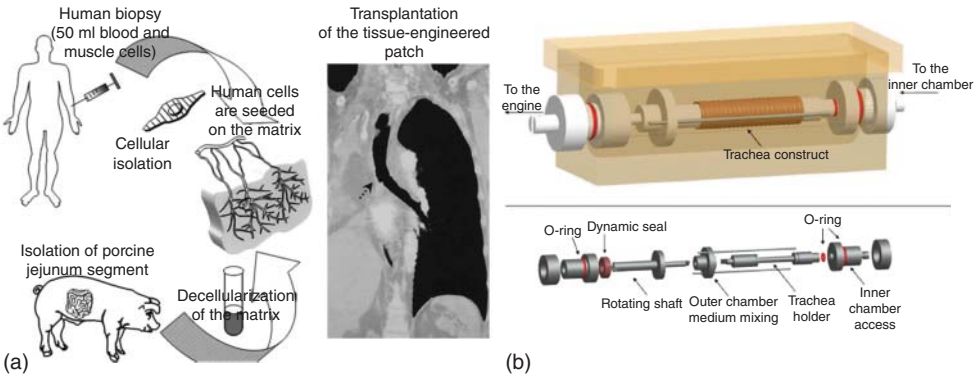

Figure 11.2 (a) The overall route of airway tissue engineering. Several different variations of this scheme have been achieved clinically during the last 10 years. (Macchariani 2004 [37]. Reproduced with permission of Elsevier.) (b) A double-chamber rotating bioreactor design to fulfill the requirements of two different cell types necessary for the tissue engineering of trachea. The outer part ensures the microenvironment for differentiation of patient's MSCs to chondrocytes within the scaffold, whereas the rotation of the system provides the air–liquid interface for the epithelial cells seeded to the scaffold lumen. (Asnaghi 2009 [38]. Reproduced with permission of Elsevier.)

tracheal lesions extended to more than half the trachea is not recommended because reconstruction using direct anastomosis is not possible. Large airway defects still are a significant clinical problem, as patients cannot survive if tracheal resection is necessary for more than 6 cm in length in adult patients or 30% of the total tracheal length in pediatric patients. For these patients, we need a tracheal substitute.

11.5.1 Replacement of the Trachea

In the last chapter of his reference textbook, Grillo detailed the results of the five schematic ways of research that have been explored by numerous teams to find a valuable tracheal substitute [36]. An ideal tracheal substitute should (i) be a strong, flexible, tubular biocompatible structure, (ii) must facilitate epithelialization, (iii) must integrate with adjacent tissues, (iv) should not cause stenosis, and (v) must resist bacterial biofilm formation. The use of various foreign materials has caused serious complications such as granulation tissue formation, undesired movement of the prosthesis, and infection. A few studies have evaluated nonviable tissues with limited functionality. Tracheal allotransplantations generally lead to necrosis and stenosis. Also, allotransplatations are not suitable for patients with malignant lesions. Because of their complications, the aforementioned methods have been largely abandoned. Use of autologous tissues such as the pericardium, skin, costal cartilage, bowel, esophagus, or bladder involve long and complex procedures and did not provide prospective clinical trials. Based on the pioneering work by Vacanti and colleagues, tissue engineering techniques have become a potential solution [39, 40] (Figure 11.2). The challenge is represented by the creation of cartilaginous tubes covered with respiratory cells [41].

11.5.2 *Ex vivo* Tissue Engineering

Birchall's group in London reported in 2008 the first tissue-engineered tracheal implantation in a patient who suffered from post-tuberculosis end-stage bronchomalacia [42]. *In vitro*, recipient' epithelial cells and chondrocytes derived from recipient's mesenchymal stem cells were cultured and then seeded onto and into (epithelial cells) a decellularized human trachea in a bioreactor system that allowed air/liquid interface culture for epithelial cells. Four months after implantation, the clinical evaluation of the engineered tissue was favorable. The shortage of human tracheal donors is, however, a major limitation of the technique. Furthermore, because of the use of the patient's epithelial cells, this method is not suitable for cancer lesions. Also, the time required (a few months) for obtaining the graft is prohibitive for its widespread use. In 2011, some members of Birchall's group replaced the decellularized trachea with a nanocomposite scaffold to circumvent the donor shortage problem [43]. The nanocomposite scaffold was seeded with the patient's bone-marrow mononuclear cells and matured in a bioreactor. The first patient implanted had recurrent and extensive cancer. The clinical observations demonstrated neovascularization, extracellular remodeling, and wound repair and also homing of stem cells *in vivo* after a follow-up of 5 months. The stem cell mobilization was boosted via growth factor administration. In 2012, the first case of a pediatric patient with tissue-engineered tracheal replacement (with autologous stem cells), with 2 years of follow-up, was published by Elliott *et al.* [44]. In 2010, a novel surgical procedure performed in a patient with extensive post-traumatic stenosis was described by Delaere *et al.* [45]. Briefly, under immunosuppression, a tracheal allograft was wrapped in the forearm fascia of the recipient. The tracheal allograft was revascularized in the fascia, and eventually it was fully lined with donor respiratory epithelium and recipient buccal mucosa. Following the cessation of immunosuppressive therapy at month 4, the tracheal allograft with the intact vasculature was moved to its orthogonal position. A 1-year follow-up gave satisfactory results. However, the need for immunosuppressive therapy and other problems in this method are similar to those of *ex vivo* engineered airways. Also, currently there are some ongoing investigations on the first engineered trachea implantations carried out in Karolinska Institute by Prof. Paolo Macchariani regarding the obtainment of patient consent and representation of the clinical results, which makes it harder to assess the real benefit of these implantations. However, recently a follow-up study of the implantation of an engineered trachea in a pediatric patient has again shown the great promise of this method for replacement, particularly in pediatric patients (Figure 11.3).

11.5.3 *In vivo* Tissue Engineering

In 1997, we proposed to evaluate the use of aortic grafts as matrices for airway transplantation. In successive experimental studies, we demonstrated encouraging results with the use of aortic autografts and fresh or cryopreserved allografts [27, 41, 46–48]. We observed *in vivo* tissue engineering with epithelial and cartilage regeneration from bone-marrow stem cells [49]. This led to the first clinical

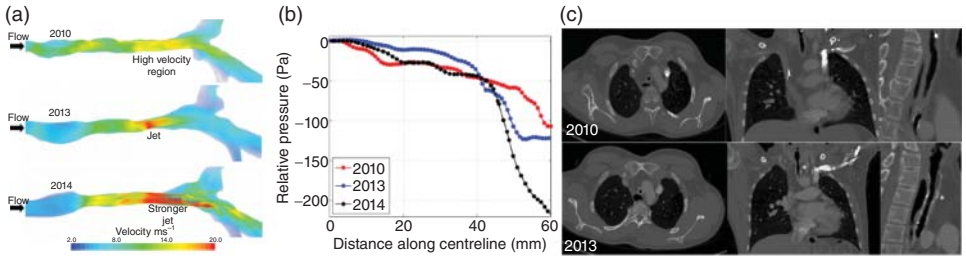

Figure 11.3 (a) Flow velocities in the engineered trachea as a function of time. Even though there is an area of constriction after 4 years, the engineered segment still allows the passage of air. (b) Relative pressure at three time points. (c) Axial, coronal, and sagittal section (CT images from 2010 and 2013, respectively). The transplanted segment (narrowed) can be seen in both the 2010 and 2013 images. (Hamilton [1], http://onlinelibrary.wiley.com/doi/10.1111/ajt.13318/abstract;jsessionid=DE9A9A963477832C139ABB6B3338CB6B.f04t03. Used under CC-BY-4.0 http://creativecommons.org/licenses/by/4.0/.)

applications in humans for extensive tracheal malignant tumors and conservative lung cancer surgery [2, 50, 51]. A prospective clinical trial (NCT01331863) is in progress. In 2014, the International Society of Cell Therapy organized the first meeting with the leading clinicians and biologists involved in airway tissue engineering to debate the issues and propose recommendations for the future [52].

11.5.4 Inherent Needs of Trachea Tissue Engineering

Although it would appear that trachea is only a simple tube that will need to be replaced, and for this reason eminently suited for tissue engineering applications, in actuality trachea is a complex organ composed of different sophisticated structures with several cellular components. Tracheal regeneration requires a cartilaginous tube lined with viable respiratory mucosa (Figure 11.2b).

11.5.4.1 Developing Strategies for Trachea Regeneration

As previously mentioned, an efficient tracheal substitute has strict requirements. Moreover, in pediatric patients, the graft should be able to keep up with somatic growth. For example, an acellular bioartificial airway patch was implanted in a case where tuberculosis damaged the patient's tracheobronchial tree [53]. However, this method cannot be applied to all airway replacement scenarios.

To overcome the different hurdles in airway engineering, several new routes are under investigation. There are other techniques, aside the ones described in the previous sections, that have not reached clinical settings but have been shown to be feasible in animal models. For example, chondrocytes (autologous) in a fibrin/hyaluronan gel have been used in rabbits to regenerate circumferential tracheal cartilage segment defects 0.5 cm wide and cervical defects 1 cm long [23]. The graft lumen was covered with a ciliated epithelium without any inflammatory reactions. The beating frequency of cilia was close to that of a healthy respiratory epithelium. Moreover, after the engineered graft implantation, the recipient rabbits breathed without stridor and there were no wound infection. In addition,

in all animals entering in this preliminary study, no stenosis or granulated tissue formation was observed, nor stenosis or granulation of the regenerated\break trachea.

Recently, the same group has made advances in the reconstruction of the trachea. Lately, a system based on a porous, degradable scaffold (poly(L-lactic-*co*-glycolic acid) (PLGA)) and fibrin/hyaluronic acid (HA) composite hydrogel has been tested for the partial reconstruction of trachea with allogeneic chondrocytes [35].The chondrocytes were obtained from rabbit articular cartilage, and they were encapsulated within fibrin/HA hydrogels. The cell-laden structures were injected into the PLGA scaffold. Following *in vitro* culture (4 weeks), the structures were implanted in eight rabbits with tracheal defects. The engineered tissue was evaluated 6 and 10 weeks after the operation. A functional ciliated epithelium was established as a regenerative event, which contributed to the absence of signs of respiratory distress on the experimental animals. Moreover, there was no stenosis, and the defects recovered well. The advantages of this new approach to trachea regeneration can be seen in comparison to a previous preliminary study, where the first fibrin/HA gel-only system was not able to maintain long-term survival of chondrocytes and did not induce neocartilage formation *in vivo* [35].

For an efficient tracheal reconstruction, there are some requirements, such as the mechanical stability to withstand negative pressure during inhalation; a respiratory epithelium as a first line of defense against infection and also to prevent the formation of intraluminal granulation tissue; and vascularization to ensure graft viability [22]. The presence of a functional epithelium is crucial, and lumeal coverage with ciliated, mucus-secreting respiratory epithelium is indispensable for successful airway tissue regeneration [54]. In addition, an intact epithelial line is crucial to prevent the in-growth of granulomatous tissues and fatal airway obstruction. The cilia are necessary for expelling microorganisms and other nano/microscale materials via the movement of the mucosal fluid. This is why tracheal tissue engineering strategies are based on the re-epithelialization of the tracheal tubes, which are crucial for the whole organ to function.

Different tissue engineering approaches for the replacement of trachea have been used in case of disease or tissue defects [21]. A recent article on the protocols on tracheal replacement models in animals demonstrates the feasibility of different approaches for such surgeries (Figure 11.4). However, the paucity of long-term data regarding the use of these strategies makes it very difficult to conduct a conclusive evaluation of which is the best in terms of clinical practice. In 2005, Omori *et al*. transplanted a long Marlex mesh tube that was covered by a collagen foam [55]. Within 2 months, epithelialization was observed, and further re-epithelialization was detected after 20 months. Walles *et al*. [56] used a decellularized a porcine jejunum patch as a carrier for cells, as the decellularization process leaves the vascular organization relatively intact. This technique has been applied so far to small defects, but its main disadvantages are the inability produce whole tracheal segments (rings) and the time required for the proper decellularization and re-cellularization of the patch [21]. In 2011, Jungebluth reported the use of a CT scan of the patient to reconstruct a resected section of the trachea

with high precision. To enhance the functionality of the synthetic graft, neovascularization has been promoted using different growth factors delivered in the graft and/or administered systemically. But the use of growth factors in cancer patients carries certain risks, and therefore this approach cannot be used for some patients. Besides, omentopexy was also performed as described for other strategies mentioned here.

Autologous tissues are the gold standard in many cases in reconstructive surgery. This is also the case for tracheal reconstruction, as several reports that have used experimental tracheal reconstruction with allotransplants have not demonstrated enough clinical amelioration for human use. As cartilage is an avascular tissue, engineered cartilage does not require large-scale vascular supply, which simplifies its production procedure.

In 2012, Jungebluth *et al*. reported the concept of *in vivo* airway tissue engineering [43]. In their study, the authors tested the concept of a "natural bioreactor," that is, using own body of the animal for engineered tissue maturation. They used decellularized pig tracheas without recellularization before transplantation. These tracheas were treated with mononuclear cells and growth factors during implantation. During the postoperative period, the *in situ* regeneration (taking also advantage from their own bodies' capabilities) was boosted as a result of the administration of bioactive molecules, which promoted the peripheral mobilization of stem cells that differentiated once within the engineered tissue and facilitated the regeneration of the implanted structure. After 2 weeks, the implanted structure had an intact respiratory epithelium with a well-defined cartilaginous component. In addition, because of the mobilization of the progenitor cells into the peripheral circulation in consequence to the administration of the growth factors and bioactive molecules, there was also an upregulation of the antiapoptotic genes.

With the advances in cell biology, and in stem cell biology in particular, new strategies have been developed in airway tissue engineering [57]. Stem cells are cells with high levels of self-renewal and differentiation capacity, and have become an indispensable tool in regenerative medicine [53]. Development of robust methods for obtaining induced pluripotent stem cells (iPSCs) has expanded the possible cell sources for tissue engineering. Recent findings related to the nonimmunogenicity of these stem cells further support their widespread use.

11.5.5 Laryngeal Tissue Engineering

The larynx is a multifunctional organ that has a function in voice production, coughing, swallowing, and breathing [58]. It is the voice box [57] of humans, and so it should be regarded with extra care in airway tissue engineering, as it allows one to speak, to sing, and to communicate. It is therefore essential for human lives.

Each year, about 136 000 individuals worldwide are diagnosed with laryngocarcinoma, some of which will be treated with total laryngectomy [59–62]). In the European Union alone, 11 826 new cases of laryngeal cancers were registered in 2006 [58].

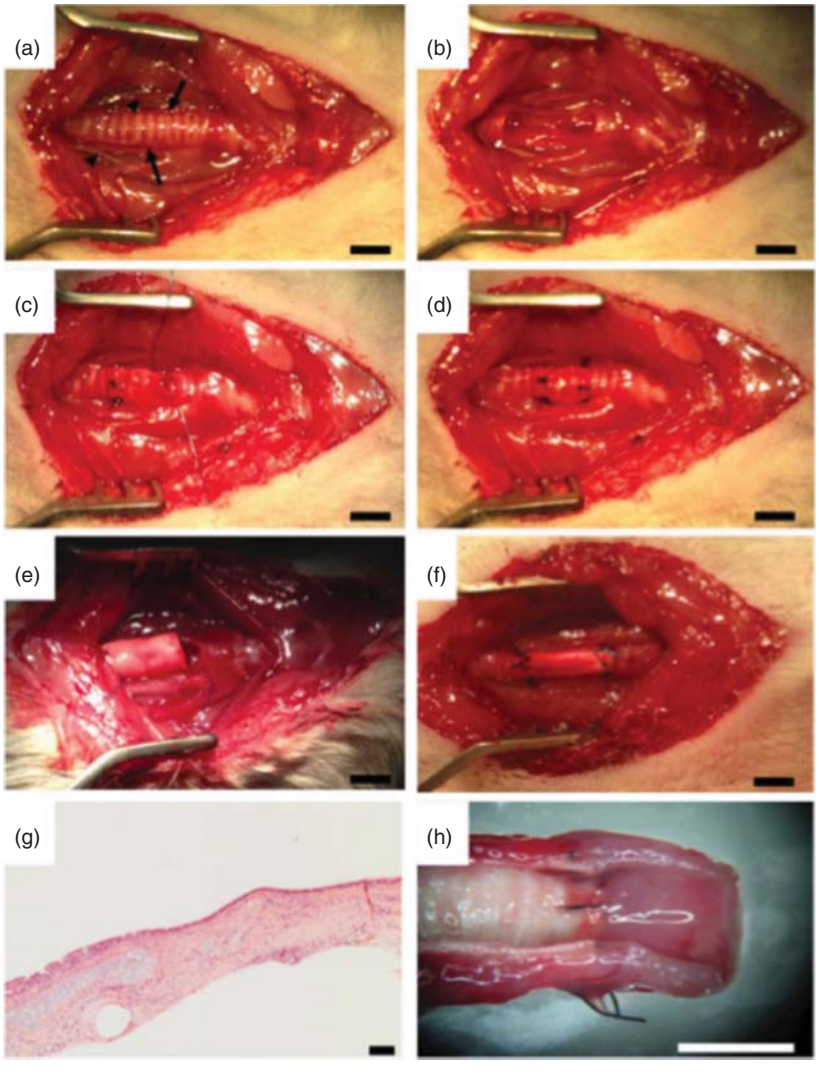

Figure 11.4 Representative examples of the application of tracheal replacements in rats. (a) Exposure of the tracheal segment. (b) Removal of the tracheal segment. (c) Replacement of the resected trachea with an allogeneic decellularized rat trachea. (d) Replacement with a bioengineered trachea. (e) Replacement with a polymer-scaffold-based engineered trachea. (f) Position of the implant prior to wound closure. (g) Histological evaluation of the engineered trachea. (h) Postmortem evaluation of the engineered trachea. (Jungebluth 2014 [63]. Reproduced with permission of Nature Publishing Group.)

Functional laryngeal reconstruction after total laryngectomy remains one of the most challenging problems. The routine treatment after a laryngectomy in clinical medicine is to perform pharynx-to-esophagus anastomosis and tracheostomy to maintain breathing, causing great pain to the patients. So far, the optimal treatment for laryngeal functional reconstruction has been laryngeal

transplantation [64]. Once the larynx is transplanted, patients must face lifelong immunosuppression and live with the risk of tumor recurrence, metastasis, and multiple infections. For this reason, researchers and medical doctors have paid much attention in exploring new avenues of treatment for these clinical cases. Theoretically speaking, the creation of a bioartificial larynx that does not elicit the immunological problems mentioned previously would solve the problem [61].

One of the main requirements of laryngeal function restoration is the presence of functional vocal cords. The muscular actions pertaining to the larynx are extremely complex, which is indirectly evidenced by the density of motor axons in the recurrent laryngeal nerve. The larynx has one of the most sophisticated actions of any muscular-based organ in the human body, as may be appreciated by listening to high-performance singing. This complexity is reflected by the comparatively high density of motor axons in the recurrent laryngeal nerve with respect to the relative size of the innervated muscles. Thus, re-innervation of any implanted larynx substitute is highly important. This can be achieved by the incorporation of neurotrophic factors or engineered nerve grafts [58, 65].

In fact, only a few cases of allotransplants have been documented, and the clinical acceptance of the method is poor because of the difficult procedure [60]. According to Baiguera *et al*. (as covered in detail in a previous section), there are two documented laryngeal transplants in humans to date [64], which seems a very small number compared to the required actual needs. This is already an indication of how difficult it is to achieve a successful transplant and how much there is still to be done. Therapies based on tissue engineering, using either biological or synthetic scaffolds, are therefore desired. Recently, the human larynx has been decellularized and characterized for its anatomical, physiologic, and biomechanical properties. The technology to develop a larynx exists, but further efforts must be made before a translation to the clinic would be possible. The development of a bioartificial cricoarytenoid unit that needs to be implanted in patients who need a total laryngectomy would probably be the most relevant undertaking.

To overcome the immunogenicity problem, a new tissue engineering solution has been developed. The extracellular matrix (ECM) has been successfully used as a biological scaffold derived from decellularized organs and tissues in both preclinical and animal studies. The ECM has low immunogenicity; thus its use decreases the need for immunosuppressants. Moreover, it provides a perfect three-dimensional architecture for tissue regeneration and induces the adherence and proliferation of cells. In order to reconstruct a full, functional bioartificial larynx, one has to take into account the fact that larynx is a complicated organ comprising multiple tissues. It has been proven that cartilage is an organ with low immunity, and therefore larynx immunogens are positioned mainly in the mucosa and muscles. Hou *et al*. in a preliminary study recellularized the decellularized laryngeal muscle with mesenchymal stem cells and constructed a low-immunity, heterogenic laryngeal graft [61].

Currently, there is a clinical trial going on regarding the efficiency and safe use of stem-cell-based engineered airways in the United Kingdom (RegenVOX study). The tested airway replacements are decellularized laryngotracheal grafts seeded with autologous stem cells. Ten patients (inclusion criteria: severe, acquired laryngotracheal stenosis (Myer–Cotton Grade 3 or 4), idiopathic,

traumatic, iatrogenic, or inflammatory caused with no possible conventional treatment method) will have been implanted with such structures during the trial. A 2-year follow-up of the patients is foreseen [57].

Further studies have been conducted with bone-marrow mesenchymal stem cells (BM-MSCs) transplanted with an artificial ECM for regenerating a scarred vocal fold *in vivo*. It was shown that the groups of rats with the transplanted BM-MSCs proliferated and showed low levels of apoptosis or myofibroblast differentiation markers. The group with BM-MSCs showed increased levels of expression of procollagen type III, fibronectin, and TGB-β1. The injections of BM-MSCs in the vocal cords of rat promoted new ECM deposition, as evidenced by the presence of fibronectin, as previously mentioned. Grafting of BM-MSCs laden HA-based artificial ECM-based scaffolds in injured vocal folds is a promising method for vocal cord regeneration [18].

A laryngeal transplant trial in mini pigs has been reported recently. Birchall *et al*. reported an early immunological implication (48 h and 1 week) in larynxes to seven pairs of NIH minipigs, which were homozygous at the major histocompatibility (MHC) locus. This is a robust model of laryngeal transplantation in the NIH minipigs, and they did not show a strong immunological response [18]. New insights seem to be available on the microstructure physiology of the vocal folds. This new knowledge, together with new surgical techniques developed such as the modern phonomicrosurgery, will provide the necessary tools for the regeneration of the vocal folds [66].

Treatments included in the laryngeal tissue engineering field comprise also the use of growth factors, implantable scaffold, or cell therapy used alone or in combination. Gugatschka *et al*. used side population cells, as they are considered to present high content of stem cells, to repair a rat vocal fold injury for a period of 5 weeks. Interestingly, the number of this specific cell population increased over time, and an early vocal fold wound healing was reported, which seems promising in larynx tissue engineering [67].

11.6 Conclusions and Future Directions

One of the problems related to the transplantation and engineering of the trachea is its shared vascularization with the esophagus. In some cases, the replacement of trachea and esophagus might be required at the same time, because of metastasis. In cases of stenosis of the esophagus, or cancer that is untreatable by chemoradiotherapy, the only possible treatment would be resection. In some circumstances, reconstruction of the esophagus is feasible by gastric advancement, colon or jejunal interposition, or a free jejunal flap. Each of these interventions has potential risks and complications especially in fragile patients, and sometimes may increase morbidity and even mortality. In patients in whom no intervention is possible, oral feeding is definitely prohibited for lifetime, being replaced by the need to use a gastrostomy tube for alternative feeding. For this reason, the creation of an engineered esophagus would be a necessary step in several cases of tracheal replacement cases. Tissue engineering would offer the possibility of preparing such systems, which would need to be pliable enough to limit the risk

of rupture of the great vessels, given the proximity of the esophagus to the aorta, and rigid enough to allow the passage of food without the risk of obstruction due to collapse.

As for the artificial and engineered larynx, total laryngectomy is generally associated with pharyngectomy, and hence it is called total pharyngolaryngectomy (except for strictly glottic cancers). Therefore, the remaining pharyngoesophageal (neopharynx) segment is limited in size, which complicates the use of artificial larynx or an engineered larynx, as the food bolus descends down slowly or may remain stuck around the prosthesis. Technical manipulations like using a vascularized flap can, however, increase the space of the pharynx under certain conditions. The possibility of producing a complete prosthesis or engineered tissue (a pharyngolaryngeal implant) reconstructing the larynx while maintaining a wide pharynx is very interesting, yet it remains under study. A more biomimetic artificial larynx, for its tracheal part, where titanium and stem cells will be present, is an objective of our team, and seems a very promising line of research. Valves allowing breathing and swallowing in the upper airway are also being studied to optimize the function of this prosthesis. It is not innate for patients rehabilitated by an artificial larynx to block their breathing during swallowing. Thus, a major rehabilitation is necessary, which is sometimes impossible for certain patients. An automated control system for opening/closing valves (pacemaker) is being considered to avoid this, sometimes tedious, rehabilitation.

It is therefore in hands of researchers and clinicians to dedicate time and effort to these questions in order to enable the clinical transfer of the technologies described above and their timely application to afflicted patient populations. However, given the recent clinical data, one can be hopeful about the use of the currently available techniques and strategies for decreasing the suffering of so many patients that each day face the difficulties of loss of a part of the respiratory system.

Declaration/Conflict of Interest

MBE is an employee of Protip Medical. NEV is an employee of Protip Medical and holds stock options of the Company. CD is a stakeholder of Protip Medical.

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12

Pulmonary Tissue Engineering

Patrick A. Link and Rebecca L. Heise

12.1 Introduction

Because of the great complexity of the lung architecture and the presence of over 40 specialized cell types, tissue engineering and regenerative medicine approaches of the lungs have lagged behind other organ systems. Recent advances in technologies such as decelluarization of native tissue, three-dimensional printing, and microfluidics hold significant promise for strategies to tissue-engineer the lung. Furthermore, cellular therapies have been introduced in clinical trials, as the need for reparative strategies for lung diseases grows. This chapter assesses the clinical need for pulmonary tissue engineering, reviews the complexities of the structure–function relationship in various components of the airway and lung parenchyma as well as the state of the art in scaffolds for lung tissue engineering, and focuses on cutting-edge science in fabrication and cellular therapies.

12.2 Clinical Need for Pulmonary Tissue Engineering

Heart diseases (including cerebrovascular disease), cancers, and lung diseases have been the three leading causes of death for decades in the United States. From 1993 to 2012, deaths due to heart diseases have decreased by 15%; lung diseases have increased significantly over the same period, more than doubling the cancer growth rate at 20% versus 9%, respectively (Table 12.1) [1, 2]. Combined noninfectious lung diseases account for 1 out of every 9 deaths in 188 countries according to reported data [3]. Tracheal, bronchiolar, and lung cancers have increased by 56.5% during the period 1990–2013, and chronic respiratory disorders have increased by 21.9% over the same period [3]. A clinical desperation exists to find a cure for these chronic diseases. Lung diseases can be broken down into three categories: restrictive, obstructive, and tumors. Tumors obtain individual classification because they can restrict, obstruct, both obstruct and restrict, or neither. Restrictive diseases impair the inhalation part of the respiratory cycle, making it nearly impossible for afflicted persons to inhale a full breath [4]. Obstructive pulmonary diseases affect the exhalation of air from lungs, leaving a markedly

Tissue Engineering for Artificial Organs: Regenerative Medicine, Smart Diagnostics and Personalized Medicine, First Edition. Edited by Anwarul Hasan.

Table 12.1 Comparison of deaths from 1993 to 2012, due to the top three causes of death in the United States [1, 2].

Cause of death	1993 Number of deaths (Est.)	1993 Percent of total	2012 Number of deaths	2012 Percent of total	Percent growth (2012–1993)
All causes	2 268 000	100	2 543 279	100	11
Cardiovascular diseases[a)]	905 420	40	787 431	31	−15
Lung[b)]	182 820	8	228 414	9	20
Neoplasms	530 870	23	582 623	23	9
All other causes	648 890	29	944 811	27	31

a) Includes cerebrovascular and atherosclerosis deaths.
b) Includes influenza, pneumonia, other acute lower, chronic lower, pneumoconiosis, and other deaths.
Source: Vital Statistics of the United States, National Center for Health Statistics (NCHS).

increased volume of air still in the lungs after a complete exhalation [4]. Categorical placement of restrictive or obstructive pulmonary disorders occurs through pulmonary functions tests.

12.2.1 Restrictive Lung Disease

Restrictive lung diseases include, among others, interstitial lung disease (ILD), sarcoidosis, obesity scoliosis, and neuromuscular disorders such as amyotrophic lateral sclerosis. These diseases affect the inhalation portion of the respiratory cycle. Sufferers cannot complete a full inhalation because of the stiffness in the lungs or muscular weakness, preventing full expansion of the chest. Though there is no cure for these disorders and the associated lung problems, treatments are available to relieve some of the underlying symptoms.

12.2.1.1 Interstitial Lung Disease

Interstitial lung disease (ILD) occurs when an abnormal healing response turns lung repair into lung scarring, over time impairing a person's ability to breathe. ILD affects the interstitium, a network of supporting scaffolds for the alveoli. ILD results in thickening and fibrosis of the interstitium, impairing alveolar inflation, and increasing diffusion distances for gas exchange, thereby decreasing respiratory efficiency. Of the 200 conditions contributing to the prevalence of ILD, occupational hazards, sarcoidosis, drug toxicity, and idiopathic pulmonary fibrosis are their most common origin [5, 6]. Once the repair mechanisms fail, the scarring continues to advance in the vast majority of patients, although there have been rare cases in which the disease resolved without treatment. There are medications that slow the progression of ILD, but frequently the people affected cannot escape the environment that initiated the cascade of events, resulting in the chronic condition.

Coal, silica, asbestos, and beryllium miners, shipbuilders, mill workers, brake workers, quarry workers, stoneworkers, glassworkers, insulators, pipe fitters, and

people in similar occupations work in high-risk environments. Long-term exposure to these trades can lead to simple pneumoconiosis, a preliminary, symptomless disorder beginning with a macrophage engulfing one of these agents [5]. Occasionally, the macrophage cannot eliminate the agent, and a macule is formed to protect the surrounding tissue, forming a granuloma [5]. Over time, the granuloma becomes fibrotic and begins to impair the respiratory function in that area [5]. Granulomas have been formed from all causes of ILD, but are most common in occupational inhalation and sarcoidosis.

In sarcoidosis, the granulomas can distribute throughout the entire body, impacting any system or organ. Sarcoidosis causes the formation of fibrotic granulomas, commonly beginning in lung tissue as pulmonary sarcoidosis [7, 8]. In pulmonary sarcoidosis, an inappropriate inflammatory response creates patches of impacted lung tissue [7]. About 50% of persons affected by sarcoidosis heal spontaneously, 30% suffer permanent lung damage, and in a fraction of people sarcoidosis becomes a chronic condition. Although rare, death from sarcoidosis usually arises from lung, heart, or brain complications. Restrictive pulmonary disorders are less common than obstructive diseases, but are no less threatening.

12.2.2 Obstructive Pulmonary Disorders

12.2.2.1 Asthma

Obstructive lung diseases include asthma and chronic obstructive pulmonary disease (COPD). Asthma is a chronic lung disease characterized by chronically inflamed bronchi, which are extremely sensitive to foreign materials. Typical foreign materials that can exacerbate this condition are smoke, pollen, cold, pet dander, and occupational inhalation hazards such as asbestos, coal, and so on. Asthma can be genetically inherited, an overreaction to allergies, a side effect of respiratory viruses, or environmental in origin, but can be easily managed by prescribed inhalant medications. Occasionally, victims of an asthma attack require further treatment in an emergency care setting with antihistamines and inhaled or oral corticosteroids, but most asthmatics lead long and otherwise healthy lives.

12.2.2.2 COPD

The World Health Organization predicts COPD to be the fourth leading cause of death in the world by 2030 [9], while in the United States COPD is currently the third leading cause of death [10]. COPD took the lives of 3 million people worldwide in 2004 [9] and accounted for 1 of every 20 deaths in the world in 2005 [9] including 120 000 Americans in 2012 [10]. Sixty-four million people were affected with COPD worldwide in 2004 [9] and 12 million in the United States in 2012 [10]. As with other lung diseases, COPD diagnoses are rising, but it can be prevented.

In persons affected with COPD, bronchioles thicken and lose elasticity, and alveolar walls separating individual air sacs are demolished, decreasing the surface area available for gas exchange (Figure 12.1) [4, 9]. As a protective measure, the lungs then produce more mucus [9], which further complicates these attributes, leaving people suffering from COPD breathless from short periods of

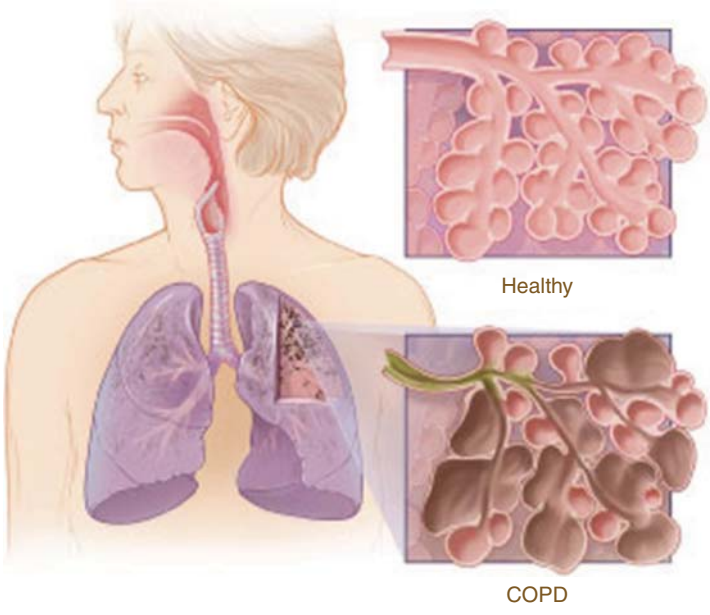

Figure 12.1 Comparison of normal alveolar sacs and COPD-diseased alveoli. Normal alveoli consist of uniform bronchioles, branching into consistently sized alveoli. Diseased sacs show an abnormal bronchiole diameter, increased production of mucus, and the alveolar wall deterioration. (Reproduced with permission from [11].)

activity. COPD is a continuously developing disease hallmarked by inflammation of bronchioles (chronic bronchitis) and the destruction of alveoli (emphysema), impairing the gas exchange ability of the lungs. COPD is caused primarily by smoking, or being exposed to smoke, but is also attributed to indoor and outdoor air pollution, occupational inhalation hazards (fumes, vapors, chemicals, and dust), and frequent recurring childhood respiratory infections [9]. A small contingent of people suffering from emphysema is afflicted by a genetic disease known as alpha-1-antitrypsin (AAT) deficiency [12]. A systemic deficiency in the production of AAT leaves the lungs more vulnerable to damage by enzymes, making AAT-deficient persons more susceptible to COPD. Unlike other causes of COPD, AAT deficiency has been successfully treated using donor AAT infusions as a replacement therapy [12].

12.2.3 Lung Cancer

Lung cancer is the leading cause of cancer deaths in both men and women worldwide. Tumors in the lungs are broken down into two major classifications: small-cell lung cancer and non-small-cell lung cancer, with the latter group again divided into the more commonly known subgroups, namely squamous cell carcinoma, adenocarcinoma, and large-cell carcinomas. These groups and subgroups can exist independently or in mixed colonies. Non-small-cell cancers account for ~85% of lung cancers, with adenocarcinomas 40%, squamous cell

carcinomas 30%, and large cell carcinomas 10–15%, leaving only 15% of lung cancers to the other major group, namely small-cell lung cancer [13].

Small-cell lung cancer is the most aggressive form of lung cancer, metastasizing rapidly to affect the brain, liver, and bones. In 75% of patients, small-cell cancer diagnosis often occurs after metastasis, leading to very poor prognosis [13]. However, if this type is found in early stages, it does respond well to chemotherapy. The lethality of small-cell lung cancer may play a predominant role in the small percentage of patients with lung cancer when compared to the non-small-cell lung cancer types.

Non-small-cell lung cancers make up the majority of cancer patients. Adenocarcinomas are the most common type of lung cancer in both men and women, smokers and nonsmokers alike, and squamous cell carcinomas are the easiest to detect. Adenocarcinomas are commonly found in mixed populations and cause no symptoms initially, but account for 40% of all lung cancers. Large-cell carcinomas contain distinctive large cells found along the outer edges of the lungs. Large-cell carcinomas and adenocarcinomas traditionally spread to nearby lymph nodes and distant organs. Squamous cell carcinomas often show up in mucus samples and often do not proliferate rapidly, making them the easiest to treat in addition to being the easiest to detect. Lung cancer has a very poor prognosis compared to other cancers because of the massive supply of vasculature, and therefore they maintain the ability metastasize easily.

12.2.4 Treatment Options – An Open Door for Tissue Engineering and Regenerative Medicine

Because of the vast range of disease severity, treatment options also vary greatly. Restrictive diseases due to obesity and general muscle weakness can potentially be cured through exercise regimes, while lung disorders due to other underlying diseases only in severe cases is lung transplantation ever an option [4]. Pulmonary rehabilitation can be used to improve or maintain spirometry (a clinical test to measure lung efficiency) stamina while also teaching patients methods to reduce energy expenditures [12]. Symptomatic treatment for most lung diseases is available through immunosuppressants specific to each disease or corticosteroids [4]. Another technique, lung volume reduction (LVR) surgery, involves the removal of severely diseased portions of lungs, with the goal of improving the respiratory ability of the remaining healthier lung [12]. LVR is commonly used to treat lung cancers as well as some other severe diseases. LVR surgery decreases hyperinflation, but it is associated with high morbidity and operative mortality [14] and is therefore a very controversial therapy for nonterminal patients. Chemotherapy is always an option for cancers, and always recommended in advanced stages of all cancers, but because small-cell cancers are known to be aggressive, they are commonly treated with volume reduction surgery and chemotherapy immediately following diagnosis. Lastly, lung transplants have not had much success, and are therefore reserved for people with only a couple of years of life expectancy remaining.

Lung transplants are regularly used in some end-stage diseases. The primary indication for patients requiring lung transplantation is idiopathic pulmonary

fibrosis. These transplantation surgeries are commonly performed; however, patient survival rates for lung transplant are the lowest among transplant surgeries, with just over 50% of lung transplant patients surviving past 5 years [15]. The shortage of donor lungs and the complexity of lung disease have indicated the need and role for either a whole tissue-engineered lung or regenerative medicine treatment approaches. In order to engineer functional strategies to repair or replace lung tissue, one must first have an understanding of the complexities of the tissue.

12.3 Structure–Function Relationship in the Conducting Airways and the Lung

In order to tissue-engineer or regenerate the lung, the key aspects of the respiratory structure–function relationship must be understood. The lung structure may be broadly classified into two main categories: (i) the conducting airways and (ii) the parenchyma. The conducting airways deliver air into the parenchyma where gas exchange occurs. These airways move air beginning from the nasal cavity, into the pharynx and larynx, into the trachea, and finally into the bronchi. The bronchi branch into approximately 60 000 terminal bronchioles to distribute the air into the lung parenchyma. The lung parenchyma consists of the respiratory bronchioles, alveolar ducts, and alveoli. This chapter's focus for tissue engineering and regeneration will begin at the trachea. Aspects of the structure–function relationship in the airways (examining the trachea and bronchi) and the parenchyma (focusing on the bronchioles and alveoli) will be examined. The mechanical behavior of all parts of the lung relies on the mechanical properties of the tissue coupled with the mechanics of the ribcage, diaphragm, and abdomen. An understanding of the basic lung anatomy is necessary for understanding the approaches utilized in pulmonary tissue engineering and regenerative medicine. Beyond the conducting airways of the nose and mouth, and continuing below the vocal cords, air moves into the trachea, where it is then diverted into the right and left main stem bronchi leading to each lung. The trachea is a tubular passageway for air about 5 in. long and 1 in. diameter. It is located anterior to the esophagus and extends from the base of the larynx to the level of T5 (thoracic vertebrae 5), where it divides into the primary bronchi. The wall of the trachea is composed of four layers: (i) mucosa, (ii) submucosa, (iii) cartilage, and (iv) adventitia [16]. The tracheal mucosa consists of a ciliated pseudo-stratified epithelium with goblet cells (commonly called the respiratory epithelium). Beneath this is a submucosa of connective tissue and mucous glands. The structural layer of the trachea has 15–20 C-shaped rings of hyaline cartilage that are arranged horizontally and stacked atop one another. The open part of the "C" faces the esophagus. It is bridged by elastic connective tissue and smooth muscle fibers called trachealis. This arrangement allows the distention of the esophagus during swallowing. The cartilage rings are composed of collagen and glycosaminoglycans. The outermost layer of the trachea is the adventitia, which is a connective tissue coating that secures the trachea within the neck anatomy.

The right lung is comprised of three lobes, so air channels from the right main stem bronchi into the three secondary bronchi. The left lung is comprised of two lobes, because of the occupation of space by the heart, so from the left main stem bronchi air follows one of the two secondary bronchi. From all the secondary bronchi, air is moved into tertiary branches, which become smaller and smaller until reaching the terminal bronchioles, which are less than 1 mm in diameter. By definition, bronchi have cartilage support rings similar to the trachea but bronchioles do not. On average, the bronchi divide 23 times to form the tracheobronchial tree. The first 16 divisions are typically considered the conducting airways. The 17 to19th divisions are lined with alveoli, and the 19+ generations will have respiratory bronchioles moving to terminal bronchioles. From the terminal bronchioles air is finally moved into the alveoli, resulting in 23 airway separations between trachea and alveoli [17]. Once air arrives at the alveoli, it is finally able to take part in aerobic respiration.

The diffusion distance from the alveolar cells to the blood in the circulatory system is generally less than 1 μm and can be as small 0.1 μm, providing an optimal site for fast and efficient gas exchange. To provide an adequate supply of oxygen to the body, the surface area of the alveoli must be large, with estimates putting it between 70 and 160 m^2 [18]. The 150 million alveoli in each lung, containing even more cells, are very delicate, specialized, and focused, requiring other cell types to provide protective measures to the delicate alveoli. The nose as the primary entry point for air protects the delicate alveolar cells by humidifying and warming the air, trapping contaminants with hairs and mucus, and using ciliated cells to move particulate to the pharynx. The nose removes contaminants of ~10 μm and larger. The pharynx utilizes lamina propria, a basement areolar tissue that secretes mucus, to support ciliated columnar cells and mucous cells. This trio of defense, namely lamina propria, ciliated columnar cells, and mucous cells, further protects the alveolar cells. The trio traps particulates of 5–10 μm in diameter, and the mucous escalator sweeps the captured objects toward the stomach. The lower respiratory system, through the bronchioles, also use the defensive trio to capture potential pathogens of 1–5 μm in diameter, with the minor difference that the captured particles are swept up the respiratory system to be coughed out or swallowed into the stomach. The diffusion distance would be severely compromised if mucus was produced in the alveoli, so in direct protection of the alveoli are roaming alveolar macrophages combating and phagocytizing all remaining pathogens [18].

The complexity of the lungs is not just restricted to the maze-like anatomical pathways but also extends to the cellular level. These cell types are depicted in Figure 12.2. In the trachea–bronchiolar passage, ciliated cells, basal cells, mucous goblet cells, serous cells, non-ciliated Club cells (formerly Clara cells), small mucous granule cells, brush cells, and neuroendocrine cells line the walls [20]. As before, the ciliated cells brush contaminants up to the esophagus to be swallowed or coughed out. Basal cells form a single cell layer surrounding the airways. Goblet cells are columnar cells responsible for secreting mucin, the primary component of mucus. Serous cells secrete serous fluid, which is responsible for lubricating and reducing friction between microvilli and lung pleura. Club cells take over the function of Goblet cells over time and become

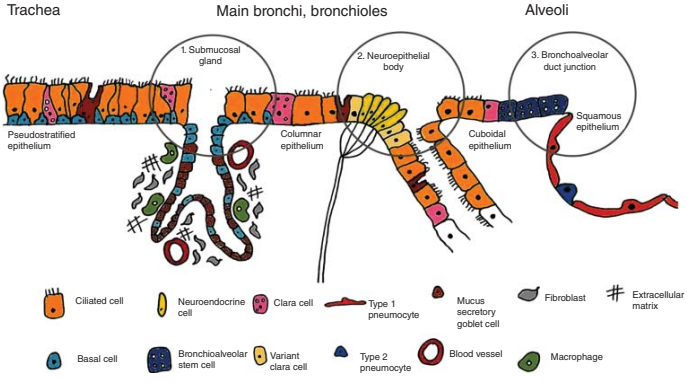

Figure 12.2 Schematic showing the location of the different cell types making up the epithelial surfaces of the airways. The stem cell niches are highlighted within circles. The mouse airway is shown, as this is where the evidence for stem cell populations originates. (Reprinted with permission from [19].)

responsible for removing pathogens from the air. Small mucous granule cells have a combination job of containing cilia and also producing mucous, and these cells are similar to a cross of goblet and basal cells. Brush cells are ciliated cells pushing debris back up the passageways, and neuroendocrine cells produce a range of paracrine hormones and proteins, creating a local response. The alveoli alone contain three functionally specific cells: type I pneumocytes, the mobile macrophages, and type II pneumocytes. Type I pneumocytes are responsible for gas exchange. The macrophages provide protection, and the type II pneumocytes produce a surfactant, a substance consisting of phospholipids and proteins that reduces the surface tension of water, allowing each inhalation to reopen the alveolar sacs. Type II pneumocytes are also the repair cells of the alveolus. Type II pneumocytes are capable of proliferation and differentiation into type I pneumocytes following injury. All of these cell types are necessary for proper lung functioning; however, studies are being performed to determine the best cell source for differentiation into this multitude of cell types. The cell source for tissue engineering is addressed later. First, we will address the scaffold on which the cells grow in a traditional engineering strategy of cell + scaffold ± exogenous growth factors to obtain the engineered tissue replacement.

12.4 Tissue Engineering and Regenerative Medicine: Approaches for the Lung

12.4.1 Scaffold Approaches for Lung Tissue Engineering

12.4.1.1 Polymeric Scaffold Approaches

The majority of synthetic scaffold approaches for pulmonary tissue engineering are centered on the larger pulmonary structures of the trachea and the main stem bronchi. A few studies have examined polymeric hydrogel approaches to grow cellular structures resembling the alveoli. This section will address how synthetic scaffolds have been utilized for the larger airway structures and the lung parenchyma.

12.4.1.1.1 Tissue-Engineered Trachea

Because of the anatomy of the trachea, consisting of cartilage, smooth muscle, connective tissue, and epithelial cells, a variety of approaches to tissue-engineer the trachea have been drawn from other organs. There are several requirements for scaffold design to tissue-engineer the trachea [21].

General requirements for any tissue engineering scaffold are as follows:

- Nonimmunogenic
- Nontoxic
- Nontumorigenic
- Allow cell adhesion, migration, proliferation, and differentiation.

Trachea-specific criteria are as follows:

- Airtight and liquid tight seals upon implantation
- Mechanical stability to both lateral and longitudinal forces

- Support airway patency
- Support adequate respiratory function.

In 2008, the first transplantation of a tissue-engineered trachea in a human was done to replace an end-stage left main bronchus with malacia in a 30-year-old woman. The implanted trachea was engineered from a decellularzed cadaveric trachea seeded with autologous epithelial cells and mesenchymal stem cell (MSC)-derived chondrocytes [22]. Five years post implantation, the tissue-engineered trachea remained viable and patent, with stenting needed in the native trachea near the implant [23]. This initial success advanced the field of clinical applications of tissue-engineered airways. While some researchers are still examining cadaveric scaffold for airway replacement, many additional approaches have utilized synthetic polymeric scaffolding materials.

Polymeric scaffolding approaches for tissue-engineered trachea seek to provide the structural and organizational cues for proper cellular behavior. Synthetic scaffolds may also be manufactured to the exact shape and size suitable for the patient. Typically, synthetic scaffolds have been classified as non-biodegradable and biodegradable. Tracheal scaffolds have been formulated from the following materials: Marlex mesh (Chevron Phillips Chemical Company LP, TX, USA), polyethylene oxide/polypropylene oxide copolymer (Pluronic F-127, Invitrogen, Ltd, Paisley, UK), polyester urethane, polyethylene glycol-based hydrogel, polyhydroxy acids, poly-ε-caprolactone, polypropylene mesh, poly(lactic-*co*-glycolic acid), gelatin sponge, and alginate gel [24]. Several of these approaches are depicted in Figure 12.3. Polypropylene meshes with collagen and/or poly(L-lactic-acid-*co*-ε-caprolactone) coating have been used in an animal model for the replacement of the left main stem bronchi [27, 28]. Greater success was achieved utilizing the poly(L-lactic-acid-*co*-ε-caprolactone) coating for epithelial regeneration. Other success has been reported in a rabbit model where tissue-engineered tracheas were formed from articular cartilage matrix and chondrocytes [29]. New advances in three-dimensional (3D) printing polymers have been harnessed for applications to the airway. A half-pipe polycaprolactone 3D printed trachea seeded with mesenchymal stromal cells

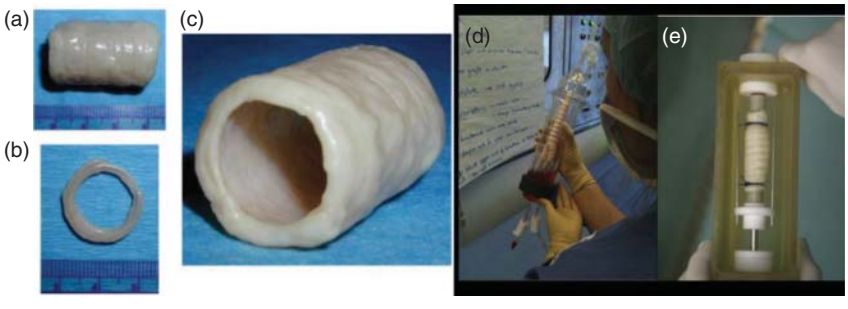

Figure 12.3 PGA mesh seeded with autologous engineered trachea chondrocytes after 10 weeks *in vitro* culture from multiple views (a–c). (Kojima 2014 [25]. Reproduced with permission of John Wiley & Sons.). Nanofiber trachea prepared for clinical use (d) and biologic tracheal scaffold (e). (Weiss 2014 [26]. Reproduced with permission of Elsevier.)

in a fibrin matrix was implanted into a rabbit model with initial success of regenerated epithelium [30]. Tissue engineering approaches using polymers for the large airways have gained momentum due to the ease of fabrication and implantation. However, smaller airways, termed bronchioles, have been attempted for *in vitro* understanding of small airway diseases such as asthma, with the eventual goal of a functional tissue-engineered lung. For the bronchiole, a type I collagen gel seeded with fibroblasts, epithelial cells, and airway smooth muscle cells was engineered utilizing a pulsatile flow bioreactor [31]. Similarly, another study used tubular bronchiole structures engineered using airway smooth muscle tubular structure collagen pulsatile flow [32]. The engineering of these smaller airway structures highlights the need for advances in bioreactor technology to go hand in hand with the advances in polymeric biomaterials.

While purely synthetic scaffolds provide structural support for cellular growth, the biologic compatibility may be increased through the addition of biologic materials engineered into or coated onto synthetic polymers. This is most commonly accomplished using materials found within the extracellular matrix (ECM), such as collagen or gelatin, in combination with growth factors to promote cellular in-growth and differentiation. Approaches that utilize natural scaffolds alone are more commonly used as support devices for tracheal stability. One approach uses gelatin and basic fibroblast growth factor (b-FGF) to promote matrix production (collagen type II and glycosaminoglycan). The growth-enhanced trachea was more resistant to collapse, suggesting that slowly released b-FGF might be useful in patients with severe tracheomalacia (airway collapse) [33]. Future use of naturally derived scaffold materials will be limited by cost and reproducibility such that batch-to-batch consistency of the naturally derived material is maintained.

12.4.1.1.2 Scaffolds for Lung Parenchyma Engineering

Following the criteria for engineering the trachea and larger airways, another specific set of criteria is necessary for engineering the lung parenchyma. The parenchyma-specific criteria are as follows:

- Maintain airtight and liquid tight alveolar-capillary barrier
- Maintain elastic recoil
- Establish differentiated epithelial cells capable of ion exchange and fluid transport
- Support gas exchange with proper capillary-alveolar distribution.

Because of the above criteria and the precise architectural needs of the alveoli to maintain a large surface area, very few *de novo* scaffolds have been synthesized to engineer the lung parenchyma. The majority of approaches involve growing organoids or spheroids of various naturally derived polymers. Embryonic lung progenitors have been seeded into foams made from Matrigel, poly-lactic-*co*-glycolic acid (PLGA), and poly-L-lactic-acid (PLLA), and have differentiated into cells expressing alveolar markers under proper growth factor supplementation [34]. An alveoli-like gelatin/microbubble scaffold has been used to support lung stem/progenitor cell proliferation and differentiation as well as angiogenesis [35]. Progenitor cells have also been seeded onto scaffolds

engineered from polyglycolic acid (PGA) or Pluronic F-127 (PF-127), which differentiated into cells with specific markers for Club cells, pneumocytes, and respiratory epithelium and organized into identifiable pulmonary structures (including those similar to alveoli and terminal bronchi) [36]. However, despite these successes, tissue engineering alveolar structures faces the hurdles of vascularization and connectivity to the airway. The most promising potential for these approaches is to utilize these biomaterial scaffold approaches as progenitor cell delivery systems to the underdeveloped lung. Additionally, these engineered alveolar constructs provide a valuable tool for studying the epithelial and mesenchymal interaction in the remodeling of alveolus or for screening compounds meant to aid in alveolar repair and regeneration.

12.4.1.1.3 A Note about Vascularization

Vascularization is often the limiting factor in tissue-engineering complex 3D tissues for lung replacement. Pulmonary epithelium has high oxygen needs, so the design of polymeric structures must allow for a highly vascularized system. Furthermore, blood supply is crucial to maintain functionality. The lung's main function is to allow gas exchange in the blood to occur. This functionality, while is seemingly obvious, is nontrivial when engineering 3D structures for replacement of lung structures. Several strategies have been employed to improve the oxygenation of cells grown *in vitro*, including the addition of perfluorocarbons and the bubbling of oxygen through the culture media. *In vivo* approaches toward vascularizing tissue-engineered scaffolds typically utilize omentum flaps, wherein the scaffold is implanted subcutaneously for several days or up to weeks. The body responds to the scaffold, causing cellular in-growth and vascularization.

In addition to the flows from the vasculature, the mechanical environment of the 3D construct must be taken into account. Furthermore, in the lung, a very complex mechanical environment exists as the lung is constantly distended and relaxed. The polymeric structures used to engineer the lung must have large elastic recoil in order to withstand this repeated deformation. Because of the complex mechanical environment and vasculature of the lung, decellularized organs may provide the best natural scaffolding material with built-in conduits for vascularization.

12.4.1.2 Decellularized Tissue Approach for Lung Tissue Engineering

Because of the complexities of engineering proper scaffolds for repairing both the airways and lung parenchyma, several groups have begun to examine decellularized cadaver lungs as a scaffold material. Research on engineering all aspects of the respiratory system *ex vivo* has been conducted, including the trachea, larynx, and the lung itself [37]. Concurrent research in both recellularization of decellularized tissues and immunomodulation has progressed together over the last 10 years [37]. Currently, extensive research is being conducted in creating bioactive lung scaffolding that can be used to increase the acceptance of cells or supplement by the lungs. Lung tissue decellularization is the primary means to create 3D lung sections that can be seeded to yield healthy, viable lung tissue. Potentially, a complete nondiseased set of lungs can be perfectly matched to any patient

using progenitor cells from those patients attached to a completely decellularized matrix, decreasing rejection by the host.

Lung regeneration utilizes decellularized lungs to be re-seeded with healthy cells so that complete transplantation can be performed. The 3D structure of lungs is essential to proper lung performance, so lung regeneration is focused on reusing the scaffolding matrix of a healthy set of donor lungs and re-seeding healthy progenitor and stem cells. This decellularized lung approach holds promise of success in engineering a structurally functional and biocompatible organ for the patient. Additionally, by decellularizing already intact lungs with low concentrations of the detergent sodium dodecyl sulfate (SDS; 0.01%), the vascular basement membrane remains unbroken [38]. However, as decellularization requires the use of harsh detergents and chemicals to ensure complete removal of native cells, higher concentrations of SDS compromise the alveolar septa [38], and it is currently unknown whether other factors are removed in the process that are required for complete refunctionalization in a new host system. Histological examination of low-SDS-treated matrixes shows no remaining nuclei or intracellular elements [38]. Murine Nkx2-1-(also known as transcription factor 1, tf1)-GFP tagged endothelial stem cells and induced pluripotent stem cells (iPSCs) have been shown to differentiate into cells expressing the characteristics of both alveolar type I and II epithelial cells. These cell types have been shown to repopulate decellularized whole-lung scaffolds, but their characterization and functional tests are not complete [37]. Cadaver rat lungs were seeded with rat fetal lung cells on the lung side, containing epithelial, endothelial, and interstitial lineages and human umbilical vein endothelial cells in the vasculature. These re-seeded lungs displayed near-physiological gas exchange, morphology, architecture, and cell distribution [38]. These lungs were transplanted into rats, which survived for 6 h without ventilation. Currently, whole-lung engineering from existing donor lungs appears to be the standard approach for lung engineering for future transplantation.

Another approach has been to decellularize rat lungs and re-seed them with human bone-marrow-derived mesenchymal stem cells (hBM-MSCs) or human adipose-tissue-derived mesenchymal stem cells (hAT-MSCs). hAT-MSCs appeared to attach to the distal airways and differentiate into non-ciliated Club (formerly Clara) cell types, expressing the Clara cell secretory protein (CCSP), or can differentiate into type II-like cells but do not express cytokeratin-5. hBM-MSCs, originally expressing CCSP, attach to proximal airways or alveolar regions, differentiating into pro-SPC (surfactant protein-C) and cytokeratin-5 expressing type II-like pneumocytes, while losing the ability to express CCSP [39].

Another approach utilizes fibroblast-derived iPSCs to recellularize decellularized rodent lungs. Human iPSC-derived endothelial cells were seeded into the scaffolding matrix via the pulmonary artery. Then iPSCs were differentiated *in vitro* into lung epithelial progenitor cells. These lung epithelial progenitors were delivered to the airways. After transplantation, histology showed red blood cell profusion of the alveolar capillary network [40].

The overall process to these key studies is to utilize a whole-lung engineering approach. Whole-lung engineering begins with the removal of healthy lungs

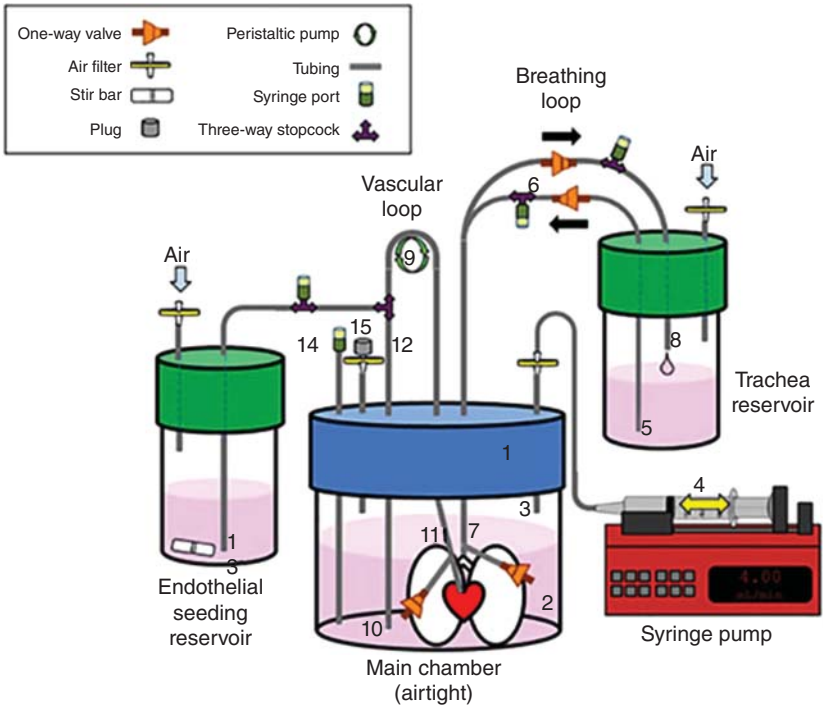

Figure 12.4 Schematic of a bioreactor used to decellularize and recellularize whole-lung regeneration. Lungs including the trachea, and sometimes including the heart, are placed in the main chamber. (1) The trachea is attached to tubes leading to the breathing loop (6) leading to the trachea reservoir containing decellularization/recellularization chemicals. The heart (if included) is attached to tubes leading to the vascular loop (9), which also contains de-cell/re-cell chemicals to be pumped through the vasculature. Once decellularization is complete, a rinsing solution is used to remove all the harsh chemicals used. Finally, the reservoirs and tubes are replaced with reservoirs containing stem/progenitor cells and media, and the solutions are pumped through the scaffolding once again. (Reproduced with permission from [41].)

from a donor. Once removed, the trachea is attached to a pump providing decellularization chemicals (SDS or CHAPS) in a slow, regular rhythm, similar to inhalation–exhalation cycles (Figure 12.4). The right and left pulmonary arteries (leading from the heart) are attached to another pump system, also supplying the chemicals needed for decellularization, but this system continuously pumps into the lungs, and tubes attached to the pulmonary veins (leading to the heart) are left to drain into another container. Chemicals used involve multiple detergents and salts, as well as rinsing. Decellularization is complete once the lungs have been stained for cells and immune factors. Once complete, the remaining lung scaffolding that is still intact is placed in a bioreactor and re-seeded with progenitor cells. Using decellularized organs, desired progenitor and stem cells can be acquired from the host or a biocompatible donor and used to re-seed and re-grow complete and intact lung systems.

12.4.1.3 Three-Dimensional Printing in Scaffold-Based Lung Tissue Engineering

Biomimetic lung scaffolding is in its infancy though, printing capabilities are advancing rapidly. The precise topography and composition necessary for scaffold materials to promote cellular growth and differentiation for lung progenitors is continually being updated through research. Three-dimensional printing provides a new technological frontier in customizing tissue-engineered replacements. Three-dimensional printing utilizes stereolithography techniques originally developed for advanced manufacturing and uses a "bioink" as the print medium. The bioink is typically composed of a biocompatible polymeric material. The bioink may be composed of cells, or cells may be seeded onto the printed structure following its creation. The benefits of 3D printing include the ability to build scaffolds of complex architectures, which addresses current limitations in traditional scaffold engineering. Additionally, 3D printing and biofabrication allows personalization of the scaffold. Magnetic resonance imaging (MRI) and computerized tomography (CT) scans may be utilized with computer-aided design (CAD) drawing tools to engineer a replacement of proper dimensions for the individual patient. Three-dimensional printing has been used successfully to engineer a tracheal splint for a toddler suffering from airway collapse [42]. Another success has been reported in a rabbit model where tissue-engineered tracheas were formed from articular cartilage matrix and chondrocytes [29]. New advances in 3D printing polymers have been harnessed for applications to the airway. A half-pipe polycaprolactone 3D-printed trachea seeded with mesenchymal stromal cells in a fibrin matrix was implanted into a rabbit model with initial success of regenerated epithelium [30]. Tissue engineering polymer approaches for the large airways have gained momentum because of their ease of fabrication and implantation. A true tissue-engineered lung will likely utilize a combination approach of polymeric materials, proper cell choices, and proper *in vitro* conditioning.

12.4.2 Microfluidics and Assist Devices

There has been significant interest in creating 3D models of functional alveolar units to examine lung physiology and to be utilized as drug screening devices. These "lung-on-a-chip" devices are an interesting application of tissue engineering in the laboratory. The devices typically employ conventional photolithography, wherein a silicon chip is patterned to become a mold for polydimethylsiloxane (PDMS or moldable silicone). The PDMS forms a microfluidic chamber, which may be then seeded with alveolar epithelial cells and microvascular endothelial cells. These chambers have been made to be distensible and have allowed the growth of the epithelial cells at an air–liquid interface. The chambers may also be utilized to grow diseased cells, such as those from COPD or lung cancer. These disease models provide a useful screening tool for drug compounds. Many of the existing microfluidic devices are best utilized to mimic the alveolar capillary interface. This interface is critical to replicate lung injury and disease for the purpose of understanding them. Furthermore, this interface is otherwise impossible to re-create *in vitro* with conventional

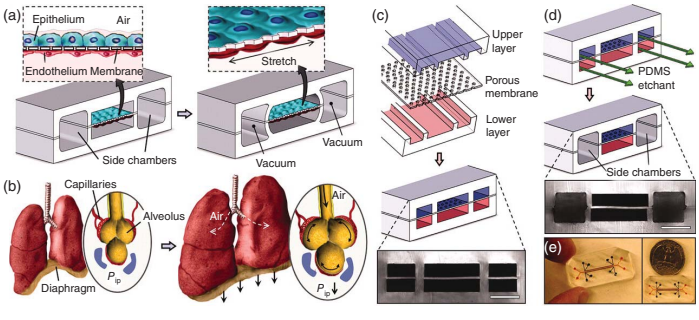

Figure 12.5 Biologically inspired design of a human breathing lung-on-a-chip microdevice. (a) The microfabricated lung mimic device uses compartmentalized PDMS microchannels to form an alveolar–capillary barrier on a thin, porous, flexible PDMS membrane coated with ECM. The device re-creates physiological breathing movements by applying a vacuum to the side chambers and causing mechanical stretching of the PDMS membrane, forming the alveolar–capillary barrier. (b) During inhalation in the living lung, contraction of the diaphragm causes a reduction in the intrapleural pressure (P_{ip}), leading to distension of the alveoli and physical stretching of the alveolar–capillary barrier interface. (c) Three PDMS layers are aligned and irreversibly bonded to form two sets of three parallel microchannels separated by a 10-μm-thick PDMS membrane containing an array of through-holes with an effective diameter of 10 μm. Scale bar, 200 μm. (d) After permanent bonding, a PDMS etchant is flowed through the side channels. Selective etching of the membrane layers in these channels produces two large side chambers to which vacuum is applied to cause mechanical stretching. Scale bar, 200 μm. (e) Images of an actual lung-on-a-chip microfluidic device viewed from above. (Reproduced with permission from [43].)

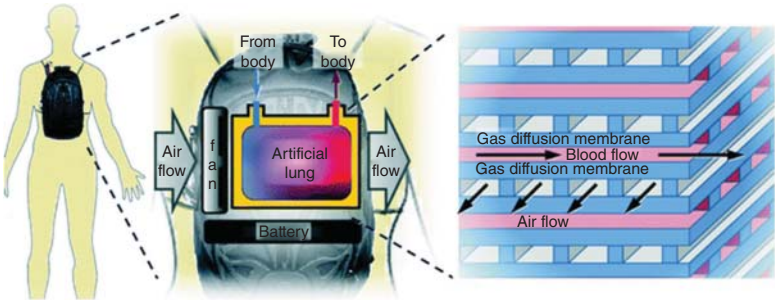

Figure 12.6 A conceptual drawing of an initial clinical application of microfluidic artificial lung technology – an ambulatory pumpless extracorporeal lung assist system. (Potkay 2014 [45]. Reproduced with permission of the Royal Society of Chemistry.)

tissue culture methods. In the most sophisticated models, collagen gels are placed outside porous membranes within microfluidic chambers. Variations of this setup may be utilized to examine the mechanical strain and shear stress environment within the alveoli. The biologically inspired design of a human breathing lung-on-a-chip is shown in Figure 12.5.

These microfluidic or lung-on-a-chip technologies have future use in advanced artificial or bio-hybrid lungs. To date, portable extracorporeal membrane oxygenation (ECMO) units have been made, referred to as *artificial lungs*, that can provide a bridge toward lung transplant. ECMO refers to a circuit of tubing, oxygenator, and a pump to carry out the work of a failing lung [44]. ECMO is largely restricted for use in intensive care units, but researchers are examining options for more portable applications. One company, Novalung, utilizes a pumpless extracorporeal lung-assist device, which is currently under clinical trials in Europe and Canada. This assist technology is highly dependent on novel biomaterials that will allow excellent oxygenation and prevention of thrombosis. A conceptual model of assist devices that combine microfluidics and hollow fiber oxygenation is shown in Figure 12.6. Current models in preclinical trials do not incorporate cellular aspects in artificial lungs; however, advances in microfluidic technology and biohybrid oxygenation may provide a pathway toward longer term bridges to transplants.

12.4.3 Cell Source, Cell Therapies, and Regenerative Medicine Approaches for Lung Tissue Engineering and Regeneration

12.4.3.1 Cellular Source for Engineered Pulmonary Tissues

In all of the aforementioned techniques, wherein a biomaterial is utilized as the support structure for cell seeding, the source of the cells is crucial. For the larger airway structures, it is recognized that ciliated epithelial cells are critical in maintaining airway patency. Other structural cell types such as chondrocytes and smooth muscle cells are of importance in maintaining airway stability and mechanical properties with growth and remodeling. The alveolar structures have a critical balance of alveolar type I epithelium, alveolar type II epithelium, and microvascular endothelium for proper gas exchange. Typically, most scaffolding

approaches utilize autologous cell seeding from the patient (or animal); however, depending upon the underlying disease state, autologous cells are not always a viable option. Most literature reports in tissue engineering the lung utilize embryonic stem cells (ESCs), iPSCs, and MSCs derived from bone marrow or adipose tissues. In some cases, fetal lung progenitor cells are isolated from rodents. All of these cell sources have shown the capability to differentiate into functional pulmonary epithelium *in vitro*; however, their differentiation capability in human lungs remains to be determined. These cell types are further discussed in the next section for use as stand-alone treatment strategies to repair the lung.

12.4.3.2 Cell Therapies for Lung Regeneration

The current view on the potential for success of stem cell therapies for lung regeneration and repair is mired in controversy. There is a large body of evidence which suggest that the distal, proximal, and alveolar regions each contains a distinct regional hierarchy for stem or progenitor cells. It is unknown how many true stem or progenitor cell types exist in the lungs [46] Endogenous tissue-specific progenitor cell therapy research will almost stand still until a hierarchy is agreed upon so that treatments may be targeted to the repair mechanisms for each affected area. Endogenous proliferation of respiratory epithelial cells begins with the identification of progenitor and stem cells. Because of the low turnover rate of the respiratory epithelium, disease processes have been induced using sulfur dioxide, ozone, or nitrogen dioxide inhalation, naphthalene, or bleomycin in mice and other animal models to identify potential progenitor or stem lineages (reviewed in [37]), There currently exists a great deal of debate on which cell types constitute progenitors, which cell types are stem cells, and if there is variation between regions. While endogenous therapies are ensconced in the basic science of trying to establish locations and cell characterization, exogenous cell therapy is experiencing some success.

12.4.3.2.1 Exogenous Cell Therapy

Exogenous stem cell therapy is focused on systemic administration via intravenous (i.v.) injection, aerosolizing stem cells (primarily MSCs) for inhalation, or delivery through an endobronchial tube. Stem cell therapy rests on the premise that inserting healthy stem cells into diseased areas will encourage repair. Stem cell simulation is focused on creating and improving drug delivery mechanisms to the lungs. Stimulation is geared to the belief that in any population of diseased cells, or nearby, there exist healthy stem cells that need more resources or a biochemical approval code to begin repairing the areas. Recent success in Phase II clinical trials for stem cell therapies with systemic distribution of MSCs into moderate to severe COPD patients showed no infusion toxicity, and no attributed adverse events over a 2-year follow-up period. Some patients with initial elevated inflammatory marker C-reactive protein did show a significant decrease in the same marker early on in trials. These results provide a firm basis for safe MSC use, including multiple infusions, in patients with chronic lung diseases [37]. Initial focus on structural engraftment following the administration of exogenous stem cells or progenitor cells has been largely supplanted by study and application of immunomodulatory and paracrine actions of MSCs (stromal) and endothelial

progenitor cells (EPCs) and by the rapidly growing field of *ex vivo* lung bioengineering [37]. Studies have shown that MSCs can express phenotypic markers of alveolar cells [47], and engraftment of respiratory epithelium can occur using several types of bone-marrow-derived cells and can express respiratory epithelial proteins [48]. MSCs obtained from adult bone marrow express phenotypic markers of airway epithelium, CCSP, CFTR, surfactant protein C, and thyroid transcription factor-1 mRNA, and to participate in airway remodeling *in vivo* [47]. Bone-marrow derived CD34+ cells showed progenitor-like alveolar differentiation, similar to resident alveolar cells [49].

ESCs have been shown in numerous studies to take on alveolar characteristics *in vivo*. Surfactant proteins, lamellar bodies, and even alveolar type II morphologies have been formed from human ESCs [14, 50]. Murine ESCs grown on an air–liquid interface have shown phenotypic and morphological markers of tracheobronchiolar airway epithelial cells [51]. Recent studies using Nkx2-1-GFP tagged ESCs have shown lung differentiation potential, and can be used to engraft an entire decellularized whole lung, although their functionality is not yet proven [52].

ESCs, iPSCs, endogenous lung progenitor cells, and MSCs (either bone-marrow- or adipose-derived) have been investigated for their lung regeneration potential. Many of these cell types have been shown to be capable of differentiating into alveolar epithelial cell populations under specific growth and media conditions *in vitro* [14, 53–55], or through genetic engineering [50]. Furthermore, these cell populations have been examined in models of lung injury and emphysema. Alveolar type II epithelium derived from ESCs rescued a bleomycin mouse experimental model of lung injury [15]. The most common stem cell therapies have utilized MSCs as regenerative strategies because of the ease of use in rodent models of COPD. Whether stem or progenitor cells can engraft and acquire the phenotype of structural lung cells following instillation remains controversial [56, 57]. However, MSCs have been shown to modulate inflammation and are easily attainable, making MSCs an attractive therapeutic option. MSC therapy was recently investigated in a Phase II clinical trial in patients with moderate to severe COPD with no adverse effects; however there were no significant improvements in the lung function [58]. These results represent a promising first step toward the use of cellular therapies for lung disease, but they also show that success in animal models does not always translate into success in human patients. Additional challenges remain in the proper cell choice for cellular therapy, along with inquiries into the proper delivery route for maximum effectiveness in repairing the lung.

12.4.3.2.2 Cellular and Drug Delivery

Hydrogel formation is a new area of interest in regenerative medicine for the lung. In addition to the cell–hydrogel constructs discussed in Section 12.1.3.2, decellularized tissue formulation into a hydrogel may provide a cellular niche for progenitor cells. Hydrogels have previously been derived from decellularized myocardium, nerve, fat, tendon, bladder, and small intestine. These hydrogels further the decellularization process, and create a native gel matrix that can be used as a platform for cellular remodeling, cellular regeneration, or drug delivery.

Many of the stem cell therapy approaches toward lung regeneration have examined i.v. injection, and a recent comparison of the approaches showed improvement when MSCs were dosed both intratracheally (i.t.) and intravenously [59]. A few studies have examined implantation of cells for lung repair in fibrin gels or collagen sponges. Furthermore, many of the lung parenchyma scaffolding strategies need to be further examined in terms of delivery strategy to the lung for integrated growth and repair. The concept behind biomaterial delivery of stem cells is to provide the cells a physiologic niche for growth, differentiation, and repair. Although this concept is logical, it presents a difficulty in the clinic. Delivery of these materials requires heavy sedation of the patient and precise placement of the material through a bronchoscope. All clinical trials to date for stem cell delivery for the lung make use of i.v. delivery strategies.

12.5 Conclusions, Remaining Challenges, and Future Directions

With the growing clinical need for lung repair and replacement, tissue engineering and regenerative medicine strategies are at the forefront in the pulmonary system repair. No longer are we able to rely on lung transplant or palliative therapeutics for lung diseases such as idiopathic pulmonary fibrosis and COPD. However, because of the complexity of the lung architecture, numerous specialized cell types, and critical life supporting function of gas exchange, a tissue-engineered lung remains far from clinical use. As material manufacturing and further understanding of the regenerative properties of progenitor cells advance, the tissue-engineered lung may be fully realized. There are significant advances in airway tissue engineering, cellular therapies, and bio-hybrid assist devices that are at the tipping point for practical and routine clinical use.

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13

Cardiac Tissue Engineering

Eun Jung Lee and Pamela Hitscherich

13.1 Introduction

Cardiovascular diseases, including myocardial infarction (MI), are the leading cause of disability and death in the Western world [2]. MI is caused by a blockage of the vasculature in the heart, leading to ischemia, which results in a large-scale loss of cardiac muscle. Initial damage to the myocardium is followed by extensive tissue remodeling, which leads to structural changes and functional alterations with a decrease in cardiac pump performance. Although the presence of cardiac stem cells (CSCs) has been identified [3, 4], the number of resident CSCs appears too small for replacement of an infarcted region, and the origin and role of these cells remain controversial. Whole-heart transplantation for end-stage heart failure patients is also limited because of the serious shortage of donor hearts.

Over the past decade, cell-based therapies utilizing multiple cell types have been used in preclinical animal models and in humans to repair or regenerate the injured heart either directly or indirectly [5–7]. Preclinical studies have suggested that transplanting bone-marrow-derived stem and side population cells [8], as well as embryonic and induced pluripotent stem cells (iPSCs) [9], in the area of the infarction has the potential to repair damaged heart tissues. However, these populations have yet to be explored in a clinical setting. In clinical trials, enhanced heart function following direct injection of both autologous and allogeneic mesenchymal stem cells (MSCs) has been reported [10]. However, long-term studies examining skeletal myoblasts alone have shown little to no improvement to left ventricular function [11]. Studies with bone-marrow-derived mononuclear and progenitor cell have also led to inconsistent results [8]. While modest improvement in heart function has been demonstrated following cell-based therapies, the enthusiasm raised by clinical trials has been dampened by extremely low retention and low long-term survival rates of implanted cells, as well as a lack of functional integration of the grafted cells within the host tissue [8, 9].

Alternatively, the development of functional cardiac tissue *in vitro* for replacing damaged heart tissues and eventually improving heart function is considered as a promising approach [8, 9]. Combination of an ideal cell type with an extracellular environment that promotes physiologic conditions is important in

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developing functional cardiac tissue *in vitro*. In addition, before replacing the damaged myocardium, engineered cardiac tissue (ECT) may serve as a powerful model system for high-throughput drug screening applications if they can reproduce key aspects of physiologic cardiac function.

In this chapter, ongoing research efforts toward ultimately developing functional cardiac replacement tissue are discussed. Current challenges include selection of a suitable and abundant cell source for constructing physiologic tissues, mimicking native anisotropic structure and function, providing appropriate biophysical stimulations, and developing vascularized tissues for better survival and integration *in vivo*. In addition, various *in vitro* models, which can be used for investigating cardiac development and pathologies as well as for high-throughput drug screening applications, are discussed.

13.2 Cardiac Tissue Architecture

The myocardium has a complex three-dimensional (3D) architecture, which consists of the extracellular matrix (ECM) composed primarily of type I and III collagen and small amounts of elastin and proteoglycans intimately associated with myocardial cells. The orientation of cardiac muscle fibers varies throughout the thickness of the wall from approximately −60° at the epicardium, parallel to the circumferential axis (0°) at the midwall, to +60° at the endocardium (Figure 13.1a) [12]. Within the layers of muscle fibers, cardiomyocytes are organized into parallel rod-shaped cells, connected by gap junctions and intercalated disks for efficient and coordinated pumping of the heart as a functional syncytium. This complex organization of cardiomyocytes gives rise to a mechanically anisotropic tissue, which is essential to the normal functioning of the heart.

A mixture of cells exists in the native myocardium, and the supportive role of non-cardiomyocytes is not only related to their function as structural components [15], but they are also involved in the allocation of essential paracrine factors or direct cell–cell contacts [16] that are critical during embryonic and postnatal development [17–20]. Seventy-five percent of myocardial tissue volume consists of cardiac cells, and the most predominant cell type in the heart is the cardiac fibroblast, which outnumbers cardiac myocytes by a ratio of 3 : 1 [21, 22]. The organization of cardiac fibroblasts in the heart was identified using anti-vimentin antibodies and discoidin domain receptor 2 (DDR2) labeling [13, 23]. Fibroblasts appear to surround cardiomyocytes, forming a three-dimensional network in the heart and interconnecting the voids between myocardial tissue layers as shown in Figure 13.1b. Cardiac fibroblasts are closely related to cardiomyocytes in their response to not only mechanical stimulation but also in conduction of electrical signals, contributing to cardiac function. Following MI, cardiac fibroblasts become activated, rapidly proliferate, and migrate into the necrotic myocardium to synthesize the ECM and to initiate the fibrosis and remodeling process [24–26]. A protein abundantly expressed in smooth muscle cells, called α-smooth muscle actin, is not found in normal cardiac

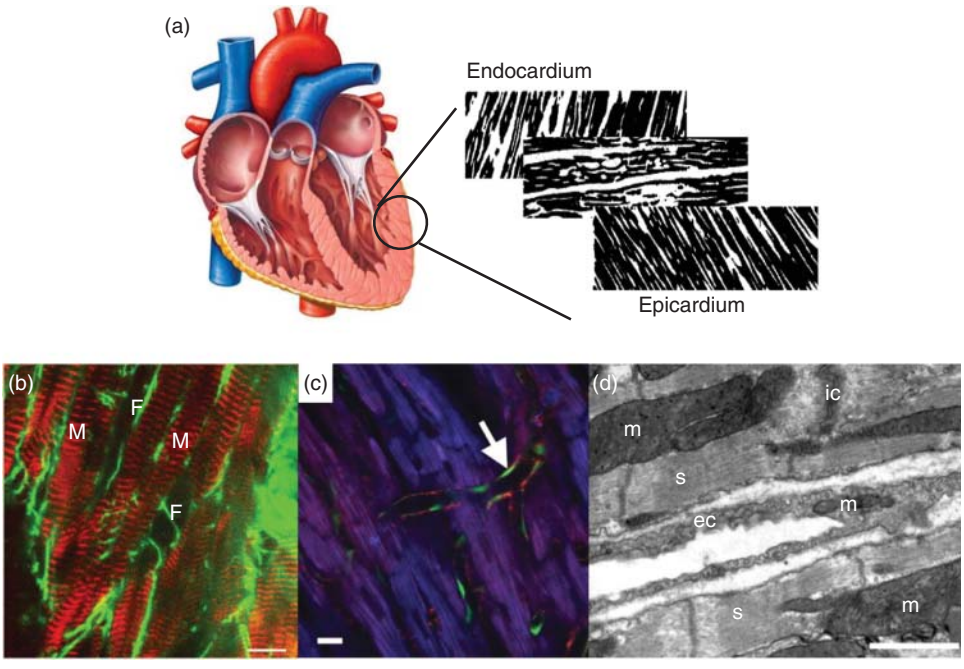

Figure 13.1 (a) Illustration of a sectioned heart showing left and right ventricular chambers with layered hierarchical organization of aligned myocardial tissue varying from endocardium to epicardium. (Adapted from Streeter *et al*. [12].) (b) Organization of cardiac fibroblasts (F) in relation to cardiac myocytes (M) in a rabbit. scale bar, 10 μm. (Kohl 2005 [13]. Reproduced with permission of Elsevier.) (c) Two-photon image of live, perfused heart tissue, demonstrating the relationship between cardiomyocytes and the surrounding capillaries. Mitochondria of endothelial cells are labeled with TMRM (red) around endothelial nuclei (calcein-AM, green, arrow). Scale bar 10 μm. (Davidson 2010 [14]. Reproduced with permission of Oxford University Press.) (d) Electron micrograph depicting mitochondria (m) in the endothelial cell of a capillary (ec) and adjacent cardiomyocytes with sarcomeres (s) and intercalated disk (ic). Scale bar 1 μm. (Davidson 2010 [14]. Reproduced with permission of Oxford University Press.)

fibroblasts, but is markedly upregulated in transdifferentiated cardiac fibroblasts in the infarcted and remodeling myocardium [27]. The matricellular protein periostin is also selectively upregulated in activated cardiac fibroblasts infiltrating the infarcted and pressure-overloaded heart and may be a promising marker for the identification of activated fibroblasts following cardiac injury [27, 28].

Vascular endothelial cells also have a close spatial relationship with cardiomyocytes, as all cardiomyocytes are estimated to be within 2–3 μm of the nearest coronary microvessel *in vivo* (Figure 13.1c,d). It is now well recognized that complex paracrine interactions between endothelial cells and cardiomyocytes exist that are important for normal physiology as well as in disease states [29, 30]. Myocardium secretes multiple angiogenic factors such as the transforming growth factor-β (TGF-β), bone morphogenetic proteins, and the vascular endothelial growth factor (VEGF), which are not only essential for the endothelial-to-mesenchymal transition that results in endocardial

cushion formation during embryonic development but also in the development, growth, and maintenance of the vasculature [31–33]. Endothelium, on the other hand, releases multiple substances including nitric oxide (NO), endothelin-1, prostanoids, and various other factors that modulate cardiac performance, rhythmicity, and growth [17]. NO secreted by endothelial cells has been recognized as an important regulator of myocardial function either by vasodilatation of coronary vessels or through direct effects on the myocardium [34].

Cardiomyocytes are subjected to a very complex combination of mechanical deformations during contraction; they undergo repeated multiaxial stretching, shortening, and substantial shear deformation [35]. These stimuli impact not only the metabolism of the myocytes and regulation of cardiac gene expression but also the formation of mature structure *in vitro*. In particular, ventricular wall stretch may be closely linked to the regulation of protein metabolism in the native heart [36]. Changes in the mechanical activities might also cause an integrated response, which ultimately leads to the hypertrophy or atrophy of the cells [37, 38]. Mechanotransduction is the process by which cells sense and transduce physical forces into biochemical signals and generate physical responses leading to alterations in cellular structure and function [39]. Since cardiomyocytes are force-generating cells, the internal loads generated are transmitted to adjacent cells and their surrounding ECM, making mechanotransduction particularly more complex. Cardiomyocyte integrins are essential for bidirectional communication between ECM proteins and the cardiomyocyte cytoskeleton via proteins such as talin, vinculin, and α-actinin [40]. There is evidence showing that mechanical forces such as passive stretch and active tension are sensed by costameres and focal adhesion complexes and are transduced into signals leading to changes in cell function [41, 42]. However, exactly how the ECM–integrin–cytoskeletal complex senses mechanical stimuli remains unclear. Moreover, the impact of structural alignment on activation of integrin-linked kinase (ILK), which may be involved in integrin-dependent mechanotransduction in cardiomyocytes, remains to be elucidated.

13.3 Cell Source Considerations

It is estimated that the adult human left ventricle contains a total of 5 billion cardiomyocytes and there is a loss of nearly one billion or more cardiomyocytes following MI [43]. Currently, there is no source of human cardiomyocytes readily available for cardiac regeneration, as cardiomyocytes have very limited ability to replicate. An ideal cell source for cardiac regeneration would possess a high proliferative capacity to allow for an abundant supply as well as the potential to differentiate and functionally integrate into the host tissue. Various cell types considered for cardiac regeneration, including MSCs, embryonic stem cells (ESCs), and iPSCs, are summarized in Table 13.1.

13.3.1 Mesenchymal Stem Cells

MSCs are an adult stem cell population originally isolated from the bone marrow [61]. MSCs have now been identified and isolated from a variety of other sources

Table 13.1 Benefits and limitations of available cell sources and their cardiac derivatives for cardiac tissue engineering.

Cell type	Advantages	Disadvantages	References
Mesenchymal stem cells	Multipotent, immune-privileged, numerous sources, potentially autologous, efficacy demonstrated in clinical trials	Cardiac differentiation efficiency unknown, mechanism of proposed paracrine benefits unknown	[44–68]
Embryonic stem cells	High proliferative potential, sustainable source, efficacy demonstrated in animal models	Ethical considerations, allogeneic source, differentiation efficiency, teratocarcinogenicity, lacking clinical data	[69–88]
Induced pluripotent stem cells	High proliferative potential, sustainable source, efficacy demonstrated in animal models, potentially autologous, possibility for disease-specific *in vitro* models	Differentiation efficiency, retains disease phenotype in need of correction for clinical use, teratocarcinogenicity, lacking clinical data	[83, 89–104]
Cardiac progenitor cells	Multipotent, cardiac commitment, potentially autologous, promotes neovascularization	Lacking clinical data	[105–108]
Cardiac stem cells	Autologous, multipotent, cardiac-committed, efficacy demonstrated in clinical trials	Specific roles in the myocardium are unknown, heterogeneous population, require isolation from the heart	[109–116]

including adipose tissue [49], amniotic fluid [53], and umbilical cord blood [50]. MSCs have the potential to differentiate into osteogenic, adipogenic, and chondrogenic lineages [55, 64] as well as into cardiac lineage under appropriate stimuli [61, 66]. MSCs are immune-privileged cells [46] and are known to secrete bioactive agents that cause trophic effects such as preventing apoptosis, promoting angiogenesis [52, 58], assisting in matrix reorganization, and augmenting circulating stem cell recruitment [117]. Recent studies examining the efficacy of MSCs in cardiac repair have demonstrated improved heart function in animal models of acute myocardial injury [47, 54, 67]. In addition, localization of MSCs to the site of cardiac injury [51, 57] and improved cardiac function [62, 68] were demonstrated in patients with heart failure [45, 48, 65]. However, it is difficult to precisely assess their role for cardiac repair, as they appear to be a heterogeneous population depending on the tissue of origin [50]. Moreover, while a previous study had shown that bone-marrow-derived MSC fuse with host cardiomyocytes to form mature cardiomyocytes, it is unclear whether MSCs can also differentiate into functional cardiomyocytes or, conversely, induce

dedifferentiation and proliferation of endogenous cardiomyocytes [44, 60]. Other studies also propose that improvements in heart function after MSC transplantation are related to secretion of paracrine factors [56, 59, 63]. Specific signals and mechanisms responsible for improvements in cardiac function and the safety of long-term MSC use for cardiac repair remain to be elucidated.

13.3.2 Embryonic Stem Cells

ESCs have emerged as an ideal source for the production of heart cells for cell-based therapy and creating functional cardiac tissue replacements. Studies have demonstrated that both mouse and human ESCs can be differentiated into functional cardiomyocytes under certain conditions, thereby opening an exciting avenue for the generation of an abundant population of cardiomyocytes [69, 70]. Differentiation of ESCs into cardiomyocytes follows a well-defined developmental lineage through mesoderm induction, as shown in Figure 13.2. Mesodermal

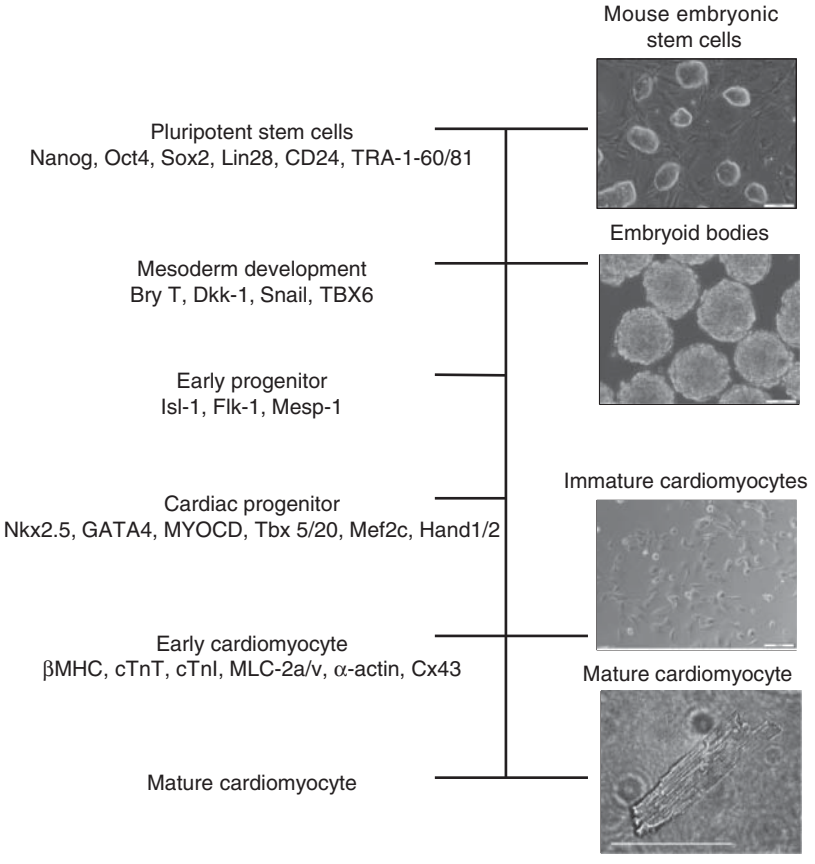

Figure 13.2 Developmental stages involved in cardiomyogenesis originating from pluripotent stem cells into mature cardiomyocytes. Changes in cell size, morphology, gene and protein expression are associated with each differentiation phases. Scale bar 100 μm. (Image sources: Dr. E.J. Lee's and Dr. L.H. Xie's lab.)

lineage cells give rise to cardiac progenitor or precursor cells exhibiting specific gene markers such as GATA4 and Nkx2.5. Cardiac progenitors then further differentiate into immature cardiomyocytes expressing genes associated with the contractile apparatus such as myosin heavy-chain β and cardiac troponin T, and ultimately into adult cardiomyocytes exhibiting mature cardiomyocyte structure, phenotype, and function, as summarized in Table 13.2.

Differentiation of mouse ESCs into cardiomyocytes involves the formation of embryoid bodies (EBs) via the hanging-drop method [69]. For human ESCs, instead of the hanging-drop method, EBs are formed using a spin, a microwell, or a micro-patterning method. Formation of EBs via spin utilizes centrifugation to encourage aggregate formation of a defined number of human ESCs in commercially available low-attachment wells to form well-defined EBs [71]. EBs are also formed in microwells coated with Matrigel [72] or agarose [73], and on

Table 13.2 Specific genes and protein markers of mature cardiomyocytes.

Gene/protein	Abbreviation	Function	References
α-Actinin	α-Actinin	Transmission of tension between sarcomeres via f-actin cross-linking	[118, 119]
Calcium-transporting ATPase	Serca2	Sarcoplasmic reticulum ion pump; controls calcium regulation	[120]
Calsequestrin 2	CASQ2	Cardiac-specific sarcoplasmic reticulum, calcium binding and release	[121]
Connexin 40	Cx40	Component of atrial gap junctions, control ion exchange and electrical coupling	[122]
Connexin 43	Cx43	Major component of atrial and ventricular gap junctions; controls ions exchange and electrical coupling	[122]
Connexin 45	Cx45	Component of atrioventricular node cell gap junctions	[122, 123]
Myosin heavy chain-α	MHC-α	Dominant isoform in earlier cardiac development in humans	[124, 125]
Myosin heavy chain-β	MHC-β	Dominant isoform in later cardiac development in humans	[124, 125]
Ryanodine receptor	RyR2	Cardiac-specific sarcoplasmic reticulum calcium binding and release channel	[126]
Troponin C	cTnC	Calcium binding protein involved in cardiac muscle contraction	[127]
Troponin T	cTnT	Involved in promoting cooperation during activation of contraction	[127, 128]

micro-patterned Matrigel islands, which offer a means of generating size-specific EBs for cardiac-specific differentiation [74]. Recently, highly purified functional human cardiomyocytes were generated from a monolayer of ESCs without EB formation through directed differentiation with the addition of activin A and BMP4 [5]. Other studies have explored the use of additional ECM components [75] and coculture with other cells, including mitotically inactive END-2 or OP9 stromal cells, for improved differentiation efficiency [76, 77].

Human ESC-derived cardiomyocytes have shown great survival, engraftment, and maturation in mouse [78], rat [79–81, 84, 85], and guinea pig [86] infarct models. Engrafted human ESC-derived cardiomyocytes provided early improvements in cardiac function [78–80] and exhibited neovascularization [81]. Chong *et al.* recently demonstrated the feasibility of clinical-scale culture of human ESC-derived cardiomyocytes, which can be engrafted and electrically coupled with a nonhuman primate heart post MI [129].

Continuous enhancements to cardiac differentiation protocols using human ESCs open an exciting avenue for the generation of patient-specific heart cells for cardiac repair and building blocks for cardiac tissue engineering approaches. However, there are still challenges remaining with *in vitro* differentiation of ESCs into cardiomyocytes, as they exhibit immature phenotype evidenced by their size, organization, and electric properties even after 2 months of standard 2D tissue culture [130]. In addition, immature cells possess inherent teratocarcinogenicity, so that new approaches to enhance maturation of these cells need to be explored to achieve clinically safe cells. A better understanding of the microenvironmental cues that regulate the differentiation and maturation of human ESCs may enhance maturation of human PSC-derived cardiomyocytes, as recently reviewed elsewhere [87, 88].

13.3.3 Induced Pluripotent Stem Cells

Unlike ESCs, iPSCs are pluripotent stem cells derived from adult cells through genetic reprogramming. Generation of iPSCs from somatic cells using four transcription factors revolutionized stem cell research [89], as the use of iPSCs not only eliminates the ethical concerns associated with ESCs but also provides an unlimited source of personalized, patient-specific cells. Since their discovery, derivation of cardiomyocytes from iPSCs has been achieved using similar differentiation methods as used with ESCs [83, 90]. It has been demonstrated that human iPSC-derived cardiomyocytes exhibit functional properties comparable to those of human ESC-derived cardiomyocytes [131]. The engraftment and integration of iPSC-derived cardiomyocytes into animal infarct models have also shown that they are comparable to ESC-derived cardiomyocytes [100, 101].

One of the major advantages of iPSCs is the possibility of generating disease-specific cells from patients with certain genetic cardiac diseases. This allows not only studies of human disease progression but also high-throughput *in vitro* drug screening. A number of iPSC lines specific for cardiac disease have been established, including cardiac sodium channel disease [91], familial dilated and hypertrophic cardiomyopathy [92], long QT syndrome [93], Timothy syndrome [94], LEOPARD syndrome [95], arrhythmogenic

right ventricular cardiomyopathy [96], and catecholaminergic polymorphic ventricular tachycardia [97].

However, initial methods of using viral vectors to reprogram somatic cells into iPSCs pose a risk due to permanent integration of viral vectors into the host genome, as well as unwanted reactivation of cancer genes within the host genome. The possibility of secondary side effects due to imperfect reprogramming is a concern for patients [103, 104, 132]. Thus, advancements have been made toward generating safer iPSCs without the incorporation of integrating viral vectors. One way to generate nonintegrative vectors is through the addition of loxP sites, generating Cre-excisable transgenes [133]. There have also been studies examining the induction of iPSCs through treatment with cell extracts from pluripotent sources [134, 135]. One study demonstrated the ability of a small molecule known as reversine to induce dedifferentiation of myoblasts [136], and another showed the induction of skin cancer cells into pluripotent cells via a microRNA, mir-302 [137]. Methods to increase the efficiency of viral transduction include the addition of low molecular weight synthetic molecules, as depicted in Figure 13.3 [138, 139]. Once iPSCs are generated, they can be banked for both allogeneic and autologous use. However, quality control is required for a bank of iPSC lines to offer human leukocyte antigen matching for patients to reduce immunological responses for clinical allogeneic use [98, 99, 104].

Moreover, recent studies have demonstrated strategies to directly reprogram somatic cells into cardiomyocytes, eliminating the need for the pluripotent stage in between (Figure 13.3). A study by Addis *et al.* describes a method to directly reprogram mouse embryonic fibroblasts into cardiomyocytes, also known as iCMs. They utilized forced expression of five different genes using a

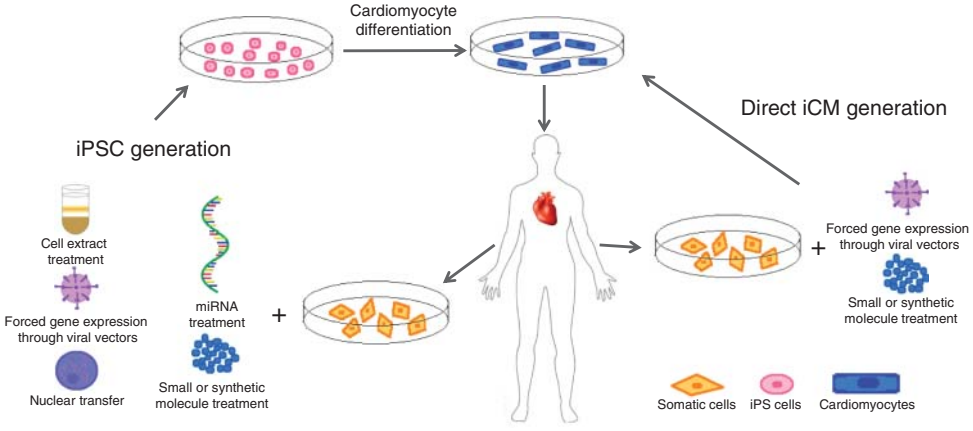

Figure 13.3 Generation of autologous cardiomyocytes using isolated adult somatic cells. Isolated somatic cells are first reprogrammed into iPSCs using one or a combination of factors such as cell extract, viral vectors, nuclear transfer, miRNA, and small or synthetic molecules. iPSCs can further differentiate into cardiomyocytes. Alternatively, isolated somatic cells can be directly reprogrammed into iCMs, bypassing the generation of the iPSC step.

lentivirus, which resulted in spontaneously contracting cells exhibiting classic cardiomyocyte markers and calcium-handling behavior [140]. Srivastava *et al.* described induction of iCMs from mouse cells similar to Addis *et al.*, but via only three gene factors [141]. They have also described a method of iCM induction from human fibroblasts utilizing retroviral infection causing forced expression of five gene factors along with small-molecule supplements, as described in Figure 13.3 [142]. This technology, although in its infant stage, has tremendous potential to eliminate the risks of teratoma formation associated with the use of PSC sources while still possessing the possibility to provide a patient-specific cell source. Further studies are needed to better understand the potential for both iCM and iPSC-derived cardiomyocytes *in vivo*.

13.3.4 Other Cell Sources

Resident CSCs have also been explored as a potential cell source for cardiac regeneration. Recently, it has been discovered that the heart is not a terminally differentiated organ but contains a small, heterogeneous population of CSCs [109, 111, 112, 115, 116]. These cells can be isolated from a human patient through a noninvasive procedure and expanded *in vitro.* CSCs can differentiate into CMs and vascular cells [111] and have been shown to improve cardiac function in small animal models [112]. Clinical trials of stem cell therapies are currently being performed with encouraging results, which show improved contractility and reduced infarct size [110, 113, 114]. However, the mechanism of action of CSCs is still unknown in the native myocardium, and their potential for cardiac tissue engineering applications has yet to be elucidated.

Cardiac progenitor cells (CPCs) are multipotent stem cells with limited ability to differentiate into only cardiac and smooth muscle as well as endothelium [107, 108]. Christoforou *et al.* has described the integration of CPC-derived cardiomyocytes into a mouse infarct model [106]. They recently reported synchronous contraction of CPC-derived cardiomyocytes within a 3D fibrin/Matrigel tissue [105], demonstrating the feasibility of these cells for cardiac tissue engineering. Other cells types have also been explored for cardiac-specific applications, including numerous bone-marrow-derived stem cell populations and skeletal myoblasts [8]. Each proposed cell type exhibits its own set of benefits and limitations, and the ideal cell source for cardiac tissue engineering remains to be determined.

13.4 Engineering for Myocardial Tissue

Engineering functional cardiac tissue *in vitro* is an alternative approach to repairing or replacing damaged areas of the heart, especially for patients with congenital heart defects or who do not necessarily require whole-heart transplant. Thus far, various strategies have been employed in developing 3D cardiac tissue constructs using numerous types of biomaterials including hydrogels, fibrous scaffolds, and cell-mediated or scaffold-free biomaterials [103, 132, 143–152].

13.4.1 Hydrogels

Hydrogels have been widely used as a candidate material for engineered cardiovascular tissues, as their chemical, mechanical and physical properties can be tuned and modified accordingly [153]. Natural hydrogels are made of natural polymers such as collagen, Matrigel, and fibrin. Type I collagen, which is biocompatible and biodegradable with minimal inflammation, has been one of the attractive options, as it is the most abundant natural structural component in the heart and vessels [154–157]. In addition, collagen provides a 3D environment for cells to remodel, and the geometry and size of the collagen based-tissues can be easily controlled using different casting molds.

Eschenhagen and colleagues established hydrogel-based cardiac tissues by seeding cardiomyocytes into a mix of collagen and Matrigel in the presence of cyclic mechanical stretch (Figure 13.4a) [154, 167]. Engineered heart tissues in various configurations such as rings, cylinders, and loops were generated. Functional improvements have been demonstrated upon *in vivo* implantation of these tissues in a rat MI model [158, 168]. Lee *et al*. have also used a mix of neonatal rat cardiac cells with type I collagen gel and Matrigel to develop an engineered cardiac organoid chamber exhibiting ventricular pump function, which can serve as an *in vitro* model for efficient evaluation of the myocardial contractile function (Figure 13.4c) [160].

As an alternative to the traditional use of collagen for ECTs, studies have demonstrated the feasibility of fibrin-based constructs [169, 170]. Birla *et al.* embedded cardiac cells within a fibrin gel to construct tubular pressure-generating tissues, and Wendel *et al.* demonstrated that fibrin-based cardiac patches reduced left ventricular remodeling and improved cardiac function upon implantation into an acute MI rat model [171]. A recent study by Zhang *et al.* reported advanced levels of structural and functional maturation of human ESC-derived cardiomyocytes in a fibrin-based cardiac tissue with elliptical pores, as shown in Figure 13.4d [161].

Synthetic hydrogels such as poly(ethylene glycol) (PEG) [172] and poly(*N*-isopropylacrylamide) (PNIPAAM) [173] have also been explored for cardiac tissue formation. Bearzi *et al.* have recently shown that the stiffness of PEG–fibrinogen hydrogels can modulate cardiac-specific differentiation of iPSCs [174] similar to what was previously shown by Young and Engler with chicken cardiomyocytes on PEG gels with time-dependent increases of stiffness [172]. Fujimoto *et al.* have shown that PNIPAAM-based injectable hydrogels support left ventricular contractility and allow host cell migration and neovascularization in a rat infarct model [173]. For *in vivo* applications, injectable hydrogels have become a popular candidate in hydrogel research, as they eliminate the need for invasive surgical procedures and hence reduce the risks associated with possible clinical therapies [175, 176].

13.4.2 Prefabricated Scaffolds

Seeding cells into various prefabricated polymer scaffolds has been extensively explored for cardiac tissue engineering applications. Various fabrication techniques were utilized to create micro- and nano-scaled, preformed scaffolds in

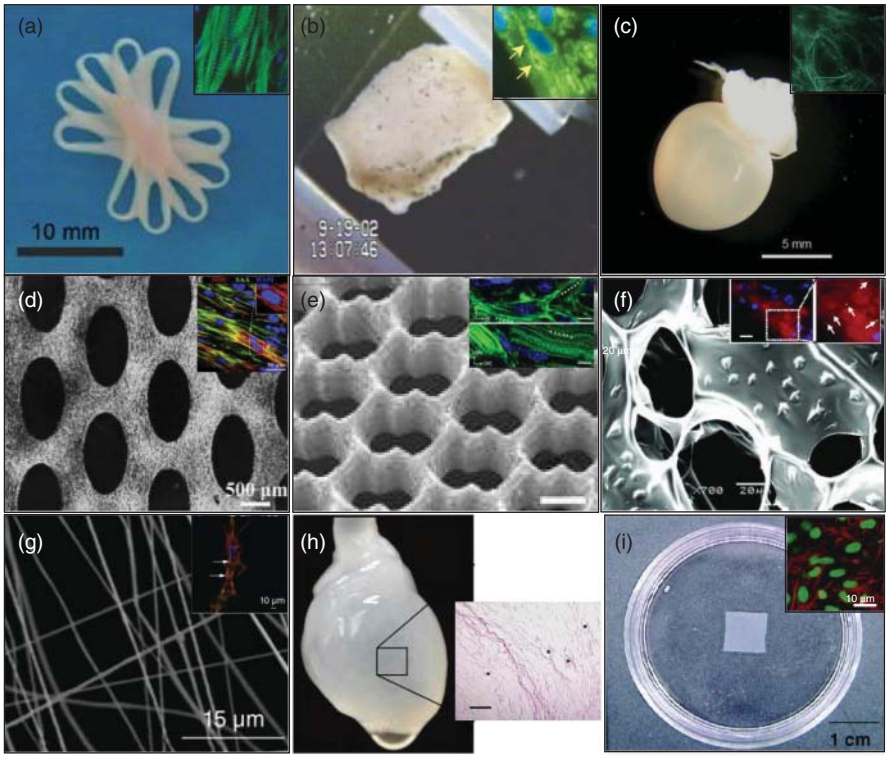

Figure 13.4 Example of engineered cardiac tissues from literature sources. (a) Collagen construct with neonatal rat cardiomyocytes. Inset shows striated actin filaments in green. (Zimmermann 2006 [158]. Reproduced with permission of Nature Publishing Group.) (b) Neonatal rat cardiomyocytes seeded onto collagen sponges after 8 days of electrical stimulation. Inset shows myosin heavy chain expression in green. (From Radisic *et al.* [159].) (c) Engineered cardiac organoid chambers. Inset shows striated actin filaments. (From Lee *et al.* [160].) (d) Fibrin cardiac patch with elliptical pores seeded with human ESC-derived cardiomyocytes. Inset shows myosin heavy chain (green) and α-actinin (red). (Zhang 2013 [161]. Reproduced with permission of Elsevier.) (e) Accordion-like honeycomb PGS scaffolds seeded with neonatal rat heart cells. Inset shows striated actin filaments in green. (Engelmayr 2008 [162]. Reproduced with permission of Nature Publishing Group.) (f) Alginate scaffold embedded with gold nanowires. Inset shows troponin I expression in red and connexin 43 in green. (Dvir 2011 [163]. Reproduced with permission of Nature Publishing Group.) (g) PCL + 10% carbon nanotube electrospun scaffold. Inset shows myosin heavy chain (green) and f-actin (red) of Aza-treated MSCs. (From Crowder *et al.* [164].) (h) Decellularized whole rat heart. Inset shows H&E staining of decellularized tissue exhibiting no nuclei. (Ott 2008 [165]. Reproduced with permission of Wolters Kluwer Health, Inc.) (i) Neonatal rat cardiomyocyte sheet generated using temperature-responsive tissue culture plate. Inset shows f-actin in red and nuclei in green. (Shimizu 2002 [166]. Reproduced with permission of Wolters Kluwer Health, Inc.)

the form of a sponge [159], fibrous mat [177], or porous scaffold [161]. Fibrous and porous scaffolds can be composed of natural polymers such as fibrin [178], alginate [163, 179], collagen [180], and gelatin [177, 181] as well as synthetic polymers including poly(caprolactone) (PCL) [164, 182], poly(lactic-*co*-glycolic acid) (PLGA) [183], poly(L-lactide) (PLLA) [184], and poly(glycerol sebacate) [162, 185, 186]. The size, structural alignment, mechanical properties, and degradation rates of preformed scaffolds can be tailored based on the polymer properties to fit their application [187, 188]. Leor *et al*. seeded fetal rat cardiac cells into cylindrical alginate scaffolds and implanted these constructs on the surface of the scar of rats with MI [179]. Bursac *et al*. cultured neonatal rat cardiac myocytes on PLGA scaffolds, creating contractile 3D tissues for molecular, structural, and electrophysiological studies [183]. Kharaziha *et al.* demonstrated that highly aligned electrospun PGS:gelatin scaffolds encouraged alignment and synchronous contraction of seeded neonatal rat cardiomyocytes [177]. Thomson *et al.* developed a microtemplated fibrin scaffold that encouraged prevascularization [178], demonstrating another example of preformed scaffolds as the building blocks for ECTs.

There has been tremendous interest recently in conductive nanomaterials, including carbon derivatives such as carbon nanotubes and graphene oxide, due to their intriguing mechanical and electrical properties [189–196]. Since cardiomyocytes exist in an electrically and mechanically dynamic and complex microenvironment in the heart, and the contractile behavior of cardiac cells is controlled by the electroconductive networks within the cardiac tissue [197], the unique features of a highly conductive material are especially attractive for cardiovascular tissue engineering applications. A few studies have shown the biocompatibility of 2D graphene films for cardiomyocyte cultures and their potential for inducing enhanced cardiomyogenic differentiation of MSCs [189, 198–200], although the 2D films do not fully mimic the complex physiological environment. Dvir *et al.* also found that gold nanowires incorporated into a lyophilized, sponge-like alginate scaffold allowed better synchronous contraction due to enhanced electrical communication between adjacent cardiac cells (Figure 13.4f) [163]. Carbon fibers embedded within a chitosan matrix has also been shown to increase metabolic activity and expression of cardiac-specific markers, including connexin43 and cardiac troponin T [201]. Crowder *et al.* developed a electrospun PCL scaffold with multiwall carbon nanotubes (MWCNTs), as shown in Figure 13.4g, and found that the addition of MWCNTs enhances cardiac-specific markers in cultured human MSCs in the presence of the DNA methylation inhibitor 5-azacytidine [164]. Recently, our group examined the biocompatibility and feasibility of 3D electrospun graphene composite scaffolds for cardiac tissue engineering application. It was demonstrated that 3D graphene composite scaffolds not only support the growth but also promote functional maturation of mouse embryonic stem cell-derived cardiomyocytes through enhanced contractility and Ca^{2+} handling behavior [202]. However, long-term effects of these conductive nanomaterials and, more importantly, their biocompatibility and integration *in vivo* need to be investigated.

Recent studies on the application of conductive materials suggest that piezoelectric polymeric materials can also be considered for tissue engineering. Electroactive piezoelectric scaffolds have the special intrinsic property of producing an electrical voltage in response to even a minute mechanical deformation such as caused by cell contraction [203]. This unique feature of the piezoelectric scaffold may be suitable for cardiac cells, as electrical stimulation has been suggested as a promising tool for solving various cardiovascular-related problems [159, 197]. In fact, Hitscherich *et al*. has demonstrated the cytocompatibility of piezoelectric PVDF-TrFE scaffolds with pluripotent stem cell derived cardiovascular cells for the first time [204]. PVDF-TrFE supported the survival, growth and function of both mouse embryonic stem cell derived cardiomyocytes and endothelial cells. However, further studies are needed to elucidate the mechanisms of piezoelectric scaffolds on functional improvements of the cells.

13.4.3 Decellularized Scaffolds

Decellularized biological tissues, retaining their native structure, may provide natural scaffold material that can be used to rebuild a tissue using autologous cells. Ott *et al*. reported a decellularized rat heart, which was processed by detergent perfusion through coronary arteries while still maintaining the intricate design and structure of the native tissue (Figure 13.4h) [165]. The decellularized heart was re-seeded with neonatal rat cardiac cells and rat aortic endothelial cells, resulting in a contractile tissue, though demonstrating only a weak fetal pump function [165]. Another group has reported repopulation of decellularized mouse hearts with human iPSC-derived CPCs which can migrate and differentiate *in situ* to form contractile muscle tissue and intact vasculature [205]. However, there was limited cell retention even after 7 days of perfusion, which resulted in minimal contractile force generation. The use of a mouse heart also limits its clinical relevance. Recently, several groups have demonstrated the decellularization of porcine hearts, which are similar in size to human hearts [206–208]. Successful re-endothelialization within the whole decellularized porcine heart has been demonstrated and shown to help increase the contractile properties of recellularized porcine left ventricles. While recellularization of partial porcine myocardial tissue with rat MSCs [209] and chicken embryonic cardiomyocytes [210] has been demonstrated, more studies are needed to achieve complete recellularization of intact whole hearts of a relevant size and with appropriate cell types.

13.4.4 Cell Sheets

Shimizu and Okano's group have pioneered a technique to detach a monolayer of cultured cells using temperature-responsive surfaces [211, 212]. Culturing cardiomyocytes and cardiac fibroblasts on temperature-responsive culture dishes for an extended period allowed extensive deposition of ECM protein by the cells, which resulted in the formation of cell sheets (Figure 13.4i). By lowering the culture temperature, the surface chemistry of the culture dishes changes to allow the detachment of intact cell sheets. Shimizu *et al.* have stacked several layers of myocytes sheets, which formed electrically coupled constructs

producing a relatively strong contractile force of 1.2 mN [166]. However, construct shrinkage, random cellular alignment, and aggregation of cells due to cell sheet contraction after several days, due to lack of physical constraints on the tissues, were observed. Their technique was enhanced by incorporating micropatterns on the temperature-responsive dishes to achieve tissues with structural alignment [213]. In addition, coculture of rat endothelial cells with rat cardiomyocytes resulted in endothelial cell networks in *in vitro* culture, which were retained after harvesting the cell sheet. Cocultured cell sheets promoted neovascularization and improvements in cardiac function when implanted *in vivo* into a rat MI model [214]. Masumoto *et al.* recently developed a new protocol to derive both cardiomyocytes and vascular cells from human iPSCs. Once a layer of cell sheets created using those cells was implanted into a rat MI model, enhanced fractional shortening and left ventricular systolic function were examined [215]. In addition, neovascularization was observed and fibrosis formation was inhibited [215]. While great promise is seen in rat infarct models, it will be necessary to expand to larger, more clinically relevant sized animal models to elucidate the efficacy of cell sheet engineering.

13.4.5 Bioreactors

The native myocardium is a highly dynamic environment, as cardiomyocytes experience both electrical and mechanical stimulation with every beat of the heart. It is thought that these native stimuli play a key role in the development, growth, and pathological remodeling of the heart [216, 217]. Many attempts have been made to replicate these dynamic stimuli *in vitro* with various bioreactor designs [147, 148]. Studies utilizing rat neonatal cardiomyocytes demonstrated that cyclic and static stretch increases the gap junction protein expression in 2D cultures [218, 219] as well as improved organization and hypertrophy with uniaxial stretching in 3D cardiomyocyte tissue constructs [167, 220, 221]. More recently, Tulloch *et al.* demonstrated that cyclic and static mechanical stretch increased the organization and alignment, cardiac protein expression, and proliferation of both human ESCs and iPSC-derived cardiomyocytes when cultured in a 3D collagen type I sponge. They also showed that the addition of endothelial cells and smooth muscle cell precursors allowed cardiac tissues to form vascular conduits, which were then perfused by the host vasculature upon implantation into a rat model [222].

Radisic *et al*. cultured cardiomyocytes with Matrigel in preformed collagen sponges/foam within a bioreactor with combined perfusion and electrical stimulation [159]. Perfusion offered higher cell viability, while electrical stimulation improved cardiac tissue morphology, contractile function, and molecular marker expression, providing a similar degree of cardiomyocyte differentiation as mechanical stimulation (Figure 13.4b). Recently, Nunes *et al.* developed a biowire technique that improved the organization, conduction velocity, and functional capacity of human ESCs and iPSC-derived cardiac tissues via electrical field stimulation [223]. In other studies, a combination of cyclic mechanical and electrical stimulation increased the functional properties of fibrin-based cardiac tissues compared to electrical or mechanical stimulation

alone [224], and resulted in increased cardiac tissue contraction and sarcomere development [225]. These early studies demonstrate the feasibility of generating *in vitro* stimulation which can closely mimic the native myocardial environment for the maturation of ECTs. Further studies are needed to examine the potential use of these combined stimulation bioreactor systems with human cell types.

13.4.6 Engineered Cardiac Tissues (ECTs) as Advanced In Vitro Models

Despite all the encouraging results with recent technologies to develop ECTs, a number of hurdles must be overcome before tissue engineers realize the long-term goal of creating living replacements for surgical implantation. Meanwhile, ECTs may find important short-term applications as *in vitro* models to bridge a wide gap between standard cell culture studies and whole-animal models. ECTs can provide a reliable platform for efficiently screening candidate therapeutic strategies, provided they reproduce key aspects of natural cardiac tissue function [226, 227]. The use of engineered tissues as an *in vitro* model of disease represents a paradigm shift in the field of tissue engineering, which has focused almost exclusively on the goal of providing a living surgical replacement for diseased or damaged tissue.

Lee *et al*. have developed a simplified *in vitro* ECT mimicking the geometry of natural cardiac trabeculae as well as a spherical tissue chamber [160, 228]. ECTs have a thin cylindrical geometry constructed with cardiomyocytes and collagen, which retain the key aspects of normal cardiac physiology. This provides a unique experimental model for evaluating muscle physiology with a higher level of control than can be achieved with traditional animal or patient studies and have longer viability (3 weeks in culture) than isolated muscles, which remain stable for only up to 72 h. In addition, Lee *et al*. have successfully created a novel cardiac organoid model of heart disease using a simple cryo-injury method, originally developed for small-animal studies. They demonstrated variations in the regional contractile function, which is qualitatively consistent with the known behavior in the natural heart (Figure 13.5a–c) [160]. This ECT can serve as a useful *in vitro* model to evaluate cell-based therapies for post-MI repair and for long-term studies examining remodeling of myocardial tissue.

Similarly, de Lange *et al*. generated engineered tissue strips using wild-type mouse cardiomyocytes and a mouse model deficient in cardiac myosin binding protein C, which demonstrated the feasibility of using murine ECTs to study the effects of rare genetic mutations on cardiac contractile function [231]. To facilitate the use of engineered tissues for high-throughput drug screening platforms, Eschenhagen and colleagues have developed an automated miniaturized setup utilizing a 24-well format to culture fibrin-based, 3D engineered tissue constructs with neonatal rat cardiomyocytes (Figure 13.5d) [229] as well as with human ESC-derived cardiomyocytes [232]. This system allows long-term monitoring of the contractile force, electrophysiological properties, and response of ECTs to various drugs. More recently, a 3D paper-based model was developed by stacking layers of multiple cell types suspended in hydrogel, which mimicked

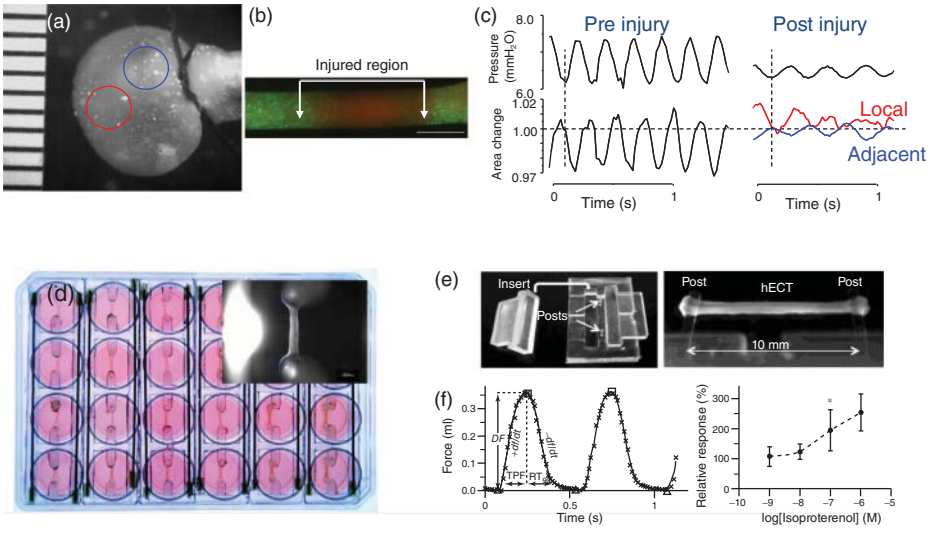

Figure 13.5 Advanced *in vitro* engineered cardiac tissue models from literature sources. (a) *In vitro* ECT of an MI model with titanium oxide makers on the surface for 2D strain analysis. Red circle indicates a local injury site, while blue circle indicates a remote uninjured site. (From Lee *et al.* [160].) (b) Montage of five digital fluorescent micrographs of an ECTs obtained 20 min post a cryo-injury. The injury site is clearly identified by dead cells labeled with etidium homodimer (red) surrounded by living cells stained with calcein (green). Scale bar 1 mm. (c) Pressure and area changes of ECTs, demonstrating regional variations of overall contractile function of the chamber pre and post injury. (d) Fibrin-based mini-engineered heart tissues (FBME) within a 24-well plate drug-screening platform. Inset shows an individual FBME in culture. (Hansen 2010 [229]. Reproduced with permission of Wolters Kluwer Health, Inc.) (e) An elastomer mold with integrated end-posts and removable inserts (left) for creating human ECTs (right). (Ma 2014 [230]. Reproduced with permission of Elsevier.) (f) Demonstration of the contractile function of human ECTs with a representative twitch tracing (left) and positive inotropic response with isoproterenol treatment (right).

aspects of cardiac ischemia [233]. This model provides an opportunity to screen new biopharmaceutical candidates efficiently in a more physiologically relevant environment through rapid data analysis at a low cost.

ECTs using human-derived cell sources [222, 232] offer additional species-specific advantages as *in vitro* preclinical models of the human myocardium. Costa and colleagues developed ECT strips using human ESC-derived cardiomyocytes embedded in type I collagen and Matrigel, exhibiting functional characteristics of the natural heart tissue [234], demonstrating its potential as a reliable preclinical model of the human myocardium (Figure 13.5e,f). Another 3D human cardiac tissue model has been developed by seeding iPSC-derived cardiomyocytes from healthy individuals and patients with long QT syndrome type 3 (LQT3) onto synthetic fibers [230]. It was demonstrated that the healthy and LQT3 tissues exhibited different levels of contractile abnormality and response to drug treatment. The generation of disease-specific 3D cardiac tissues opens exciting opportunities for the discovery and development of patient-specific therapies.

13.5 Conclusion and Future Directions

Despite promising advancements, there are still challenges that remain to be overcome in the field of cardiac tissue engineering. Numerous cell types have been explored, from adult stem cells including MSCs to pluripotent ESCs and iPSCs for cardiovascular tissue engineering applications. For potential clinical use, a large number of cells are required to efficiently repopulate the damaged tissue. Further studies are needed to improve the differentiation efficiency of pluripotent and multipotent stem cells into cardiovascular cells. Moreover, the degree of maturation and functional capacity of pluripotent stem-cell-derived cardiomyocytes should be carefully considered and examined prior to clinical application. Functional integration capacity of stem-cell-derived cardiomyocytes, exhibiting varying degrees of maturation, into the host also awaits further investigation. In the search for an ideal biomaterial candidate, complete characterization of the mechanical, electrical, topographical, and degradation properties is needed to determine the biocompatibility *in vitro* as well as *in vivo*. In addition, as a combination of physiological stimulations is considered beneficial in developing functional tissue, the degree of dynamic stimulation necessary for proper development of functional tissues should be examined. There is also a very restrictive size limitation for engineered tissues without the incorporation of vascular networks, which have only just begun to be developed. Finally, genetic disorders with more complex genomic mutations have yet to be explored via the generation of disease-specific iPSCs. The development of these cell lines will aid in the understanding of complex disease development *in vitro*. Further sophistication of *in vitro* model systems is also required to understand the biological mechanisms of cardiac regeneration and to develop therapeutic approaches to regenerate and restore function to human cardiovascular tissue following an injury. Although it is unreasonable to expect 3D tissue models to respond exactly as a native tissue, such models can recapitulate specific physiologic functions and be used in early stage research efforts to investigate the safety, efficacy, and mechanism of therapeutic agents.

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14

Approaches and Recent Advances in Heart Valve Tissue Engineering

Anna Mallone, Benedikt Weber, and Simon P. Hoerstrup

14.1 Introduction

This chapter aims at describing approaches and recent advances in heart valve tissue engineering (HVTE), opening a window to today's most relevant improvements and challenges and offering an outline on the state of the art in the field. As established in the last decades, the driving force behind the science of tissue engineering (TE) primarily resides in the detailed anatomical study of natural structures and in the desire to reproduce them as close as possible to the reality. Herein, and in accordance with this idea, first a description of the native morphology and functionality of heart valves is given, followed by a description of the different TE approaches and their rationale. An overview of "the tools of the trade" will follow, which will be mainly focused on polymeric materials both from synthetic and natural sources and on the different cell sources used for tissue-engineered heart valve (TEHV) fabrication.

14.1.1 Heart Valve Biology

Human heart valves are thin membranes, which are remarkable structures attached to the cardiac wall, opening and closing intermittently in order to move the blood flow unidirectionally. The pulmonary, aortic, tricuspid, and mitral valves are anatomically located within a matrix of dense, collagenous fibers recognized as the base of the heart [1]. This functionally and evolutionarily selected design ensures the necessary stability to the valvular leaflets, allowing dynamic valve function throughout their entire life. Human heart valves share several similarities in their microstructure, mainly encompassing their micro architecture and histological profile, modeled by evolution to ensure functionality in performing their fundamental role in cardiac function. To better exemplify the opening and closing behavior over a wide range of hemodynamic conditions, the aortic valve will be considered as a benchmark [2–4]. The latter shall be evaluated in its supporting unit, the aortic root, which sustains the aortic valve, providing the anatomic connection between the aorta and the left ventricle. The hemodynamic junctions between the left ventricle and the aorta are, instead, the valve leaflets. The leaflets are organized in a specific three-layered structure: the ventricularis, the spongiosa, and the fibrosa layer [1, 5].

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These composite paper-thin structures are coated by a tight and peculiarly organized endothelial cell layer connected by junction proteins, also found on endothelial cells elsewhere in the vasculature but with a different arrangement [6] (orthogonal with regard to the flow direction). Furthermore, the layers exhibit different microstructural compositions, reflecting their different mechanical characteristics [1] (Figure 14.1); the most external layer directly in contact with the inward blood current is the ventricularis, which is characterized by radially elongated elastin fibers, while the spongiosa, the mid layer, is mainly composed of glycosaminoglycans (GAGs) and collagen fibers, resembling the structure of the basal lamina. The fibrosa layer is rich in collagen fibers and forms the macroscopic bendings that extend parallely to the flexing edges of the leaflets. Here, the force applied to the leaflets is directly transferred to the walls of the aortic root [5, 7]. In a diastolic–systolic cycle, the kinetic energy of the blood flow is transferred to the leaflets, which open as a result of a passive, synergic movement. In diastole, the valve closes and the elastin in the ventricularis relaxes, transferring the main energy load to the collagen of the spongiosa and fibrosa layers. At the same time, it has been proposed that GAGs in the spongiosa, with their hydroxyl groups, bind and interact with water molecules, lowering the pressure differences applied on the two sides of the leaflets and mitigating the overall shear stress [8]. The leaflet-residing interstitial cell compartment

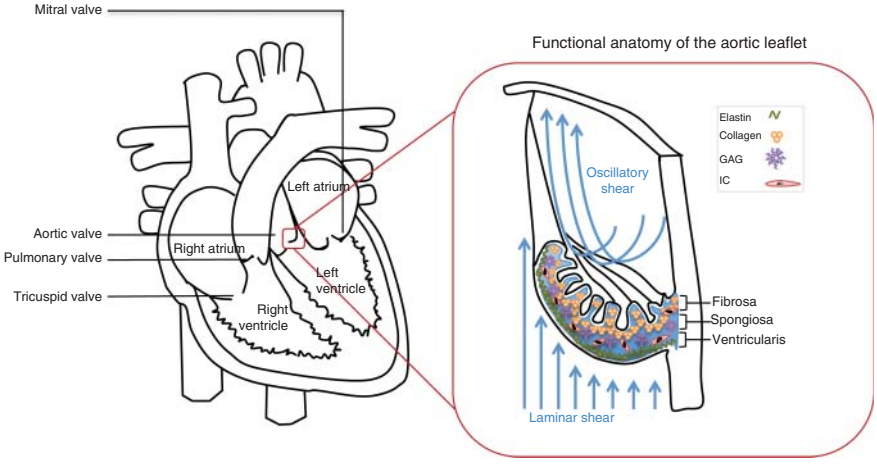

Figure 14.1 Schematic representation of the human heart and aortic valve leaflet. The arrows indicate the position of the four heart valves. In the red panel is given a focus on the functional anatomy of the aortic valve. The extracellular matrix components are shown to be organized in the typical tri-layered structure. In the ventricularis, the radially elongated elastin is considered to be the predominant component, while the ECM of the spongiosa is mainly enriched in GAGs. Valvular interstitial cells (ICs) and collagen are generally widely distributed within the three layers, but the latter shows a peculiar circumferential alignment in the fibrosa. Insights on the biomechanical forces acting on the liflet walls are provided. During the systolic phase, the aortic liflets are pushed in their open conformation and are exposed to both laminar and oscillatory shear stress. In diastole, the leaflets are stretched and closed, allowing the filling of the chambers and preventing blood back-flow.

has been widely investigated: not only smooth muscle cells but also fibroblasts can be found, the latter being considered to be mainly responsible for the extra cellular matrix (ECM) deposition and maintenance. Interestingly, about 60% of the fibroblasts are classified as myofibroblasts, a hybrid cell type expressing markers from both smooth muscle cells and fibroblasts [9].

14.1.2 Heart Valve Disease and Valve Replacement

Each heart valve has dual functions: open correctly so that blood can flow out of the chamber, and close properly so as to avoid back-flow of blood from the ventricles to the atria. When the valves and chambers fail in ensuring proper flow, clinical symptoms may eventually develop. As shown in recent studies, valvular heart disease has a high incidence, especially in the developed countries, with a dramatically growing number of patients [10, 11]. It is necessary to point out that medical treatments of valvular disease have improved with time, increasing the life expectancy and ameliorating the life perspectives of patients. The best treatment for late-stage valvular dysfunctions at the moment is heart valve replacement, with a constantly increasing number of cases, which is expected to triple in the next 50 years [12, 13]. Nowadays, various alternatives for valvular replacement are available, but the best option must be chosen considering various important parameters, such as thromboresistance, hemodynamics, implantability, durability, and quality of life (Figure 14.2). A good example of valvular substitutes is mechanical devices mimicking the leaflets in a pivot cavity [14]. Despite their excellent structural durability, these devices are prone to thromboembolic

	Bioprosthesis	Mechanical heart valve	Tissue engineered valve
Durability	✗	✔	✔
Hemodynamic profile	✔	✗	✔
Thromboresistance	✔	✗	✔
Quality of life	✔	✗	✔
Remodeling abilities	✗	✗	✔

Figure 14.2 Comparison of the today's alternatives for valvular replacement with tissue-engineered valvular grafts. Different parameters are considered: durability, thromboresistance, hemodynamic profile, quality of life, and remodeling abilities. Among the different options, bioprosthesis appears to be optimal in terms of hemodynamic profile and thromboresistance but still shows high tendency for structural deterioration and calcification. Furthermore, bioprostheses do not exhibit the remodeling abilities necessary to adapt to the changing organ anatomy of a child. In the future, these limitations will be overcome by introducing tissue-engineered valvular grafts in clinical practice.

complications caused by high shear stress and non-physiological flow profiles [15, 16]. Therefore, their implantation is followed by life-long anticoagulation therapy with related hemorrhagic complications. Other options are bioprosthetic valves derived from animals or directly from humans (xenografts or homografts), which can simulate the flow patterns of the native heart valve, with low risk of thromboembolism and allowing life without anticoagulation treatment. Nowadays, these grafts are modified with a high-radial-strength frame in order to promote full expansion of the leaflets. Hemodynamic features are optimized via low rates of structural valve deterioration by using flexible acetal homopolymer stents [17] or other stentless valves naturally designed to offer hemodynamic conditions close to the physiological milieu [18]. The disadvantage of these devices is mainly related to their limited durability, high impact by calcification, or deterioration [19]. The best option for valvular replacement available at the moment is cryopreserved valves obtained from human donors [20]. On the other hand, these can be considered a less suitable option because of the extreme scarcity of donors worldwide. Even though replacement surgery is effective and the heart valve substitutes available nowadays show excellent durability [11], the absence in remodeling capabilities is indeed remarkable upon transplantation. As one can imagine, there is constant search going on for alternative heart valve replacement. TE technology can, on one hand, reduce the costs of reoperations [21, 22]; and on the other hand, it can allow implantation of malleable grafts that are able to grow and adapt even to the changing organ anatomy of a child. One of the main advantages of the tissue-engineered valvular grafts is the ability of self-healing, remodeling, and lack of thrombogenicity, which are advantageous features when compared to the ones shown by valvular replacement listed above. The bioengineered grafts would potentially be able to adapt to the patient's growth behavior and to cope with a variable and constantly alternating hemodynamic environment (e.g., due to physical activity).

14.2 Principles of Tissue Engineering: Shaping the Valvular Construct

TE is the science of tissue manufacturing, which is deeply rooted in the fields of biotechnology, cellular biology, and biochemistry and combining them for the purpose of multicellular system manufacturing. The key steps in today's HVT are (i) cell isolation from the patient, expansion, proliferation; (ii) cell seeding and tissue formation on a biodegradable support matrix shaped like a trileaflet heart valve; (iii) ECM deposition with concomitant scaffold degradation; and, lastly, (iv) implantation of the valvular graft with further *in vivo* tissue remodeling [23–25]. These four key points can be fulfilled following different culturing techniques and using *in vitro* bioreactor systems, with the final goal to stimulate tissue formation by reproducing physiological mechanical stimuli given by blood pressure and flow, an approach used for producing bioengineered artery models [26]. The four-step procedure is commonly recognized as the *ex vivo* (or *in vitro*) tissue engineering strategy, but different approaches for valve-substitution TE have

been additionally investigated. Nowadays, a second promising approach is the *in vivo* (or *in situ*) strategy based on the intuitive concept that the most suitable scaffold for an engineered tissue should be the ECM of the target tissue itself in its native state. Following this idea, natural tissue-derived matrices obtained from decellularized tissues [27] as well as synthetic scaffold matrices seeded with cells are directly implanted into the patient. This allows the cells spontaneous transmigration and the active recruitment of circulating endogenous cells to reach an *in vivo* repopulation of the tissue-derived scaffolds [28–30]. Nowadays, the *in vitro* production of heart valves through conditioned systems, namely bioreactors, is the most widespread methodology aiming at stimulating adequate cellular proliferation and ECM deposition via a combination of both biochemical and biomechanical stimuli. The second type of stimuli implies the introduction of a medium flow rate as well as pressure variation within the system for an alternation of static/dynamic intermittent cycles in order to better mimic the human physiological environment. This pressure variation has been shown to be beneficial for myofibroblasts seeded onto the scaffolds, allowing their alignment in the biomaterial meshes during the early phases of tissue organization [31, 32]. In 2008, Engelmayr and collaborators introduced the flex–stretch–flow bioreactor system into the common TE practice [33], proving for the first time the beneficial effects of these mechanical constraints on TEHV formation and the possibility to obtain constructs with mechanical properties similar to those of the native ones.

14.3 *In Vitro* Bioengineering of Heart Valves: Scaffold Materials

The design and the chemical composition of the scaffold matrices employed and the valvular fabrication process can be considered as critical steps for the success of the biological substitute production in both the *ex vivo* and *in vitro* approaches. Therefore, the choice of the best material should be done by appreciating these criteria together with other peculiar parameters, for example, cell types used for tissue production (in the *ex vivo* strategy) and the ability of scaffold degradation and the concomitant ECM deposition [20, 34]. In addition, the biocompatibility of the scaffold is crucial for a successful TE graft production [35, 36]. The predominant and most commonly used scaffolding approach supports cell growth on interconnected pore networks of biodegradable scaffolds [30], allowing nutrient supply and the release of waste compounds produced by cell metabolism and cycles. Today's attempts in developing scaffolds fulfilling strict requirements for valvular tissue engineering are proceeding in two directions, which are based on the use of more or less complex polymers from different sources (recently reviewed by Generali *et al.* [37]): (i) fully synthetic scaffolds, or (ii) biological matrices. An overview of the options of biomimetic and biodegradable scaffolds is given in the following paragraphs (Figure 14.3).

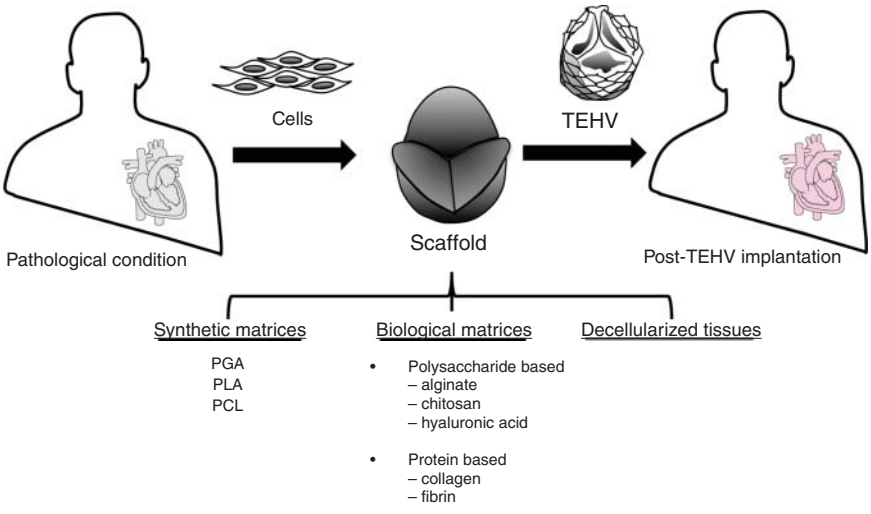

Figure 14.3 Schematic view of the "tissue engineering" approach and the scaffold matrices used for this purpose. Cells are seeded on support matrix shaped like a tri-leaflet heart valve. In the figure, peculiar scaffold materials for tissue engineering are summarized, which include (i) synthetic matrices, (ii) biological matrices, and (iii) decellularized tissues. The final goal is the production of a TEHV characterized by optimal durability, thromboresistance, and hemodynamic profile.

14.3.1 Polymeric Scaffolds from Synthetic Sources

Polymeric scaffolds are currently the most used matrices for *ex vivo* HVT mainly because of their high durability, high reproducibility, and low cost, as well as their remarkable mechanical strength and predictable physical properties. The latter strictly relies on the local scaffold stiffness, which is also considered one of the main modulators for ECM production and remodeling; the cells indeed are exposed to different local stresses according to the scaffold's biomechanical profile. Many studies have investigated various fabrication techniques in order to generate novel structures for TE applications, such as polymers assembled with porous [38, 39], woven, nonwoven, and electrospun designs [40, 41]. A large variety of biodegradable synthetic polymers have been synthesized and historically proposed as suitable TE matrices [42], such as polyhydroxyalkanoates (PHAs), polyglycolic acid (PGA), polylactic acid (PLA), polyglactin (PLGA), poly(vinyl alcohol), polycaprolactone (PCL), and poly-4-hydroxybutyrate (P4HB) [37], all suitable for tissue engineering, being biodegradable and varying in manufactured shapes.

14.3.1.1 Aliphatic Polyesters: PGA, PLA, and PCL

Though materials with a lower tendency for calcification, such as polytetrafluoroethylene (PTFE), polyethyleneterephthalate (PET), and polyurethane (PUR), were initially used for the production of bioengineered vessels [43], these show many disadvantages as they are nondegradable, rigid, and prone

to thrombogenesis, and therefore are useless in terms of both vascular and valvular graft production when compared to the newer polymers available on the market. Nowadays, a large number of flexible and biodegradable polymers can indeed be found in the literature; among these, aliphatic polyester composite structures are considered one of the best options for TE. One of their main attractive features is their relative resistance to rapid hydrolytic degradation, a remarkable point since living systems are cultured in aqueous media [44]. PGA, PCL, PLGA, and PLA are all synthetic polymers approved for clinical use by the Food and Drug Administration (FDA) and belong to the aliphatic polyester family; their erosive degradation originates from the presence of ester bonds in their polymeric backbone and, once used for TE purposes, these compounds undergo macromolecular degradation by random hydrolysis and concomitant enzymatic esterase activity [45, 46]. It is necessary to underline that monomers produced as a result of the degradative process are nontoxic and can be easily bioresorbed by cells and reintroduced into normal cellular metabolic pathways (e.g., oxidized to pyruvic acid). Therefore, these compounds are considered optimal starting materials for the art of *in vitro* tissue shaping.

14.3.2 Polymeric Scaffolds from Biological Sources

Biologically derived polymeric scaffolds are used alone, as plain material, or blended with synthetic polymers as described in the previous paragraph (e.g., PLGA) [47, 48]. The large family of bioscaffolds includes polysaccharides such as alginate, chitin/chitosan, hyaluronic acid derivatives, and proteins such as collagen, fibrin, and silk, as well as polyhydroxyalkanoates (PHAs) and decellularized ECM [49–52]. Huge progress has been made using these biomaterials; recently, Duan and collaborators managed to bioprint in three dimensions living alginate/gelatin hydrogel aortic valves, concomitantly incorporating within the root sinus both smooth muscle cells (SMCs) and aortic valve leaflet interstitial cells (VICs) [53]. Despite the great efforts made in introducing these natural polymers in the TE field, synthetic materials are still considered a better choice due to their better strength and durability. Moreover, a parallelism with the functional capabilities of natural tissues is inevitable and demanded; still, the polymeric scaffolds derived from biological sources have to be mechanically improved mainly because of their pronounced weakness, an important point when considering the pressure conditions experienced by a native human heart valve under physiological conditions. Of great relevance in the field of HVT are the commercially produced PHAs [54], which are microbially derived polyesters that can be generated with desired monomer composition, degradation rate, and adjusted production times by engineering and tuning the microbial (e.g., *Escherichia coli*) metabolic pathways of interest [55]. This family includes poly-3-hydroxybutyrate (PHB), copolymers of 3-hydroxybutyrate and 3-hydroxyvalerate (PHBV), poly -4-hydroxybutyrate (P4HB), copolymers of 3-hydroxybutyrate and 3-hydr oxyhexanoate (PHBHHx), and poly-3-hydroxyoctanoate (PHO) [37]. PHO and P4HB have been largely investigated and utilized in TE to create trileaflet heart valves [38, 39, 42]. These polymers are generally combined with other synthetic polymers (e.g., PGA) for the final manufacture of a hybrid scaffold; P4HB coating

of PGA meshes, indeed, has been reported in the literature as a successful and promising combination of biophysical material characteristics, coupling the high porosity of PGA with the thermoplasticity of P4HB [27, 56]. Furthermore, PHA family members can be also used for covering xenogeneic decellularized aortic valves [57, 58]. Unfortunately, an unpleasant drawback of these compounds is their slow degradation, which can ultimately impair tissue growth and the expected histologic composition of the final bioengineered construct.

14.3.2.1 Polysaccharide-Based Scaffolds

Polysaccharides are one of the most accessible, cheap, hemocompatible, and nontoxic sources of materials for TE. The monosaccharide units constituting the long polysaccharide chains can be tuned. By varying the final monosaccharide composition, interesting physical properties are transferred to the scaffold structure, such as solubility or gelation [37]. Because of their high flexibility, polysaccharides have been largely used for a variety of purposes in TE, for example, blood vessels, myocardium, heart valves, bone, and other tissue-engineered constructs, but also for encapsulation and delivery of pancreatic islets and ovarian follicles [59]. Among the large polysaccharide family, alginate and chitosan are today's best investigated and used materials for TE and the production of TEHV [53]. Alginate is a biodegradable and bioresorbable polysaccharide derived from brown algae; it is composed of repeating guluronic and mannuronic units that can be easily cross-linked into a solid gel form at room temperature by simply adding bivalent metallic ions such as Ca^{2+} or Mg^{2+}. Its mechanical and physical behavior are highly influenced by the percentage of different monomers in the final construct and by their chain length. Some disadvantages of alginate have been monitored and reported in literature: that is, its mechanical weakness and the poor cell adhesion on the meshes. However, some of these obstacles, like the low cell adhesiveness, have already been overcome [60]. A second polysaccharide of interest is chitosan, derived from chitin, which a biopolymer found, for example, in the exoskeletons of insects. Unfortunately, as with alginate, its use as scaffold for TE is limited because of its poor mechanical properties, and therefore studies on chitosan fiber reinforcement has been recently carried out to increase the polysaccharide's strength and stiffness in proportion to the fiber/scaffold mass ratio [61], reducing the possibility to use this polymer for TEHV. In addition, polysaccharides have been coupled with other polymers – for example, the synthetic biomaterials considered in the previous paragraph – in order to increase chitosan's strength and cell-adhesive property [62]. In the long run, scaffolds as similar as possible to the native ECM environment will be used for TEHV. From this point of view, especially the investigation and study of the polysaccharide hyaluronic acid (HA) will be largely considered for TE applications. HA is commonly found in the ECM of connective tissues, it is a high molecular weight, non-sulfated GAG, and it has been coupled with other biomaterials, for example, alginate, to produce 3D bioprinted hybrid scaffolds for TE purposes [63]. The idea of using the non-thrombogenic, organizational, structural, and space-filling physiological properties of HA for TE is outstanding; on top of this, its mechanical weaknesses have been recently overcome by copolymerizing it with other relevant synthetic or natural polymers [64]. Interestingly, the small disaccharides

derived from HA degradation, which are released concomitantly with tissue formation, were shown to increase ECM production [65], an advantageous point for the improvement of the mechanical features of HA and for its potential use in TEHV manufacturing.

14.3.2.2 Protein-Based Scaffolds

Protein-based scaffolds not only have the advantage of mimicking several features of the ECM but also have the potential to positively influence the growth and native organization of cells during tissue formation [5]. Collagen (mainly collagen type I) and fibrin are today's best options among protein-based matrices for TE and, especially, for TEHV production because of their bioresorbable properties and malleability. Furthermore, collagen, thanks to its fibrillar nature, can be shaped according to structural requirements into sponges [66], sheets [67], or also gels [68] and fiber-based scaffolds [69]. These polypeptide scaffolds are characterized, on one hand, by their low degradation ability and, on the other, by their scarce availability, since collagen is extremely difficult to obtain from patients. Fibrin is composed of fibrinogen units and, like collagen, can be derived directly from the patient, carrying the advantages of autologous scaffold production [70]. Fibrin is once again an extremely malleable polymer possibly modified in hydrogels, beads, and glue [71]. Degradation of these scaffolds can be slowed down or stimulated by varying the amount of fibrinolysis blocking agents in the medium, such as the serin protease inhibitor aprotinin [50]. The high softness, the tendency to shrink, and the rapid degradation are still major issues to be overcome. Much effort has been made in the field, for example, the use of poly(L-lysine) chemical treatment of the scaffold, in order to improve the mechanical properties, but still more has to be done in view of the use of this interesting biomaterial for *in vitro* bioengineering of heart valves.

14.3.3 Scaffolds Derived from Decellularized Tissues

The excursion on the different scaffolds used (or possibly used) for *in vitro* engineering of heart valves ends at decellularized tissue-derived matrices. The goal of a good bioresorbable scaffold, besides providing a stable substrate for the growth of cells, is to encompass as many features of the tissue-specific ECM as possible, allowing cell differentiation with concomitant tissue morphogenesis [72]. The use of native-tissue-derived templates composed of structured ECM proteins directly organized in their native architecture is inevitably seductive; additionally, the idea that hemodynamic and biomechanical properties are already similar or even identical to those of their native counterpart is even more interesting in terms of TE applications [24]. One of the main issues when speaking about tissue-derived ECM scaffolds for TE is the tissue donor source. The concerns about using ECM directly derived from either xenogeneic or allogeneic donors are mainly related to the possible immune reactions that may be evoked. Other problems are related to the high risk of disease transmissions, for example, prion diseases and retroviruses, due to the use of living substrates [73]. Therefore, a strategy for easy and safe retrieval and use of tissue-specific ECM as starter matrices has been recently optimized, which is based on living tissue decellularization; the use of this technique has been proven to decrease the immunological

response without limiting the remodeling capacity, thereby positively impacting the long-term durability of the TE constructs [27]. Homografts or xenografts (the latter obtained from animal sources) are today's most used choices as scaffold matrices. Furthermore, Weber *et al*. investigated the repopulation capacity of decellularized, tissue-engineered heart valves (dTEHVs) in a nonhuman primate model in up to 8 weeks of follow-up studies. They showed for the first time a remarkable and rapid cellular repopulation of the decellularized constructs and high remodeling capacities, proving at the same time how such an approach would be feasible for translation into clinical practice. The different decellularization protocols described in the literature are set up to ultimately preserve the resident ECM with its mechanical characteristics [74–76] and to remove all cells entrapped in the tight, low-porosity, and branched protein network [77]. Given the complex ECM structure and the desire to preserve matrix integrity and functionality as much as possible and to maintain the biosafety of the construct, some precautions have to be inevitably taken. Those are, for example, the use of protease inhibitors [78] or the addition of a nucleic acid digestion step to the decellularization protocol in order to get rid of residual and harmful RNAs and DNAs [79].

14.4 Cells for Valvular Bioengineering

One of the most important variables to be considered for a fruitful *in vitro* tissue-engineered valvular production is the cell type that is seeded onto the trileaflet molds [80]. Further points are the cell-type composition, meaning either single cell type or in coculture (e.g., myofibroblast/fibroblast-like cells and endothelial cells), and the regeneration ability of the latter to interlacing tissues and link to the ECM. The optimal cells to be used for TEHV manufacturing should be, indeed, first, easy to be isolated from the donor, ideally via a noninvasive procedure or derived from surgical waste material. Second, cells should be of autologous origin, nonimmunogenic, and with pronounced plasticity. Despite the difficulties in finding the cell type encompassing those ideal characteristics, many improvements have been made through research on the best candidate [81], including the attempt to understand the real requirements of scaffold seeding. Recent discoveries have proven that circulating blood cells or cells from adjacent tissues can easily migrate and grow within the scaffold *in vivo* [82, 83]. However, considering the case of sole scaffold implantation, further challenges/problems have to be approached, such as the issue of immune response activation by a foreign material [84, 85] or the danger associated with molecular pattern molecules (DAMPs) that are released into extracellular space by necrotic cells within the tissue [85, 86]. A wide panel of cell types have been investigated for TE, such as vasculature, adipose tissue, umbilical cord, chorionic villi, amniotic-fluid-derived cells, or cells directly differentiated from the patient, that is, induced pluripotent stem cells (iPSCs) [5]. Here we focus on the most used and most promising sources for valvular bioengineering.

14.4.1 Cells Derived from Vasculature

Cells harvested from vascular donor tissues (e.g., from peripheral arteries) and mixed vascular cell populations are so far the cell types preferred for scaffold seeding in TEHV production experiments [13]. Usually, two main cell lines are isolated from the donor tissue: myofibroblasts (or fibroblast-like cells) and endothelial cells. The first are responsible for ECM deposition and assembly, while the second are used to produce a tightly interconnected and confluent layer [87]. The advantage in choosing these cell types as a seeding option is that they can be directly harvested from the vessel of the recipient (patient). However, this implies the use of an invasive procedure. For this reason, much effort has been directed toward the use of circulating endothelial progenitor cells (EPCs). EPCs were identified by Asahara *et al*. [88] in peripheral blood, and subsequently proposed as a potential cell source for TE. The use of these cells allows for a milder and noninvasive isolation procedure, making them a highly attractive alternative. Nevertheless, the endothelial progenitors had already been employed for *in vitro* production of TEHV and their potential to provide both interstitial and endothelial function had been confirmed [89].

14.4.2 Umbilical-Cord-Derived Cells

The human umbilical cord protects and insulates umbilical blood vessels, namely one vein and two arteries. These are embedded in mucoid Wharton's jelly, a substance of gelatinous consistency derived from extra-embryonic mesoderm. The latter is today recognized as a great source of mesenchymal progenitor cells, which are peculiar adult stem cells retaining the potential of multilineage differentiation and of particular interest for TE applications [90]. The umbilical cord is one of the best cell sources for HVT, given the vast cellular phenotypes that can be ultimately retrieved from it. Not only Wharton's jelly mesenchymal stem cell (MSC) progenitors can be isolated from umbilical cord, but also endothelial cells (ECs) and myofibroblasts from both arteries and vein and EPCs from the umbilical cord blood [91, 92], therefore revealing it as the best cell source available so far. An additional advantage in comparison to the vascular-derived cells is the possibility of umbilical cord cryopreservation, providing a life-time, expandable cell source.

14.4.3 "Stem Cells": A New Source for Valvular Bioengineering

Stem cells are one of today's most attractive option for HVT [93] mainly because of their high growth rate and their potential allowing for fate-tuning toward different cell types such as endothelial cells, fibroblasts/myofibroblasts, and SMCs [94]. Mesenchymal stem cells, for example, share vast similarity in phenotype to valvular interstitial cells and were proven to stimulate *in vivo* endothelialization and recruitment of autologous host cells by paracrine secretion [95]. Bone-marrow- and adipose-derived stem cells (BMSCs and ADSCs), among the others, have been widely studied as amenable cell sources. They are currently used for THEV production, and show satisfactory *in vivo* functionality, native like histological profile, and ECM organization [96–99]. MSCs retain indeed a

higher cellular potency and can be isolated without severe interventions (by a simple puncture, e.g., of the iliac crest), which makes them highly comparable with vascular-derived endothelial cells.

14.4.3.1 Pluripotent and Induced Pluripotent Stem Cells

The most important goals in tissue engineering are the prevention of *in vivo* tissue deterioration and the production of a valvular graft with intrinsic regeneration capacity. The best candidates in these terms are embryonic stem cells (ESCs). ESCs are pluripotent, undifferentiated, and self-renewing cells, able to generate all terminally differentiated adult cell types, with the exception of extraembryonic tissues [100]. During embryo development, in the blastocyst stage, a small number of cells constitute the inner cell mass (ICM), which then further differentiates into the three germ layers (endoderm, mesoderm, and ectoderm) that are considered to be the developmental starting point for adult tissues formation. ESCs are directly derived by the expansion of the ICM *in vitro*. Despite the great interest and the initial success, the use of ESCs is constantly fraught with ethical issues, together with immunogenic and tumorigenic problems [101]. In the past years, another interesting cell source has become available thanks to Takahashi and Yamanaka. They demonstrated the possibility to reprogram terminally differentiated cells, such as mouse fibroblasts, to a multipotent status, obtaining iPSCs [102]. The use of iPSCs in TE could potentially overcome all the ethical problems due to their nonembryonic origin. However, since they retain a gene expression panel close to that of ESCs, they are still associated with a high risk of tumor formation. The research in this field is now focusing on brand new transdifferentiation approaches, allowing the direct production of the desired cell type (e.g., endothelial cells) from another (e.g., fibroblasts) by adjusting and fine-tuning the biochemical environment, overcoming the harmful iPSC transition step [103].

14.5 Challenges and Limitations

The art of *in vitro* valvular bioengineering has undergone remarkable changes since its first steps in the early 1990s and, like never before, is grasping for the transition to clinical reality. Given the tight link between morphology and functionality, today's biggest goal in TEHV fabrication is to mimic the shape and biomechanical function of native heart valves and to provide a native-like graft sustaining long-term functionality after implantation. Nowadays, TE represents the most promising and exciting approach for the generation of functional replacements of cardiac valves being able to grow and adapt to the changing and challenging anatomy of patients with congenital heart defects. Despite the excitement, many difficulties still have to be surmounted, including (i) optimization of the scaffold material, (ii) discovery of the ideal cell source for *in vitro* seeding and *in vivo* survival, (iii) overcoming ethical and logistic hurdles related to the isolation, culture, and use of autologous cells, and (iv) reduction of dangerous phenomena such as thrombosis, massive inflammation, or calcification.

14.6 Conclusion and Future Directions

The biodegradable synthetic polymers described in this chapter provide multilevel advantages over biological materials mostly in terms of degradation speed, reproducibility, and mechanical properties. Despite this, the best scaffold material remains the tissue-specific ECM, and in the future either autologous or synthetic ECM may be used for *in vitro* TE. This idea is supported by some promising results obtained with reseeded, decellularized allografts after 10 years of follow-up studies [104]. These advances are unfortunately limited by the prominent dichotomy of high demand and lack of donors. Therefore, much effort has been focused on finding an off-the-shelf alternative [27] allowing the production, storage, and availability of valvular constructs in large numbers. However, the first clinical trials using nonseeded valves underlined the weaknesses of this approach, which resulted in heart valves with structural failure, mainly due to strong foreign body reactions [83]. In this regard, experimental approaches have been established both for the investigation and the production of heart valves and vascular grafts. Only future will tell whether these efforts will enable TE a rapid translation into clinical phase.

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15

Musculoskeletal Tissue Engineering: Tendon, Ligament, and Skeletal Muscle Replacement and Repair

Jorge A. Uquillas, Settimio Pacelli, Shuichiro Kobayashi, and Sebastián Uquillas

15.1 Introduction

Soft tissue injuries are an important clinical problem for trauma, orthopedic, and reconstructive surgeons around the world [1]. Figures from tendon, ligament, musculotendinous interface, and articular capsule injuries are high: it is estimated that nearly 32 million Americans experience musculoskeletal trauma annually [2]. Of those, 325 500 suffer soft tissue disorders that require hospitalization with an associated estimated cost to the society of $23.3 billion per year [3]. However, the intangible costs such as personal suffering and low quality of life are the most difficult problems to bear for patients suffering soft tissue disorders [4]. In the case of tendons, ligaments, and skeletal muscle, the causes for trauma can be associated with lacerations, contusions, ischemia, sport accidents, or recurring and intense use [5–8]. Despite the injury source, the body has the ability to initiate a well-orchestrated process of recovery and deposition of scar tissue that evolves through three well-defined phases: hemostasis with inflammation, proliferation with fibroplasia, and remodeling with maturation [5, 7, 9, 10]. Nonetheless, the result of the healing process varies substantially from patient to patient, and relies heavily on the injury characteristics [11–13]. In fact, in certain cases the natural healing process cannot restore the normal functional capability of musculoskeletal tissues, removing their capacity to no longer bear daily loads. Hence, orthopedic surgeons have developed a wide variety of surgical procedures and grafting techniques to fix injured soft tissues. In the case of tendons, the traditional method of repair is the use of core sutures or wire loops to hold both ends of lacerated tendons together [14–17]. For example, in Achilles tendon repair, Kirschmayer core or Kessler-type sutures, combined with cross-switch epitenon [18] or apposition sutures [19], have proven to be a good alternative treatment since the bridged tendon can withstand weight-bearing exercises shortly after surgery. Evidence has demonstrated that the number of suture knots used to connect the lacerated tendon when combined with rehabilitation has no effect on the strength at the repair site [20]. Nonetheless, suture knots are normally the weakest points, especially when chronic degenerative injuries are being treated [5]. Specifically, in 38% of chronic cases, important tears appear because

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Table 15.1 Success criteria for the design of tissue-engineered tendons and ligaments.

What makes a good soft tissue-engineered bioscaffold?
1. Tissue-engineered tendon/ligament/muscle should withstand *in vivo* unidirectional stresses without deformation or rupture [33]
2. Tissue-engineered tendon/ligament/muscle should allow rapid proliferation rates and guarantee survival of any cells seeded on them [34–36]. Additionally, tissue-engineered skeletal muscle is required to anastomose with the host vascular system for efficient exchange of nutrients, O_2, CO_2, and waste by-products [37]
3. The degradation rate of tissue-engineered tendon/ligament/muscle should match *in vivo* growth rates of endogenous neo-tendon without compromising the functionality at the repair site [38]
4. Tissue-engineered tendon/ligament/muscle should possess sufficient porosity (200–400 μm) and pore interconnectivity to allow the adhesion, infiltration, and proliferation of cells, and permit the expression of tenogenic, ligamentogenic, and musculogenic phenotypes [37]
5. Tissue-engineered tendon/ligament/muscle should be reproduced in large quantities, while following sterile good manufacturing practices (GMP)
6. Tissue-engineered tendon/ligament/muscle should have good handling properties so the surgeon can trim the bioscaffold on site to fit it into the defect site [39]

of excessive tension in the sutures, tendon weakening, and muscle atrophy [21, 22]. Regarding, instead, skeletal muscle, strategies designed to fix the musculotendinous junction of a gluteus medius tear often require endoscopic surgery [23].

The consequences of surgical procedures to repair soft tissue lesions often promote local tendon weakness, periods of inactivity and physical therapy, and the potential need of a second surgical intervention due to reinjury [24–26]. Thus, surgical techniques combined with early postoperative tendon mobilization, electrical stimulation, ultrasound evaluation, and injectable growth factors (GF) have improved soft tissue strength and restored joint stability [27, 28]. Specifically, early mobilization (i.e., physical rehabilitation) has been shown to improve the mechanical properties of the injured tendon, and in some cases has even enhanced tendon-gliding function [29, 30]. Nonetheless, the frequency and cost of soft tissue injuries, combined with suboptimal results of surgical interventions, clearly indicate the need of a new and inventive approach to overcome these problems. Tissue engineering represents a valuable alternative to traditional methods for the treatment of soft tissue injuries [7, 31, 32], because it has unveiled the possibility to create functionally robust tendon-like constructs.

A good tissue-engineered tendon/ligament/muscle has to fulfill the design criteria explained in Table 15.1. In clinical settings, successful tendon/ligament/muscle tissue-engineered scaffolds should provide excellent structural support for cells and become a source of biological factors (GFs and cytokines) to accelerate repair.

15.2 Biology of Tendon, Ligament, and Skeletal Muscle

15.2.1 Structure and Biology of Tendon

Tendons are connective tissues that link muscles to bones, allowing the transmission of force from muscles to skeletal structures for locomotion and joint stability [40, 41]. Tendons have a hierarchical structure that begins with collagen molecules, tendon fibrils, tendon fiber bundles, tendon fascicles, and ultimately tendon units [40] (Figure 15.1). This hierarchical architecture is sufficiently strong to resist tensile loads along the long axis of the tendon structure. The organization of the molecules in the extracellular matrix (ECM) of tendons at the micrometer and nanometer level is responsible for the physiological function and mechanical strength of tendons.

At the microscale, tendons are crimped (Figure 15.2a) with a waveform appearance, which plays an important role in the mechanical properties of this tissue (Figure 15.2b). The angle and length of the crimp pattern are important in the mechanical properties of tendons. Fibers with a small crimp angle are mechanically weaker than those with larger crimp angle [44].

The molecular composition of tendons is important in providing mechanical robustness and elasticity. Collagen type I protein is the most abundant molecular component in tendons [45, 46]. Collagen type I constitutes approximately 60% of the tendon's dry weight, and corresponds to 95% of the total tendon collagen content (Figure 15.3). The other 5% corresponds to collagen types III and V [40]. Other types of collagens such as type II, VI, IX, X, and XI are present in tendons at very low quantities [47]. Collagen type I molecule self-assembles into highly organized and aligned collagen fibers [48, 49]. These anisotropic collagen fibers are cross-linked *in situ* and confer tendon its high tensile strength [50].

Proteoglycans in the ECM enhance the mechanical properties of tendons (Figure 15.3). For example, aggrecan helps to retain water, increasing tendon's resistance to compression [51]. Decorin's function within tendon's ECM is to facilitate fibrillar slippage during mechanical deformation along the long axis of the tissue [52, 53]. Tendons also contain glycoproteins, including fibronectin and tenascin-C. Fibronectin, an adhesive glycoprotein located on the surface of collagen, is responsible for the regeneration and repair of tendons [54, 55]. Fibronectin also provides cell attachment sites, which prevent cell detachment during gliding friction [56]. Tenascin-C is another component of the ECM of tendons and contributes to their mechanical stability as it interacts with collagen fibrils and decorin [57]. Tenascin-C is not widely expressed in healthy musculoskeletal tissues, but is locally overexpressed in sites subjected to demanding mechanical forces or where elasticity is required [58]. Tenascin-C is an elastic protein, just like elastin – another important component of tendon [59] that comprises approximately 2% of the dry weight of tendons.

Tendons, which possess less vasculature than muscles or bone, also have low cellularity compared to tissues such as cartilage or muscle [60]. However, two major cell types coexist in tendons: tenocytes and tenoblasts. Tenoblasts, with spindle-shaped morphology, are immature tendon cells with high metabolic activity. The length of tenoblasts ranges from 20 to 70 μm [61]. They are the

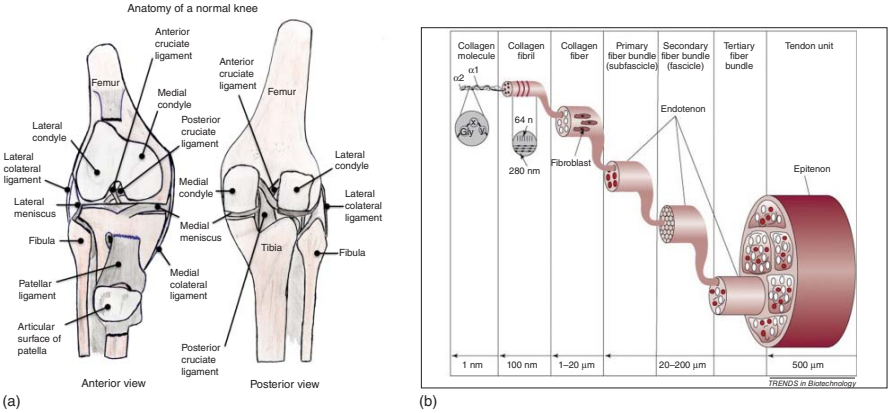


Figure 15.1 (a) Anatomy of the knee. (b) Hierarchical structure of tendons and ligaments. Each collagen molecule is made of three peptide chains, coiled into a triple helix, that form the 280-nm-long molecule. Collections of collagen molecules aggregate in longitudinal and lateral directions to form collagen fibrils. Collections of fibrils form collagen fibers. Collections of fibers form tendon fiber bundles, and several fiber bundles form larger secondary and tertiary bundles wrapped by the endotendon. A tendon unit is formed by the aggregation of tertiary fiber bundles wrapped by the epitendon. (Reprinted with permission from [42].)

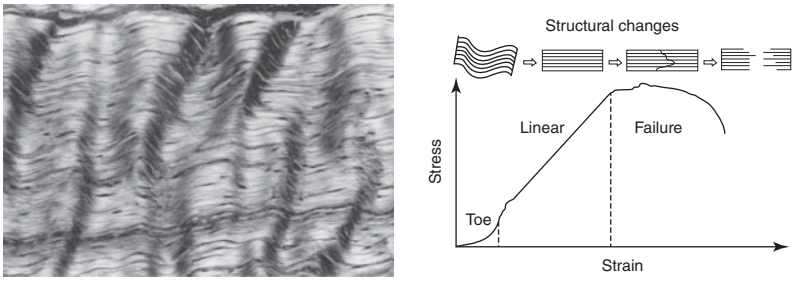


Figure 15.2 (a) Crimp pattern in tendons and ligaments. (Reprinted with permission from [43]) . (b) When the tendon/ligament is mechanically elongated along the direction of the collagen fibers, structural changes occur. The monotonic stress versus strain curve indicates a toe region, where the tendon/ligament is crimped. The linear region is a state where the collagen fibers are under tension. The transition between the linear and failure region is a state where the collagen fibers start to break and the tendon/ligament structure fails.

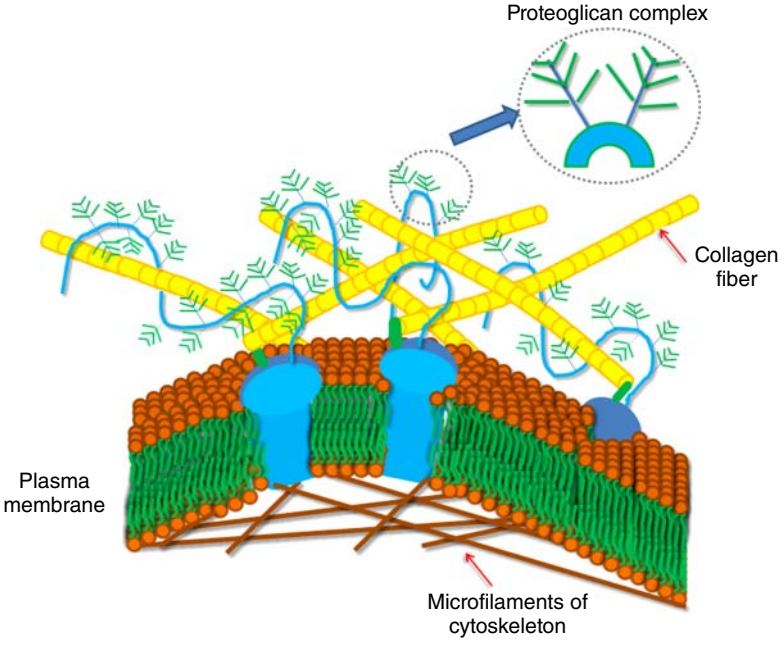


Figure 15.3 Proteins and proteoglycans in the tendon/ligament ECM. The ECM is connected to the cytoskeleton through peripheral and integral proteins embedded in phospholipid bilayer.

predominant cells and can be found as clusters in the pericellular region of tendons. Tenoblasts can mature into tenocytes, which have fibroblastic morphology, very low cytoplasmic organelles, and, therefore, low metabolic activity [62, 63]. Tenocytes are more elongated than tenoblasts, ranging from 80 to 300 μm in length [61]. Tenocytes are differentiated cells with limited proliferation capacity and are distributed throughout collagen fibers [64]. Other

types of cells present in tendons are progenitor cells [65], endothelial cells, synovial cells, and even chondrocytes, although all these other types are less predominant [66]. A subpopulation of myofibroblast-like contractile cells can be present in healthy tendons. These cells are involved in the modulation of the contraction–relaxation in the muscle-tendon complex [67, 68]. One of the most studied tendons, due to their relevance in the stability of the shoulder, is the rotator cuff [69–71]. Nonetheless, other important tendons like the Achilles tendon and the flexor tendon in the hand have also received the attention of tissue engineers [36, 72, 73].

15.2.2 Structure and Biology of Ligament

Ligaments are dense fibrous connective tissue that connects bone to bone [74]. Ligaments are characterized by the presence of fibroblasts and fibrocytes and abundant ECM mainly composed of collagen type I [75]. Two-thirds of ligament's dry weight is composed by water. The remaining one-third consists of other types of collagen (types III and V), elastin, glycosaminoglycans (GAGs), and other biochemical molecules [76]. Ligaments, like tendons, have a hierarchical structure with levels of organization that start with collagen molecules, fibrils, fibril bundles, fibers, and fascicles that run parallel to the long axis of the tissue [1]. Ligaments also display a crimp pattern that repeats every 45–60 μm [77]. Ligaments contain more proteins, less total collagen, and greater proportions of type III collagen and GAGs than tendons [76]. Ligaments are very similar to tendon, as they have the same hierarchical structure, which plays an important role in their mechanical properties. However, the major function of ligaments is purely mechanical, as ligaments passively stabilize joints and help in guiding joints through their normal range of motion when a tensile load is applied [74]. Capsular ligaments act as mechanical reinforcements, while extracapsular ones join together and provide joint stability. Two of the most studied ligaments, due to their importance and injury incidence, are the anterior cruciate ligament (ACL) and the medial collateral ligament (MCL) of the knee [78–80].

In general, both tendons and ligaments are metabolically active with incessant cell renewal and matrix turnover, although at a relatively slow rate [78].

15.2.3 Structure and Biology of Skeletal Muscle

Skeletal muscle is an anisotropic, highly vascularized, and hierarchically organized tissue (Figure 15.4). The macroscopic tissue bundles are organized by lateral aggregation of multinucleated muscle cell fibers, which are made of myofibers that ultimately contain filaments. Muscle fibers are innervated by skeletomotor neurons (Figure 15.4a). Each motor neuron innervates a subset of muscle fibers, and the complex motor neurons and muscle fibers is referred to as the *motor unit*. The motor neuron represents the functional unit of muscle contraction. Motor units may contain up to a thousand or more muscle fibers in large skeletal muscles. Motor units vary in contractile, biochemical, and fatigue properties [81]. Nonetheless, since most mammalian skeletal muscles are composed of many motor units, a high proportion of slow contractile motors, as in the soleus muscle, will make the muscle contract slowly. On the other hand, an abundance of

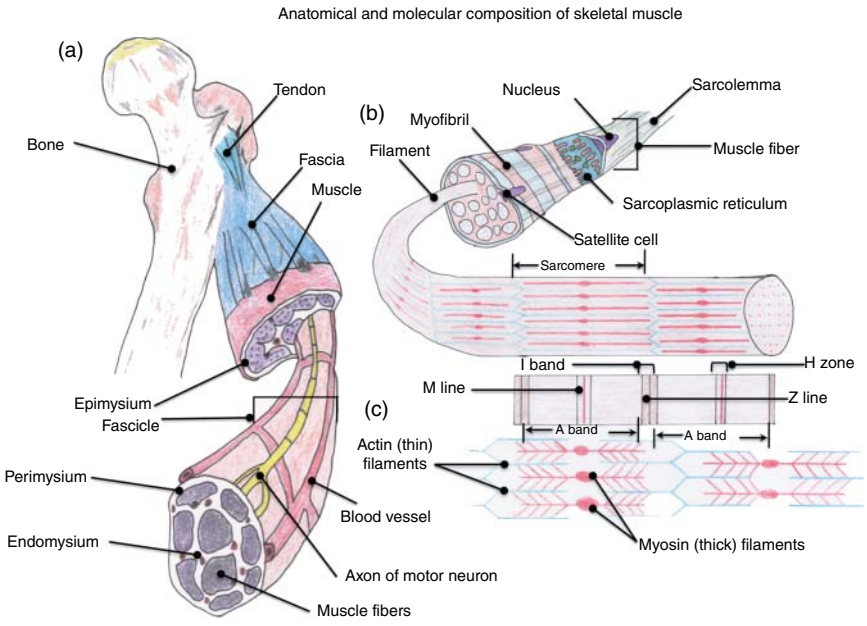


Figure 15.4 Anatomical and molecular composition of skeletal muscle. (a) Anatomical organization of skeletal muscle. A progressively magnified view of a whole muscle demonstrates the organization of the filaments composing the muscle. The organization of the connective tissue within muscle is also visible. The whole muscle consists of the epimysium surrounding it, the perimysium encasing smaller bundles of muscle fibers, and the endomysium that covers individual muscle fibers. (b) Ultrastructure of the muscle myofibril. The sarcolema, sarcoplasmic reticulum, and satellite cells are visible. The sarcomere organization with repeating units of A and I bands is present. The Z line is part of the I band. The H zone has an M line in the center. Contraction is the displacement of I filaments into the A space. (c) Organization of actin and myosin within a muscle fiber. The arrangement of the actin and myosin chains in two adjacent sarcomeres within a fiber produces the characteristic striations of skeletal muscle.

fast motor units, as in the gastrocnemius muscle, will make the entire muscle contract fast.

Individual units called *muscle cells* compose skeletal muscles. Smooth muscles are usually mononucleated and small (a few micrometers wide and 10–100 μm long). By contrast, the units of skeletal muscle are larger, such as multicellular syncytia, which appear during development from the fusion of originally mononuclear myoblasts yielding myotubules, or from the fusion of myoblasts with previously formed myotubules [82, 83]. The resulting muscle fibers are typically 10–100 μm thick and several centimeters long. These muscle fibers may have a thousand or more nuclei, usually of normal diploid constitution. In between these muscle fibers there are satellite cells, which, while in contact with differentiated muscle fibers, remain as quiescent myoblasts even in the adult animal (Figure 15.4b). If the muscle fibers are damaged, the satellite cells begin to differentiate and merge, and become responsible for muscle regeneration [83]. Cardiac muscle, by contrast, consists of branching muscle

fibers, compartmentalized by intercalated disks of fiber-like arrangements of cardiocytes. These intercalated disks are 20–100 µm in size. In this chapter, we will mainly focus on the musculoskeletal muscle. For a comprehensive review on cardiac and myocardial muscle tissue engineering, refer to [84–87].

The sliding filament theory of muscle contraction (Figure 15.4b,c): Muscle fibers are cross-striated, as their constituent fibrils, resulting from an alignment of *sarcomeres*. As a result, contraction of the muscle as a whole is the sum of single contraction events occurring within individual sarcomeres. A sarcomere is a single unit, measured from one fibrillar Z disk or Z line (in longitudinal sections) to the next. It is the smallest element containing the whole property of contractility. In 1682, the Dutch scientist Leeuwenhoek discovered that a musculoskeletal periodic element, usually of 2–2.5 µm, was evident microscopically [88]. Immunostaining or the use of polarization optics can accentuate the visibility of these elements. The A-band, the central part of the sarcomeres, is of greater density and shows anisotropy in polarized light. By contrast, the I-band appears isotropic and is bisected by the Z line. The periodicity can also be visualized and measured by light diffraction from a laser beam, since the regular sarcomere spacing constitutes an optical diffraction lattice. Sarcomeres contain two types of longitudinally oriented myofilaments: actin and myosin (Figure 15.4c). Contraction results from the formation of cross-bridges between the myosin and actin filaments, causing the actin chains to "slide" on the myosin chain. Contraction is initiated by an electrical stimulus from the associated motor neuron, causing depolarization of the muscle fiber. When the fiber is depolarized, calcium is released into the cell and binds with the regulating protein troponin. The combination of calcium and troponin acts as a trigger, causing actin to bind to myosin, beginning the contraction. Cessation of the nerve's stimulus causes a reduction in calcium levels within the muscle fiber, inhibiting the cross-bridges between actin and myosin. Therefore, the muscle relaxes. If the stimulation of the muscle fiber occurs at a sufficiently high frequency, new cross-bridges form before prior interactions are terminated, causing a fusion of succeeding contractions. Ultimately, a sustained or tetanic contraction results.

Actin filaments are fixed in their middle at the Z disk and extend into the two adjacent sarcomeres (Figure 15.4c). When they are present alone (close to the Z disk), the sarcomeres are optically isotropic (I-band). When myosin filaments overlap with actin filaments (in the middle part of the sarcomeres), they form the A-bands. Myosin filaments are held together by structures appearing as the M-line. At normal rest length (2–2.5 µm), the sarcomere contains only myosin filaments in middle portion, giving rise to the H zone.

Actin consists of a double-stranded linear assembly of globular molecules, G actin, forming filamentous F actin. This chain is twisted and contains 13–15 actin pairs per full turn of 750 µm. Polymerized tropomyosin with attached troponin molecules is found in both grooves of the actin double strand. Each myosin molecule consists of a tail, a neck, and two movable heads which under conditions of rest stick out 90° from the longitudinal axis. The tails of many myosin filaments are aggregated in the middle sarcomere region, while the heads stick out between the actin filaments to potentially bind to them at specific binding sites. The heads contain ATPase, which is activated during contraction

and can split adenosine triphosphate (ATP), which is bound to the head during inactivity.

As mentioned previously, skeletal muscle fibers are composed by long myofibrils. These are typically 1–2 μm in diameter. The myofibrils are surrounded by the sarcoplasm containing the *sarcoplasmic reticulum* (SR), which comprises (i) the terminal cisternae and longitudinal elements; (ii) the transverse (T) tubules, which are in contact with the terminal cisternae in triads; (iii) glycogen granules; (iv) mitochondria; and (v) occasionally fat droplets [89].

15.3 Grafting Practices for Tendon, Ligament, and Skeletal Muscle Repair

In the orthopedic field, naturally derived grafts have been effectively used to repair and reconstruct musculotendinous injuries [90]. These biomaterials can be either seeded with site-specific cells before their utilization or used in their acellular form. Here, different types of grafts used in soft tissue replacement and repair are discussed.

15.3.1 Acellular Grafts

The first scaffold material accepted by orthopedic surgeons and approved by the Food and Drug Administration (FDA) was the porcine-derived graft RESTORE™. This scaffold is obtained from porcine intestinal submucosa, and has been used to reinforce ruptures of the rotator cuff tendon [70]. Subsequently, collagen-rich ECM bioscaffolds, such as GraftJacket™ and TissueMend™ (developed by Stryker Orthopaedics), ACI-Maix developed by Matricel® Germany, or the Zimmer® Collagen Repair Patch, have been introduced in the market. For example, The Zimmer® patch is an acellular collagen/elastin-based reinforcement patch that has been used in rotator cuff repair surgeries. This patch has four times as much tensile failure load as that for small-intestine submucosa scaffolds.

Achilles tendon ruptures can be repaired using GraftJacket™, an acellular human dermal tissue matrix [91]. Nine patients (smokers or suffering diabetes mellitus) with neglected Achilles tendon injuries underwent surgery in which the tendon segments were brought together using the Krackow suturing technique. With the sutures in place, the GraftJacket™ matrix was wrapped around the repair site, and results were evaluated. Most of the patients exhibited complications such as swelling, pain during walking, and deep-vein thrombosis. However, based on the manufacturer's specifications, this graft was designed to repair ulcerations in the lower extremities. Nonetheless, Barber *et al.* [92] found that the suture pullout force to cause the breakage of the GraftJacket™ device was as high as 455 N, making it appropriate for clinical studies in Achilles tendon repair. Although human cadaveric legs were used to study the strength of this graft, for making the study clinically relevant, the results need to be further validated by using larger groups (i.e., only eight pairs of cadaveric legs were used in this study).

As for the use of acellular graft to repair skeletal muscle fibers, basal lamina (BL) was employed as a scaffold for *de novo* tissue formation and re-cellularization in an important study by Vracko and Benditt [93]. Successful BL grafting and vascularization was evident in rabbit and rat anterior tibial muscle 8 days after induced ischemic necrosis. However, acellular grafting of ischemic rectus femoris muscles with intact nerves and blood vessels intended for large tendon repair resulted in malfunction of the musculotendinous joint [94].

15.3.2 Autografts

Autologous tendon grafts (also called autotransplants) are commonly used when the injury site is sufficiently small that it can be reinforced with tendon extracted from the same patient; or when primary repair has been neglected or delayed because of infections [95, 96]. Autografts present disadvantages such as donor site morbidity and long operative rehabilitation periods [97]. Moreover, autografting flexor digitorium profundus (FDP) and flexor digitorium superficialis (FDS) show significant adhesions. They appear as a consequence of intrinsic or extrinsic fibrosis which limit joint flexion causing joint contracture [95]. Yet, the use of autologous bone–patellar tendon–bone grafts is the gold standard in ACL repair [98, 99]. Prevention of adhesions after ACL surgery can be minimized with an early physical rehabilitation program applied to the repaired knee.

To treat acute rupture of Achilles tendon injuries [100], 314 patients received a triceps surae tendon autograft to reinforce the reattachment of the tendon using the Kessler end-to-end suturing method. A total of 28 patients (8.9% of the entire population) presented complications such as tendon infection, delayed wound healing, sensory medial and lateral sural nerve wounds, or deep vein thrombosis. Fascia lata grafts combined with an overlaying deltoid flap have been used to repair rotator cuff tears in a rabbit model [101]. This study, although encouraging in terms of the remodeling and revascularization activity evidenced in the repair site 3 months after surgery, may not be applicable in irreparable rotator cuff tears where muscle retraction and adhesion are regularly observed.

15.3.3 Xenografts

Tendon xenografts are required when the supply of available autologous grafts is not readily available, when repairing large tendon defects requires autografting large areas of tendon, or when donor site morbidity is clinically relevant [102, 103]. Consequently, animal-derived grafts (i.e., xenografts) are generally proposed as tendon and ligament replacements in order to avoid the difficulties mentioned earlier. Johnson *et al.* [102] prepared a collagenous xenograft from kangaroo tail and treated with nitrous acid to control the degree of cross-linking achieved using glutaradehyde (GA). The collagenous xenograft was sterilized with γ-radiation, and implanted in an ovine model to repair digital extensor tendon injuries. A biomechanical evaluation after 26 and 52 weeks post implantation showed signs of swelling and increased cross-sectional area in

nitrous-GA-gamma xenografts. These results gave rise to extensor tendons with suboptimal tensile strength.

In another study, Whitlock *et al.* [104] decellularized Leghorn chicken's FDP tendons, determined the DNA content in these scaffolds, and tested them monotonically to failure. The DNA content of decellularized tendon scaffolds was 67% lower than that of untreated FDP tendon controls but the mechanical properties were affected by the decellularization method. Namely, the ultimate tensile stress, elastic modulus, and stiffness values were 75%, 78%, and 78% of the values observed for FDP tendon controls. The decellularization process proved to be partially effective in removing host DNA and could decrease the chance of inflammation and disease transmission after grafting. Hence, the degree of decellularization that could ensure proper integration without side effects upon implantation needs to be further evaluated.

Chen *et al.* [105] followed another approach to solve large rotator cuff ruptures in a rabbit model. They seeded autologous rabbit tenocytes on RESTORE™ or ACI-Maix (collagen type I matricel) matrices and evaluated their histologic/immunologic responses upon implantation in the same rabbit. Results indicated that tenocytes loaded onto both scaffolds were able to maintain their phenotype by expressing collagen type I, collagen type III, and EpHA4 genes during cell culture [106]. The tenocytes were able to produce type I collagen during *in vitro* culture but displayed important inflammatory responses 4 and 8 weeks after implantation. Evidence of inflammation is a genuine concern with these types of bioscaffolds. Sclamberg *et al.* first reported inflammation evidence [107], and later Malcarney *et al.* [108] and Zheng *et al.* [109] corroborated that pig small intestinal submucosa (SIS) cannot be classified as an acellular collagen xenograft as it contained pig DNA traces. This is specifically true for the RESTORE™ bioscaffold. Consequently, surgeons should proceed with caution if they want to continuously use porcine small intestine submucosa and porcine-derived type I/III collagen scaffolds as orthopedic biomaterials.

Nonetheless, xenotransplantation has proven a successful strategy in the regeneration of musculoskeletal tissue in a rodent abdominal wall model [110]. Porcine SIS replacement triggered restoration of functional skeletal muscle with shape, composition, and histologic characteristics similar to those in native muscle. Additionally, porcine SIS was used for abdominal wall repair in a canine model [111]. The SIS implants were replaced by host tissue at 4 months. The repaired tissue resulted in a dense, well-organized, and smooth collagenous connective tissue that was well incorporated into the fascia and the skeletal muscle fibers.

15.3.4 Allografts

Acknowledging the problems with xenografts presented above, researchers have investigated biophysical characteristics and fabrication methods of human tensor fascia lata as an alternative allogeneic source of tendon grafts [112]. Fascia lata was employed in the treatment of glenohumeral instability associated with capsular deficiencies [113], its biomechanical properties have been elucidated

[114], and several pilot animal and clinical studies have demonstrated its feasibility as a tendon/ligament repair graft [115, 116]. Concern still exists about the traces of DNA left in the allograft after the decellularization treatment, which can increase the risk of disease transmission and immunogenic response. Bacterial infection from patellar and Achilles tendon allografts obtained from cadaveric donors represents a main reason of morbidity and mortality in beneficiaries [117]. The *Clostridium* bacteria can cause infections associated with tendon allograft transplantation. It is important to mention that the root of the problem lies in the lack of aseptic conditions in some tissue banks while handling allografts. Specifically, some of the common failures contributing to tendon/ligament allograft contamination and fatal clostridium sepsis are (i) refrigeration of donor bodies >20 h after death, (ii) handling of allografts without using strong disinfectants and/or thermal sterilization, and (iii) no bacteriostasis testing at the time of the final sterility evaluation [117]. Clinically, researchers and surgeons have used Achilles tendon allografts to repair chronically ruptured Achilles tendon with gaps longer than 5 cm [118] or ruptured proximal hamstring lesions [119]. In these studies, although the duration of follow-up was either brief or not done at all, rejection of the allograft or major inflammatory complications due to bacterial contamination was not reported.

Derwin *et al.* [112] found that a decellularization treatment by detergents and DNAse combined with an antibiotic bath reduced the DNA contents of freshly dissected human fascia lata by 10-fold (bioscaffold dimensions: $6 \times 12\,cm^2$). Denser and thicker scaffolds could be more difficult to decellularize and would require more time to integrate into the surrounding host tissue [120]. This decellularization process has been intended to prevent immunogenic problems associated with SIS. Additionally, biochemical and architectural similarities between fascia lata and tendon have been reported [112], which made fascia lata appealing as allograft in tendon applications, as mentioned previously. Namely, hydroxyproline and chondroitin/dermatan sulfate GAG contents were comparable between tissues. However, it is important to point out that the architectural configuration of fascia lata orthogonally orients its collagen fascicles in a bilayer configuration [114], while tendon arrangement uniformly orients its fascicles along the direction of load in a d-staggered manner [121]. Yet, the d-staggered (d-banding) configuration in the bilayer architecture in fascia lata is similar to that found in tendons.

As for the case of allografts in muscle regeneration, allogeneic tissues are promising alternatives in cancer patients. It is well known that patients undergoing organ transplantation acquire a permanent dependence on immunosuppression drugs. These medications increase the risk of skin cancer in renal transplant recipients [122–124]. Decreasing or withdrawing the immunosuppression drug does not increase patient survival, and could result in rejection of the allogeneic graft. However, researchers have reported a successful reconstruction of a scalp wound resulting from resection of scalp cancer with a vascularized allogeneic skeletal muscle [125]. Allografts for skeletal muscle reconstruction are being used in the clinic today; however, the problems associated with allogeneic grafts still limit their widespread application.

15.4 Factors in Musculoskeletal Tissue Engineering

15.4.1 Development of Natural Scaffolds in Tendon, Ligament, and Skeletal Muscle Tissue Engineering

Based on the previous requirements for tissue-engineered tendons and ligaments, choosing an adequate scaffold seem to be of primary concern. Collagen fibers, gels, and collagen-based 3D scaffolds have been traditionally chosen as adequate biomaterials in tendon/ligament engineering applications [126–128]. Collagens are key components in the ECM of tendons and ligaments, and many biochemical factors and cytokines can be decorated onto it [33, 129]. Moreover, collagen (specifically type I) represents 70–90% of tendon's dry weight [6, 130]; thus a bioengineered mimic of native tendon would necessarily include polymeric collagen as fundamental building block. Collagenous synthetic analogs (CSAs), defined as biomaterials with randomly oriented fibers intended to substitute collagen-rich tissues, have demonstrated to be excellent cell-carrier materials [131–133] but weak load-carrier analogs for the repair of soft connective tissues [36, 134, 135]. In fact, CSAs possess mechanical properties similar to those found in viscous fluids and weak solids, which make them unsuitable as load-bearing tissue-engineered bioscaffolds. Consequently, improving the strength of CSA can facilitate their transition to become effective replacements for tendinous and ligamentous tissues.

To improve the biomechanical properties of CSAs, several methods have been developed that aim to increase the hierarchical ordering of collagen fibers. These CSAs can be aligned using magnets, liquid crystallography, or electrospinning, but these techniques have demonstrated to be time consuming [136–139]. In fact, liquid crystallography methods have rendered weak and inhomogeneous fibers [140], and the electrospinning route may yield denatured collagen in the form of gelatin [141]. On the contrary, microfluidic alignment of collagen fibrils produced using photolithography techniques has created constructs with microscopic size, which are still too small to be used in the clinic [142].

More than 7 years ago, an electrochemical method was modified to increase the hierarchical ordering of CSAs and improve its mechanical strength [143]. The application of direct current to collagen solutions has demonstrated to produce oriented [144] and highly packed constructs in the form of fibers [145], called electrochemically aligned collagens (ELACs), with mechanical properties converging to those of native tendons [146] (human central patellar tendon maximum stress value is 19.2–64 MPa and elastic modulus ranges from 305 to 660 MPa [114, 147]). This technique has the potential to produce highly oriented collagenous fibers in short periods, which can be further processed to render mechanically strong fibers [143]. Although collagen-based synthetic bioscaffolds have been demonstrated to have hierarchical fibril arrangement similar to that found in their natural counterparts, their material properties (i.e., maximum stress, strain at maximum stress, and elastic modulus) need to be improved substantially. The ELAC route represents an innovative alternative to circumvent the limitations inherent to CSAs. The pioneering electrochemical route has been shown to be versatile enough to produce ELAC bioscaffolds that were

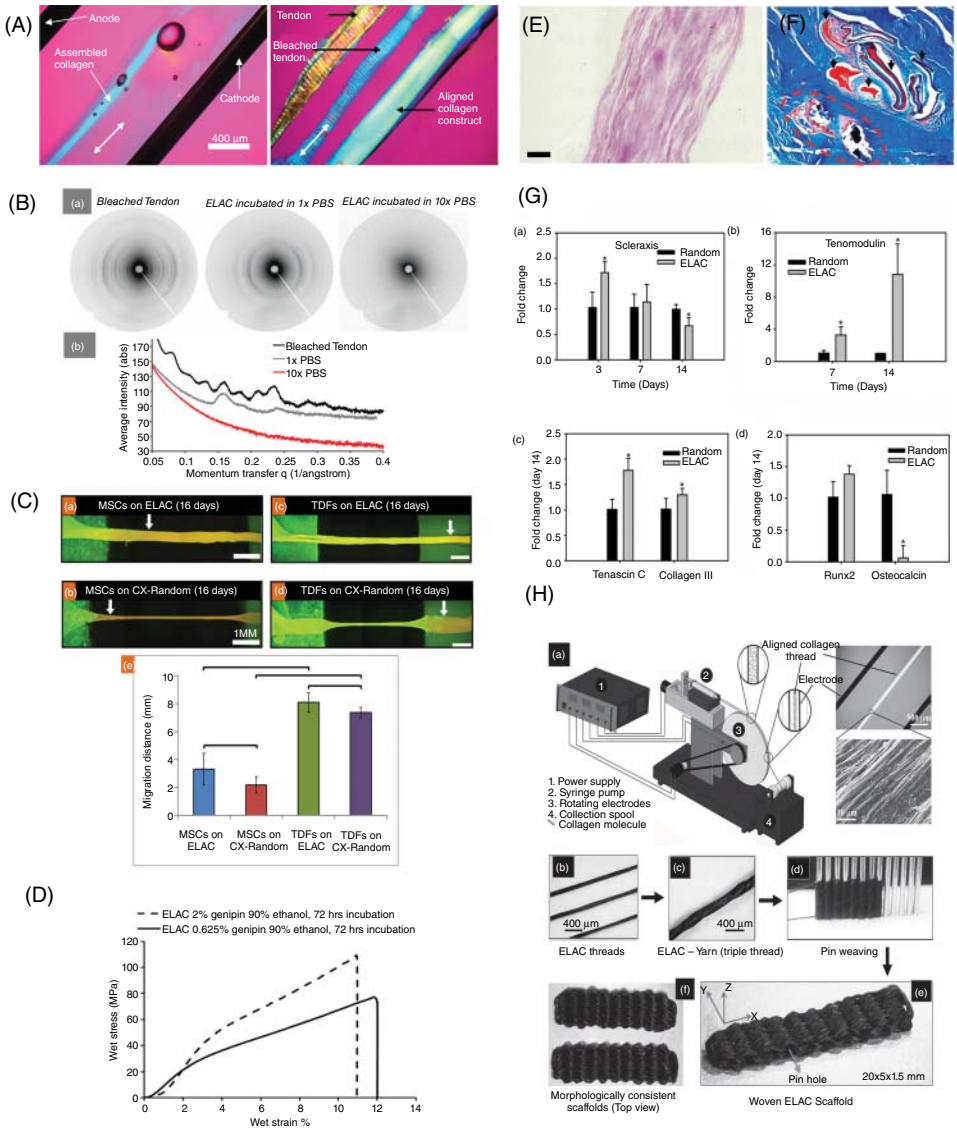
Figure 15.5 (A) An electrochemical route to produce ELAC fibers. (Reprinted with permission from [143].) (B) Small angle X-ray scattering (SAXS) of the ELAC fibers showed a d-banding pattern similar to that found in native tendons (a). Average Intensity vs. peak position of X-ray scattering data of the patterns (b). (Reprinted with permission from [145].) (C) Anisotropic ELAC fibers accelerate the migration of mesenchymal stem cells (MSCs) and tendon-derived fibroblasts (TDFs) Fluorescent images displaying f-actin filament staining with FITC-Phalloidin at day-16 of (a) MSCs on the ELAC, (b) MSCs on the CX-Random, (c) TDFs on ELAC and (d) TDFs on CX-Random. (e) Graph showing the migration distance (mm) of MSCs and TDFs on the ELAC fibers and the CX-Random collagen bundles at 16 days. (Reprinted with permission from [148].) (D) ELAC fibers exhibit similar mechanical properties as native tendons. (Reprinted with permission from [146].) (E) Cryo-section stained with DMMB dye to indicate the presence of decorin mimetic in ELAC fibers. (Reprinted with permission from [52].) (F) H&E staining of ELAC fiber implanted in a rabbit patellar tendon. Arrows indicate the presence of ELAC fibers 4 months after surgery. (Reprinted with permission from [72].) (G) ELAC fibers induce the upregulation of tenogenic genes and the differentiation of MSCs to tenocyte-like cells. Specifically, the fold increase expression for (a) Scleraxis, (b)Tenomodulin, (c) Tenascin-C and Collagen-III was higher in the ELAC fibers. On the contrary, the expression of bone differentiation markers (d) such as Osteocalcin is significantly lower compared to random fibers. (Reprinted with permission from [149].) (H) 3D biotextile technology allows the continuous production of ELAC fibers to create woven scaffolds for the repair of tendons and ligaments (a) Schematic of the rotating electrode electrochemical alignment device. (b) Picture of a collagen fiber made by a rotating electrode electrochemical aligning device, (c) yarn consisting of twisting three collagen threads and (d) pin-setup for weaving the collagen scaffold. (e-f) Images displaying the resulting woven collagen scaffold and two distinct scaffolds respectively. (Reprinted with permission from [150].)

not only strong to withstand loads when implanted *in vivo* [72] but also able to promote tenocyte migration [148], activate tenogenic differentiation pathways of mesenchymal stem cells (MSCs) [149], facilitate the incorporation of decorin mimics [52], and fabricate 3D woven bioscaffolds [150] for the functional repair of tendons and ligaments (Figure 15.5).

As for skeletal muscle tissue engineering, the use of natural and biodegradable scaffolds such as collagen has been an acceptable choice since the aligned topographic feature of its fibrils can induce the alignment of cytoskeletal proteins and muscle myoblasts [151, 152]. A biodegradable 3D scaffold was fabricated from acellular muscle ECM [153]. A disadvantage of this biomaterial is its low mechanical properties, which can be correlated to poor handling properties when used in the clinic. Another natural biodegradable 3D biomaterial was fabricated using polymeric fibrin, which is a globular protein involved in the blood clothing cascade. Here, satellite cells were mixed in a fibrin/ thrombin hydrogel [154–158]. Host muscle progenitor cells produced *de novo* ECM proteins after 3–4 weeks post implantation and degraded the original fibrin matrix [159, 160]. The limitation of this tissue engineering strategy is that the progression of tissue repair is comparable to wound healing. After wound healing, no vascularization or innervation was reported.

15.4.2 Tissue-Engineered Collagenous Bioscaffolds for Tendon Repair

Over the past 25 years, tissue engineering strategies have been a way to provide cell- or molecular-based therapies in an attempt to obtain controlled restoration



of tissue's morphology and function. In order to generate the right biomimetic tendons, researchers have looked at the properties of native tendon/ligament and transfer their characteristics to models developed in the laboratory. Thus, the right biological scaffold must be compatible with the requirements regulating functional tissue engineering principles underlined previously (Table 15.1).

One of the first seminal studies in which tendon tissue engineering strategies were used consisted in MSCs grafting to repair Achilles tendon pathologies [161]. Briefly, MCS were seeded in a collagen hydrogel, mechanically preconditioned using a spring wire device, and implanted in a rabbit Achilles tendon defect.

This study showed that the organization and elongated morphology of MSCs seeded onto collagen matrices was parallel to the direction of the mechanical tensile load. Besides being uniaxially oriented, polarizing images showed that these cells could synthesize collagen fibers with some degree of hierarchical arrangement and d-banding pattern. Differentiation of MSCs along different cellular lineages have been proposed to depend on the interaction between the ECM, surrounding cells, local cues, and the genomic potential of MSCs [1, 162–165]. Therefore, although not identified as fibroblasts, some cells seeded on collagenous scaffolds could have been characterized as such. Additionally, typical stress–strain curves showed that the values of maximum tensile stress and maximum percent strain were 12 MPa and 20%, respectively. Maximum tensile strain values agreed very well with native canine tendon data [143], but the maximum tensile stress value was 10-fold lower. Nevertheless, this study showed that implantation of well-organized MSC-populated collagen matrices could be used to repair tendon injuries.

Mechanical conditioning organizes MSCs along the loading force, and the resulting synthesized ECM has an architectural arrangement similar to that of native tendon. But, can this cellular orientation and subsequent ECM production be optimized based on cell seeding density? This question was answered by Awad *et al.* [126]. Their study evaluated *in vitro* the effects of initial seeding density on the nuclear orientation of MSCs. The results indicated that after 72 h of culture conditions, almost 60% of the cells seeded on mechanically preconditioned collagen-based scaffolds (i.e., 8×10^6 cells ml^{-1} of collagen) had their nucleus oriented parallel to the loading force. These results were later validated in a subsequent study in which MSC–collagen scaffolds were grafted in patellar tendon defects of New Zealand rabbits [166]. Biomechanical evaluation of the explanted scaffolds showed that varying the cell seeding density had no effect on the maximum tensile stress, maximum strain, or elastic modulus of the explanted repair tissues. However, tissues recovered after 26 weeks upon implantation exhibited maximum stress, elastic modulus, and maximum strain values significantly lower than those of a normal rabbit patellar tendon. These results correlated well with the low percentage of MSC nuclei oriented along the fibers of the implanted scaffold (17–22% at 26 weeks after surgery). Interestingly, H&E staining showed ectopic bone formation in the central region of nearly 30% of explanted scaffolds. Consequently, it was possible that the population of cultured MCS could have included subpopulations of osteogenic progenitor cells. To understand the source of bone formation, the seeded cells were examined for positive alkaline phosphatase (ALP) by Harris *et al.* [167]. ALP levels where elevated when high cell densities were used in collagenous constructs.

In order to further improve the repair biomechanics in patellar tendon defects and reduce ectopic bone formation, cell-to-collagen ratio was optimized by Juncosa-Melvin *et al.* [135]. Their results showed that cell-to-collagen ratios ranging from 0.04 to 0.08×10^6 cells mg^{-1} collagen prevented the formation of the ectopic bone. Additionally, the best material properties of patellar tendon repair constructs with low cell-to-collagen ratio were 30 MPa for maximum stress, 107 MPa for elastic modulus, and 51.8% for maximum strain. Normal material properties of patellar tendon were 70% and 88% higher for maximum

stress and elastic modulus when compared to those of recovered constructs. Moreover, the recovered constructs with low cell-to-collagen ratio (i.e., 0.04 million cells per milligram collagen) were much more compliant than their natural counterparts [135]. Nonetheless, histologic studies of the recovered constructs showed slight presence of collagen types I and V, but strong presence of collagen type III, which implied that *in vivo* ECM synthesis did not reached maturate levels. Similar studies to repair Achilles tendon defects in young New Zealand rabbits have been evaluated. An interesting result from these experiments showed that after 12 weeks of implantation, the retrieved constructs (i.e., mechanically conditioned before implantation) stained positive for fibronectin, decorin, and collagen type III. On the contrary, normal rabbit Achilles tendon showed strong staining for fibronectin and decorin, and mild staining for collagen type III. As mentioned earlier, this *in vivo* synthesized ECM differs from its natural counterpart, and efforts should be made to accelerate the maturation rate of these newly synthesized ECMs to match the biochemical characteristics of synthetic tendon to that of native equivalents.

Although the material properties of bioscaffolds composed of mildly contracted collagenous constructs in combination with autologous MSCs were similar to those of rabbit's Achilles tendon, the structural properties were not. Therefore, Juncosa-Melvin *et al.* [168] decided to combine MSCs (cell density of 0.1×10^6 cells ml^{-1} of collagen) diluted in bovine collagen gel with collagen sponges. These sponges were introduced to improve the biomechanical characteristics of tissue-engineered constructs, which were then implanted to repair rabbit patellar tendon defects. The cell–gel–sponge construct was incubated under static tension conditions (i.e., during incubation, MSCs impose contractile force over the construct). The results indicated that maximum stress and elastic modulus of the cellular constructs were 50% and 68% lower than those of rabbit's patellar tendon. Interestingly, the maximum strain of the cellular constructs was 62.5% greater than the corresponding value for normal rabbit's patellar tendon. Additionally, the cellular constructs showed MSC alignment parallel to the tendon's load-bearing axis with modest crimp pattern.

15.4.3 Development of Synthetic Bioscaffolds in Tendon, Ligament, and Skeletal Muscle Tissue Engineering

Along with the design of natural scaffolds mentioned previously, synthetic polymers have been widely used as materials for the preparation of scaffolds in tendon/ligament and skeletal muscle tissue engineering applications. In particular, synthetic polymers offer several advantages over the natural ones, as they can be produced in large scale at low costs. Additionally, their mechanical properties as well as their degradability can be modulated to match specific requirements. Moreover, the biological scaffold obtained from human or animal tissue carries also the risk of disease transmission, which is not present while using a synthetic scaffold [169].

Along these lines, polyhydroxyesters such as poly(L-lactide) acid (PLLA), poly(L-lactide-*co*-glycolide) (PLGA), and poly(ϵ-caprolactone) (PCL) represent interesting choices for the preparation of biodegradable scaffold for

tendon/ligament repair. In fact, several parameters including the molecular weight as well as the type and ratio of monomers can be modified to tailor the degradation rate of the synthetic scaffold. In addition, their biodegradation products are normally present in the human body and are easily metabolized and removed without potential risks of toxicity. For example, Langer *et al.* proposed an engineered vascularized skeletal muscle starting from 3D biodegradable polymer scaffolds [170]. A scaffold made of 50% PLLA and 50 % PLGA with porosity of 225–500 μm was cultured with three different cell lines including myoblasts, embryonic fibroblasts, and endothelial cells in an attempt to pre-vascularize the engineered skeletal muscle tissue. This approach could be useful in promoting anastomosis with the host vasculature and potentially resolve the grafting limitations explained previously.

In this section, a brief explanation on the main techniques to engineer synthetic scaffolds will be presented with particular attention on their relevance in the field of tendon/ligament and skeletal muscle engineering.

15.4.3.1 Synthetic Tendon, Ligament, and Skeletal Muscle Bioscaffolds

The strategy to repair tendon, ligaments, and skeletal muscles using synthetic materials are generally based on the fabrication of structures that mimic the native tissue organization and its functional properties. To achieve this goal, several methods have been proposed so far, including electrospinning, knitting or braiding, and micropatterning.

15.4.3.1.1 Electrospinning

This useful technique has been widely investigated because of its versatility and scalability in the fabrication of synthetic fibers with diameters varying from a few nanometers to several hundreds of micrometers [171]. Briefly, electrospinning involves the application of a high voltage to a capillary, which contains a viscous polymeric solution (Figure 15.6). The voltage creates a repulsive electrostatic force opposite, which counteracts the surface tension of the polymeric fluid. As a result, the liquid elongates, creating a *Taylor cone* at the tip of the capillary tube, which overcomes the droplet surface tension, and creates a polymeric liquid jet.

In general, electrospinning is more suitable for processing synthetic polymers than natural ones, as the harsh condition can denature the triple-helix in structure like that of collagen [172]. The peculiar advantage of this strategy relies on the precise spatial orientation of the fibers, which can affect the final mechanical properties of the construct. In the specific case of tendon and ligaments, a dense parallel packing of electrospun fibers is generally required to achieve the biomechanical properties of native tendons/ligaments. Moreover, these fiber alignments seem to be involved in defining the biological response of cells and promote their differentiation [173–175]. In the case of skeletal muscle tissue engineering, the precise organization of biocompatible polymers into thin fibers matrices can provide temporary support for muscle regrowth, allowing satellite cell adhesion, migration, and myotubes alignment. It is then important that the fabricated scaffold does not degrade overtime as the new tissue grows, facilitating the process of myogenesis [176]. Moreover, electrospinning can also be used to fabricate scaffolds that mimic the complicated architecture at the

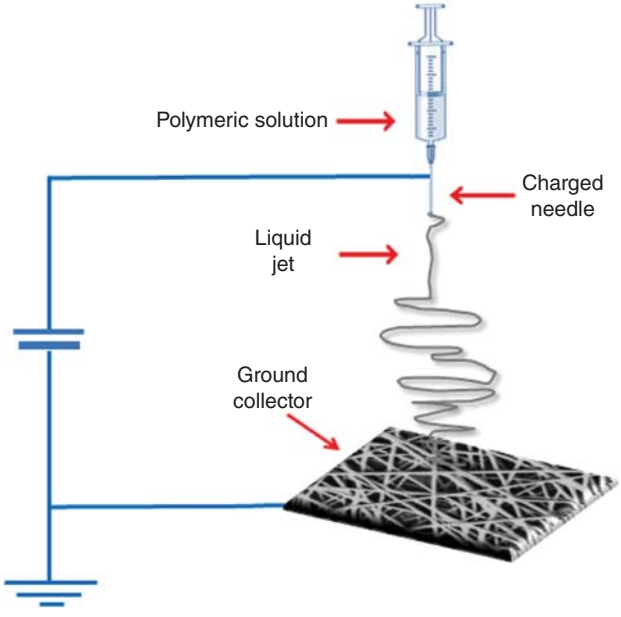


Figure 15.6 Schematic of electrospinning fabrication technique for the preparation of porous bioscaffolds.

interface between different tissues as in the case of musculotendinous junctions [177].

Several parameters should be taken into account during the electrospinning process, as they can deeply influence the final characteristics of the fibrous scaffold. First of all, the polymer solution has to possess the right viscosity, charge density, and surface tension to avoid the formation of droplets before the solvent evaporation occurs. Moreover, the volatility and polarity of the solvent can modulate the morphology and the diameter of the electrospun fibers. Once the scaffold has been prepared, it can be seeded with cells. One of the main challenges in the seeding of cells in synthetic bioscaffolds for tendon, ligament, and skeletal muscle engineering is the heterogeneous cell distribution and negligible cell infiltration among the synthetic fibers. This problem limits the clinical applicability of these materials, and several strategies have been proposed to increase cell infiltration by introducing higher porosity or sacrificial fibers inside the internal network, although a loss in mechanical properties is generall y observed [178].

Bio-electrospinning could represent another possible solution to engineering synthetic tendon/ligaments, as it can offer the solution to electrospray cells and polymeric fibers in one single step and overcome the problem of cell infiltration within a well-packed fibrous scaffold. However, the process itself can be limited by the shear stress during spraying on the cell membrane; therefore, assessing the biological activity and viability of the cells is a fundamental requirement during the production of bioelectrospun tendons and ligaments [179].

15.4.3.1.2 Knitting

Together with electrospinning, knitting has been used as a complementary strategy to modulate the mechanical properties of a bioscaffold, thereby improving the bioscaffold's capacity to sustain and redistribute the loading stress along its long axis [61]. In particular, in a knitted mesh or network, a high level of arrangement and interlocking loops is achieved, allowing control over the microstructure of the scaffold. According to the direction of these loops, knitting can be classified into weft or wrap knitting (Figure 15.7).

In weft knitting, the wales are perpendicular to the course of the yarn, while in warp knitting the wales and the courses run roughly in parallel, leading to structures of different mechanical properties. In fact, the weft knitting structures stretch more easily, whereas the warp ones can provide higher stability. In this sense, an optimized scaffold having knitted mesh can be designed to match specific tissue requirements following two different ways of fabrication including the one-step molding or by assembling several units together [180].

15.4.3.1.3 Micropatterning

With this technology it is possible to mold polymeric solution into nanoscale channels with a well-defined structure and higher surface area. Micropatterning

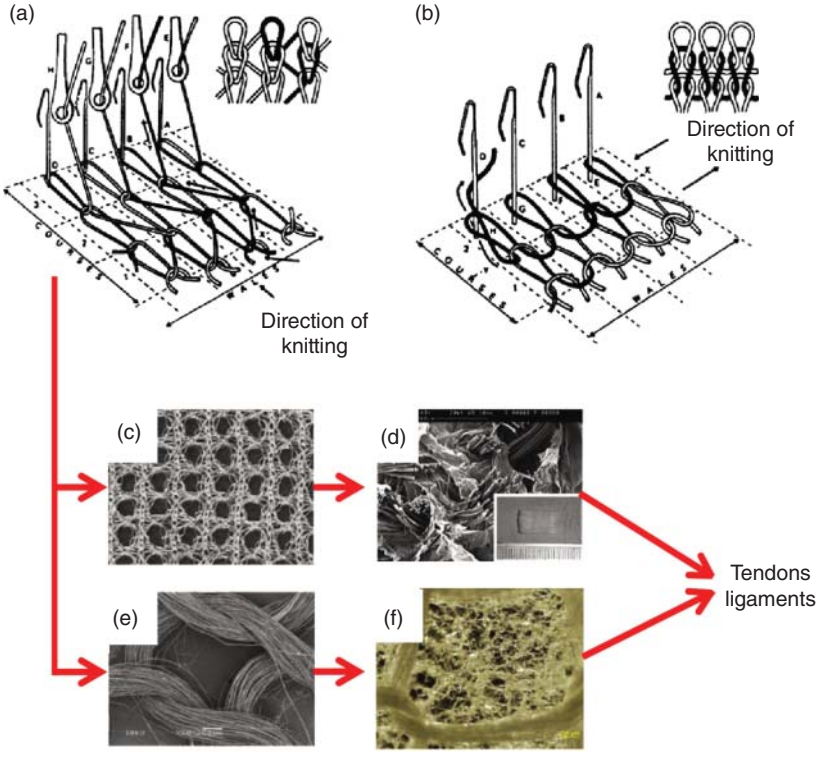


Figure 15.7 Schematic of (a) weft knitting and (b) wrap knitting (c) Gross image displaying silk knitted mesh, and (d) corresponding SEM image showing their integration with collagen scaffold. (e) SEM picture of silk knitted mesh, and (f) phase-contrast image of porous silk sponge integrated within the mesh. (Reprinted with permission from [180].)

offers the possibility to design 2D scaffolds particularly useful in skeletal muscle tissue engineering, which can mimic the parallel alignment of myotubes of the native tissue. In particular, the presence of well-organized nanoscale channels can promote satellite cell motility and myoblast alignment and fusion. At the same time, the whole construct can sustain significant longitudinal forces [181, 182]. However, micropatterning is not a good technique while considering the fabrication of large 3D constructs. In fact, the removal of cell monolayers from the micropatterned surfaces using biodegradable hydrogels is still a limitation, which allows only the obtainment of small construct [183].

A brief summary of the synthetic polymers used and the design strategy proposed for the engineering of the main tendons, ligaments, and skeletal muscles is given in Table 15.2. For a more complete review, refer to [200–202].

15.4.4 Mesenchymal Stem Cells in Tendon, Ligament, and Skeletal Muscle Tissue Engineering

Cell-based therapies with or without the presence of a scaffold represent an alternative strategy for tendon and ligament repair as well for skeletal muscle regeneration. Among the multiple cell types available, stem cells have been widely investigated because of their ability for self-renewal while maintaining their multipotency. Stem cells in tendon/ligament and skeletal muscle tissue engineering can be divided into three main classes: embryonic stem cells (ESCs), induced pluripotent stem cells (IPSs), and MSCs.

ESCs are totitpotent primary embryonic cells, which have not been extensively studied for tendon and ligament repairs, as their use still raise several ethical issues [203]. However, they provide a potential cell source for tendon/ligament tissue engineering, and several protocols have been established recently to promote their efficient differentiation into tenocytes through an MSC transition stage [204].

IPSs are stem cells obtained by reprogramming differentiated cells in order to convert them into a multipotent cell type. This technique has gained in importance, although their use in clinical trials is limited by the large amount of processing time that these cells must undergo [5].

On the contrary, MSCs are widely used in regenerative medicine and represent the most important source for the treatment of tendon, ligament, and skeletal muscle repair [205]. In fact, MSCs have been discovered in a variety of tissues like bone marrow, adipose tissue, synovium, periosteum, muscle, tendons, articular cartilage, and periodontal ligament. They can be easily differentiated into several connective tissue phenotypes such as osteocytes, chondrocytes, adipocytes, tenocytes, and myoblasts [206]. Several studies have been carried out in the past decade to figure out which source of stem cell would be more suitable for tendon and ligament repair and their advantages over tenoblasts/tenocytes and fibroblasts [207, 208]. Bone-marrow-derived mesenchymal stem cells (BMSCs) can be easy and quickly isolated and represent a widely investigated stem cell source in tendon/ligament engineering [209]. At the same time, adipose tissue stem cells (ADSCs) have been extensively used for tendon regeneration, as they can be easily differentiated into several lineages and their harvesting procedure can cause minimal donor site morbidity [210, 211]. Most recently, tendon

Table 15.2 Synthetic scaffolds and their use in tendon, ligament, skeletal muscle repair.

Ligament/tendon/muscle	Polymer	Scaffold preparation	Cells types *in vitro*	Animal model *in vivo*	References
Anterior crucial ligament (ACL)/Medial collateral ligament (MCL)	PGA/PLLA/PLGA	Braiding	ACL rabbit fibroblast	N/A	[184]
	PLGA	Braiding	ACL rabbit fibroblast	N/A	[185]
	PLGA	Knitting	ACL canine fibroblast	Nude mouse	[186]
	PLCL	Knitting Electrospinning	Rat BMSCs	N/A	[187]
	PU	Electrospinning	Human ligament fibroblast	N/A	[188]
	PLGA		Porcine BMSC	N/A	[189]
Rotator cuff	PCL	Electrospinning	N/A	Rat	[190]
	PLGA	Electrospinning	Human tendon fibroblasts	N/A	[191]
	PLGA	Electrospinning	Rabbit patellar tendon fibroblast	Rat	[192]
	PLA	Patch	N/A	Dog	[193]
	PGA	Sheet formation		Rabbit	[194]
Achilles tendons	PLGA	Knitting	BMSCs	Rabbit	[195]
Skeletal muscle	PCL/PANi	Electrospinning	Mouse skeletal myoblasts	N/A	[196]
	PLGA	Electrospinning	Murine C2C12 myoblasts	N/A	[197]
	PDMS/pHEMA	Micropatterning	Murine C2C12 myoblasts	N/A	[198]
	PDMS	Micropatterning	Murine C2C12 myoblasts	N/A	[199]

PGA, poly(glycolic acid); PLLA, poly(L-lactic acid); PLGA, poly(lactide-*co*-glycolide); PLCL, poly(L-lactic-*co*-ϵ-caprolactone); PU, polyurethane; PCL, poly(ϵ-caprolactone); PANi, polyaniline; PDMS, poly(dimethylsiloxane); pHEMA, poly(2-hydroxyehyl methacrylate); BMSCs, bone-marrow-derived mesenchymal stem cells; N/A, not available.

stem cells (TSCs) have been isolated and their multipotency investigated and compared with BMSCs by evaluating the expression of tenogenic, osteogenic, and chondrogenic markers in both cell lines [212, 213]. Finally, satellite cells, which are muscle-specific stem cells, represent the primary choice for skeletal muscle tissue engineering applications. In fact, this type of stem cell is generally involved in the process of muscle repair and can differentiate specifically into myoblasts. Specifically, satellite cells are present in quiescent form between the sarcolemma and the BL of the mature muscle fiber, and they express a transcriptional factor Pax7 that is considered a required marker to identify their stemness. After an injury, which can be induced by mechanical loading, satellite cells proliferate and differentiate to myoblasts and start the whole process of muscle repair, defined as myogenesis. [214]. A more complete review on their role in muscle regeneration can be found elsewhere [215].

In general, the therapeutic efficacy of MSCs relies on their ability to trigger a series of biological factors including chemo-attraction, angiogenesis, immune modulation, anti-scarring, and production of a structural matrix, which are crucial steps in successful tendon, ligament, and skeletal muscle repair [216, 217].

MSCs can be delivered to the site of injury using injectable carriers, scaffolds, or cell sheets. In the case of injectable cell carriers, natural polymers are often used to deliver cells without membrane rupture during the injection while maintaining cell functionality when they come in contact with the host tissue [218]. Among natural polymers, collagen and fibrin are widely investigated as injectable carriers of MSCs for the repair of tendon and ligaments due to their low antigenicity and immunogenicity as well as the presence of cell recognition signals that promote adhesion, migration, and proliferation [219–222]. At the same time, in the case of synthetic polymers for local delivery of stem cells, a recent study investigated the possibility to promote healing in the ligament–bone interface using an injectable hydrogel made of poly(ethylene glycol)diacrylate (PEG-DA) to supply periosteal progenitor cells (PPCs) tethered with bone morphogenic protein-2 (BMP-2) [223]. Injection of cell-carrier polymers is generally suitable to promote healing in small tendon/ligament defects or for the treatment of ruptures in bone–tendon junctions. Regarding skeletal muscle repair, injectable spherical scaffolds of PLGA were used as the carrier of ADSCs to demonstrate their potential in muscle regeneration in mice [224].

On the contrary, tissue grafts and MSC-seeded bioscaffolds are the most useful strategies for the reconstruction of large tendon injuries. This approach can provide a three-dimensional environment for cell infiltration and growth and facilitate directional cell spreading and new tissue formation *in vivo*. Tendon grafts can undergo a step of decellularization and be reseeded with different kind of cells including BMSCs, ADSCs, fibroblasts, and tenocytes [225, 226]. The presence of BMSCs in tendons has been investigated and found to be important in collagen metabolism inducing the expression of metalloproteinases while facilitating the differentiation of BMSCs to the tendon phenotype *in vivo* [227, 228]. Recently, several approaches have promoted the differentiation of TSCs and ADSCs into the tenogenic linage as an alternative source of stem cells for tendon grafts seeding [229–231].

Together with tissue grafts, collagen-based hydrogels [166, 232] and synthetic degradable polymers [233–235] have been widely used as carrier for MSC therapy of tendon and ligaments. Moreover, ECM scaffold have also been recently proposed in combination with MSCs to repair skeletal muscle injury in rats [236].

Another interesting idea coined as a scaffold-free strategy aims to produce cell sheets of MSCs that can be wrapped around the tissue or synthetic graft [237–240] to enhance the process of healing of tendons [241, 242]. Although cell sheets have not found much use in the field of tendon/ligament tissue engineering so far, they represent a possible area of study, as they can provide more natural cellular junctions and microenvironments suitable for ligament and tendon treatment. Along with all the findings described so far, the use of MSCs still presents several drawbacks. For example, their multipotency still creates concerns when it comes to controlling their differentiation to tenocytes and fibroblasts. In fact, the formation of ectopic bone and cartilage at the tendon/ligament site has often been observed [167], and this major issue can be avoided by a better understanding of the biological signaling events governing MSCs' differentiation to tendon-like cells. Another possibility to guide stem cell differentiation is to co-culture them with a different cell population in order to establish intimate contact between different cell types, promoting an efficient transduction of molecular signals involved in tenocyte differentiation. For example, a co-culture of MSCs and ACL fibroblasts showed an increase in stem cell differentiation [243]. Additionally, ESCs' differentiation can be improved by a co-culture with periodontal ligament fibroblasts (PLFs) [244], although further investigation is needed to better clarify the reason behind this accelerated differentiation mechanism. Regarding skeletal muscle repair, scaffold-free strategies are generally based on satellite cells and fibroblasts in co-culture to self-assemble into cylindrical muscle constructs. Specifically, fibroblasts obtained from muscle provide support to satellite cells, which can organize into axially aligned myotubes [245]. Moreover, these 3D scaffold free myotubes can be interfaced with engineered tendon to form functional myotendinous junctions [246] or with nerve tissue to fabricate nerve-skeletal muscle scaffolds *in vitro* [247]. Although this strategy provides biocompatible engineered tissues that can be potentially vascularized *ex vivo* [248], still two major concerns remain to be resolved. The first is the difficulty to scale up the biofabrication process of these tissue constructs, and the second is the time (3–4 weeks) required for their fabrication *in vitro*.

15.4.5 Growth Factors in Tendon/Ligament and Skeletal Muscle Tissue Engineering

A crucial point in the design of a bioscaffold for tendon, ligament, and skeletal muscle repair is the combination of a biomaterial with biological clues that can activate a cascade of healing events. Cytokines and GFs should be delivered at the injured site in the right amount and in a controlled manner to achieve a successful treatment [249, 250]. Several GFs are recruited during tendon and ligament regeneration, including the transforming growth factor (TGF-β), insulin-like growth factor-1 (IGF-1), platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF), basic fibroblastic growth factor (bFGF),

and growth differentiation factor (GDF). Each one of these has several roles in the different stages of ligament and tendon healing. A complete description of their function in controlling the inflammatory, proliferative, and remodeling phase is given elsewhere [251]. Regarding skeletal muscle, the same GFs can play an important role in the proliferation and differentiation of satellite cells during the myogenesis process [252]. For this reason, they can be potentially used to promote muscle healing after injury [253, 254].

Specifically, TGF-β is involved in cell proliferation, migration, and synthesis of both collagen and proteoglycans. In particular, TGF-β signaling is fundamental for the formation of tendons, and the isoforms TGF-β2 and TGF-β3 seem to have crucial roles in this process. This was confirmed when TGF-β2 and TGF-β3 knockout mice were unable to form mature tendons and ligaments [255]. At the same time, TGF-β is also involved in skeletal muscle homeostasis, and overexpression of its signaling pathways could be responsible for a variety of acquired or inherited neuromuscular disorders [256]. Another interesting GF to study is bFGF, which has shown particular efficacy as a promoter of tendon/ligament regeneration. In a recent study, bFGF was successfully incorporated within electrospun PLGA nanofibrous bioscaffolds seeded with BMSCs. The release of the GF was able to influence BMSCs proliferation and upregulate gene expression of ligament-specific ECM proteins. Moreover, bFGF induced BMSCs to differentiate into tendon fibroblasts over 14 days [257], and another study confirmed the importance of controlled delivery of bFGF on ligament and tendon regeneration *in vivo* [258].

In an another study, a combination of IGF-1 and bFGF was able to improve muscle healing and increase fast-twitch and tetanus strength compared to controls groups after 1 month [259].

Together with the GFs mentioned earlier, GDF has been studied to determine its role in signaling events during tendon development. In particular, ectopic subcutaneous implantation of human GDF-5, GDF-6, or GDF-7 in rats can lead to the production of a connective tissue rich in type I collagen fibers that show characteristics similar to those of tendons [41]. Other studies have shown that the deficiency of GDF can lead to weaker Achilles tendon in mice, reducing the content of collagen in tendons, which affects the mechanical integrity of the tissue [260–264].

An interesting source of GFs can be obtained using platelet-rich plasma (PRP). PRP has been reported recently as a useful strategy to induce tendon and skeletal muscle healing [265–268]. PRP is a bioactive component of whole blood, which contains 1 million platelets or more per microliter [269]. When platelets are activated both *ex vivo* and *in vivo*, they release GFs and proteins that are present within their alpha and dense granules (Figure 15.8). The alpha granules contain cytokines including PDGF, TGF-β1, and VEGF, among many others [249]. Platelets can be commonly activated with thrombin, which leads to a rapid release of the content of the granules. On the contrary, slow platelet activation and clot formation have been achieved using soluble type I collagen, making possible the administration of PRP through a small-gauge needle [271]. In addition, release of GFs from the activated platelets can be promoted by changing the pH of the PRP, especially in an acidic environment (pH 5) [272].

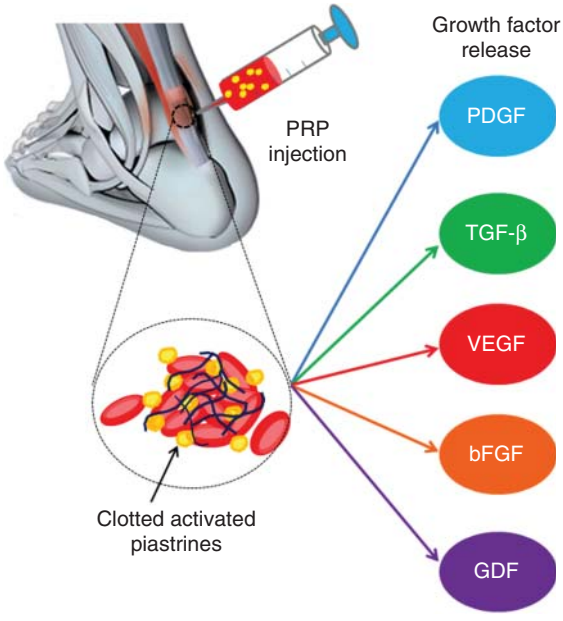


Figure 15.8 Representation of PRP treatment in tendons and the local delivery via injection of growth factors obtained from activated platelets to accelerate the regeneration process. (Reprinted with permission from [270].)

PRP is generally used to treat tendon disorders including tendinosis or complete tendon tears, in combination with surgery. Moreover, GFs contained in PRP can be potentially used to promote muscle healing. In a study involving 140 patients affected by chronic elbow tendinosis, treatment with buffered PRP prior to surgery was able to reduce pain in 81% of the patients [265]. In another trial in athletes who, instead, received PRP injection after surgery in the Achilles tendon, showed better and faster recovery respect to the control group without any treatment [273]. In an additional example of the use of PRP for arthroscopic repair of large rotator cuff tears, 48 patients were randomly assigned to receive PRP, and their effects were compared with those of a control group who did not receive any PRP augmentation treatment. The application of PRP significantly improved structural outcomes with improved clinical benefits for patients after a 1-year follow-up evaluation [274]. Concerning muscle healing using PRP, human trials on athletes who have suffered muscle injury returned to sport in half the expected recovery time without any evidence of excess fibrosis [275]. However, PRP treatment of skeletal muscle injuries relies on limited experimental data, and no randomized controlled trials and meta-analyses have been carried out to evaluate their safety [254]. For a complete summary on the recent therapies for soft tissue repair using PRP treatment, refer to [268].

Finally, PRP can be also potentially used as an injectable cell carrier to promote tendon and ligament healing [276]. PRP mixed with ADSCs were injected in a rabbit Achilles tendon defect model and compared with a control group treated only with acellular PRP gel. The combination of ADSCs and PRP produced increased tensile strength of Achilles tendons, evidencing that the mixture of platelets and stem cells could be beneficial in tendon regeneration. In the same way, a synergistic healing effect was found in injured rat Achilles tendons treated

with an injectable mixture of PRP and TSCs [277]. However, several concerns regarding the use of PRP as a suitable treatment for tendon and muscle healing have emerged because of the undesirable effect of TGF-β in PRP, which is responsible for the differentiation of various stem cells toward bone and cartilage lineages [278, 279]. Moreover, other recent studies indicate that a high concentration of leukocytes in PRP may result in persistent expression of inflammatory factors associated with scar tissue formation [280]. According to these findings, a long-term preclinical study should be carried out to assess and evaluate PRP's safety for clinical treatment [281].

Another main problem in the use of GFs is their restricted biological half-life, which can limit their use in clinical therapy. Growth-factor gene therapy can overcome this limitation [282]. In fact, with this strategy it is possible to maintain a high amount of GF at the injured site by continuous expression of certain genes. GFs can be delivered using viral carriers (lentiviral adenoviral, and retroviral) or nonviral vectors to target cells, causing a variation in the normal biological function of cells [237, 283, 284]. BMP-12 cDNA affected positively the Achilles tendon stiffness [285], and transduction of BMP-14 in Achilles tendon promoted a greater number of tenocytes and higher tensile strength when compared to untreated sham control [286]. Improvement in biomechanical properties and faster recovery in Achilles tendon defects have been also observed when BMSCs were transfected with adenovirus carrying human TGF-β cDNA [76]. At the same time, expression of collagen genes was upregulated when the adeno-associated virus (AAV) vector of the bFGF gene was transferred [287, 288]. Wang *et al.* included plasmid-containing PDGF cDNA to rat tenocytes of intra-synovial tendons, which showed significant upregulation of type I collagen gene.

15.4.6 Bioreactors in Musculoskeletal Tissue Engineering

In tissue engineering, bioreactors can provide ideal conditions for cell culture to facilitate dynamic exchange of oxygen, nutrients, and waste products compared to traditional 2D static culture conditions. Bioreactors in tissue engineering can also facilitate the control of environmental variables such as pH, temperature, and pressure. Additionally, bioreactors are versatile because they allow the application of physiological forces and stresses to stimulate tissue-engineered tendons and ligaments. Therefore, bioreactors can closely mimic *in vitro* the native environment of musculoskeletal structures.

Table 15.3 presents a summary of studies where mechanical stimulation has been applied on scaffolds populated with a particular cell type to be used in tendon, ligament, and skeletal muscle engineering.

Several benefits regarding the use of bioreactors in tendon/ligament engineering can be highlighted over traditional static culture:

1) *Nutrient transport*: Under static culture condition, uptake of nutrient, cytokines/GFs, and oxygen by cells and the removal of metabolic products occur only at the surface of the scaffold. Consequently, cells are less metabolically active and not viable at the core of scaffold.

   Perfusion chambers are common bioreactor configurations to facilitate the transport and exchange of nutrients, oxygen, and waste products [298, 299].

Table 15.3 Bioreactor use and outcomes in tendon, ligament, and skeletal muscle engineering.

Cell type	Stimulation	Scaffold	Results	References
Bone marrow mesenchymal stem cells (BMSCs)	Cyclic stretching at 2% strain	Human umbilical vein	Increased cell proliferation, ultimate tensile stress, and elastic modulus	[289]
Tenocytes	Cyclic stretching at 9% strain	Small intestine submucosa (SIS)	Increased cell proliferation and scaffold stiffness	[290]
Tracheal fibroblast	Cyclic stretching at 10% strain	Polyurethane (PU)	Increased deposition of collagen type I and elastin; increased elastic modulus	[291]
Mesenchymal stem cells	Cyclic stretching at 2.4% strain	Collagen sponge	Increased deposition of collagen type I; increased linear stiffness	[292]
Human embryonic stem cells	Cyclic stretching at 10% strain	Knitted silk–collagen sponge	Increased deposition of collagen types I and III	[293]
Tenocytes	Cyclic stretching at a constant force of 1.25 N	Rabbit hind-paw tendon	Increased ultimate tensile stress and elastic modulus	[294]
Human dermal fibroblasts	Dual dynamic loading at 0.625 or 10 N	Human flexor tendon	Increased ultimate tensile stress and elastic modulus	[295]
C2C12 (skeletal muscle myoblast cell line)	Cyclic stretching at 60 rpm	Agarose gel	Highly dense, highly oriented hybrid muscular tissue	[296]
Human myogenic cell	7.5% or 15% rapid or cyclical ramp strain	Collagen	MMP-2 expression was higher in experimental group	[297]

Interestingly, the number of cells in bioreactors functioning under continuous perfusion regimes increases significantly when compared with static cultures [289, 292].

2) *Mechanical stimuli*: The main role of the bioreactor in tendon/ligament engineering is to mimic, as much as possible, the physiological environment in the body. When tendon/ligament bioscaffolds are cyclically stimulated along the long axis of the scaffold, the newly deposited ECM fibers usually deposit along the direction of the mechanical force [300]. Proper mechanical stimulation can facilitate growth of BMSCs, as well as ECM synthesis and the upregulation of collagen type I and III genes [301]. Fibroblast and BMSCs proliferation, collagen deposition, and tenogenic gene expression have also been shown to increase under cyclic uniaxial stimulation [302, 303].

Although the advantage of bioreactors in tendon/ligament tissue engineering has been proven at small scale and in several animal models, the optimal functioning conditions to translate its use to the clinic are still unknown. The use of bioreactors as part of the strategy to fabricate engineered tendons and ligaments is still restricted by the size of engineered tissue, scale-up limitations, cell source, and production costs. Nonetheless, the use of bioreactors in tendon and ligament engineering is promising and will be a common practice in the clinic over the next decade [304]. For a comprehensive review on the use and design of bioreactors in tissue engineering, refer to the work of Bilodeau and Mantovani [305].

To replace large muscle tissue defects, functional substitutes could be prepared *in vitro*, using bioreactors that mechanically and electrically precondition the construct in order to better replicate the native target tissue. Studies have shown that electrical stimulation can not only influence muscle cell phenotype [306] and myosin expression [307] but also modulate the fiber-type switch [308] and induce contractility in differentiated myotubes [309]. Liao *et al.* showed that a combination of topographical and electrical cues can significantly improve myoblast alignment, maturation, contractility, and protein expression of skeletal myoblasts on electrospun polyurethane fibers [310]. Electrical stimulation can also be effective for the expansion of myogenic progenitor cells in 3D scaffolds without losing their myogenic potential [311]. This fact can be extremely important for satellite cells, which can lose their regenerative potential after expansion in culture.

Mechanical conditioning has been used to greatly influence myogenesis muscle remodeling and fiber thickening that occurs following exercise [312]. Corona *et al.* and Moon *et al.* reported that mechanical-stimulation-associated improvements in structural organization can be translated into force-generating muscle constructs when implanted *in vivo* [313, 314]. Okano and Matsuda encapsulated C2C12 myoblasts in a collagen gel, and after subjecting it to cyclic mechanical stretching, observed an assembly of highly dense and oriented myotubes resembling native muscle tissues [296]. Auluck *et al.* seeded myoblasts on porous collagen scaffolds and subjected them to either continuous or cyclic uniaxial strains [297]. They reported that relative to unstrained controls, continuous mechanical straining induced significantly higher expression of matrix metalloproteinase-2 (MMP-2), indicating the onset of muscle ECM remodeling.

15.5 Recent Advancements in Musculoskeletal Tissue Engineering

15.5.1 Animal Models in Musculoskeletal Tissue Engineering

Animal models can contribute to the understanding of the biology of tissue regeneration. Several models of tendon/ligament defects have been investigated in mouse, rat, rabbit, sheep, goat, pig, and horse. The most relevant animal models have been summarized in Table 15.4.

Mouse and rat models are relatively cheap compared to other animal models, and rat shoulder models have an anatomy very similar to that of the human shoulder. Therefore, rat models have been widely used in rotator cuff research. Nonetheless, the size of tendons and ligaments in these animal models cannot be compared to those in humans. Results from rabbit models are also often reported, but in this case, the anatomy differs from that of humans because the flexor digitorum superficialis is not present in humans and the flexor digitorum longus does not exist in rabbit [332].

Therefore, some investigators have attempted to study engineered tendon and ligament models directly in large animals. Sheep has been preferred as a knee model because its corresponding force curves display shapes similar to those of the human knee when sheep knees are loaded throughout the gait cycle [333]. Additionally, the healing and recovery process in pigs is similar to that of humans [334], so researchers have chosen the pig's knee as preferred ACL repair model [335]. Horse superficial digital flexor tendon has been used as an important model since this tendon injury and Achilles tendon tendinopathy have similar features to those of humans as shown by ultrasonography and magnetic resonance imaging (MRI) evaluation [336].

Regarding muscle regeneration studies, rat skeletal muscle injury models are commonly used to evaluate how the process of healing can be modulated by the injection of cells or specific muscle microRNAs, which are an important modulator of skeletal muscle development and homeostasis [337, 338].

Specifically, von Roth *et al.* demonstrated restored function in damaged muscle tissue after intra-arterial infusion of mesenchymal stem cells (MSCs) in Sprague Dawley rats that received an open crush trauma in the left soleuls muscle. Interestingly, MSCs were not found at the injury site, supporting the hypothesis of a paracrin effect via secretion of soluble factors, which can play a crucial role in the functional regeneration of the muscle [321]. Analogously, in another study, Pecanha *et al.* injected adipose-derived stem cells into the soleous of female rats to promote muscle healing, which had the maximum effect 2 weeks after the treatment [323]. Alternatively to rat, also rabbit models have been used to promote the regeneration of skeletal muscle following several strategies such as implantation in the defect site of atelocollagen sponge scaffolds [339] or intramuscular injection of naked plasmid-DNA to improve angiogenesis and muscle regeneration [340].

In summary, all these examples highlight the importance of animal models to better understand the possible strategies to repair soft tissue injuries. However, it is important to consider that every animal model has unique pathological

Table 15.4 Animal models in musculoskeletal tissue engineering.

Animal	Ligament/tendon/ skeletal muscle model to be repaired	Cell type	Scaffold	Cytokine	Results	References
Mice	Achilles tendon defect	Human embryonic stem cells	Connective tissue	N/A	Higher load to failure	[315]
Rat	Patellar tendon defect	Tendon-derived stem cells	Cell sheet	Connective tissue growth factor	Higher collagen deposition, ultimate stress, and Young's modulus	[241]
Rat	Achilles tendon defect	TDSCs	Collagen scaffold	PRP	Higher collagen type I and III when compared to control group	[277]
Rat	Rotator cuff transaction	BMSCs	N/A	Fibrin and Mt1-MMP	Higher load to failure	[316]
Rat	Achilles tendon detachment	BMSCs	N/A	N/A	Accelerated healing and high load to failure	[317]
Rat	Patella tendon window	BMSCs	N/A	PRP	Higher collagen type I and III expression	[318]
Rat	Patella tendon window	Human ES	Fibrin	N/A	Increased collagen formation and tendon strength	[204]
Rat	Ectopic site	MPCs	ECM	N/A	Increase in myosin protein content, formation of myofibers	[319]
Rat	Dorsal skinfold window chamber	Myoblast	Fibrin and Matrigel	N/A	In-growth of blood-perfused microvasculature and improved structural organization of engineered muscle	[320]

(*Continued Overleaf*)

Table 15.4 (Continued)

Animal	Ligament/tendon/skeletal muscle model to be repaired	Cell type	Scaffold	Cytokine	Results	References
Rat	Crush injury	BMSCs	N/A	N/A	Improved muscle force but no change in fibrosis. No BMSCs found in the injury site after 21 days	[321]
Rat	Muscle resection	BMSCs	ECM	N/A	Reduction in functional deficit	[322]
Rat	Muscle laceration	ADSCs	Matrigel	N/A	Repair accelerated but no change in amount of fibrosis	[323]
Rabbit	Achilles tendon defect	ADSCs	N/A	PRP	High collagen type I, FGF, and VEGF detection in Immuno-histochemistry	[324]
Rabbit	Patella tendon window	BMSCs	N/A	N/A	Increased maximum load	[325]
Rabbit	Rotator cuff	TDSCs	Silk-collagen scaffold	N/A	High collagen deposition, stiffness, and load to failure	[326]
Sheep	ACL	BMSCs	N/A	N/A	Increased collagen content, stiffness, and tangent modulus	[327]
Goat	ACL	Skin fibroblasts	Collagen	N/A	Sharpey's fibers and fibrocartilage were observed at bone–ligament interface	[328]
Pig	ACL	BMSCs	Silk	N/A	Improved ligament-to-bone insertion was observed	[329]
Horse	Superficial digital flexor tendon	N/A	N/A	PRP	High collagen production, tensile properties, and elastic modulus	[330]
Horse	Superficial digital flexor tendon	Embryonic stem cells	N/A	N/A	Increase in cell density, collagen pattern, and repair size	[331]

N/A, not available.

conditions and anatomical features that differ from those of humans. Therefore, clinical trials are always necessary to clearly delineate possible tissue-engineered strategies in humans.

15.5.2 Current Musculoskeletal Tissue Engineering Repair Strategies in Clinical Trials

Although tissue engineering is a promising alternative to current clinical practices for soft-tissue regeneration, the gap between basic laboratory research and the clinical need is still large. A thorough search in clinical trial databases indicates that engineered strategies in tendon/ligament repair are very limited.

PRP is currently used for clinical treatment, but the reported results are still conflicting. Almeida *et al.* reported the effect of PRP in ACL repair in a randomized control trail. MRI was performed 6 months after surgery and injection of PRP, and it was found that the visual analog scale score for pain was lower in the PRP-treated group when compared with the untreated group [341]. Kesikburun *et al.* subjected patients with rotator cuff tendinopathy to one injection of PRP. Evaluation after 1 year showed no significant difference in the quality of life, pain, disability, and range of motion between those patients who received the PRP treatment ($n = 25$), and the placebo group ($n = 21$) [342]. Therefore, more studies on the real effect of PRP for tendon/ligament healing are required.

Ellera Gomes *et al.* investigated the effect of bone marrow mononuclear cells (BMMNCs) on rotator cuff tears during 12 months after injury. Autologous BMMNCs were injected in the injury site, and MRI screening showed good tendon repair ($n = 14$); nonetheless, the experimental design in this study lacked a control group [343]. Obaid *et al.* injected skin-derived fibroblast into Achilles tendons of patients suffering from tendinosis [344]. Six months after injection, Victorian Institute of Sports Assessment (VISA) and Visual Analog Score (VAS) quantification score sheets were used to evaluate tendons. All cell-treated unilateral cases (only one knee received the cell treatment, $n = 12$) showed improved healing. However, no significant difference was evidenced between bilateral cases (both knees received the cell treatment, $n = 8$) and the control group.

Biophysical modalities such as low-intensity pulsed ultrasound (LIPUS) and extracorporeal shockwave treatment (ESWT) can help in healing tendons and ligaments. Walsh et al. showed that LIPUS improved the peak load to failure and stiffness of the tendon-to-bone junction in sheep ACL models [345]. Wang *et al.* and Chow *et al.* found that load-to-failure and tensile strength increased significantly after ESWT [346, 347]. However, LIPUS treatment did not show any benefit beyond that of a placebo effect in a randomized trial. Warden et al. found that there were no differences between the active-LIPUS group ($n = 17$) and the inactive-LIPUS group ($n = 20$) using the VAS score for chronic patellar tendinopathies [348].

Concerning the topic of muscle regeneration, no clinical trials are yet available on the application of MSCs for muscle injury. However, several studies are showing promising results in this area of research. Specifically, in a study by Torrente *et al.*, muscle-derived CD133+ stem cells were injected into eight boys with Duchenne muscular dystrophy [349]. Patients reported an increase in the content

of their fast myosin-positive fibers and the capillary density of their muscle fibers. Another alternative for achieving muscle regeneration is the use of acellular ECM scaffolds. In a recent clinical case study by Mase *et al.*, acellular ECM scaffolds were applied for constructive remodeling of the skeletal muscle within the volumetric muscle loss site (VML) of the quadriceps femoris muscle [350]. At 16 weeks post implantation, the patient showed a 30% increase in muscle function concomitant with the presence of soft tissue resembling skeletal muscle. Finally, in another study, Sicari *et al.* evaluated the effect of ECM implantation at the site of VML in 80 patients. They reported that multipotent perivascular cells, neovascularization, and regeneration of skeletal muscle tissue (centrally located nuclei) existed throughout the ECM scaffold [351].

15.6 Conclusions and Future Directions

Research in tissue engineering and regenerative medicine involves deep understanding of cell and molecular biology, developmental cell biology, immunology, polymer chemistry, and engineering design. Mastering these complementary fields is taking medicine to a direction where three strategies are important: transplantation of cells to induce neo-tissue formation at the transplant site, grafting of bioartificial tissues fabricated in the laboratory, and induction of replacement and repair of neo-tissue using biological cues from native tissue adjacent to the injury [352]. Regarding the first strategy, the use of stem cells will be preferable over ESCs or differentiated cells harvested directly from the donor, because stem cells have potentially unrestrained proliferation and growth capacity. These stem cells can acquire tenogenic and ligamentogenic linages and provide regenerative tissue able to recapitulate the embryonic linage transitions originally involved in soft-tissue formation. The topics of this chapter show that the use of tenocytes or ligament fibroblasts is still viable, but further research is needed to develop strategies capable of maintaining viable cell for long periods of time at the injury site. Cell delivery vehicles and smart bioscaffolds decorated with GFs and cytokines will be necessary to maintain stem or differentiated cell populations active during injury repair.

Alternatively, stem cells can be utilized to fabricate a bioartificial tissue to restore and repair the tissue at the injury site. Still, concern arises while considering the *in vitro* biofabrication process of these tissue constructs, which is often too slow and does not allow the possibility to produce large, practically useful 3D scaffolds. Specifically, in the case of muscle tissue engineering, the challenges yet to be overcome are the scaling up of these 3D scaffolds and their lack of vascularization and innervations at the implanted site.

Moreover, a particular area that will require special attention is the design of a scaffold mimicking the osteotendinous or osteoligamentous interface, which corresponds to a gradient section of a highly mineralized tissue and a poorly mineralized one. Engineering these interfaces will be extremely useful in promoting the host integration of these 3D tissue constructs at the implantation site, speeding up the process of healing and soft tissue regeneration.

To achieve all these challenging steps, a possible combination and improvement of the biofabrication techniques described in this chapter will be necessary in order to resurface large osteotendinous or osteoligamentous defects, leading to the possibility of developing tissue-engineered prostheses for soft-tissue reconstruction.

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16

Bone Tissue Engineering: State of the Art, Challenges, and Prospects

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16.1 Introduction

Bone is a dynamic tissue with its own blood vessels. It contains living cells, aiding in its growth and self-repair. During the years preceding adulthood, cartilaginous tissues grow and are thereafter replaced by a mineralized matrix (bone). The major functions of bone tissue include (i) provision of structural support for the rest of the body, (ii) protection of vital organs, and (iii) provision of an environment for blood cell production and a compartment for the storage of minerals. Bones are composed of a hard outer layer (compact or cortical bone) and a spongy inner compartment (trabecular or cancellous bone) softer than the compact bone. Bone also harbors osteoblasts, osteocytes (cells forming bone), osteoclasts (cells resorbing bone), and a nonmineralized collagen matrix (osteoid, with non-collagenous proteins) [1].

Bone cells are responsible for bone production, its maintenance, and remodeling. The osteoblasts originate from mesenchymal stem cells (MSCs), which synthesize and mineralize bone matrix proteins. In grown-ups, most of the bone surface areas do not undergo cycles of formation and resorption (i.e., remodeling), and they are populated with bone-lining cells. Osteocytes and osteoblasts, which are embedded in the *de novo* formed osteoid, are eventually turned into hard bone. Osteocytes, being positioned in the center of bones, stay connected to osteocytes in the osteoid as well as osteoblasts and lining cells on the surface. They all constitute an intricate network of connecting cells, which is optimally situated to respond to altered impact of mechanical forces, transducing messages that initiate net remodeling responses. Osteoclasts are voluminous and multinucleated cells emanating from the hematopoietic lineage, and they resorb mineralized tissue. They express a "ruffle border," on which mineral resorption is effected, with the ensuing secretion of enzymes resorbing the mineralized matrix [1].

Bone tissue formation (osteogenesis) is accomplished via the so-called intramembranous and endochondral ossification. The former process encompasses replacement of connective tissue structures in the production of flat

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bones, while the other process provides the exchange of hyaline cartilage with mineralized tissue types such as the femur, tibia, and humerus/radius. Bone modeling takes place when resorption occurs on separate surfaces. In contrast, remodeling is characterized as the exchange of the old bone tissue with newly formed bone. This occurs in adults in order to preserve bone mass and is effected by coupling bone formation and resorption. The process sequence consists of the following steps: (i) activation, when immature osteoclasts differentiate into multinucleated osteoclasts; (ii) resorption, when these cells digest "old" bone; (iii) the reversal phase, which represents the termination of matrix resorption; (iv) formation, when the osteoblasts produce *de novo* bone matrix; and (v) quiescence, when the osteoblasts are "transformed" into resting, that is, inactive, "bone-lining" cell units [1].

Bone engineering by regenerative medicine is generally designed to restore the form and function of bone lesions. One critical factor is the size of the osseous "deficiency," which fails to regenerate spontaneously, thus necessitating intervention through surgery. The need for successful rebuilding (i.e., reconstruction) of bone defects with critical size has brought about the search for efficacious tissue engineering over the past 20 years. This chapter focuses on the art of "bone tissue engineering," its challenges, and future prospects.

16.2 Factors Important in Tissue Engineering of Bone

The process of bone renewal includes knowledge about the triad, namely cellular entities, molecular signaling, and artificial 3D scaffolding. However, pertinent questions must be addressed. The important issues are (i) the type of cells, biologicals, and matrices to be used; (ii) the selection of optimal physiological and therapeutic doses; (iii) temporal and/or spatial distribution of the mentioned criteria for tissue reconstruction; (iv) their dynamics and kinetics; (v) application related to the display of customized, performance-related design specifications, and, finally, (vi) the manipulation of pathways involved in the requirement for sophisticated tissue engineering therapeutics. All of these aspects may be summarized as constituting the epigenetic machinery, encompassing epigenators, initiators, and maintainers, where humoral factors, DNA-binding factors (transcription factors (TFs)), noncoding RNAs (micro-RNAs), and histone modifiers (like histone deacetylaces (HDACs) and histone acetyl transferases (HATs)) play important roles (see Figure 16.1).

16.2.1 Scaffolds and Biomaterials

These 3D structure provides a lattice for the migration of regenerating cells, as well as their specialization and sequestration in scaffolding materials and proliferative and differentiating environments. The support is multidimensional, and provides a basis for the attachment, proliferation, and phenotype development of the cells in question. The physiology of induced mineralized tissues also necessitates that biomaterials are guided to the sites to be rebuilt and eventually are able to withstand the loads associated with bone compression.

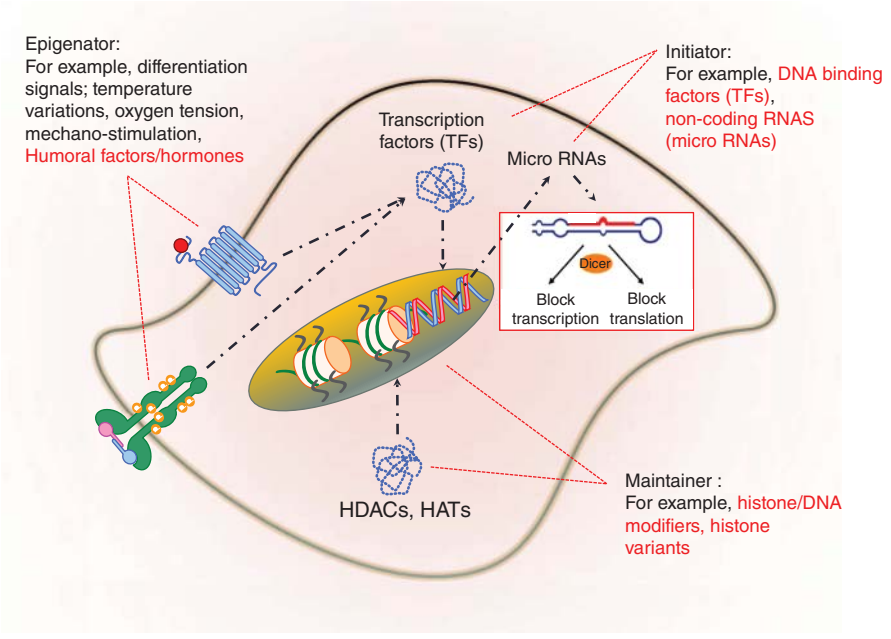

Figure 16.1 Modern concepts of epigenetics. The epigenetic machinery consists mainly of factors like epigenators (i.e., differentiation signals, such as temperature variations, oxygen tension, mechano-stimulation and humoral factors) initiators (such as DNA-binding factors (transcription factors) and non-coding RNAs (micro-RNAs)), and maintainers, including histone/DNA modifiers (like HDACs and HATs) and histone variants.

Inorganic materials (e.g., such β-tricalcium phosphate, hydroxyapatite (HA), and glass scaffolds) have been long utilized for tissue replacement purposes because of their structure and composition identity with the bone mineral composition [1, 2]. Their main deficiency is their brittleness, which is a drawback when high load-bearing capacities are required. Naturally derived biomaterials (such as collagen [3], hyaluronic acid [4], cellulose [5], silk, alginate [6] chitosan, and others) display characteristics of true bone tissues, which allow cell adhesion and migration within their pores.

Synthetic materials with tunable features are under development. On the microscale, they feature 3D composition, architecture, and active molecular reactive groups. On the other hand, macroscale characteristics include porosity, stiffness, and elasticity. Regarding constituents, polymer materials applied in tissue regeneration include polymeric compounds of lactic and polyglycolic acids (PGAs), polycaprolactone (PCL), polyethylene, poly(ethylene glycol) (PEG), and poly(methyl methacrylate). However, these materials lack bioactivity and hamper the required biomaterial–host interactions. Furthermore, their degradation products often include detrimental acidic products, hampering tissue regeneration.

Eventually, smart scaffold design is accomplished by using both organic and inorganic materials (e.g., electrospun collagen nanofibers + PCL micro strands,

or chitosan + HA), which have furnished scaffolding materials with appropriate mechanical characteristics, pore size, and biological activity, supporting cell in-growth and *de novo* bone formation [7]. Many similar examples can be found in the literature [8–10]. A large body of data, moving this field of research forward, has been published by the Department of Biomaterials, Institute for Clinical Dentistry, University of Oslo, Norway [11–15].

16.2.2 Sourcing

The lack of clinical success may be attributed to several issues. First, the quality or quantity of used cells and preculture conditions of cells seeded onto scaffolds are variable or limited. Second, the seeded cells may be subjected to inflammation and nutrient scarcity because of tissue damage and the diffusion of nutrients and oxygen from adjacent vessels [16, 17]. The current state of the art in cell-based bone tissue engineering/regenerative medicine (BTE/RM) in terms of critical procedures and efficacy of mono and coculture approaches encompasses stem cell (SC) sources (such as non-adult SCs, adult SCs, bone-marrow-derived MSCs (BM-MSCs), adipose-tissue-derived MSCs (AT-MSCs), and dermal papilla-derived MSCs (DP-MSCs)), isolation and expansion of MSCs, and cell-seeding parameters (i.e., techniques and preculture on scaffolds, cell-seeding efficiency, cell-seeding density, perfusion of nutrients, and pre-differentiation of cells).

In vivo preclinical studies encompass cell sources, scaffolds, seeding density, *in vitro* preculture time, *in vivo* time, species' repair site evaluation methods (i.e., histology, micro-CT, and X-ray), efficacy of bone formation in terms of bone percentage (%), bone volume (mm^3), and bone mineral density (BMD) [16].

Bone regeneration may be compromised in some cases, such as in patients with risk factors like smoking or diabetes [18]. Cell-based BTE/RM has thus proven to as an attractive approach for bone regeneration in many preclinical studies [16, 17]. Standardized methods of cell-based tissue engineering in clinical work entails protocol-based cell isolation and cultures, proper characterization of the isolated cells, and efficient cell seeding with optimal cell sources, scaffold types, and cell seeding parameters. A proper pre-differentiation scheme is also mandatory.

Based on the promising outcomes from preclinical studies, in which cell-based constructs enhanced bone formation compared with cell-free constructs, one took the translational step from the bench to the bedside. Because of the limited clinical experience and nonvalidated examination methods (i.e., biopsy), few studies could show the efficacy of cell-based strategies for BTE/RM in the clinic. Hence, ongoing active research is most relevant to the translation of cell-based constructs for bone regeneration into clinics: that is, development of bioreactor systems, scaling up to clinically sized bone constructs, evaluation of cell-based grafts in large animal models, and swift establishment of vascularization of implanted bone grafts [16]. An overview of the opportunities and challenges of cell-based tissue-engineered constructs for clinical applications in BTE/RM is given elsewhere [16].

The essence is the mobilization of "engineered" or adapted cells to restore a continuous tissue microstructure, thus ensuring proper osseous infrastructure and functionality. Implantation of exogenous pluripotent precursor cells (e.g., adipose-derived, mesenchymal, dental-pulp-derived, and/or induced pluripotent stem cells (iPSCs)) has advanced the field of bone engineering. Genes, whose expression needs control when producing iPSCs, encompass oncogenes, Kruppel-like factor 4 (KLF4), and c-MYC, as well as OCT4 and SRY-box 2 (SOX2) [19]. But, an "adjustment" of the genomic imprint raises doubts concerning enhanced chances for the development of cancer-inducing implants.

16.3 Fabricated Tissues by 3D Printing of Suspensions of Cells on Micro-Carriers

A new strategy addressing these demands is combining bio-printing with micro-carrier technology. The present strategy enables the optimal expansion of phenotype-controlled cell phenotypes, forming multicellular clusters. Here, living constructs are produced via bio-printing of cell-laden micro-carriers. MSC-laden polylactic acid (PLA) micro-carriers would be trapped in gelatin methacrylamide–gellan gum bio-inks and fabricated as composite material, demonstrating both enhanced cell number and survival rate. This technology has improved several characteristics such as the hydrogel constructs' compressive modulus, degree of cell adhesion, and sustained osteogenic differentiation of the osseous matrix containing the MSCs. Three-dimensional osteochondral tissues could be generated with the aid of cell-laden artificial material, generating bone compartments [20–22].

Several challenges emerge in relation to the fabrication of hydrogel-based tissue implants, for example, the introduction of powerful hydrophilic microenvironments, where cells favor attainment of a round shape irrespective of their original morphology [23]. Hence, cues to predict cell fate should be sought. Next, the printing of large osseous grafts necessitates the encapsulation of a large number of cells [24]. Therefore, tedious 2D expansion steps are mandatory, reducing the healing potential of the cultured cells by altering their characteristics [25]. Furthermore, hydrogels appear too soft to be applied in load-bearing organ structures [26]. A solution would be to load composite bioactive microspheres onto a hydrogel matrix.

16.3.1 Cellular Behavior: A Scrutiny of the Cell–Biomaterial Interface

Generally, cell material is sampled from the receiver patient (autologous cells), which displays a few downsides: (i) tedious procedure, (ii) requirement of surgical access to a sampling site, (iii) low cell output, (iv) low expansion level, (v) limited viable cell quantity, and (vi) absence of bone remodeling. An alternative to the application of the patients' own cells could be cells from heterologous donors or xenografts. Unfortunately, the present procedures also represent "obstacles" such as (i) enhanced immune reactions, (ii) possible transmission of putative

infectious agents, and (iii) controversial moral and religious issues. In this context, the biology of SCs emerges and truly represents the more trustworthy solution (e.g., see [27]).

16.3.2 Some Interesting Recent Discoveries

MSCs, isolated from the bone marrow, were differentiated into both premature chondrocytes and osteoblasts via stimulation with the transforming growth factor (TGF)-β1. In another report, isolated MSCs were incubated in a polylactic matrix for 10 days in an osteogenic medium. Then, the differentiated cells were transferred to surgically induced bone lesions of the mandible. At 6 weeks, histologic, clinical, and radiographic analyses were performed. The defects now harbored dense, newly made tissue, mimicking osseous nanostructures in all aspects of trabecular bone, including blood vessels. Furthermore, cells taken from the bone marrow, which were multiplied *in vitro* and grown on an HA lattice, fully "mended" a prefabricated bone lesion. A complete integration with the repaired bone was seen after 6–7 years of treatment. This "achievement" indicates the long-term durability of bone regrowth accomplished by the presently described rebuilding concept.

Much effort has been funneled toward the reconstruction of the periodontal ligaments, cartilage regeneration, regeneration of teeth, induction of the mineralizing environment (i.e., nucleation of HA with carbonates), and the making of functional dental crowns mimicking naturally "grown" teeth. Generally, one may acknowledge that the requirement for cellular growth preferably encompasses phenomena such as the following: (i) specific bio-activating "growth factors" or hormones within each tissue; (ii) well-characterized physical, biochemical, and/or chemical features of the carriers, that is, surface energy characteristics, loading capacity, and the extent of hydrophobicity; (iii) morphology of "vehicle" materials (which are effective in stimulating the cell activity such as the degree of porosity and roughness/resilience); and (iv) their ability to serve as mechano-stimulators with or without piezoelectric modulatory contraction/relaxation frequency (e.g., see [27]).

16.3.3 Behavior of Cells as an Approach to Understanding Interactions Between Cells and Biomaterials

A cell's response to a given bioactive compound describes an important concept featuring the activation of a membrane receptor, signal transduction, and an ordered cascade of events that leads to altered expression of specific genes, thus mastering a cell's behavior. It was recently discovered that these signaling pathways may interpret the biochemical and topographic (3D) characteristics of the certain substances, even when variations take place at the nanoscale. The novel class of "intelligent" materials can be constructed relying on preferential cell responses only, ensuring a predefined outcome, with reference to the restructuring of the cytoskeleton in a controlled fashion. This involves signals stemming from different angles, involving key proteins. Examples of such factor are MAPK2, PP2A, cofilin, and HSP-27 (e.g., see [27]).

Subsequent to integrin activation, defined molecular classes are evoked for structuring lattices of focal adherence. Such signaling molecular arrays involve focal adhesion kinase (FAK) and Src activation. Phosphorylation at specific sites significantly activities these enzymes, thus altering cell behavior. Moreover, a low molecular weight protein tyrosine phosphatase (LMW-PTP) controls Src activity through dephosphorylation (e.g., see [27]).

16.3.4 Microarrays of Peptides as Useful Tools to Predict Signaling Mechanisms

By applying PepChip-based strategies, it was shown that glycogen synthase kinase (GSK)-3b serves as a centrally modulating "device" during the adherence of osteoblastic cells to a scaffold surface. GSK-3 appears to be critical in a survival path involving PI3 kinases/cell Akt. Another molecule that is active during the adherence of osteoblasts is Rap1A. Rap1A yields phosphorylation arrays 8–10 times higher in the course of cell adherence, when compared to a reference group. It appeared feasible to define a network of proteins induced by HA exposure, and it was shown that HA triggers pathways yielding the reconstruction of the osteoblastic cell cytoskeleton. This could be attributed to the stimulation of ADD1 (Adducin 1), protein kinases A and C, and vascular endothelial growth factor (VEGF), as well as HDAC1, showing that HA kick-started the differentiation of osteoblastic cells while the metabolic pathways of cell survival were still active.

Some comprehensive and exhaustive reports on biomaterials and growth factors supporting the differentiation and phenotype acquisition of pre-osteoblast and osteoblast differentiation from SCs should be mentioned, which encompass pore-graded biomimetic TiO_2 bone scaffolds without or with alginate hydrogel, enamel matrix derivative, PPARδ agonist GW501516, simvastatin-coated TiO_2, surface hydrid or hydrofluoric acid treatment of titanium or titanium dioxide surfaces, and SiO_2 and CaP coatings. For references, see [27].

16.3.5 Growth Factors

Growth "hormones" for bone regeneration include fibroblast growth factor (FGF), bone morphogenetic proteins (BMPs), VEGF, platelet-derived growth factor (PDGF), and PRP (platelet-rich plasma). Delivery of exogenous growth factors is stalled as a result of poor penetration of tissues, arbitrary migration/movement to desired sites and less efficient internalization (endocytosis) by the cells [1, 28, 29], and exposure to hypoxic/acidotic inflammatory environments with residing neutrophils and macrophages [30]. Rather, one should pursue the expression of temporally and dynamically interplaying growth factors present during the osteo-inducing sequence of events. As previously stated, the major players are growth factors such as PDGF, FGF, VEGF, and the BMPs. Interestingly, BMP-2 and VEGF work in a synergistic fashion in bone regeneration when added sequentially to a PLGA (poly(lactic-*co*-glycolic acid)) scaffold [31]. VEGF displays no osteo-induction alone, but it is angiogenic. Recombinant BMP-7 (rhBMP-7) in the presence of rhPDGF-BB has been shown

to sustain a better bone production than each factor by itself when applied to mice with osteoporosis-induced critically sized defects [32].

16.3.6 Bioreactors and Mechano-Chemical Stimulators of Bone Tissue Engineering

The way microgravity is impacting the biology of human cells, and in particular bone cells, during the formation of 3D cell cultures during genuine and artificial microgravity (r- and s-μg) is of major interest to biomedical appliances. During r- and s-μg, different cell entities are directed into 3D structures. Tissue engineering facilities in space and on earth apply systems exemplified by the random-positioning machine (RPM), the 2D clinostat, or the NASA-developed rotating-wall vessel (RWV) bioreactor to produce 3D tissues from MSCs and osteoclasts [33]. The present devices also facilitate the impact of various growth hormones, differentiating factors, and/or a variety of drugs. Much new information has been gained and accumulated/achieved in bone engineering using the RWV system, but also multicellular tumor spheroidal (MCTS) clusters, formed in both r- and s-μg, have recently been reported [34].

To date, the research encompassing intricate cellular interactions between bone-producing cells engaged in mechanical and biochemical induced phenomena remain obscure because of a lack of adaptable *in vitro* model systems, capturing its "coupled" nature. By applying the newly invented "rotating coculture" methodology, a large mineralized 3D tissue construct from a coculture of primary osteoblast and osteoclast precursor cells, without the assistance of any auxiliary bone-inducing scaffolding material, could be generated. In general, mature osseous tissues are made of an outer region populated by osteoclasts and osteoblasts residing within a central core harboring osteocytes engulfed/embedded in an extracellular mineral matrix (ECM). "Constructed" bone tissues display various features (morphology, mineral contents, and biochemistry-related) close to human remodeling trabecular bone tissue while expressing mRNAs for factors like Sclerostin (SOST), Bone GLA-Protein (BGLAP), acid phosphatase 5, tartrate resistant (ACP5), and various BMP species. The bone constructs also secrete BMP-2 protein into the surroundings [33, 34]. A summary of microRNA manipulations, scaffold materials (PLA, HA, or TiO_2), and exposure to mechano-stimulation of human SCs isolated from belly adipose tissue (hADSCs) for 20 days is given in Figure 16.2.

Furthermore, Figure 16.3 sketches a comprehensive check list of how to proceed when evaluating the characteristics of biomaterials and cells and the array of analyses to be conducted, guiding the researcher toward successful bone engineering. Tables 16.1 and 16.2 present some of the results obtained by following the guidelines summarized in Figure 16.3. Here we show the effects of various scaffold material with or without mechano-stimulation on osteoblast parameters, mineralizing properties, their ability to communicate with osteoclasts, and levels of marker microRNA species (Table 16.1), and the ability of hADSCs to produce osteoblasts, recruit osteoclasts, and mineralize within the tibial muscle of SCID mice, after having been exposed to cytokines, normally secreted from inflammatory T-helper (Th) cells (Table 16.2).

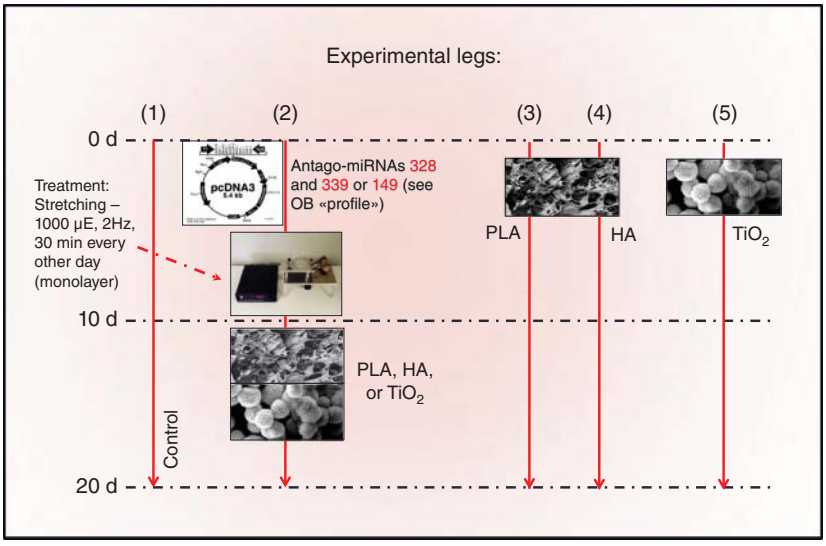

Figure 16.2 Experiments featuring microRNA manipulations, scaffolds (PLA, HA, or TiO_2), mechano-stimulation (1 µE equals 1 ppm enhancement of cell diameter), and mechano-stimulations of human adipose-tissue-derived stem cells (hADSCs) for 20 days. Leg 2 represents a "permutation," that is, a pretreatment of the hADSCs using microRNA manipulation, mechano-stimulation (3D culture), and a final growth in different scaffold material (3D culture).

16.4 Recent Advances in Bone Tissue Engineering

Bio-printing using inkjet printing may turn out to be a promising concept for artificial mineralized tissue production. The types of cellular sources and scaffold biomaterials are deemed to be critical factors to secure a successful bio-printed tissue. Human MSCs dispensed in PEGDMA were mixed with bioactive glass or HA and subjected to an identical polymerization process. The MSCs cultured on HA demonstrated the highest cell viability (87%) and augmented compressive modulus (358 kPa) after 3 weeks in culture. The highest production of collagen and larger alkaline phosphatase (ALP) activity were observed in the PEG-HA group, which shows a good correlation with quantitative polymerase chain reaction (Q-PCR) analyses. Finally, Masson's trichrome coloration indicated that PEG-HA scaffolds produced most collagen [20–22].

However, both the thermal and chemical pretreatments of commercially available scaffolds reduce their applicability to cell specimens and humoral growth-inducing molecules as 3D building units. Hence, both cells and stimulatory humoral macromolecules are added to the scaffold separately. However, both the migration and penetration of live cell entities into the scaffold material remain unsatisfactory. In order to circumvent such drawbacks, inkjet bio-printing has been designed to deposit all components mentioned above to their respective 2D or 3D application sites. The detailed procedure can be found elsewhere, but it was, however, confirmed by analyses such as bio-printing and

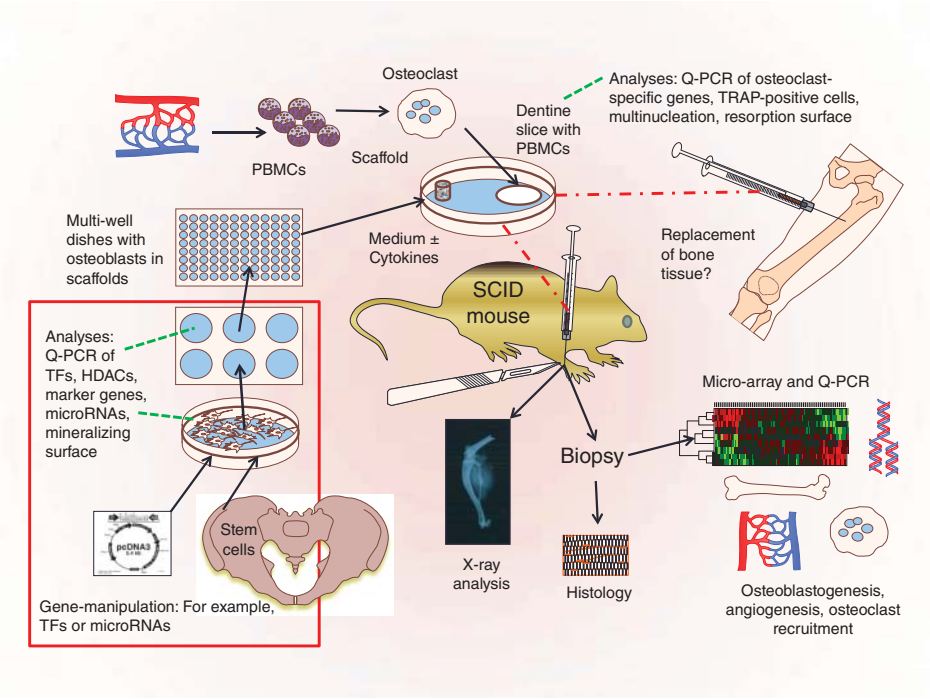

Figure 16.3 One comprehensive scheme showing the possibilities of manipulating human stem cells, prior to injecting them into the tibial muscle of SCID mice. This model system allows checking for the differentiation potential, ability to produce bone, ability to recruit osteoclasts, ability to develop own vasculature, and finally to ensure proper epigenetic profile of the cells retrieved within the scaffold material.

tissue culture, swelling characterization of printed structures, mechanical characterization of printed cell-laden material, RNA extraction and gene expression via RT-PCR analyses of Runx2, Collagen-I, Osteocalcin (OCN), Collagen 10A1, and MMP13, as well as by biochemical analyses (DNA, ALP) and histology scores [20–22].

16.4.1 What Are the Next Steps for 3D Bio-Printing?

For 3D bio-printing to meet its true potential, further refinement of a multitude of aspects and understanding of both the underlying biology and biophysics of healing, that is, regenerative processes *in vivo*, are needed. Issues to be addressed are the following: (i) bio-printer technology, (ii) enhanced resolution and speed, as well as large-scale implementations, (iii) biomaterials' complex "iterations" of gradients to obtain optimal functional, mechanical, and supportive characteristics, (iv) cell sources, that is, well characterized, stable, and reproducible cell sources, (v) combinations or mixing of sets of cell phenotypes demonstrating specific functionalities, (vi) vascularization, that is, well-adapted vascular features mandatory for large-scale tissue generation and survival, (vii) innervation, that is, nerve in-growth required to maintain basal and stimulated tissue functions, and

Table 16.1 Results of using scaffold materials PLA, HA, or TiO_2 alone in 3D cultures of hADSCs compared to a permutation scheme.

Parameters	Control = 100%	Permutation	PLA/HA	TiO_2
Runx2	100	467	161	412
Collagen1α1	100	367	155	324
Osteocalcin	100	543	173	445
Osterix	100	488	182	432
Mineralizing surface	100	232	95.3	198
OPG/rank-L ratio	**100**	**435**	**112**	**411**
Trap+ cells ≥ 3 nucl.	**100**	**21.1**	**14.8**	**15.1**
Eroded surface	**100**	**28.7**	**10.9**	**31.7**
Mir - 149	100	16.3	18.5	22.2
Mir - 328	100	7.87	16.9	10.9
Mir - 339	100	8.56	17.9	13.6

The data presented encompass (i) a prior manipulations of microRNAs and mechano-stimulation, (ii) RT-PCR analyses of osteoblast markers of differentiated cells isolated from the scaffolds, and (iii) the presence of osteoclasts, as estimated by their OPG and RANK-L, number of TRAP-positive cells, and eroded surface in an "osteoclast-on-dentine" resorption assay. Bold values refer to ostecclast-related characteristics.

(viii) maturation, pinpointing minimal time slots required for proper assembly and stable maturation [35, 36].

16.4.1.1 A Typical Process for Bio-Printing of 3D Tissues

Biomimicry, ensuring tissue self-assembly, and the creation of mini-tissue building blocks are design approaches employed as such (alone) or in various combinatory schemes. The selection of appropriate materials and cell sources is essential and specific to each and every tissue form and functionality. Commonly applied materials are synthetic and natural polymers, as well as cell-free ECM. Sources of cells may be either allogeneic or autologous. The components should be designed to be compatible with the bio-printing system, for example, inkjet, micro-extrusion, and/or laser-assisted printer entities. Some of the designed tissues may require maturation in a bioreactor before being transplanted, or the 3D tissue may be reserved for *in vitro* applications. A few references to different procedures are Self-assembly images; The printing of 3D organs; Tissue spheroids serving as building units; Mini-tissue images; Scaffold-free vascular tissue engineered by using bio-printing; Assessment by collecting ECM images;

Table 16.2 The ability of differentiated hADSCs to express osteoblast markers (RT-PCR), induce calcifications (verified by RT-PCR of Osterix, Ca^{2+} contents, and X-ray analyses), and the ability to recruit osteoclasts, in the presence or absence of osteoclasts (coculture), a mixture of cytokines (i.e., IL-1, IL-6, TNF-α, IL-17), and a set of antago-miRs (anti-miR-149 and anti-mir-miR-328).

Parameters	Control	Osteoblasts + osteoclasts	Osteoblasts + cytokines + osteoclasts	Osteoblasts + antagoMirs + cytokines + osteoclasts
Collagen1α1	100	113	53.8	98.8
Osteocalcin	100	127	33.6	123
Runx2	100	88.8	27.5	129
Osterix	100	95.9	22.1	112
Ca^{2+} contents	100	97.2	18.9	89.9
Verified on X-ray	Yes	Yes	Yes	Yes
Calcitonin receptor	–	100	267	88.8
TRAP	–	100	361	113
Cathepsin K	–	100	322	97.3
Carbonic anhydrase II	–	100	276	116

Evidence for the presence of human lung SCs; Laser-assisted images; High-speed 3D printing of cells and biomaterials for tissue engineering [35, 36] using laser technology.

16.4.1.2 Materials Suitable for Bio-Printing for Generation of Artificial Tissues

The choice of suitable basic materials for bio-printing, as well as their behavior in selected applications, relies on characteristics, such as follows: (i) *printability*, that is, their properties should facilitate the handling and deposition by the bio-printer including fluidity/viscosity, gelation methodology, and rheological characteristics; (ii) *biocompatibility*, that is, the selected bio-compounds should not initiate untoward local or systemic host reactions, and should spontaneously and predictably sustain both biological and functional constituents of the replacing tissue; (iii) *metabolic transformation and waste products*, that is, the degradation velocities should sustain the cells' ability to synthesize own ECM, the waste products must be nontoxic, and materials must show close to natural swelling or contractile characteristics; (iv) *structural versus mechanical features*, that is, the material must be selected according to optimal mechanical and geometrical features of the biomaterials such as rigid thermoplastic polymer

fibers (ensuring sufficient strength) and compressible hydrogels (ensuring cell compatibility); (v) *biomimicry of the constructs*, that is, the design of optimal material properties (i.e., structural/functional/dynamical) must closely resemble those of tissue-compatible endogenous biomaterial constituents [35, 36].

16.4.1.3 3D-Shaped Biomaterials and Scaffold-Building Substances for Bone Tissue Engineering

The design, printing, and application of 3D scaffolds constitute as a milestone toward the creation of functional artificial 3D bone materials. This especially relates to the induction of angiogenesis, which is necessary for bone tissue to survive throughout the generation and remodeling phases, ensuring preservation of the newly synthesized bone material. Blood vessel production (angiogenesis) of 12 scaffold varieties with a range of wall thicknesses and pore sizes was evaluated for various core features such as the length of induced blood vessel network (LIBVN), mean cell invasion depth (MID), maximum cell penetration depth (MPD), number of induced anastomoses (NOIA), and percentage of true sprouts (POTS). A certain wall thickness was demonstrated to sustain almost all parameters describing angiogenesis. In contrast, a smaller wall thickness or increase in pore size did not influence POTS, LIBVN, or MID. High-porosity (50%) scaffolds, as well as a small range of wall thickness and pore sizes gave the optimal vascularity metrics [35, 36].

16.4.2 Nanobiotechnology and Bone Regeneration

This section reviews the current status in the engineering of osseous tissues, with focus on the intriguing effects of nanobiotechnology. This fusion of nanotechnology and biotechnology offers unlimited possibilities in modulating biological processes both at the molecular and atomic scale. First, a multiscale hierarchical structure of bone and its implication on the design of new scaffolds and delivery systems are discussed. Then, a brief presentation of different types of nanostructured scaffolds is given, and finally nanoparticle delivery systems and their potential use in promoting bone regeneration are summarized [37]. Tissue engineering and regenerative medicine concepts, collectively known as *TERM*, are emerging as potential alternative approaches to deal with difficult clinical situations caused by severe bone loss.

For this to be successful, intricate interplay between three main features of the regenerative triad, that is, bone-residing cells (osteoblasts/osteocytes/osteoclasts), unchanged ambience, and scaffold material, must be re-created. Adequate and sufficient blood supply and revascularization are other element shown to be very important adjuncts [38]. Finally, one of the critical parts of the TERM approach is to ensure a porous 3D scaffold (lattice), which may render sufficient support for cells and, consequently, ensure generation of fresh and resilient osseous material.

Biomaterials exhibiting nanoscale organization have been used as release reservoirs sustaining adaptable and physiological drug delivery and artificial matrix substances for successful *de novo* tissue construction. The definition "nanotechnology" is "a set of manipulation of matter with at least one dimension sized

from 1 to 100 nm." In this context, it is noteworthy that gravity becomes relatively unimportant while surface tension or van der Waals forces become very important [37].

16.4.2.1 Nanostructured Scaffolds for Bone Repair

Changes in material microstructure affect the strength and toughness in different ways, tending to be mutually exclusive. That is, nanostructure reinforcement enhances the strength (i.e., resistance to nonrecoverable deformation) and decreases the toughness (i.e., measure of damage tolerance). Consequently, designing nanocomposite scaffolds neglecting architectural design inevitably compromises the mechanical properties of a given scaffold. Furthermore, ceramic-based materials have been in use in medicine for decades, and their toughness is able to shield a crack by deflecting it. By using nanotechnology, it is feasible to create alumina/zirconia nanocomposites with much more favorable mechanical properties. These materials are a dispersion of small amounts of tetragonal zirconium particles ($\approx$ 200 nm in size) in an alumina matrix, yielding materials that can sustain twice higher workloads compared to pure materials. For references, see [37, 39, 40].

It has been concluded that the nanoscale topography is "the essential factor" influencing the adsorption of proteins and modulating the interactions of cell-based environments and the cell or tissue surfaces of a given biomaterial. Also, a thorough knowledge of the size, density, and distribution (ordered vs random) of the topographical features is essential for the design of scaffolds with appropriate surfaces. By using advanced lithographic fabrication techniques (multiphoton lithography, nanoimprinting, laser origami, etc.), it is possible to make a variety of topographical features, which in turn modify cell–material interactions. For references, see [37, 39, 40].

Recent studies have shown that immunological responses to a given biomaterial surface could be changed by the introduction of nanometer-sized grooves and crevices on the surface of a substrate. The best example is surfaces modified by nanosized Ti, Ti6Al4V, or CoCrMo powder in the production of orthopedic implants. When osteoblasts were seeded on such surfaces, they showed improved functions such as adhesion, proliferation, and deposition of calcium-containing minerals [38]. Other nanostructured materials, for example, ceramics (aluminum, zirconium), calcium phosphates (HA, tricalcium phosphate), and glass ceramics, also exhibit improved osteogenic capabilities compared to microstructured ones. For references, see [37, 39, 40].

Synthetic polymers are suitable for designing 3D scaffold structures. When seeded with cells, they sustain cell proliferation and matrix deposition. Any material to be used in tissue engineering should have favorable biocompatibility, minimal immunogenicity, and inhibition of infections, while also expressing sufficient mechanical stability to allow containment and orientation of the seeded cells. Collagen exerts good biocompatibility and can be successfully seeded with cells; however, its mechanical properties are suboptimal. Other agents with better mechanical properties include polymers of α-hydroxyl esters, for example, PLA, PGA, PCL, or copolymers of same (e.g., PLGA). When used as nanocomposite materials (e.g., nano-HA/polymer scaffold), they show improved functioning

of the seeded osteoblasts. A recent study showed that electrospun PCL/HA/gel composite nanoscale scaffolds/gels enhanced both the cell doubling rate and the generation number, as well as the number of mineralizing osteoblasts, ensuring proper and resilient bone formation *in vitro*. For references, see [37, 39, 40].

Carbon nanotube (CTN)-reinforced composites are another class of materials designed for bone engineering [37]. Furthermore, a group of self-assembling proteins (SAPs) made of only natural amino acids was developed, which have been shown to serve as unsurpassed biomaterials for various 3D cultures of a plethora of human cells phenotypes, stimulating cell migration into the scaffold. Many different types of SAPs have been designed in the last few years, including EAK16, RAD16-I, DN1, and KLN12, which readily undergo gelation in a polarized solvent such as a saline solution [37].

16.4.2.2 Nanoparticle Delivery Systems for Bone Repair

In parallel to the provision of temporary 3D lattices, nanoscale scaffolds function as effective carriers for bioactive cues, promoting both osteogenesis and angiogenesis. With this technology, a drug can be efficiently delivered to its destination, keeping its concentration optimal. Nanoparticles for the delivery of drugs and/or cells can be categorized as organic and inorganic remedies. Organic supply modes generally encompass polymerized nanoscale particles, such as PLGA, but inorganic remedies mainly contain silica-based mesoporous material and hydroxide in double layers [37]. These systems vary in terms of efficacy, biocompatibility, and the size of molecules, or the genetic sequences being delivered [37]. For bone regeneration or repair, both genes and proteins can be delivered to promote osteo- and angiogenesis.

For example, it was shown that the injection of the peptide amphiphile (PA), similar to BMP-2, into the dorsal subcuticular rat dermis layer stimulated the production of a 3D hydrogel and brought about a substantially homogeneous ectopic bone production. It was also anticipated that the supplementation of biological cues with cellular entities will instigate a more efficient repair because the molecules can directly act on the cells in their vicinity. Regeneration and repair of tissues are generally construed as a recapitulation process occurring during embryogenesis and later developmental phases. They involve both spatial and temporal signaling, directing all types of cell behaviors, including differentiation [37].

In this study, Gusic *et al.* used cell-free constructs loaded with both BMP-2 and Noggin in a mouse model of calvarial dysfunction [37], and demonstrated that 3D bio-patterning of a growth-stimulating factor/hormone along with a growth factor modifier can direct cell differentiation *in vitro* and tissue formation *in vivo* adhering to printed patterns. Another nano-based approach is the creation of chemical or physical gradients that can be directly incorporated into the design of specific scaffolds to obtain tissue and organs exhibiting enhanced structure and function.

16.4.2.3 Connection between Mechanics and Bone Cell Activities

A plethora of models describing bone turnover models have been proposed. These include model systems based on ubiquitous optimality criteria, related

to the maintenance of states of homeostatic stress versus strain energy, models founded on accumulation versus repair, mechanistic models that consider both mechanical and metabolic conditions connected to remodeling loops, as well as models molded around interstitial fluid flow. Some systems deal with 2D simulations, describing a reaction–diffusion system under the influence of mechanical forces. The latter have enabled the prediction of normal bone architecture. However, they are based on mechanical stress versus strain in a system control model, where continuous adaptation is propelled by the discrepancy between mechanical set points versus mechanical stimuli to predict the remodeling of bone, not taking into consideration the inherent "biophysical" features of the bone-forming and bone-resorbing cells. For reference, see [40].

There are only a small number of mathematical models describing the turnover in basic multicellular units (BMUs). Komarova *et al.* [41] launched a mathematical model featuring nonlinear auto-regulation involving both osteoblasts and osteoclasts responsible for both autocrine and paracrine bioactive molecules, respectively. Rattanakul *et al.* [42] described a model proposing parathyroid hormone (PTH) serving as the most important regulatory factor in bone turnover. Moroz *et al.* [43] proposed another (mathematical) model based on the Michaelis–Menten variant of feedback mechanisms. Finally, they arrived at an advanced model system describing a direct interaction (auto-regulation) involving osteoblasts (bone formation) and osteoclasts (bone resorption/turnover). This concept relies on the RANK–RANKL–OPG (ostoblast-osteoclast) activation cascade, PTH, and TGF-β. Developing the Lemaire model system further, Maldonado *et al.* [44] constructed another model involving bone–cell interactions, which took into account the impact of osteocytes activated by mechanical forces (also called the "mechanostat" principle). Finally, it should be mentioned that Pivonka *et al.* [45] described a more refined model, adding the impact of the RANK/RANKL/OPG pathway, as well as PTH and TGF-β to the "old" bones, but based on Hill functions. Hence, this model system is considered better suited to describe the binding equilibrium and dynamic states between the ligand and the receptor.

Finally, the model system proposed by Ryser *et al.* [46] describes a more sophisticated modeling of both autocrine and paracrine biomolecules, compatible with Komarova's concept. And, related to the spatiotemporal dynamic observation of BMU behavior, this model integrates the RANK/RANKL/OPG pathway, as well. A scrutiny of all the previous BMU-related modeling systems indicates that the Komarova/Moroz/Rattanakul models include only a few parameters, while the Lemaitre/Pivonka/Ryser variants are more sophisticated and superior, featuring more than 90 parameters. Despite the fact that the present cell-based model systems provide new insight into the regulation dynamics of bone homeostasis (i.e., metabolic turnover), none of them has been implemented to predict bone turnover looking at the mechano-biological angle and considering the cell-based activities interacting with the mechanical forces within the bone tissue itself.

The last modeling of the interactions described above with biological factors suggested by Komarova *et al.* was finalized by Bonfoh and coworkers [47], who added the impact of an external loading modulus (i.e., a stimulating component

impinging on osteoblast/osteocyte dynamics). Finally, one research group combined Komarova *et al.*'s model system with that of Bonfoh *et al.* (i) to implement the mechanical properties of the osseous tissue, such as fatigue/damage, growth/repair, mineralization/porosity, and osseous tissue properties, including evolution, and (ii) to describe the concept of cell–cell accommodation, asserting that the reference measures for bone turnover stimuli are only dependent on the history of mechanical loading. Furthermore, a sensitivity assessment (SA) was undertaken to reveal the impact of the model compounds/factors' set points on the calculated mineral density of a given region of interest (ROI) (e.g., the femur neck).

16.4.3 Novel Comprehension of the Role of Osteocytes in Bone Growth and Regrowth

Osteocytes comprise the majority of cells in bone and represent its only true "permanent" resident cell population. The osteocyte is now recognized as a major coordinator of skeletal turnover, sensing and integrating various signals to emulate bone turnover rates. Previously, it was demonstrated that osteocytes regulate their effector cells (i.e., osteoblasts and osteoclasts) directly within their "habitat" – entombed within the mineralized matrix of bone and connected to each other by a multicellular network. Osteocytes synthesize bone matrix non-collagenous proteins including osteopontin (OPN), OCN, and dentin matrix protein 1 (DMP1), as well as proteoglycans and hyaluronic acid.

16.4.3.1 Osteocytes as Mechano Sensors

A schematic representation of the key microstructural features of osteocytes, implicating them in mechano-sensing, reveals bundles of F-actin. These bundles increase osteocyte stiffness and limit membrane deformation. Furthermore, osteocytes demonstrate proteoglycan tethering elements, bridging the cell process to the bony canalicular wall. Proteoglycans, such as perlecans, are spaced approximately every 40 nm along the osteocyte process, and they exert a resistance to loading-induced fluid flow. The resulting drag force is finally sensed at the cell process membrane. Immunohistochemical staining shows β1-integrins being located only on osteocyte cell bodies. Photomicrographs demonstrate discrete protrusions from the canalicular wall, contacting the osteocyte processes. Furthermore, β3-integrins are present as a punctate pattern along osteocyte processes. For references, see [48].

16.4.3.2 Osteocyte–Osteoblast Communication

Osteocytes communicate directly with osteoblasts at bone surfaces by via gap junctions and indirectly via extracellular, paracrine signaling pathways. The signal transmission is subject to constraints, such as the signaling molecule size, gap junctional selectivity, interstitial fluid flow, and resistance of the pericellular matrix. The signals common to osteocytes and osteoblasts are small "ubiquitous" molecules, that is, NO, nucleotides/nucleosides, prostanoids, as well as peptides such as insulin-like growth factors (IGFs). Osteocytes also express the PTH/PTHrP receptor, rendering them responsive to an "endocrine regulator,"

like PTH. Paracrine signaling from osteocytes encompass anabolic signals, being enhanced by mechanical stimuli. This concept is consistent with bone adaptation, responding to mechanical demand. Cyclic AMP [98] and PGE_2 are the early signals upregulated by mechanical loading of bone cells. PGE_2 production in bone tissue is also stimulated by mechanical loading in conjunction with enhanced expression of the type-2 cyclooxygenase (COX-2). Osteocytes, in particular, have been shown to increase COX-2 expression and PGE_2 synthesis in response to diverse mechanical stimuli including fluid flow, substrate deformation [105], and hydrostatic pressure. For references, see [48].

Most importantly, osteocytes are the major producers of sclerostin (encoded by the SOST gene). Sclerostin inhibits the canonical Wnt pathway, which is the major stimulator of osteoblast activation. Sclerostin binds to Wnt co-receptors like LRP4/5/6, thus blocking the interaction between Wnts and their co-receptor Frizzled (Frz). Osteocytes also produce DKK-1, another inhibitor of the Wnt signaling pathway that binds LRP4/5/6. Both sclerostin and DKK-1 apparently act synergistically to hamper osteoblast activation, even though they do not bind simultaneously to the LRP5/6 co-receptors. Mechanical loading decreases the expression of both sclerostin and DKK-1 in conjunction with enhanced bone formation, while unloading stimulates sclerostin/DKK-1 secretion, inhibiting bone formation. For references, see [48].

16.4.4 Advances in Vascularization of Tissue-Engineered Bones

Purified factors, such as VEGF, showed diminished efficacy when analyzed in clinical settings, which was attributed to their very short half-lives. However, efficacious and stable overexpression might favor aberrant blood vessel induction and the development of hemangiomas. To ensure a clinically applicable concept, a fluorescence-activated cell sorting (FACS)-based method was launched, identifying cellular entities displaying predicted transgene features in a heterogeneous cell population. When implemented in VEGF-producing muscle cells, the methodology ensured the induction of sufficient and phenotypically "correct" blood vessels in the skeletal muscle *in vivo*. These vascular cells were also devoid of any untoward or aberrant growth. Finally, the "designed" BMSCs boosted the production of a blood vessel network ("neovascularization") of critically sized osteogenic implants, significantly reducing the necrotic central core and increasing osteogenesis.

16.4.4.1 The "Flap Prefabrication" Approach

This concept applies implantation of the tissue-engineered constructs in a vascularized environment by creating a flap, favoring the in-growth of a vascular network. Then it can be transplanted into an osseous tissue site harboring the defect. The bone-generating ability of this ectopic prefabrication of bone-marrow-derived stem cell (BMSC)-based bone "chips" was proven successful in animal models. However, these flap prefabrications have the major clinical disadvantage of requiring a two-step surgery, which clearly limits their bone-forming ability in clinical setting. For references, see [49].

16.4.4.2 Strategies Involving the "Pre-Existence of Vascular Networks"

This method features the application of local host blood vessels. These are manipulated to invade the osseous construct, creating arteriovenous (AV) loops, bundles, or "flow-throughs." The present strategy represents a challenge, which is related to the geometrical features and molecular constituents of the osteo-inducing material. To overcome this problem, one may form a cell-containing fibrin construct with ceramic granulates, molded around the AV loop. Proofs of the principle were collected, in part from cocultures of myogenic C2C12 cells together with human umbilical vein endothelial cells (HUVECs) and embryonic fibroblastic cells. Furthermore, an application of an artificial skin graft sustained a comparable acceleration of blood perfusion of hypoxic areas of the graft. Many other studies enhancing blood flow to bone grafts have been conducted with great success, some of which are referenced underneath. For references, see [49].

16.4.4.3 Fat Tissue May Transform Into Vasculo- and Osteogenic Cells

For a long time, fat tissue is deemed to serve as a suitable source of cells in regenerative applications, in accordance with its abundance and accessibility, low morbidity, and enhanced frequency of progenitor cells yielding clonogenic mesenchyme, in comparison to bone marrow. Furthermore, there is a consensus that this cell fraction also contains a large fraction of endothelial and progenitor cells, implying that they constitute a useful bank of autologous vasculogenic and osteogenic progenitor cells.

16.4.4.4 Vasculogenic Progenitors

It has been demonstrated that adipocytes differentiated from stromal cells (ASCs) are able to differentiate into endothelial cells and attain vasculogenic features. In this fat tissue stromal vascular fraction (SVF), there exists a CD34+/CD31− cell population, which displays features compatible with immature endothelial progenitors, both *in vitro* and *in vivo*. VEGF and FGF-2/ascorbic acid have been demonstrated to be essential for the preservation and differentiation potential of these cells. Lately, it was shown that these cells, being present in the SVF, represent a cell population able to create a blood vessel network *in vivo*. This suggests that adipose-derived cells constitute a reservoir of vascular progenitors. For references, see [49].

16.4.4.5 Osteogenic Progenitors

Numerous investigators have studied the ASCs' osteogenic potential by applying *in vitro* assays only. Bone formation is a complex process involving interactions between cell types like osteoprogenitors, osteoblasts, and osteoclasts, but also endothelial and vascular cells. However, no exhaustive minimal, but sufficient, set of parameters has yet been launched to link *in vitro* "osteogenic" ability to *in vivo bone* formation. A useful criterion to assess intrinsic *in vivo* bone-forming ability of MSCs is the ectopic osseous tissue production assay. However, only a few published articles featuring this ectopic model could corroborate true bone-forming activity *in vivo*. In accordance with the specific conditions described, one might

hypothesize that only two features are deemed essential for inducing and/or sustaining the bone-forming potential of ASCs:

1) *Osteogenic transformation of ASCs*: Early analyses where ASCs showed osseous tissue development *in vivo* applied *in vitro* predifferentiated adipocytes exposed to a standard osteoblast-inducing medium or cells transfected with BMP-2. Other investigations did not report on typical bone-inducing factors/hormones, but accomplished cell population expansion on ceramic substrates with flow-induced shear stress, using freshly harvested SVF cell populations.
2) *Including mineral components into scaffolds*: The application of β-tri-calcium-phosphate (β-TCP) or HA is considered a critical requirement to guarantee the successful production of artificial bone, and trials with fibrin or PEG/PCL-based scaffolds were not able to ensure *in vivo* true ectopic bone production using ASCs as the cell source. The necessity for a mineral component was proven in cultures of human ASCs incubated for 2 weeks in the presence of a standard osteoblastic differentiation medium within various collagen-based scaffolding substances. However, in a follow-up experiment, a similar approach, but based on a ceramic β-TCP scaffold, was conducted. Histologically "correct" or proper bone formation inside the *in vivo* implanted 3D scaffolds after 2 months could now be demonstrated. In another report, ASC-induced osseous tissue production was not observed in the absence of a mineral compartment. However, in the presence of a platelet-rich gel structure as cell carrier, proper bone structures were verified. For references, see [49].

16.4.5 Advances in the Understanding and Control of Intracellular Signaling Pathways of Osteoblast Differentiation

Human skeletal mesenchymal stem cells (hBMSCs) are characterized as stromal cells devoid of ability to initiate hematopoiesis. hBMSCs may *in vitro* adhere to plastic surfaces and express a cluster of surface biomarkers such as CD146/44/63 and CD105, and display the ability to attain osteoblast, adipocyte, and chondrocyte phenotypes. The "stemness" of hBMSCs characterizes their ability to create heterotopic osseous tissue, including marrow substance, as a consequence of serial *in vivo* transplantations.

By applying genetic approaches, cell phenotype-characterizing TFs such as Runx2 and Osterix (SP7), (PPARγ2), and Sox9 determine osteoblast-, adipocyte-, and chondrocyte-like lineages, respectively. Furthermore, other factors instrumental in osteoblast differentiation are BMPs and TGFs (stimulatory), and Dlk1/Pref-1 and Noggin (inhibitory). Furthermore, a plethora of studies indicate the presence of a reciprocal correlation between BMSC differentiating into bone (osteoblastic) cells versus fat (adipocytic) cells. A number of molecular entities, including Wnt3a/Wnt10b, Wnt5a, BMP2, Shh, TGF-β1, Nell-1, Wwtr1, Spry1, Rb1, MITR/HDAC9, NFI-C, KDM6A, Bmi1, Msx2, TAZ, Ho-1, SPARC, FXR, Sirt1, SERPINF1, Sox2, EZH2, TLE3, S100A16, Y1R, Tmsb4x, and CMKLR1, have been shown to be implicated in the acquisition osteoblastic or adipocytic phenotype markers from SCs differentiating into either osteoblasts or

adipocytes. Furthermore, in this context, the differentiation toward osteoblasts or adipocytes seems to be a process of mutual exclusion. For references, see [50].

It should also be mentioned that some regulatory loops exist between microRNA species and TFs, encompassing SP1, SP3, ETS1, RUNX1, and miR-328, miR-149, miR-23, miR-27, miR-29, and miR-133 (the Gordeladze and Stein microRNA "signatures"), respectively [51–53]. Interestingly, vitamin K2, acting via its nuclear factor PXR = SXR = NR1I2, interfers with these regulatory loops, fortifying the osteoblast phenotype, which has become resistant to the negative influence of immune cells (Th cells) (Gordeladze, unpublished data).

16.4.5.1 LRP5 (Low-Density Lipoprotein Receptor-Related Protein 5) Signaling in BMSC Differentiation

Signaling conveyed by LRP5 and Wnt. The Wnt molecules have been characterized at length and shown to modulate many cell-specific processes like cell differentiation, proliferation, migration, and acquisition of polarity. Wnt signaling may be divided into a so-called canonical (shown to be β-catenin-dependent) pathway and a non-canonical (i.e., β-cateninin independent) pathway. Canonical Wnt signaling is kick-started when a Wnt ligand associates with the FZD receptor and LRP5 or LRP6 as co-receptors. The present cluster of molecules shelters β-catenin from being phosphorylated by the glycogen synthase kinase (GSK-3β), followed by a degradation via the ubiquitin/proteasome metabolic pathway. Thereafter, the stable β-catenin entity is funneled into the nucleus, associates with the T-cell factor/lymphoid enhancer binding factors (TCF/LEF), and modulates the gene expression of downstream target genes. The non-canonical Wnt pathway encompasses both the Wnt/Ca^{2+} and the Wnt/planar cell polarity signaling mechanisms. Within the Wnt/Ca^{2+} pathway, given Wnt molecules interact with specific FZD receptors, thus activating the Ca^{2+}/calmodulin-sensitive kinase II (CamKII) and protein kinase C (PKC), as well as a "nuclear-factor-of-activated T" cell (named "NFAT-transcription-factor"). The Wnt/planar pathway consequently stimulates RhoA/Rac GTPase, c-Jun N-terminal kinase (JNK), and nemo-like kinase (NLK) stimulated pathways in order to master the cells' lining or orientation, according to the "polarity," and thus cellular movement in an nonhomogenous cell cluster (i.e., tissue). For references, see [50].

16.4.5.2 Signaling Protein Kinases and Impact on hBMSC Adaptation/Differentiation

Protein kinase (PK) signaling orchestrates the initiation, modulation, and termination of many biological functions, as well as the differentiation of SCs. So far, some 500 PKs conveying a plethora of localized reactions have been characterized, constituting ~2% of the human genome. Furthermore, some 30% of every cellular protein is phosphorylated in one or more sites. An uncontrolled PK-induced modulation is associated with the development of a stunning number of human diseases. Hence, the PKs (as a family of hormones and growth factors controlling signaling pathways) have been suggested as targets for the development of novel drugs.

Thus, several PKs have been characterized, regulating the initiation and adaptation of the osteoblast phenotype, as well as osteoblast-related cyclical

changes, characterizing bone formation and turnover. These are Akt, AMPK, BIK, CaMKII, CK2, c-src, ERK1/2, FAK, GSK3B, JNK, LIMK2, MLK3, NLK, p38MAPK, PRKA, PRKC, PRKG1, PTK7, Pyk2, ROCK, and TAK1. Furthermore, several osseous anabolic growth "hormones" show cognate receptors with inherent kinase activity. BMPs convey their signals via their cognate receptor serine/threonine kinases, phosphorylating cascade-specific proteins, such as smad1/5/8, and TGF-β activated kinases (like TAK1). This leads to controlled osteoblastic cell differentiation; in addition, many kinases modulate osteoblastic cell functioning via downstream modulation (activation or inactivation) of selected osteoblastic TFs. Runx2, serving as a master modulator of osteoblastogenesis, is activated by the phosphorylation of mitogen-activated protein kinase (MAPK) and extracellular signal-regulated kinase (ERK). Osterix (Osx or SP7), being a "zinc-finger" variety of osteoblast-specific TFs, is "turned on" by phosphorylation of the p38MAPK. ATF4, among many others, is another TF that is modulated post-translationally via the phosphorylation by RSK2 (ribosomal S6 kinase 2), which serves as one requirement for osteoblastic differentiation from precursor cells and functioning through all its developmental and bone-remodeling phases. For references, see [50].

16.5 Conclusion and Future Prospects

This chapter has addressed a number of factors deemed necessary for the completion of successful bone tissue regeneration *in vitro*. These include a mixture of scaffolds, SCs and/or osteoprogenitor cells, osteogenic media containing a minimum, but sufficient, cocktail of growth- and differentiation-inducing molecules. To understand the interaction of cells, scaffolds, and/or nanoparticles of different makes and surfaces with growth factors, it may be necessary to develop computerized bioinformatics model systems encompassing all these variables into a predictive dynamic interaction system. The use of the Mir@nt@n algorithm [54] and Ingenuity™ is recommended here.

In particular, it is necessary to emulate the impact of mechano-stimulation in scaffolds behaving like a live bone (i.e., piezoelectric-sensitive scaffolds) loaded with SCs being able to differentiate into osteoblasts osteocytes, nerve cells, micro-vessels, and osteoclasts, in order to create a micro-environment enabling the response to mechano-stimulation and neuronal input necessary to activate osteocyte-like cells to take part in the bone remodeling cycle in a proper manner. We have focused on several parameters in this chapter, but it may it also be necessary to benefit from the impact of microRNA exposure/delivery to boost the epigenetic machinery closer to the genome [55].

In Figure 16.4, we show a comprehensive model for a modern concept of bone engineering, based on 3D bioprinting. Here we incorporate many aspects deemed necessary to ensure a vital and functional bone formation, like a timely assembly of scaffold nanoparticles, SCs, osteoblasts, osteocytes, osteoclasts, differentiation cocktails (enabling differentiation of cells to become bone-producing cells, vascularizing epithelial cells, as well as nerve

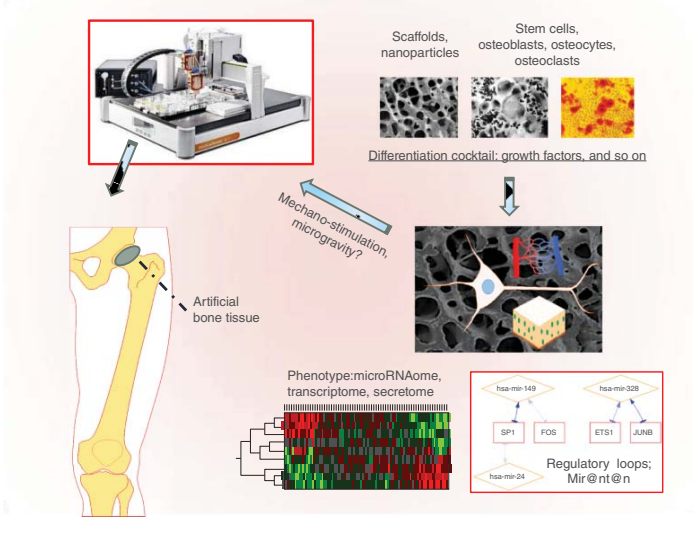

Figure 16.4 A comprehensive model for modernized production of artificial bone tissue, showing the necessity for using, selected manipulations. Here, we focus on factors like the use of scaffold material, stem cells, osteoblasts, osteocytes, and osteoclasts having been adapted to show the wanted characteristics, defined phenotype screening (microRNAome, transcriptome, and secretome), as well as reinforcing the presence of elements (transcription factors and microRNAs) constituting regulatory loops (envisaged by the Mir@nt@n algorithm). Furthermore, we focus on the production (as least as intermediates) of cells being able to differentiate into osteoblasts/osteocytes, vessel-building epithelia cells, and nerve cells. Finally, one may subject the cells to a coculture system, applying mechano-stimulation and/or micro-gravitation forces prior to a defined 3D printing, to produce a viable, healthy, and self-renewing bone tissue.

cells). Furthermore, we recommend the timely use of microgravity and/or mechano-stimulation, aided by osteoblast microRNAome, transcriptome, and secretome analyses, as well as checking for the stabilization of microRNA–TF regulatory loops.

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17

Tissue Engineering of the Pancreas

Masayuki Shimoda

17.1 Introduction

The pancreas has two major functions, endocrine and exocrine. Dysfunction in its exocrine functionality comprises various conditions, such as exocrine pancreatic insufficiency (EPI). In this condition, a deficiency of exocrine pancreatic enzymes causes improper food digestion. Management of EPI is based primarily on pancreatic enzyme replacement therapy, lifestyle modifications, and vitamin supplementation. Endocrine function is provided by the clusters of cells in the pancreas called islets of Langerhans. These islets comprise four main cell types: α cells, which secrete glucagon to increase the blood glucose level; β cells, which secrete insulin to decrease the blood glucose level; δ cells, which secrete somatostatin to regulate α and β cells; and PP cells, which secrete pancreatic polypeptide. In this way, islets play a critical role in blood glucose homeostasis and diabetes. This chapter discusses the treatment of endocrine dysfunction.

Diabetes mellitus, which is the most prevalent endocrine disease in many countries [1, 2], represents a major public health problem. Diabetes can cause many complications including ophthalmic, kidney, nerve, brain, heart, and peripheral vascular diseases [3]. Treatment of diabetes and its secondary complications and the resultant loss in productivity cause considerable economic burden [4]. There are two main types of diabetes mellitus, type 1 (T1D) and type 2 (T2D). T1D results from the autoimmune-mediated destruction of pancreatic β cells [5]. The blood glucose level increases while the level of metabolic fuel is insufficient and cells are starved. The factors causing this autoimmune destruction of β cells in T1D are unclear, but viral infections or environmental factors have been considered potential triggers of β-cell autoimmunity and T1D. In T2D, the body can still produce at least some insulin in the pancreas, but cells fail to respond properly to insulin. In the late stage, a lack of insulin may also occur, as it can in T1D. If an artificial pancreas could be used to supply insulin properly, it may effectively treat T1D and T2D patients requiring exogenous insulin injection.

There are several treatment strategies available for T1D, including exogenous insulin injection with continuous glucose monitoring, whole organ pancreas transplantation, and islet cell transplantation; more recently, a mechanical artificial pancreas and a bioartificial pancreas have been developed. As discussed

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by Kizilel *et al*. [6], among these treatment options, a bioartificial pancreas could provide as rapid a reaction to blood glucose levels as a well-functioning native pancreas and therefore eliminate the need for immunosuppressive agents. Additionally, if such a bioartificial pancreas could be produced using easily acquired cells, it could help alleviate or resolve the current shortage of donors.

17.1.1 Biology of the Pancreas and Islets

The pancreas is a soft, elongated organ located in the upper dorsal abdominal area. The pancreas comprises ductal, exocrine, and endocrine cells, which collectively synthesize and secrete the enzymes and hormones necessary to sustain nutritional balance. The pancreas develops from the dorsal and ventral protrusion in a region of the primitive gut epithelium. Several transcription factors, including Ngn3, Pdx1, Isl1, NeuroD1, Pax4, Pax6, and Nkx2.2, play important roles in the control of pancreatic endocrine cell differentiation [7]. The islets constitute approximately 1–2% of the mass of the pancreas, and the combined mass of the islets is 1–1.5 g. The pancreas of an adult human contains ~1 million islets, each approximately 50–300 μm in diameter. Each islet is separated from the surrounding pancreatic tissue by a thin capsule of fibrous connective tissue. Islets are rich in capillary vessels.

17.2 Treatment Options for T1D

17.2.1 Exogenous Insulin Treatment

T1D was an acute and deadly disease for many years, and effective treatments are still very limited. Following the discovery of insulin in 1921, exogenous insulin administration has been the therapy of choice. It can save the lives of T1D patients, effectively extending their longevity. But since maintaining perfect glycemic control is difficult, secondary complications of diabetes can occur and reduce quality of life [8]. Therefore, a novel alternative or additional treatment for T1D has long been sought.

17.2.2 Whole Pancreas Transplantation

Since the first whole organ pancreas transplantation in 1966 [9], more than 30 000 pancreas transplants have been performed worldwide [10]. In many countries, pancreas transplantation is the standard treatment option for T1D patients, especially those with chronic renal failure. Pancreas transplantation has the potential to be a long-term treatment that restores glycemia without the risk of severe hypoglycemia and also prevents secondary complications, but it requires major surgery. The graft survival rates are excellent, at almost 85% at 1 year and 60% at 5 years [11]. However, the benefits of this approach come at the cost of major surgery and lifelong immunosuppressive agents to prevent rejection [11]. These factors and the shortage of human donors limit its application to a very select subpopulation of T1D patients. Even though there is room for improvement in surgical techniques and immunotherapy, it would be difficult to dramatically increase the number of whole organ pancreas transplantations.

17.2.3 Pancreatic Islet Transplantation

As discussed by Pareta *et al*. [12], islet transplantation is a potential treatment that is regarded as much less invasive than whole pancreas transplantation. Islets are infused into the portal vein without major surgery. However, there are several problems with this procedure in addition to the whole pancreas transplantation, such as the loss of islets during their isolation and transplantation and the need for immunosuppression. Islet isolation, the current procedure by which islets are extracted from the pancreas with collagenase, is still a difficult technique; only 50% or fewer islets in the whole pancreas can be isolated successfully. In addition, many islets infused into a portal vein are immediately destroyed by an immune reaction, inflammation, loss of vascularization, and other issues during the early post-transplantation period. Thus, islet transplantation sometimes requires more than one donor pancreas. Recently, great improvement has been made with the introduction of the Edmonton protocol [13], and single-donor transplantation has become possible in top facilities. This has dramatically improved the outcomes of islet transplantation, bringing them closer to the outcomes of whole pancreas transplantation. However, as with whole pancreas transplantation, the necessary lifelong adherence to immunosuppressive drugs is related to side effects. Another problem is the shortage of donors, which results in only a limited number of patients being able to receive this treatment.

Thus, the two current therapeutic options that reproducibly achieve normoglycemia, namely pancreas transplantation and islet transplantation, have two major barriers: requirement of immunosuppressive drugs and donor shortage. Therefore, many studies have tried to develop new therapies to solve these two problems.

17.2.4 Artificial Pancreas

To address the problems of the shortage of human donors and the inadequacies of insulin treatment, the development of an artificial pancreas has been energetically pursued. The artificial pancreas functions like a healthy pancreas, enabling patients with T1D to control their blood glucose automatically.

An artificial pancreas can be one of several types:

1) *Medical device*: Currently, automatic insulin infusion devices (insulin pumps) are widely used. Moreover, continuous glucose monitoring devices to check real-time blood glucose level are also clinically used. A mechanical artificial pancreas is a combination of the insulin infusion pump, the glucose sensor, and a computer that controls the infusion rate of insulin according to the blood glucose levels [14, 15]. This is a potential approach, but there are some technical problems such as hypoglycemia by infusing an overdose of insulin [16].
2) *In vivo gene therapy*: This involves therapeutic delivery of a genetically engineered vector (typically a viral or plasmid vector) into a diabetic patient, overexpressing a specific gene, causing the transformation of other cells into glucose-responding and insulin-producing cells [17, 18]. Although this approach has great potential, it is still at the experimental level with several unanswered questions associated with its safety and mechanism.

3) *Bioengineering approach*: A bioartificial pancreas is used that includes specifically bioengineered cells to act as a substitute for the endocrine portion of the pancreas. Its use would be to mimic the secretion of insulin by a healthy pancreas. In addition, a device or capsule is used to encapsulate the transplanted cells in a synthetic biocompatible membrane, thus avoiding the need for lifelong immunosuppression or gel [19].

17.3 Bioartificial Pancreas

A bioartificial pancreas can be used to implant islets or alternative functional cells generated from islets or other cells. Such alternative cells would secrete insulin automatically in response to blood glucose levels like natural islets. However, a T1D patient still has an autoimmune reaction against β cells and insulin [20], and a simple transplantation is not tenable even if the implanted cells are generated from the patient's own cells. Therefore, the use of islets or cells requires immunosuppressive agents or a protective barrier to prevent immune attack. By using such a barrier with encapsulation technology, allo- and even xenotransplants may be applied safely, thereby addressing the lack of human donors as well as the burden of immunosuppressive agents.

The use of a bioartificial pancreas with tissue engineering technology is one of the most promising available approaches. This chapter mainly focuses on the design and implementation of bioartificial pancreases.

17.3.1 Cell Sources

A bioartificial pancreas is a potential approach, but there are still some hurdles for its broad application. At the present time, the most effective and safe source of cells is human islets from cadaver donors; however, they are very limited. Therefore, other sources of insulin-producing cells have been sought, leading to the identification of several promising sources.

17.3.1.1 Pig Islets

One option is animal islets (xenotransplantation). Pigs (Figure 17.1a) are the major candidate source for several reasons. They have similar physiological features to humans, in particular, in terms of glucose metabolism. They are cattle, so there are fewer ethical concerns, and they are available in large numbers. High quality and quantity of islets can be isolated from a pig. Moreover, porcine insulin is very similar to human insulin (only one amino acid is different) and has been used for the treatment of diabetes for many years [21, 22]. However, xenografts require a heavy immunosuppressive regimen to prevent xenorejection, even when using specific antigen knock-out pigs or neonate pig islets [23]. Therefore, a tissue engineering approach, in which pig islets are encapsulated inside a semipermeable membrane and in an immunoisolated condition, may be a potent method. It is necessary that such a semipermeable membrane shields the graft cells from the host's immune system and allows the release of small molecules such as insulin for a longer period, thereby controlling glucose metabolism without the need for immunosuppressive drugs.

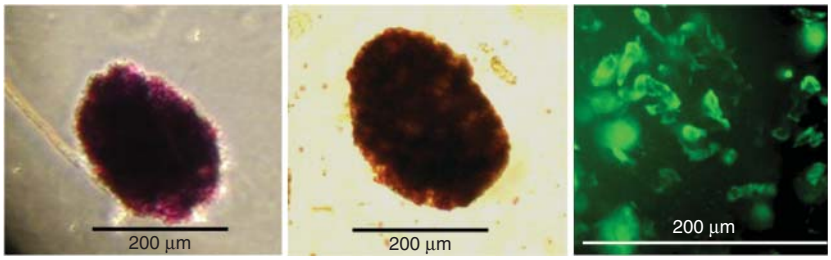

Figure 17.1 Representative images of the different types of cells for use in a bioartificial pancreas. (a) Porcine islet (dithizone staining). (b) Human islet (dithizone staining). (c) Human iPSC-derived insulin-producing cells (C-peptide staining). Scale bar: 200 μm.

17.3.1.2 Human Islets

An approach in which human islets (Figure 17.1b) from donors are encapsulated and transplanted has been studied. The advantage of this method is that it does not require the use of immunosuppressive agents, although it will not solve the problem of donor shortage. A Phase I clinical study reported that four patients with T1D received an intraperitoneal transplant of microencapsulated human islets without immunosuppressive drugs and it was effective for 3 years [24]. In another Phase I clinical study, it was shown that microencapsulated human islets could be transplanted intraperitoneally and were safe for 3 years [25]. These studies indicated that transplantation of microencapsulated human islets could be safe, but its efficacy was still less than that of standard islet transplantation with immunosuppression.

17.3.1.3 Islet Isolation

One of the most important factors that determines the efficacy of a bioartificial pancreas is the viability and quality of the used islets or cells. For successful human and animal islet transplantation, methods to reduce damage during their isolation and transplantation processes are critical. For pig islet isolation, it is particularly important because very little peri-insular capsule is present and the structural integration of porcine islets in the exocrine pancreas is almost exclusively determined by cell–cell adhesion [26]. Thus, it is important to establish a good method to isolate pig islets for their use in a bioartificial pancreas. This method might be applied when neonatal porcine islets are used.

17.3.1.4 Human Pluripotent Stem Cells

Another promising cell source is pluripotent stem cells (PSCs), which are immortal, stable cell lines that can be differentiated into any type of cell, including insulin-producing β cells. Much interest has been focused on the possibility of using these derived insulin-producing β cells to treat diabetes. There are two types of PSCs: embryonic stem cells (ESCs), which are derived from blastocyst-stage human embryos [27], and induced pluripotent stem cells (iPSCs), which are derived by reprogramming somatic cells to an ESC-like state [28]. The differentiation potential, proliferative capacity, morphology, and gene expression profiles of iPSCs are highly similar to those of ESCs, but the use

of the former avoids the ethical complications associated with deriving ESCs from human embryos [29]. Another advantage of iPSCs is that human iPSCs, which are a genetic match to the person from whom they are generated, could theoretically circumvent the issue of immune rejection [30].

There are numerous protocols published by many groups for the differentiation of PSCs toward either pancreatic progenitors or endocrine cells (Figure 17.1c). One of the most influential methods was published recently by the biotechnology company ViaCyte, Inc. (http://viacyte.com). In their protocol, which was developed by D'Amour *et al*., undifferentiated PSCs were transformed into insulin-expressing cells [31, 32]. In 2014, this group initiated a clinical trial using their bioartificial pancreas device. In addition, many other laboratories have published their own modifications of pancreatic differentiation protocols [33–39]. They are all promising, but there are still some problems. In contrast to the successful generation of pancreatic progenitors from PSCs, the overall yield of end-stage, differentiated, insulin-producing β cells remains low. In addition, the safety of the transplanted cells also needs to be considered. In particular, undifferentiated PSCs have the potential to form teratomas, which is an ongoing concern [40]. Therefore, a promising approach is that cells could be encapsulated in a device that restricts their dispersion and facilitates their retrieval in the event of unwanted growth or differentiation, similar to the xenotransplantation approach.

17.3.1.5 Other Cell Sources

The direct lineage conversion of adult cells, which is called *direct reprogramming*, is a promising approach. Cell sources for this approach include fibroblasts [41], adipose-tissue-derived mesenchymal stem cells [42], PSCs isolated from human amniotic fluid [43], bone-marrow-derived mesenchymal stem cells [44], liver cells [45], pancreatic stem cells derived from adult human pancreas ducts [46], cultured pancreatic islet cells [47], and human pancreatic nonendocrine cells after islet isolation [48]. The direct reprogramming approach could be faster, more efficient, and potentially safer than differentiation from PSCs; however, one problem is that direct reprogramming protocols generally do not produce large numbers of differentiated cells.

17.4 Biomaterials/Encapsulation

As mentioned previously, to prevent immune attack without the need for immunosuppressive drugs, which cause unwanted side effects, islets or insulin-producing cells are immunoisolated by encapsulation with biomaterials that are biocompatible and semipermeable. This semipermeability allows the free exchange of hormones, glucose, nutrients, oxygen, and insulin, but at the same time blocks immune cells, immunoglobulins, and complement. In addition, the capsule should also stop the inner graft cells from escaping the capsule, preventing these cells from scattering and creating tumors elsewhere. Any substance that is identified as nonself will cause a foreign body reaction, but some materials induce a weaker reaction, so the biocompatibility of the biomaterials used is a crucial consideration. Biocompatibility is usually assessed

mainly by fibrosis and revascularization around the graft after transplantation. A smooth contact surface or hydrogel coating may improve biocompatibility [12] through the absence of interfacial tension, leading to reduced adsorption and cell adhesion. The devices may be implanted into a vascularized site [49], peritoneal cavity [50], or other sites.

A large number of biomaterials have been examined for their use in bioartificial devices [6]. The most commonly applied materials include alginate [51], chitosan [52], agarose [53], cellulose [54], Hydroxyethyl methacrylate -methyl methacrylate (HEMA-MMA) [55], copolymers of acrylonitrile [56], and poly(ethylene glycol) (PEG) [57]. These materials induce a weak foreign body reaction after implantation, but for clinical application several factors should be considered. Particularly, hydrogels can provide high permeability of low molecular weight substances and metabolic products [12], so a considerable effect is expected from a hydrogel microcapsule with soft and smooth properties that do not damage the surrounding tissue at the implant site [58]. Macrocapsules have been produced using many materials such as nitrocellulose acetate, acrylonitrile, a copolymer of polyacrylonitrile, and polyvinylchloride, sodium methallyl sulfonate, and alginate [59]. Alginate is a polysaccharide extracted from seaweed. It is one of the most studied and attractive hydrogels for cell encapsulation because it has many advantages over the other available substances. Alginate can be processed easily for its application as a three-dimensional scaffolding material, including hydrogels, microspheres, microcapsules, sponges, foams, and fibers. Alginate consists of linear homopolymeric blocks of α-L-guluronic acid and β-D-mannuronic acid and undergoes gelation in the presence of divalent cations such as Ca^{2+} and Ba^{2+}. Alginate gel provides a useful biological condition for islet encapsulation and cell immobilization [60]. Alginate encapsulation of islets or cell clusters is typically performed by dropping a 1–2 wt% sodium alginate solution into a 1 wt% calcium chloride solution. Beads are formed rapidly because the drops are round and cross-linking begins. Figure 17.2 shows porcine islets encapsulated in an alginate hydrogel macrocapsule.

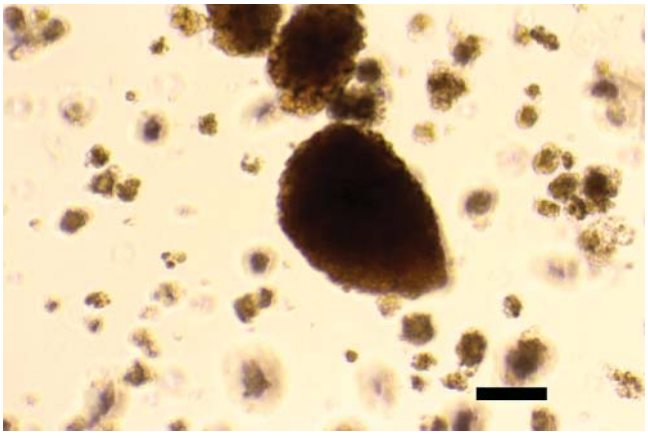

Figure 17.2 Encapsulated porcine islets in an alginate macro-planer capsule. Scale bar: 100 μm.

17.4.1 Macroencapsulation

For the encapsulation of islets or insulin-producing cells, a large device that can sufficiently enclose all the cells in one is an attractive and useful approach that is both easy to handle and inexpensive. The two types of macrocapsule systems differ according to the implant site [12]: extravascular and intravascular. In the intravascular approach, islets or insulin-producing cells are seeded between hollow fibers (such as dialysis cartridges) that are connected with the host blood vessels when implanted. Low molecular weight substances can be exchanged freely through the permeable fibers, whereas the cells are blocked and isolated. These devices have successfully induced normoglycemia in diabetic animal models [61, 62]. In terms of nutrient exchange, this is regarded as one of the most attractive approaches. However, anticoagulation treatment is required because of the device being in direct contact with the blood and there is a risk of thrombosis.

In contrast, extravascular macrocapsule devices (Figure 17.3) have an advantage from a safety viewpoint. They can also be retrieved in case of complications. However, the disadvantage of these devices is their imperfect biocompatibility, which causes nonspecific inflammation, leading to fibrosis that impairs the exchange of substances and leads to the death of the encapsulated cells [50]. For the extravascular type of macrocapsule device, the implant site is a very important factor; for example, it should have sufficient space, easy access for implantation and removal, a weak foreign body reaction, no adjacent delicate organs, and an abundance of surrounding blood vessels.

The first study of macroencapsulation of islets and its implantation was reported in 1943 [63]. Since then, various studies have been reported. As discussed by Dufrane and Gianello [64], a subcutaneously implanted macrocapsule device (TheraCyte device; Baxter Healthcare), with the size of a postage stamp and produced from a bilayer polytetrafluoroethylene membrane, was used recently. Neonatal pig islets inside the device, which was transplanted to nondiabetic cynomolgus monkeys, continued to be viable for 8 weeks without

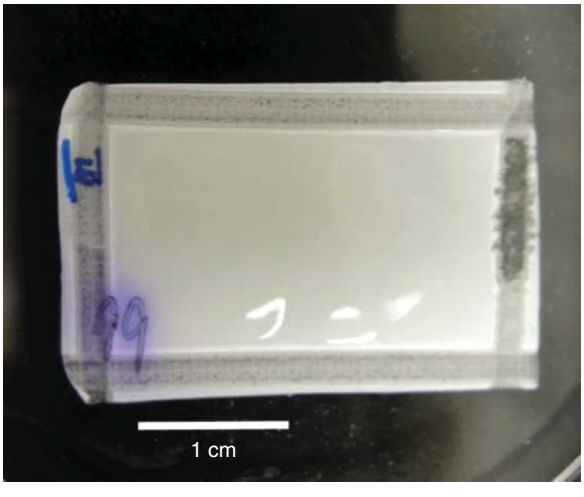

Figure 17.3 Macrocapsule device constructed using a semipermeable biocompatible polymeric membrane. Scale bar: 1 cm.

significant inflammation of the surrounding tissue [65]. Moreover, a number of groups have reported that such a device could support the growth and differentiation of human fetal β-cell precursors or PSC-derived pancreatic progenitors, and this composite bioartificial pancreas could restore glucose control in mouse models [66–70]. A clinical study reported an immunomodulation approach. In this study, porcine islets encapsulated in hollow fibers coupled with porcine Sertoli cells were transplanted without immunosuppressive drugs. One of 12 adolescents achieved insulin-free status and five children had reduced insulin requirements after transplantation [71]. One of the problems of macrocapsules or devices is the relative low surface area to volume ratio, which disturbs the suitable spread of nutrition and oxygen. This low surface area to volume ratio also impairs glycemic control because of the slow exchange of substances such as glucose and insulin. Recently, some studies have overcome these drawbacks by using new strategies. Monolayered macroencapsulated pig islets inside a device transplanted subcutaneously significantly improved diabetes control in primates for 6 months without immunosuppression [72]. More recently, as mentioned previously, ViaCyte, Inc. started a Phase I/II clinical study using human ESC-derived pancreatic progenitor cells in a planar macrocapsule device implanted subcutaneously in patients with T1D (http://viacyte.com/). This is the first clinical trial using ESC-derived cells in diabetic patients.

17.4.2 Microencapsulation

Another encapsulation approach is the microencapsulation of 1–3 islets or spheroidal insulin-producing cell clusters in a semipermeable and immunoprotective microcapsule. This type of capsulation has several advanced features, making it superior to macrocapsulation, especially with respect to viability of the inner cells. The spherical shape of these microcapsules has a higher surface area to volume ratio than the tube or planar disk-type of macrocapsules or devices, leading to a higher diffusion rate [73]. In addition, microcapsules can be implanted easily by injection, because they are typically less than 1 mm in size. However, microcapsules have an increased overall volume and are difficult to remove after transplantation. This disadvantage may be significant in case of transplantation of PSC-derived cells in particular. A smaller microcapsule (300 μm in diameter) has been developed because it can reduce the total graft volume for clinical application [74]. In terms of the implantation site, the intraperitoneal cavity, under the kidney capsule, and subcutaneous space are suitable. In the past two decades, some clinical trials using pig islets have been performed. One of the most promising series was shown by a New Zealand company, Living Cell Technologies (LCT). As explained by Dufrane and Gianello [64], LCT reported a clinical study, starting in 1996, using microencapsulated pig islet cells [75]. The grafts were transplanted to T1D patients intraperitoneally, because of sufficient space for the capsules and good exchange of substances by ascites. Surprisingly, laparoscopic examination revealed that some of the transplanted pig islets were still alive and stained positive for porcine insulin even 10 years after transplantation. This study marked a huge step forward

for bioartificial pancreases, showing the potential for safe clinical application. From 2007, LCT performed a Phase I/II clinical study in Moscow using alginate and poly-L-ornithine-alginate microencapsulated neonatal porcine islet cells (commercially named DIABECELL). The porcine islets and encapsulation technique were similar to those used in the previous trial. Seven subjects with T1Ds received 1–3 implants of DIABECELL including 5000 or 10 000 islet equivalent (IEQ) per kilogram body weight. Five patients continued to have normal blood glucose levels and two patients did not require exogenous insulin injections, showing no remarkable adverse events for up to 96 weeks. All patients improved their glycemic control and HbA1c concentrations. After this clinical study in Russia, Diatranz Otsuka Ltd. conducted a Phase I/IIa clinical trial in New Zealand using LCT's technology [76]. Microencapsulated neonatal pig isles were transplanted by laparoscopy into the peritoneal cavity of 14 patients with unstable T1D without any immunosuppressive agents. The patients received microencapsulated islets of 5000–20 000 IEQ per kilogram body weight. The results showed that transplantation of microencapsulated neonatal pig islets was safe and could reduce hypoglycemic events.

17.4.3 Cell Surface Coating

Even smaller microcapsules still increase the total volume of the graft, which limits the number of transplantable cells and transplant sites. A technique to coat islets or insulin-producing cell clusters with a very thin membrane or conformal polymer coating can significantly reduce the size of these microcapsules. The potential materials are alginate, agarose, tissue-engineered chondrocytes [77, 78], and PEG. The surface of islets was reportedly modified with thin membranes made from PEG-conjugated phospholipid and polyvinyl alcohol [79]. A conformal PEG layer may form on the islets or cell surface at the nanometer level, and this approach can significantly reduce the total graft volume; however, the long-term stability of the PEG layer is still a problem to be solved.

17.4.4 Prevention of Fibrosis

In general, there are two types of host reaction toward an implanted bioartificial pancreas: an inflammatory reaction against the biomaterials of the capsules, and a response against the allogeneic or xenogeneic cell-derived bioactive substances or antigens coming through the capsules.

Fibrosis caused by implanted bioartificial devices, both macro- and microencapsulated, is a major unavoidable problem in bioartificial pancreas technology. The biomaterials used for encapsulation are not perfectly biocompatible and can cause inflammatory reactions to a greater or lesser degree. This fibrotic overgrowth can diminish the spread and exchange through the capsule of nutrients, oxygen, cytokines, and waste products, leading to the destruction of the implanted cells due to hypoxia, starvation, and cytotoxic factors from macrophages [80]. It is critical to develop a capsule with optimal biocompatibility that will not cause fibrotic overgrowth.

17.4.5 Protection from Immune Attack

It is also important to regulate the host immune reaction. What is an optimal capsule or membrane design for bioartificial pancreases from the aspect of immunoisolation? One of the most critical factors is the pore size of the capsule or device membrane [81]. Although alginate is the most common material used for the capsule, uncoated alginate reportedly has high permeability (more than 600 kDa). Therefore, immunoglobulin G (IgG) (150 kDa), small molecules, and cytokines from macrophages and T cells, such as interleukin (IL)-1β, tumor necrosis factor (TNF)-α, and interferon (IFN)-γ, can easily penetrate the alginate capsule and damage the encapsulated cells. Indeed, alginate microbeads became positive for IgG and C3 component at 1 week after transplantation into the peritoneal cavity [82]. To provide immunoisolation, it is first necessary to improve the biocompatibility and selective permeability of capsules. For macroencapsulation, the material of the capsule itself should function as an immunoisolation barrier. Conversely, for microcapsules, a coating of microcapsules can be an option. Applying a positively charged polyamino acid polymer layer outside the capsule surface typically creates such a barrier, which not only lowers the pore size of microcapsules but also provides mechanical stability, which prevents immune cells from invading the capsule [83]. The most studied biomaterial is poly-L-lysine. Recently, it was shown that poly-L-ornithine significantly reduces the immune reaction [84].

Can such capsules protect inner islets or cells? The capsules must stop the invasion of immune cells and antibodies. In addition, it is reported that islets secrete cytokines under some kinds of stress [85], and even encapsulated islets under stress also secrete cytokines such as MCP-1, MIP, nitric oxide, and IL-6, which contribute to the recruitment and activation of inflammatory cells [86]. Activated macrophages produce cytokines such as IL-1β and TNF-α when cultured with encapsulated islets but not when cultured with empty capsules [87]. These cytokines are deleterious to the islets or cells; therefore, the proper permselectivity of capsules is crucial. However, it is important to consider that the pore sizes of polymer membranes are not uniform but vary by nature. This indicates that thorough immune protection may be difficult to achieve with the current permeable polymer membranes or capsules. Further studies are needed to solve this problem.

Some studies utilizing new strategies to reduce inflammation and immune attack have been performed, including macrophage depletion with clodronate liposomes [88], blockage of T-cell costimulation with CTLA4-immunoglobulin to inhibit CD28/B7 and with an anti-CD154 antibody to impede CD40/CD40-ligand interactions [89], and functional capsules that can slowly release an immunosuppressive drug [90].

17.4.6 Transplantation Site

One of the most important features of encapsulation is the lack of revascularization because endothelial cells cannot enter the capsule. This hampers both the function and viability of grafts. Therefore, it is crucial to find an optimal site at which the grafts are as close to the blood stream as possible, especially for

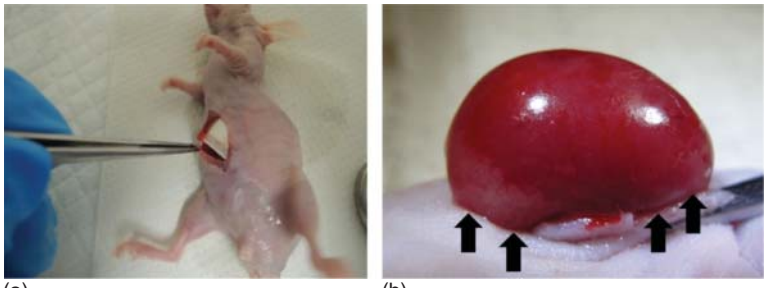

Figure 17.4 Examples of the different cell sources and their transplantation sites. (a) Porcine islets in a microcapsule device transplanted in the peritoneal cavity of a diabetic nude mouse. (b) Human pancreatic nonendocrine cell-derived insulin-producing cells transplanted under the kidney capsule of a mouse. The arrows indicate the implanted cells under the capsule.

clinical application. However, such a site is very rare because it is necessary to have enough volume to implant a large graft as well as be near blood vessels. The liver is a common site for clinical islet transplantation (portal vein infusion), but it does not meet these requirements because of the lack of space for a bioartificial pancreas. Therefore, most transplantations of encapsulated islets, including clinical trials, are performed intraperitoneally (Figure 17.4a), as this approach offers the advantage of a minimally invasive procedure such as laparoscopic implantation or injection [24, 75]. However, the peritoneal site is not necessarily optimal. Its most important disadvantage is that capsules implanted intraperitoneally are attacked by intraperitoneal T cells and macrophages [86, 89]. In addition, the intraperitoneal site has less access to the vasculature [58], leading to an increase of fibrotic growth over the grafts, loss of graft function, and a delay in response to blood glucose concentrations. Therefore, other transplantation sites have been sought, such as under the kidney capsule [91] (Figure 17.4b), subcutaneous space [92], and the omentum pouch [93]. While many studies have been performed to identify suitable implant sites, long-term graft survival is limited because of the lack of vasculature. One potential idea is to design capsules to provide the release of agents in a controlled manner to activate angiogenesis, such as fibroblast growth factor 1 [94]. Another promising approach is the subcutaneous implantation of agarose gel rods including basic fibroblast growth factor and heparin before transplantation; after vascularization of the site, the rod is replaced with a bioartificial pancreas [95].

17.5 Conclusion

A bioartificial pancreas made of encapsulated islets or insulin-producing cells may overcome the two primary problems of allogeneic islet transplantation from cadaver donors, namely, donor shortage and the need for immunosuppressive drugs. Indeed, bioartificial pancreases have already been used in the clinical

Table 17.1 Summary of clinical studies of bioartificial pancreases.

Study	Cells	Type of graft	Type of encapsulation	Islet equivalent (IEQ) per kilogram body weight	Transplant site
Soon-Shiong *et al.* [81]	Human islets	Allograft	Microcapsule	15 000	Intraperitoneal
Valdés-González *et al.* [71]	Neonatal porcine islets with Sertoli cells	Xenograft	Macrocapsule	13 927–20 833	Subcutaneous
Elliott *et al.* [75]	Porcine islets	Xenograft	Microcapsule	15 000	Intraperitoneal
Tuch *et al.* [25]	Human islets	Allograft	Microcapsule	98 200–227 900 total IEQ	Intraperitoneal
Basta *et al.* [24]	Human islets	Allograft	Microcapsule	5 000–15 000	Intraperitoneal
Matsumoto *et al.* [76]	Porcine islets	Xenograft	Microcapsule	5 000–20 000	Intraperitoneal
ViaCyte, Inc. (http://viacyte.com)	Human ESCs	Allograft	Macrocapsule	Not applicable	Subcutaneous

setting (a summary of these studies is presented in Table 17.1). All studies showed promising outcomes; however, in spite of the simple concept and strong need for the elimination of immunosuppression, advances in the field over the past 20 years have fallen short of expectations. This is due to several factors, including the lack of knowledge of the developmental biology of endocrine cells and the properties of biomaterials (e.g., capsule structure and immunoisolation technology) and their combined effects on biocompatibility. In order for a bioartificial pancreas to be used in preclinical and clinical studies, improvement of the implanted cells, maintenance of selective permeability by the biocompatible material, effective methods for oxygen and nutrition delivery, and methods to curb inflammation and for immunoisolation are all required. To control glycemic metabolism, the encapsulation device must be able function well mechanically and the encapsulated cells must maintain viability and function over the long term. In addition, the production cost of these devices should be considered for their broad application. The performance of bioartificial pancreases is being targeted by a number of strategies, which may act synergistically. These strategies include the improvement of cell differentiation methods, better encapsulation materials and capsule structure, induction of immune tolerance, enhancement of neovascularization at the implant site, suppression of oxidative stress, and transplantation of a sufficient amount of cells. The most recent studies with promising results are shown in Table 17.2 (including a couple of the same studies shown in Table 17.1). These improvements should allow a bioartificial pancreas to become the "standard" treatment for diabetes.

Table 17.2 Summary of recent promising studies of bioartificial pancreases.

References	Source cells	Type of encapsulation	Host	Transplant site	Duration of graft function
Pagliuca *et al.* [36]	Human ESCs	Macrocapsule	Mouse	Intraperitoneal	112 d
ViaCyte, Inc. (http://viacyte.com)	Human ESCs	Macrocapsule	Clinical trial	Subcutaneous	Ongoing study
Bruin *et al.* [37]	Human ESCs	Macrocapsule	Mouse	Subcutaneous	24 wk
Raikwar *et al.* [38]	Human iPSCs	Not applicable	Mouse	Under the kidney capsule	150 d
Toyoda *et al.* [39]	Human iPSCs	Not applicable	Mouse	Under the kidney capsule	150 d
Matsumoto *et al.* [76]	Porcine islets	Microcapsule	Clinical trial	Intraperitoneal	1 yr
Shimoda *et al.* [48]	Human pancreatic nonendocrine cells	Not applicable	Mouse	Under the kidney capsule	30 d

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18

Tissue Engineering of Renal Tissue (Kidney)

Raquel Rodrigues-Díez, Valentina Benedetti, Giuseppe Remuzzi, and Christodoulos Xinaris

18.1 Introduction

Chronic kidney disease (CKD) is a devastating disease that is reaching epidemic proportions. Most patients eventually progress to end-stage renal disease (ESRD), a destructive disease characterized by progressive and irreversible nephron loss. The only treatment for ESRD is renal replacement therapy, which consists mainly of dialysis (hemodialysis or peritoneal dialysis) and kidney transplantation. Although dialysis keeps patients alive, their quality of life is severely affected by the associated complications, while concomitant events such as accelerated cardiac disease and infection raise CKD's annual mortality rate to over 25% [1].

Besides the complications, global dialysis costs, which will exceed $1 trillion this decade, added to the current 7% annual increase in patients needing dialysis, indicate that dialysis is set to become unaffordable even for developed countries.

The alternative is kidney transplantation. Renal allotransplantation improves the quality of life and extends the lives of ESRD patients. However, apart from the complications associated with the use of nonspecific immunosuppressants and chronic allograft dysfunction, the main drawback here is the shortage of donors. With a kidney transplant waiting list of 101 170 people in the United States alone [2], there is a critical need for alternative or complementary approaches to allogeneic transplantation.

In the last decade, the regenerative potential of stem cells (SCs) has been explored as an alternative to classic kidney disease treatment. The exogenous administration of SCs isolated from various sources has been shown to stimulate kidney regeneration in renal diseases in which the renal structure is preserved [3–9]. This occurs mainly due to paracrine actions and less frequently through the differentiation/transdifferentiation of SCs into renal cell types [6, 10–13]. Therefore, these strategies cannot be applied in situations where renal structure is severely damaged, such as in ESRD.

The most efficient approach in these conditions would be to create an on-demand functional, nonimmunogenic kidney in the laboratory, ideally using the patient's own cells, as is being attempted with other tissues and organs

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[14, 15]. However, reconstructing a new kidney with the appropriate 3D structure and renal function is an arduous task because of the complex anatomy of the mammalian kidney, which is made up of a large number of highly specialized cells that are essential to renal function, and which consists of a sophisticated network of basement membranes, nerves, and vessels.

There are currently various viewpoints regarding renal tissue engineering. Several authors encourage mimicking kidney development by exploiting the capacity of renal progenitor cells to self-organize into 3D kidney tissue *in vitro*. These approaches make use of 3D culture systems in which fragments from embryonic kidneys or progenitor cells from various sources can be grown to generate immature kidney tissue. Here, we refer to these approaches as "developmental engineering strategies". Alternative strategies use decellularized renal scaffolds as templates to seed stem or progenitor cells called bio-scaffold-based technologies here.

18.2 Biology of the Kidney

18.2.1 Renal Anatomy

The kidneys are a pair of bean-shaped organs located at the back of the abdomen on either side of the vertebral column. The concave sides of the kidneys face each other and possess a slit known as the renal hilum, which provides the space through which the renal artery and vein, as well as the nerve plexus and ureter, enter and exit the kidney. The kidney surface is covered by a thin layer of connective tissue known as the renal capsule. Under the renal capsule there are two areas that can be identified on the cut surface of a bisected kidney: (i) the renal cortex on the outside, and (ii) the deeper medulla region [16] (Figure 18.1a). In humans, the medulla is organized into 8–18 cone-shaped structures, the renal

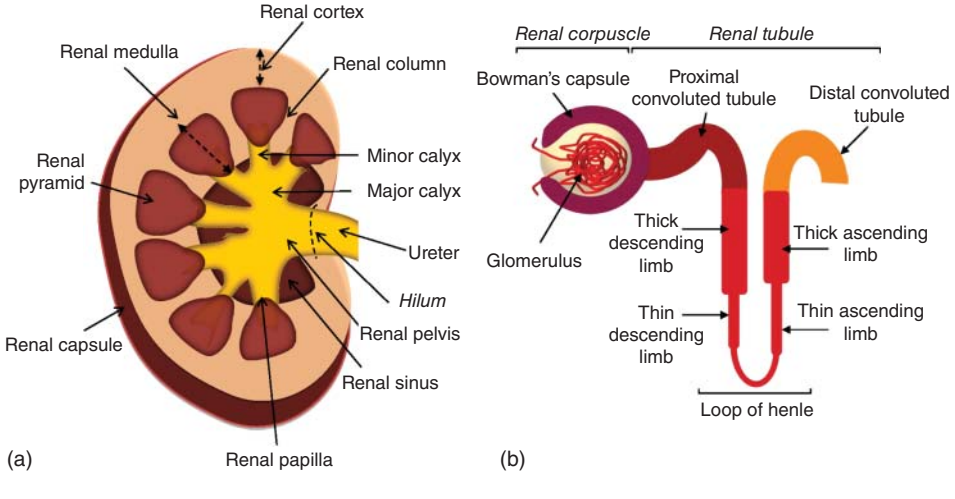

Figure 18.1 Basic schematic representation of (a) kidney anatomy and (b) nephron.

pyramids, which are divided by a downward extension of the cortex, the renal column. The bases of the renal pyramids are aligned with the renal cortex, while their peaks extend inward toward the renal pelvis, forming the papilla. The surface of each papilla has many small openings that correspond to the distal ends of the collecting ducts, which empty the urine formed by each pyramidal unit into its minor calyx. Several minor calyces merge to form the major calyces (2–3 in humans), which finally converge into the renal pelvis. This hollow structure exits the kidney through the renal hilum and funnels the collected urine into the ureter (Figure 18.1a) [16]. Unlike humans, some laboratory animals, such as rats, have kidneys with a single renal pyramid named unipapillate, which extends directly into the renal pelvis.

18.2.2 The Nephron

The basic structural and functional unit of the kidney is the nephron, which is composed of the renal corpuscle, the filtering structure, and the renal tubule, which is responsible for reabsorption and secretion (Figure 18.1b). The renal corpuscle is composed of a tuft of capillaries lined by a thin layer of endothelial cells (the glomerulus), a central mesangial region of mesangial cells, the visceral epithelial layer of Bowman's capsule and the associated basement membrane, and the parietal layer of Bowman's capsule with its basement membrane. The narrow cavity between the two epithelial cell layers is called Bowman's space or the urinary space.

The glomerulus[1] connects with the renal tubule at the urinary pole, whereas the renal arterioles enter and leave the glomerulus at the vascular pole. The renal tubule comprises (i) the proximal convoluted tubule formed by cuboidal epithelial cells with a brushed border which help increase the area of absorption; (ii) the loop of Henle, a U-shaped conduit formed by epithelial cells, which is divided into the descending limb and the ascending limb; and (iii) the distal convoluted tubule that drains urine into the collecting system (Figure 18.1b). Each of these segments of the nephron has different structural and functional properties [16].

18.2.3 Renal Functions

The kidney is a highly specialized organ that regulates the composition and volume of body fluids. It filters the blood, removing waste substances including creatinine, ammonia, uric acid, urea, and toxins, finally producing urine, and then reabsorbs water and essential nutrients.

Urine formation is a process that begins with blood filtration in the glomerulus. The filtration barrier between the blood and the Bowman's space is composed of the fenestrated endothelium of the glomerular capillaries, the peripheral glomerular basement membrane (GBM), and the slit pores between the foot processes of the visceral epithelial cells (also called podocytes) that are wrapped around the capillaries (see also Section 18.3.2) [17]. This barrier functions as

1 While the term "renal corpuscle" is more precise anatomically than the term "glomerulus" when referring to that portion of the nephron composed of the glomerular tuft and Bowman's capsule, the latter term is used throughout this chapter because of its common use.

a sieve or filter that allows the passage of water and small molecules from the blood into the Bowman's space, but almost completely restricts the passage of molecules about the size of albumin or larger. The product of filtration, the ultrafiltrate, leaves the corpuscle and enters the renal tubule of the nephron, to be further altered by tubular reabsorption and secretion, finally forming urine. In the proximal convoluted tubule, ~70% of the ultrafiltrate, consisting of water, electrolytes, amino acids, glucose, vitamins, and other macromolecules, is reabsorbed and returns to the blood through the peritubular capillaries that surround the proximal convoluted tubule. Then this isotonic ultrafiltrate moves to the loop of Henle, where water and ions are reabsorbed by the descending and ascending limb, respectively, as a result of the osmotic difference between the hypotonic filtrate and hypertonic medullary interstitium. The loop of Henle plays a key role in the maintenance of a hypertonic medullary interstitium and in the concentration/dilution of urine. Finally, the ultrafiltrate enters the distal convoluted tubule, where sodium chloride, calcium, and magnesium are reabsorbed, and potassium is secreted [18]. At this point, the ultrafiltrate passes through the connecting tubule and flows to the collecting duct, where bicarbonate is reabsorbed, resulting in acidification of the ultrafiltrate. At the end of the collecting duct, the ultrafiltrate, now called urine, is composed of waste products and a small amount of water.

By regulating the absorption/secretion of ions and water, the kidney controls water and electrolyte homeostasis, the pH level of the blood, and blood pressure. The latter is also regulated by renin, a proteolytic enzyme secreted by the juxtaglomerular cells of the juxtaglomerular apparatus located at the vascular pole of the glomerulus where the distal tubule contacts its glomerulus. Renin activates the renin–angiotensin system, causing an increase in blood pressure. In addition to these functions, the kidney has endocrine functions, secreting two hormones: (i) erythropoietin, which is produced by cortical interstitial fibroblasts in response to anemia to stimulate erythropoiesis, and (ii) calcitriol, the activated form of vitamin D produced by proximal convoluted tubular cells, which regulates calcium reabsorption in the distal tubules and collecting ducts, contributing to the maintenance of calcium homeostasis. Calcitriol is also a suppressor of renin production [19].

18.3 Overview of Kidney Development and Vascularization

18.3.1 Early Kidney Development

Developmentally, the kidney derives from reciprocal inductive interactions between two components of the intermediate mesoderm (IM), namely the Wolffian duct (WD) epithelium [20] and the metanephric mesenchyme (MM) (Figure 18.2a). At 10.5 embryonic days (E10.5) in mice, and 5 weeks in humans, an outgrowth of the WD, the ureteric bud (UB), invades the MM. This process gives rise to the metanephros, the embryonic kidney that persists as the definitive kidney. The MM induces the growth and sequential cycles of UB

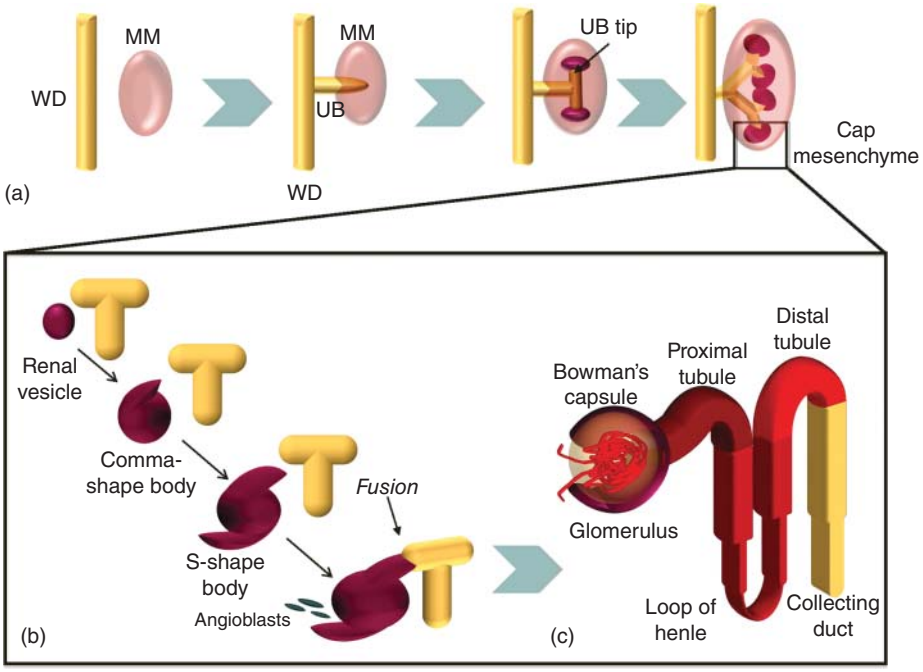

Figure 18.2 Schematic representation of kidney development and nephron formation. (a) Metanephric kidney development starts when an outgrowth from the WD called UB invades the MM. Reciprocal interactions between MM and UB induce UB growth and branching and drive MM to condense around the UB tips (cap mesenchyme). (b) The first epithelial structure of the nephron is the renal vesicle, which derives from cap mesenchymal cells. The renal vesicle grows and progressively forms comma- and S-shaped bodies. At this stage, endothelial progenitor cells (angioblasts) invade the proximal cleft of the S-shaped body and develop the glomerular capillaries, while the distal end of the S-shaped body fuses with the UB and forms the distal tubule. (c) This eventually gives rise to the mature nephron. WD, Wolffian duct; UB, ureteric bud; MM, metanephric mesenchyme.

branching, which will finally generate the collecting duct network. In turn, the UB induces the MM to condensate around the UB tips, and subsequently to undergo mesenchyme-to-epithelial transition, leading to the formation of a primitive polarized epithelium, the renal vesicle. This vesicle grows to give rise to the comma-shaped body, which further develops into the S-shaped body (Figure 18.2b). The distal end of the S-shaped body fuses with the UB and becomes the distal tubule, and the intermediate portion becomes the proximal tubule and the loop of Henle, while the proximal end develops the glomerulus (Figure 18.2c) [21]. MM cells may also differentiate into stromal cells, a spindle-shaped mesenchymal population that surrounds the MM after UB invasion [22]. The stromal cells can differentiate into endothelial cells, which give rise to peritubular capillaries [23], vascular supportive cells such as mesangial cells [24], and interstitial fibroblasts, which include collagen- and erythropoietin-producing cells [25–27]. Genetic studies in mice with stromal gene mutations have demonstrated that stromal cells play pivotal

roles during kidney development, contributing to renal capsule formation, nephron progenitor and smooth muscle cell differentiation, and UB branching morphogenesis [27]. In this way, the MM generates nephrons and renal stromal cells, whereas the UB gives rise to the collecting system.

18.3.2 Development and Maturation of the Glomerular Filtration Barrier

Glomerulogenesis is one of the most complicated developmental processes and involves reciprocal interactions between epithelial and endothelial components [28]. The proximal end of the S-shaped body contains epithelial cells, which further differentiate into parietal cells that form the Bowman's capsule and podocytes [29]. The developing podocytes produce the vascular endothelial growth factor (VEGF), a soluble factor that attracts the endothelial cells, which express the VEGF receptor, into the vascular cleft on the other side of the podocyte basement membrane (Figure 18.2b). The migrated endothelial cells then proliferate, generate the capillary loops, and produce a basement membrane that fuses with that secreted by differentiating podocytes to finally form the GBM [30]. At this stage, podocytes begin to migrate around the capillary loops and develop primary processes and secondary foot processes. Adjacent podocyte foot processes interdigitate and generate a cell–cell junctional structure called the slit diaphragm, which plays a key role in glomerular filtration. In parallel, glomerular endothelial cells develop fenestrae, the pores that open on both sides of the GBM. Podocytes, fenestrated endothelial cells, and the GBM constitute the glomerular filtration barrier, the highly specialized structure responsible for selective blood filtration. Another glomerular cell population is mesangial cells, which localize among the capillary loops and exert multiple functions in the glomerulus, such as structural support to the capillaries, regulation of capillary flow, and phagocytic functions [31].

18.3.3 Kidney Vascularization

The kidney is a highly vascularized organ that receives about 20% of the cardiac output in the normal adult. The vascularization of the kidney begins early on E13 in mice, when MM-derived capillaries first appear around the S-shaped bodies. At the same time, a branch of the dorsal aorta invades the embryonic kidney and branches into smaller arteries, forming the glomerular arterioles [32]. Nevertheless, the origins of the renal microvasculature are still controversial. The issue is whether the smaller vessels and glomerular capillaries arise exclusively from the dorsal aorta (angiogenesis) [33–37] or from endothelial precursors resident in the MM (vasculogenesis) [38–41]. In all these studies, avascular kidneys were xenotransplanted into different sites in host animals in order to evaluate the origin of the graft's microvasculature. When avascular kidneys were transplanted into host sites that contain angioblasts (e.g., the omentum), graft vascularization was predominantly of host origin, supporting the angiogenesis theory. On the contrary, when the implantation sites did not contain angioblasts (e.g., anterior eye chamber), the glomerular endothelium originated from the graft, promoting the theory of vasculogenesis [42]. This observation suggests that both angiogenesis

and vasculogenesis may act in a coordinated manner to finally establish kidney vascularization.

18.4 Developmental Engineering

Early developmental studies documented that MM and UB cells have the ability to instruct each other to form 3D kidney tissue *ex vivo*, raising interest in exploiting these properties to engineer renal tissue. Some of these approaches make use of embryonic kidney fragments or progenitor cells, obtained either from embryonic kidneys or pluripotent SC (PSC)-directed differentiation, and here are divided into two major groups, tissue-based and cell-based strategies.

18.4.1 Tissue-Based Strategies

More than half a century ago, Grobstein demonstrated that MM and UB tissues can be grown *ex vivo* and are capable of recapitulating, at least to some extent, the kidney's developmental program – including nephron formation and branching morphogenesis of the collecting system [43]. Building on this knowledge, several groups achieved nephrogenesis *in vitro* by using isolated MM in coculture with different "inductors" [44, 45]. Ekblom *et al*. showed that isolated MM differentiated into glomerular, proximal, and distal nephron segments when cultured with embryonic spinal cord, used as an exogenous inductor of nephrogenesis [44]. In another study, isolated MM underwent nephrogenesis when cultured in contact with UB cell pellets, without the use of exogenous tissues [45].

As far as UB branching morphogenesis is concerned, Qiao *et al*. showed that uninduced rat UBs underwent branching morphogenesis when cultured in gel enriched with a medium conditioned by BSN cells – an immortalized cell line derived from E11.5 mouse MM – and glial cell-derived neurotrophic factor (GDNF), a factor essential for kidney development [46]. Under these conditions, the UBs developed 3D tubular structures in the absence of direct contact with the MM. The cultured UB was also able to induce nephrogenesis when recombined with intact MM, which led to the formation of primitive nephrons connected with the cultured UB. These data clearly indicated that cultured UB retained its induction competence. On the other hand, the MM induced cultured UB branch elongation, an important process that occurs during *in vivo* UB development [46]. It has been shown that it is possible, by exploiting this UB culture system, to propagate isolated rat UBs *in vitro* through several generations [47]. Specifically, isolated UBs were cultured for 7 days and subdivided into thirds, which were subsequently cultured for 7 days. This second generation of rat UBs was cultivated and sectioned in the same manner for several generations. Remarkably, the recombination of these propagated rat UBs and the freshly isolated rat MM formed "neokidneys" that developed tubular structures morphologically identical to the cultured whole-rat kidney [47]. Based on these studies, it has been demonstrated that not only the UB but also a single isolated WD bud retained the capacity to branch into a 3D culture [48]. In addition, when an *in vitro* branched structure was recombined

with freshly isolated MM, it induced 3D nephron formation similar to that observed with branched UB. These MM-derived tubules expressed transporters, suggesting tubular differentiation, and were able to absorb a fluorescent organic anion compound, indicating tubular function. Furthermore, after implantation under the renal capsule of a rat host, recombined kidney-like tissue displayed multiple vascularized glomeruli, containing red blood cells. Finally, the authors demonstrated that MM-derived nephrons had gene expression patterns similar to the E17–E18 rat kidney [48]. Given that these studies employ no immunosuppressive strategy and grafts were maintained *in vivo* for only 2 weeks, long-term viability and functionality remain to be determined.

18.4.2 Cell-Based Strategies

18.4.2.1 Generation of Kidney Tissues from Embryonic Kidney Cells

The Grobstein group pioneered studies on generating tissue *in vitro* from single cells, by developing a method in which suspensions of dissociated MM cells are reaggregated with spinal cord cells to generate 3D tissue containing rudimental nephron-like structures *in vitro* [49].

More recently, using the immortalized UB and BSN cell lines obtained from E11.5 transgenic mouse embryonic kidney UB or MM, respectively, Sakurai *et al*. set up a method to establish branching tubulogenesis *in vitro* [50]. The authors reported that the UB cell line formed cell processes and multicellular cord-like structures with clearly visible lumens – early features of *in vitro* tubulogenesis – when cultured in a 3D gel in the presence of BSN cell-conditioned medium. Furthermore, a recent *in vivo* study showed that when MM and UB cell lines were grown in a 3D coculture in Matrigel implants in immunodeficient mice, they organized into spheroid and tubuloid structures that matured over time to finally form organoid profiles surrounded by capillary-like structures [51]. Although the implants displayed epithelial structures with apical vacuoles, microvilli, and junctional complexes, and developed linear basement membranes, the more mature proximal tubule features, such as brush borders and the expression of aquaporin 1, were not observed in these studies [51].

Over the last few years, several authors have demonstrated the possibility of generating fetal kidney tissue starting with single cells isolated from the embryonic kidney [52–56]. Osafune *et al*. showed that single MM cells that expressed a high level of Sall1, a zinc-finger nuclear transcription factor essential for kidney development [57], were able to form colonies when cultured on a feeder layer composed of the mouse embryonic fibroblast cell line NIH3T3, which was genetically modified to express Wnt-4 [52], a protein necessary for nephrogenesis [58]. Sall1-expressing colonies reconstituted a 3D kidney structure containing tubular- and glomerular-like structures positive for the proximal tubule marker Lotus tetragonolobus lectin (LTL) and the glomerular podocyte marker Wilm's tumor 1 (WT1), respectively [52].

Based on Grobstein's studies, Unbekandt and Davies developed a new method wherein the dissociation of whole E11.5 mouse kidneys into a single cell suspension followed by reaggregation led to the formation of immature nephrons and collecting ducts *in vitro*, without using any supportive exogenous tissue [53].

Furthermore, the authors introduced a transient treatment with a Rho-associated kinase (ROCK) inhibitor (for 24 h) to prevent apoptosis induced by the dissociation process [53, 59]. Despite these advances, this method produced tissues that had an anatomical deficiency: the UB re-formed in multiple small-branched UB trees, instead of a single branched collecting duct tree, as occurs in normal kidney development. To overcome this limitation, they combined dissociated MM with a single re-formed UB fragment, derived from a previous round of dissociation/reaggregation. This new approach efficiently generated organ rudiments with a single branched collecting duct [54].

Although it has been shown that the dissociated and reaggregated MM (drMM) retains nephrogenic potential, it undergoes apoptosis if nephrogenesis is induced after the dissociation step [60, 61]. To overcome this limitation, in 2014, a new protocol was reported in which drMM was cultured in the presence of bone morphogenetic protein 7 (BMP7) and fibroblast growth factor 2 (FGF2) – two growth factors that promote the survival of MM – for at least 24 h before tubule induction [62]. Under these conditions, uninduced MM cells survived, and initiated nephrogenesis when cultured with the dorsal piece of an E11.5 embryonic spinal cord as the inductor. The induction resulted in the formation of well-segmented nephrons, expressing markers of proximal and distal tubules, the loop of Henle, and podocytes. Furthermore, BMP7/FGF2-treated drMM was combined with intact UB, previously exposed to GDNF, to form an explant. After 9 days of *in vitro* culture, the explant displayed Pax-2-positive developing nephrons, with nephrin expressed in renal corpuscle-like structures, as well as UB branching, as occurs during normal kidney development. This novel engineering technology has the advantage of extending the *in vitro* culture time of uninduced MM cells without apparent apoptosis, allowing for the manipulation of MM cells before nephrogenesis induction [62].

The complete reproduction of the glomerular structure is a critical step in any kidney engineering approach and is necessary for functional kidney tissue development. Nevertheless, the avascular *in vitro* environment does not allow the formation of functional glomeruli. By optimizing previous methods [49, 52]. Xinaris *et al*. created renal organoids using suspensions of mouse embryonic kidney cells that were integrated into living recipients, and grew them to form vascularized glomeruli and functional nephrons (Figure 18.3) [55]. This method introduced two new key steps: the first was the generation of large cell aggregation cultures that allowed organoids to survive and grow beneath the kidney capsule of athymic rat hosts, leading to the formation of complete nephrons. The second step was the use of VEGF to pretreat the organoid, and the injection of VEGF into the recipient animals after the organoids were implanted in the kidney, thus restoring the podocyte–endothelial VEGF axis that is required for glomerular capillary endothelial induction [63]. This method provided well-vascularized organoids (Figure 18.3b–d) with glomerular endothelium of donor (mouse) origin, demonstrating the capacity of engineered tissue for endogenous vascularization. The newly formed organoids also presented glomeruli with fully differentiated podocytes and capillary walls, including endothelial fenestrae, foot processes and slit diaphragms, and functioning tubules (Figure 18.3e–h). Consistent with this high degree of

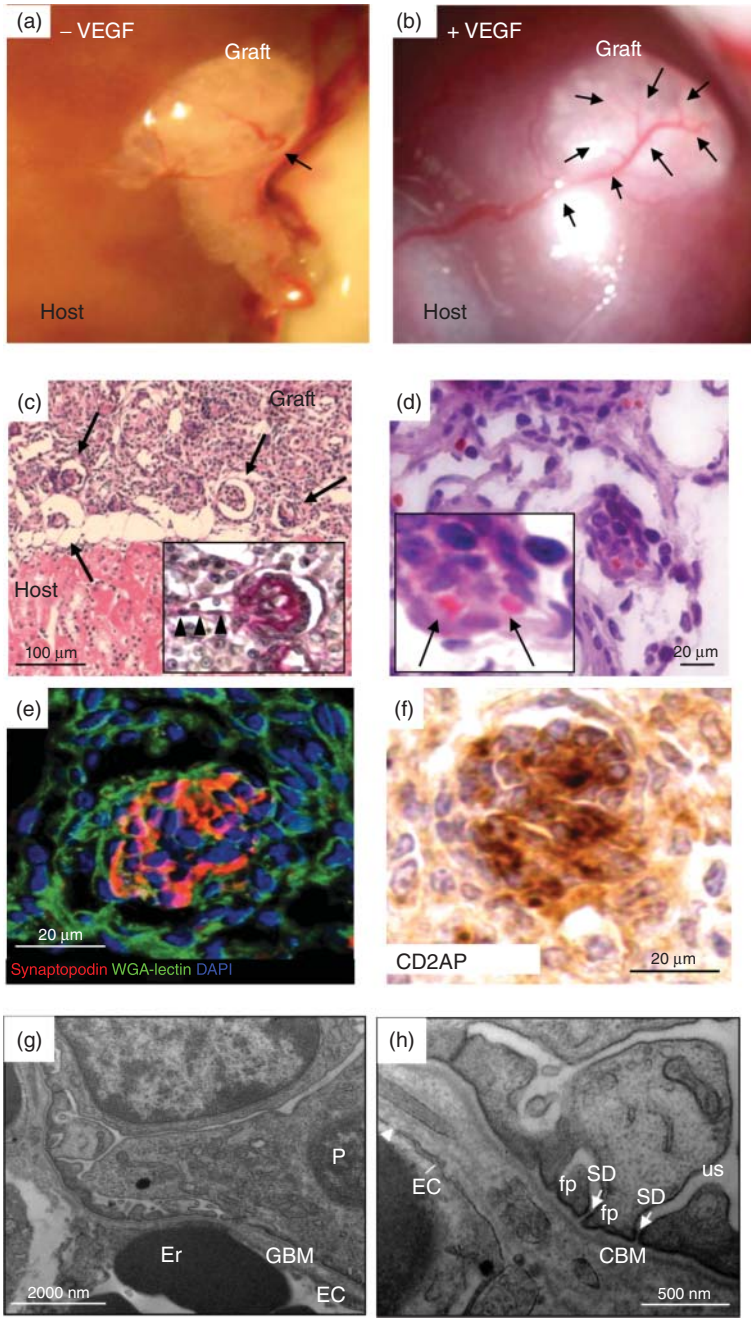
(a)
– VEGF
Graft
Host
(b)
+ VEGF
Graft
Host
(c)
Graft
Host
100 µm
(d)
20 µm
(e)
20 µm
Synaptopodin WGA-lectin DAPI
(f)
CD2AP
20 µm
(g)
P
Er
GBM
EC
2000 nm
(h)
EC
fp
SD
fp
SD
us
CBM
500 nm

Figure 18.3 *In vivo* development of renal organoids made from suspensions of mouse embryonic kidney cells. (a, b) Macroscopic image of a renal organoid 3 weeks after implantation beneath the renal capsule of uninephrectomized athymic rats. (a) Key configuration for obtaining sufficient vascularization (arrows) was obtained by soaking the organoids in a solution containing VEGF, and then injecting VEGF into the recipient animals after the organoids were implanted in the kidney (b). (c) Histology shows several glomeruli (arrows; H&E) with proper glomerular arterioles (arrowheads in inset; PAS). (d) Glomerular structures contained erythrocytes (arrows; H&E). (e,f) Glomeruli were positive for podocyte markers, synaptopodin (e) and CD2AP (f). (g,h) Electron micrographs of intragraft glomeruli. (g) A capillary structure containing erythrocytes (Er) was lined by the endothelium (EC). (h) Complete foot processes (fp) and slit diaphragms (SD; arrows) separated the glomerular basement membrane (GBM) from the urinary space (us). The endothelium shows fenestration (arrowhead). H&E, hematoxylin and eosin; PAS, periodic acid–Schiff. (Adapted from [55].)

structural maturation, the implanted organoid displayed specialized physiologic functions, including proximal tubular reabsorption of tracer macromolecules that gained access to the tubular lumen by glomerular filtration. Moreover, erythropoietin-producing cells were detectable in the interstitium, indicating hormonal competence [55]. By integrating electron microscopy analysis and macromolecular tracing experiments, the authors further documented that organoids could recapitulate, *in vivo*, the complex 3D filtering structure of glomerular slits and accomplish selective glomerular filtration [64]. One limitation of this study was that the organoids were viable for only 3–4 weeks and began to involute thereafter. Thus a challenge will be to achieve greater longevity, possibly by using anti-rejection therapy and testing other potential implantation sites that might enhance survival and growth.

Taken together, these studies suggest that progenitor cells derived from undifferentiated MM and unbranched UB maintain their developmental potential and, therefore, under appropriate culture conditions, could be used to generate nephron-like structures and branching collecting ducts *in vitro*, as occurs during normal kidney development. Nevertheless, whether it is feasible to establish renal progenitors from patient cells that can act as the MM and the UB remains unknown.

18.4.2.2 Generation of 3D Kidney Tissue from Pluripotent Stem Cells

The experiments described above suggest that, if renal progenitor cells could be derived from patient cells and exposed to a defined developmental niche [47, 48, 50, 53, 55], they could subsequently spatially self-organize to generate 3D renal tissues. To explore this possibility, a subset of studies used embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs), with the concept that results obtained with ESCs can be translated to iPSCs. Though it is still unknown whether these cells can be differentiated to such a degree that they can faithfully recapitulate the behavior of either the UB or MM, several studies have reported differentiation of ESCs or iPSCs into renal progenitor-like cells according to marker expression and the capacity to integrate into developing kidneys [65–70].

The first decisive step toward inducing renal progenitors was taken by the Osafune group by differentiating PSCs into the IM, the embryonic germ layer that

gives rise to the kidney [65]. Initially, PSCs were differentiated into mesoderm (ME) through stimulation with Activin A and the Wnt-agonist CHIR99021, and then treated with a combination of BMP7 and CHIR99021 to give rise to cells that highly expressed OSR-1$^-$, the earliest marker of IM. These OSR-1$^+$ cells also expressed a wide range of genes and proteins specific to the developing kidney, including the nephric duct, UB, MM, and metanephric stroma markers, as well as markers of the adult kidney. However, when hiPSC-derived OSR-1$^+$ cells were cocultured with dissociated mouse embryonic kidney cells in 3D conditions, they showed only a modest ability to form renal polarized tubular-like structures, and rarely differentiated into UB, indicating low efficiency in renal lineage cell commitment [65]. To improve the efficacy of the method, the authors bypassed the ME differentiation step through the exclusive use of small molecules, which enhanced the ability of the hiPSC-derived OSR-1$^+$ cells to form tubular-like structures (10-fold) [66].

Recently, a new protocol described by Xia *et al*. allowed the differentiation of PSCs into UB progenitor-like cells in 4 days [67]. Human ESCs or iPSCs clustered in Matrigel were costimulated with BMP4 and FGF2 for 2 days to induce ME commitment and then exposed to a combination of retinoic acid (RA), Activin A, and BMP2 to generate UB progenitors. Differentiated cells expressed the UB-related transcription factor HOXB7 and GDNF-receptor – essential for UB invasion and branching – and were positive for nephric duct markers. To promote further maturation, differentiated cells were aggregated with mouse embryonic kidney cells in a 3D culture, where they integrated into chimeric UB structures and, consistent with the absence of MM markers, did not contribute to the MM compartment [67, 68].

Almost simultaneously, Taguchi *et al*. differentiated PSCs into MM by first defining the developmental origins of MM cells and then by carefully replicating them *in vitro* [69]. Through *in vivo* lineage-tracing studies, they observed that MM precursors were localized in the Brachyury (T)-positive posterior nascent mesoderm, whereas UB derived from T-negative anterior nascent mesoderm. Initially, they defined the best combination of factors for inducing the differentiation of the mouse-isolated T$^+$ population into MM. Then, they used these factors to differentiate MM cells from both mouse ESCs and human iPSCs. First, embryoid bodies (EBs) were incubated with Activin A, followed by BMP4 and CHIR99021 to induce posterior mesoderm differentiation, and then stimulated with Activin A, BMP4, RA, and CHIR99021 to acquire an IM identity. Finally, differentiation toward MM progenitors was achieved through exposure to CHIR99021 and FGF9 [69]. When cocultured with mouse embryonic spinal cords [49], the differentiated EBs reconstituted the 3D structures of the kidney, including renal tubules with both proximal and distal regions as well as glomerular structures with cells that expressed podocyte and foot process markers. Notably, implanting differentiated murine EBs together with spinal cord *in vivo* underneath the renal capsule of immunodeficient mice resulted in rudimental renal tissue with vascularized glomeruli containing red blood cells, demonstrating connection with host circulation [69].

In a different approach, Takasato *et al*. proposed a three-stage protocol that allowed for simultaneous differentiation of human PSCs into both UB and MM

cells [70]. The stepwise protocol began with the differentiation of hESCs growing in a monolayer into the posterior primitive streak (from which IM derives) through Wnt-1 activation, either with CHIR99021 or with a combination of BMP4 and Activin A. Subsequent exposure to FGF9 induced IM specification. These IM cells were exposed to FGF9, BMP7, and RA treatment, giving rise to a mixed cell population that expressed UB or MM markers, as well as the renal stroma markers. Reaggregation after dissociation of the monolayer cultures upon UB and MM commitment enabled the formation of a small, self-organized kidney organoid-containing nephrons. Moreover, when ESC-derived renal progenitors were reaggregated with dissociated mouse embryonic kidneys, they integrated into all developing renal structures, including the ureteric epithelium, renal vesicles, nephron progenitor mesenchyme, and renal stroma [70].

These studies demonstrate that human PSCs can be differentiated into the major renal epithelial cell types, and that they self-organized into immature 3D tissues when exposed to the appropriate developmental signals. However, there are still several challenges to be met before functional renal tissue can be generated from SCs. One of these is the efficient vascularization of engineered tissues to achieve further maturation to a status which resembles that of adult organs. To date, the most promising solution is still the transplantation of these tissues, which can stimulate invasion from the host vasculature [55, 69]. Another important issue is that the PSC-derived kidney tissue should develop in such a manner that it contains a single collecting duct tree draining into the ureter, which can potentially be connected to the host's ureter.

18.5 Bio-Scaffold-Based Technologies

The extracellular matrix (ECM) is a complex meshwork present within all tissues and organs and it is formed by the material secreted by the resident cells. The ECM provides not only the mechanical framework for the adjacent cells but also initiates biochemical signals that are required for tissue morphogenesis, differentiation, and homeostasis [71]. Based on these important functions, it has been proposed that a kidney that has been properly decellularized, in a manner that retains the renal ECM signals, could act as an inductive template for seeded stem and progenitor cells to migrate, proliferate, and differentiate in order to reconstitute organ structure (Figure 18.4) [72].

18.5.1 Decellularization to Generate Whole Kidney Scaffolds

Traditionally, decellularizing biological tissues for engineering approaches has been limited to thin tissue structures such as the intestines [73], blood vessels [74, 75], the bladder [76, 77], and the respiratory tract. Recently, based on the techniques described by Mertsching *et al*. [78], several studies have reported that it is feasible to obtain decellularized, intact ECM scaffolds from more complex organs, including the heart, liver, and lung [79–83]. Concerning the kidney, different decellularization protocols have been developed in order to obtain a renal scaffold that maintains the 3D ultrastructure, physicochemical composition, and

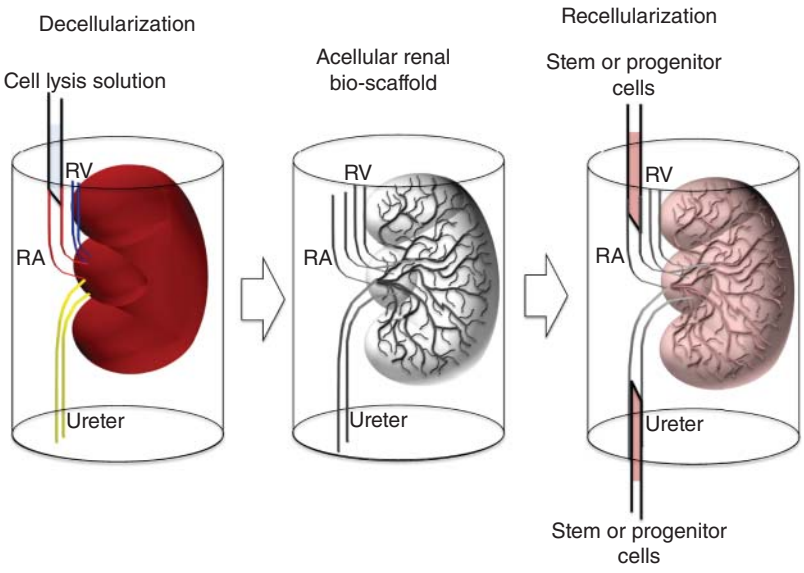

Figure 18.4 Decellularization/recellularization protocol overview. Human or animal whole-kidney ECM scaffolds can be obtained by perfusing cell-lysis solutions via the renal vasculature. Subsequently, these scaffolds can be repopulated by injecting different cell types via the renal artery and the ureter. RA, renal artery; RV, renal vein.

biological activity of the ECM. Most reported protocols include the perfusion of cell-lysis solutions (detergent or enzymatic) through the renal vasculature, along with some supporting techniques such as freeze–thaw cycles, osmotic shock, or nuclear material degradation with deoxyribonuclease (DNAses).

Based on preliminary renal decellularization protocols [84] – originally designed for the morphologic study of GBM – Ross *et al*. described the first decellularization method for obtaining an intact bioscaffold for subsequent recellularization. The protocol consisted of a 5-day, stepwise, gravity-based arterial perfusion with different fluids directed to cell lysis, nuclear material degradation, and cell debris elimination. In order to identify the best protocol for renal ECM preservation, two ionic detergents were tested: sodium dodecyl sulfate (SDS) and sodium deoxycholate (NaDOC). SDS treatment showed better disruption of intact cells and debris removal than NaDOC, maintaining the connective tissue pattern throughout the cortex and medulla. The resulting 3D scaffold presented a well-preserved glomerular, tubular, and vascular ECM microstructure, expressing laminin and collagen IV [85, 86].

Building on this study, Song *et al*. presented a protocol that significantly simplified the decellularization steps through arterial perfusion at constant pressure using two solutions containing ionic (SDS) and non-ionic detergents (Triton-X-100), respectively, which allowed total decellularization in under 13 h. The whole-kidney scaffold showed complete cellular component removal with a total DNA content per kidney of less than 10%. Preservation of both tubular basement membrane and GBM structures, assessed through laminin- and collagen IV-positive staining, were similar to those of native kidneys. Finally,

the function of the acellular kidney was tested *in vitro* through perfusion with modified Krebs–Henseleit solution at physiologic pressure (80–120 mmHg). As expected, the obtained filtrate solution contained high levels of proteins, glucose, and electrolytes, due to an increase in glomerular and tubular hydrostatic filtration, caused by the loss of cell-mediated functions such as macromolecular sieving and solute transport [87].

Recently it has been shown that, after decellularization, the levels of ECM-related growth factors, including hepatocyte growth factor (HGF), connective tissue growth factor (CTGF), transforming growth factor-beta (TGF-β), and VEGF present in the decellularized kidney were similar to those observed in intact kidneys, suggesting that renal acellular scaffolds could retain some cytokines at concentration levels suitable for contributing to renal regeneration [88].

The use of non-ionic detergents such as TritonX-100 may be traumatic to tissue and could damage the collagen structure [89]. Because of this, Bonandrini *et al*. applied a short (17-h) protocol for rat kidney decellularization, using 1% SDS exclusively (Figure 18.5a,b). The obtained whole-kidney scaffolds presented a well-preserved glomerular ultrastructure, including capillary segments, Bowman's capsule, and tubular matrix, and featured a basement membrane with collagen IV, laminin, and fibronectin staining patterns similar to those of the native kidney. Vascular network integrity and permeability, as well as connection of the arterial tree with the venous tree, were also observed [90].

Successful kidney decellularization has also been reported in large mammals such as pigs [91–93] and even humans [94], thus overcoming an important challenge in scaling up the decellularization process into more clinically relevant models. Most of the reported protocols employed a perfusion system that allowed continuous rinsing of different solutions under controlled pressure, using SDS as the principal component of the lysis solutions. Treatment with SDS solutions in porcine kidneys [91] produced decellularized renal matrix that retained the 3D structure and microarchitecture, including renal microvasculature and basement membranes, and preserved the ECM components (collagen, laminin, and glycosaminoglycans) in patterns similar to those found in native kidneys. In accordance with this report, a comparative study of swine kidneys perfused with different detergents demonstrated that SDS solution was the most efficient at cellular clearance and at preserving ECM components [93]. After SDS decellularization, the DNA for porcine xenoimmunogens including galactose-α-1,3-galactose (1,3-α-Gal), porcine endogenous retrovirus (PERV), swine leukocyte antigen 2 (SLA-2), and swine leukocyte antigen DR alpha (SLADRA) were undetectable, and Gal epitope staining decreased [93], denoting that porcine renal scaffolds would be less immunogenic than native pig kidneys.

Every year, a large number of kidneys designated for transplantation are discarded for various reasons [95]. Some authors have proposed using these discarded kidneys as scaffolds for bioengineering applications. Based on their previous studies on swine kidneys [91], Orlando *et al*. successfully decellularized a transplantable discarded human kidney through SDS that was perfused via both the renal artery and the ureter. As in pigs, the decellularized human renal scaffold presented well-preserved 3D structures, an intact vascular network, and

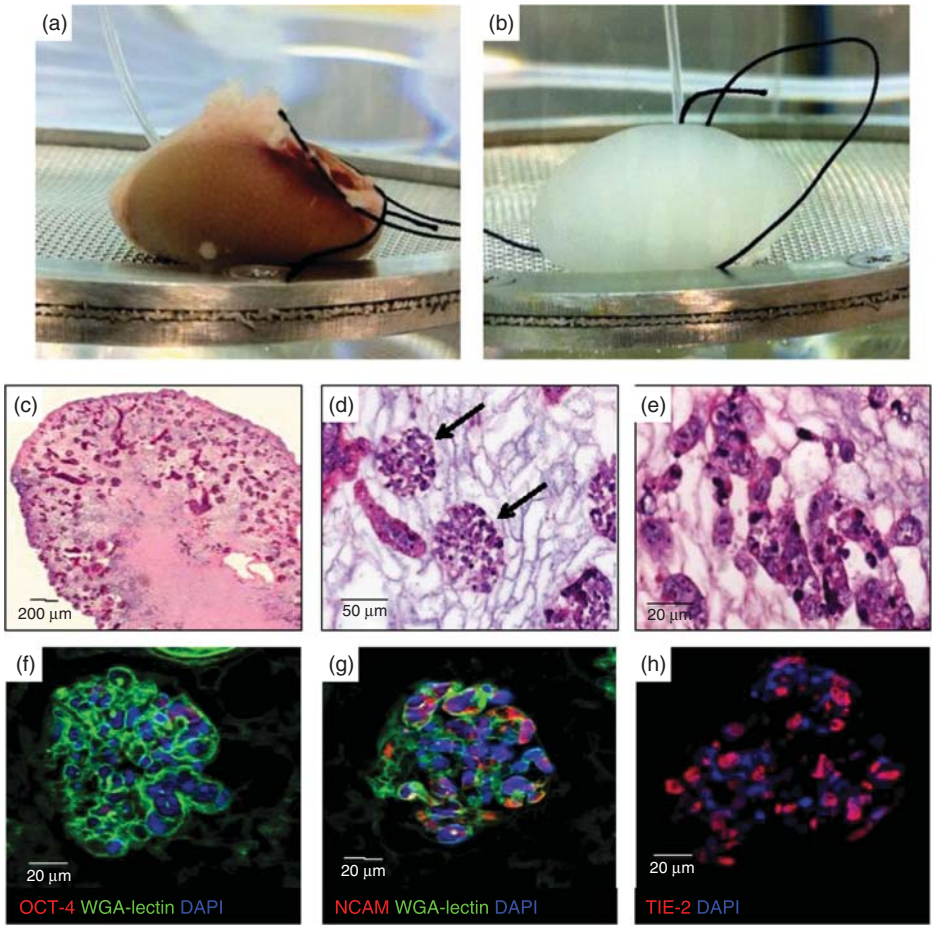

Figure 18.5 Repopulation of rat kidney scaffolds with mouse embryonic stem cells (mESCs) by renal artery infusion. Whole rat kidney at the beginning (a) and after decellularization (b). (c–e) H&E staining of recellularized kidney shows homogeneous distribution of cells into glomerular (arrows) and vascular structures (c,d), and peritubular capillaries (e). (f–h) Immunofluorescence analysis of repopulated glomerular structures 72 h after infusion. (f) Engrafted cells were mainly negative for Oct-4, demonstrating the loss of embryonic stemness. Infused cells start to express NCAM (g) and Tie-2 (h), indicating differentiation toward mesoderm-derived endothelial precursors. H&E, hematoxylin and eosin; Oct-4, octamer-binding transcription factor 4; NCAM, neural cell adhesion molecule. (Adapted from [90].)

ECM protein expression. In addition, the decellularization process removed the two major histocompatibility complex antigens HLA-ABC (I) and HLA-DR (II), which are key factors in human immune rejection. However, this protocol also removed the transmembrane receptor Integrin α3/β1 – an essential player in glomerular morphogenesis and the maintenance of glomerular filtration barrier integrity [94].

Altogether, these data indicate the feasibility of obtaining an acellular renal scaffold that preserves its 3D architecture and ECM structure and composition,

with the added advantage of attenuating tissue antigenicity, which is particularly useful for xenotransplantation.

18.5.2 Recellularization of Whole-Kidney Scaffolds

The capacity of the remaining scaffold to support cellular adhesion has been tested by seeding scaffolds with different cell types using the renal artery (antegrade) or the ureter (retrograde) as infusion routes. Ross *et al*. first reported the feasibility of repopulating an acellular rat kidney ECM scaffold with murine ESCs. After decellularization, the remaining whole-kidney scaffolds were infused manually with murine ESCs via the renal artery and cultured in a static medium or perfused through a pulsatile system (270–300 beats per minute, 120/80 mmHg). The authors reported that *in vitro* culture in a static medium did not preserve cell viability, and it was necessary to slice the pre-seeded scaffold. In both culture systems, the cells were initially distributed into vascular structures including small vessels, arterioles, glomerular capillaries, and their associated glomerular tuft. After progressive incubation, epithelialized areas, which are defined by the presence of pan-cytokeratin-positive cells, were observed adjacent to vascular basement membranes, in some areas of Bowman's capsule, and on the kidney capsule. Evidence of cell division and differentiation was provided by positive staining for Ki-67 and by the expression of the renal embryonic markers Pax-2 and Ksp-cadherin [85]. Subsequently, in rat bio-scaffolds seeded with mouse ESCs, this group further demonstrated vascular endothelialization, assessed through staining for endothelial-specific markers *Griffonia (Bandeiraea) simplicifolia* lectin I, isolectin B4 (BsLB4), and VEGF receptor-2 (VEGFR-2/Flt-1). Seeded mouse ESCs also contributed to basement membrane remodeling through the secretion of ECM proteins such as laminin β1 [86].

In an attempt to facilitate access of cells to the collecting system, the authors injected ESCs through the ureter, which resulted in repopulation of the calyxes and tubular structures. However, cell retention was less than that observed after renal-artery seeding, and cells were not uniformly distributed throughout the kidney, possibly because of the particular architecture of rat renal papilla, which could be a hurdle to uniform retrograde cell dispersion [85].

The recellularization protocol described by Song *et al*. combined both paths (renal artery and ureter) and the perfusion of two cell types into the decellularized rat kidney: human umbilical venous endothelial cells (HUVECs) through the renal artery, and rat neonatal kidney cells (NKCs) through the ureter. Negative pressure was applied to allow NKCs to reach the entire renal parenchyma. Optimal vacuum pressure was established at 40 cmH_2O, where no tissue damage or cell outflow was observed, while pressure over 70 cmH_2O damaged calyxes and parenchyma. On day 4 of perfusion, the renal scaffold had been repopulated with both epithelial and endothelial cells engrafted in their appropriate compartments. The average percentage of repopulated glomeruli per regenerated kidney was ~70%, but the glomerular diameter was smaller than that of cadaveric kidneys. Glomerular structures presented podocin$^+$/nephrin$^+$ cells (podocytes) and CD31$^+$ (endothelial) cells, and electron microscopy

revealed glomerular capillaries with engrafted podocytes and foot process formation. Na/K-ATPase and aquaporin-positive cells were observed in structures resembling the proximal tubular epithelium, whereas E-cadherin-expressing cells were observed in the distal tubular epithelium and collecting duct-like structures, as happens in the normal kidney. Although native kidney levels were not reached, functional *in vitro* assays indicated partial restoration of renal function in recellularized kidney scaffolds, assessed through creatinine clearance (23%) and an increase of approximately 50% in albumin retention and glucose and electrolyte reabsorption, compared to decellularized kidneys. In addition, vascular resistance was decreased compared to decellularized kidneys, but remained high compared to cadaveric kidneys [87].

Recent studies by Bonandrini *et al*. on recellularizing renal scaffolds through antegrade mESC seeding via constant renal artery perfusion at low controlled pressure showed that the seeded cells were almost completely retained in the organ and uniformly distributed in the vascular network and in glomerular capillaries, without major signs of apoptosis. Hardly any cells were observed in the peritubular capillary and tubular compartments (Figure 18.5c–e). The seeded ESCs progressively lost their pluripotency, as demonstrated by a lack of octamer-binding transcription factor 4 (Oct-4) expression (a marker of embryonic stemness) and started to differentiate into meso-endothelial lineage as observed through the expression of the neural cell adhesion molecule (NCAM, a marker of mesoderm precursor) and endothelial cell markers including Tie-2 and CD31, suggesting positive participation of the 3D renal structure and ECM signals in inducing cell differentiation in the infused cells (Figure 18.5f–h) [90].

Kidney recellularization is a key step toward whole-kidney regeneration. Although the described methodologies to repopulate the kidney are promising, for the efficient recellularization of all kidney compartments, there are still limitations to be overcome. One problem is the recellularization of the renal parenchyma, because the most common routes of infusion, namely the ureter and renal artery, do not allow seeding cells to reach the parenchyma easily. In addition, the actual conditions required for injected cell types to proliferate and differentiate into all renal cell lineages need further investigation. Finally, in order to translate this technology into clinical application, bioreactors need to be upscaled and cell seeding protocols must be optimized for human-size scaffolds.

18.5.3 Recellularization of Acellular Renal Sections

Although the recellularization of slices of decellularized kidneys presents limited clinical applicability, these studies can provide a powerful tool for understanding the interaction mechanism between seeding cells and the bioscaffold.

Sullivan *et al*. analyzed renal scaffold cytotoxicity by seeding isolated primary human renal cells onto 5-mm cortical sections of pig scaffolds decellularized with SDS or Triton X-100. They observed that cells seeded onto the scaffolds treated with SDS proliferated over 4 days, whereas the cells seeded in the scaffolds decellularized with Triton X-100 died after 1 day [92].

Isolated kidney stem cells seeding in the tree regions (renal cortex, medulla, and papilla) of porcine renal scaffolds displayed significant differences in proliferation, metabolic activity, and cell morphology, whereas this was not observed with mesenchymal SCs, indicating a significant degree of recognition and specificity between adult kidney SCs and their extracellular environment [96]. This was also supported by the finding that human ESCs seeded in monkey lung and kidney scaffolds did not differ significantly in the expression of lung- or renal-specific markers [97]. These data suggest that cell commitment toward specific kidney lineages prior to seeding could be necessary to facilitate interactions between the cells and the ECM, and to eventually achieve efficient cell differentiation.

18.5.4 Implantation and Vascularization of Kidney Scaffolds

Vascularization is essential for the viability and functionality of bioengineered solid organs. Some authors therefore transplanted acellular whole-kidney scaffolds orthotopically to evaluate whether the scaffold's vascular network could support host blood pressure and recipient tolerance [87, 91]. Decellularized porcine kidneys have been transplanted to nephrectomized pigs through the renal artery and vein anastomosis to the recipient aorta and vena cava, respectively. Despite the presence of inflammatory cells, no immune rejection was observed, showing the low immunogenicity of acellular scaffolds. The acellular scaffold was blood-perfused for 1 h without signs of hemorrhage, but 2 weeks after transplantation the renal vasculature was completely obstructed by thrombi and clotted red blood cells, possibly due to the absence of endothelium within scaffold vasculature ECM [91]. In contrast, transplanted rat renal scaffolds previously recellularized with HUVEC and NKCs were well perfused, with no hemorrhage or thrombosis, and they exhibited higher urine production compared to the decellularized kidney [87]. These results suggest that vascular pre-endothelialization of the renal scaffold before transplantation is essential for preventing thrombosis and, at least, partially restoring renal function. Following this line, the Atala group recently reported a novel protocol for re-endothelializing a porcine renal vascular matrix by pretreating it with CD31 antibodies prior to cell seeding, to improve murine endothelial cell attachment and retention in the matrix. This seeding approach resulted in a homogenous, recoated endothelium, leading to vascular patency of a tissue-engineered whole kidney for 4 h after transplantation [98].

In another approach, a decellularized rat kidney section was surgically engrafted with the remaining part of a partially dissected kidney in order to regenerate nephrectomized tissue. After 1 week, cells positive for nestin – a marker of renal stem/progenitor cells – were observed in the renal tubules and in the interstitium near the regenerative area, but nestin^{+} cells progressively disappeared and became undetectable after 8 weeks. The kidney graft showed some macroscopic regrowth and bloodstream evidence. In addition, a partial recovery of function was observed [88].

The complete vascularization of kidney scaffolds is still a major issue in the field. However, *in vitro* pre-endothelization of the vascular matrix seems to be a promising option to facilitate graft vascularization *in vivo*.

18.5.5 Organ Printing

3D bioprinting or organ printing usually involves precise layer-by-layer positioning of living cells together with biomaterials and growth factors, to fabricate 3D macrotissues or organ constructs. Organ printing commonly includes three sequential steps: designing the "blueprint" for an organ, printing the organ, and finally organ conditioning in order for it to mature and potentially become functional [99, 100].

Although a kidney has not yet been constructed through organ printing, a promising step toward printing a vascularized solid organ has been reported recently [101]. Using a customized 3D printer that allowed simultaneous printing of multiple materials and cells and a gel that liquefies when cooled, Kolesky *et al*. manufactured a 3D tissue composed of various patterned human cell lines and biomaterials, interlaced with hollow tube-like structures that were subsequently seeded with vascular cells (HUVEC).

Despite the smallest tubule diameter being about 75 μm, which is far bigger than the tiny (5–10 μm) glomerular capillaries, this method may enable the construction of the main blood vessels that could potentially branch and develop into smaller ones within the appropriate developmental environment.

18.6 Conclusions and Future Directions

The extensive progress made over the last two decades in the fields of stem cells, developmental biology, and tissue engineering, have undoubtedly brought the goal of generating a functional kidney a little closer, giving new hope to ESRD patients waiting for a transplant. Nevertheless, several challenges must be addressed before functional renal tissues with clinical applicability can be obtained: (i) engineered tissues must develop into 3D organs of the size and with the anatomy of human kidneys, able to sustain life in the long-term; (ii) they need to become fully integrated into the host, in terms of inducing no immune response and being vascularized by host vessels; and (iii) the new tissue should be linked to a draining collecting system.

Future strategies will focus on solving oxygen supply and vascularization problems, which are key issues for engineered tissues developing into 3D organs. For example, strategies based on the use of chemical sources of oxygen, including supplementing tissue-engineered kidneys with oxygen-carrying molecules, or employing oxygen-generating biomaterials as scaffolds, could provide sustained oxygen release before and immediately after implantation, while neo-vascularization is being established. In addition, neo-vascularization of engineered tissue can be promoted though coculture with endothelial cells or by incorporating pro-angiogenic molecules, such as VEGF and epidermal growth factor (EGF).

Applying mechanical forces to culture renal organoids would improve oxygen and nutrient delivery, as well as waste elimination. For example, it has been proven that the use of an intra-tissue perfusion system enhances the viability and functionality of other tissues, such as liver slices, *in vitro* [102].

Although the renal tissue engineering field is still developing and it is obvious that it will take several years of experimentation before it can be applied to regenerative medicine, the systems described here have enormous potential to, in the short term, be applicable to the study of kidney development and disease, and as a tool for drug testing. Evaluating these systems within preclinical models of renal disease will be of the utmost importance in the coming years.

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19

Design and Engineering of Neural Tissues

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19.1 Introduction

Neural tissue engineering (NTE) is the subsection of tissue engineering that essentially explores and develops technology and methods for remediation or elimination of the effects of disease, aging, or injury of the nervous system. These methods are primarily based on implantation of grafts and scaffolds, which may serve as the platform for damage repair and regeneration of the nervous system, more specifically neural tissue. These artificial foreign objects tend to cause inflammation and fibrosis after implantation. Search for remedies for such inflammation and fibrosis also falls under the scope of NTE. The process of neural tissue repair and regrowth or regeneration is known as *neuroregeneration*. In this chapter, we discuss natural and synthetic biomaterials as well as the fabrication and development of nanostructured scaffolds used for NTE.

Tissue engineering, by definition, is the study of cells, engineering methods, and materials to produce artificial biocompatible means either to improve or to replace or regenerate biological functions. The study of materials is crucial, and requires multidisciplinary knowledge and research. Material selection is the first step of tissue engineering while developing new strategies or methods. The selection of material is constrained by some fundamental properties needed for it to function and serve its purpose in tissue engineering. These properties are (i) biocompatibility, (ii) surface property or surface chemistry, and (iii) biodegradability [1]. Biocompatibility ensures little or no tendency of unwanted tissue response due to the presence of the implanted material inside the living tissue. The surface chemistry of the material has to possess the ability to be functionalized for proper cell adhesion and functioning. The material used for regeneration of the tissue must degrade and deliquesce without producing any toxic effect on the host tissue. To be a suitable material for tissue engineering, the material has to be biodegradable. There are different types of materials that possess most or all of these properties, to some extent, to be considered for tissue engineering. This includes metal, polymer, or ceramic, or a composite of these materials [2]. Not all of these materials are suitable for all types of tissue engineering applications. Thus material selection is very much application or field-specific (subfields of tissue engineering).

Tissue Engineering for Artificial Organs: Regenerative Medicine, Smart Diagnostics and Personalized Medicine, First Edition. Edited by Anwarul Hasan.

Polymer-based materials are the preferred and most used materials for tissue engineering especially for neural tissue engineering because metal and ceramic-based materials are less suitable for nerve regeneration. The main issue, regarding these two types of materials, is the level of biodegradability required for neuroregeneration. Metal- and ceramic-based materials possess low biodegradability and machinability to fabricate the scaffolds. Both natural and synthetic polymers are extensively used for not only NTE but also in different subfields of tissue engineering. Collagen, glycosaminoglycan (GAG), and starch [3] are the widely used natural polymers for NTE. Some other natural polymers that are generally used are fibrin, agarose, alginate, and hyaluronan [4]. The advantage of natural polymers is their close simulation of the native cellular milieu, whereas the disadvantages are the large variation of their properties

Table 19.1 Natural and synthetic biomaterial for neural tissue engineering.

Source	Type	Material	Application to other tissue engineering	References
Natural	Protein	Collagen	Bone, cartilage, heart, ligament, vasculature	[6–17]
		Fibrin	Cartilage, vasculature	[18–24]
	Polysaccharide	Agarose	Cartilage, heart	[25–31]
		Alginate	Cartilage, vasculature, liver	[26, 32–39]
		Hyaluronan	Adipose, skin, cartilage, vasculature	[40–46]
		Chitosan	Bone, cartilage, skin	[33, 47–49]
Synthetic	Polymer	PLGA	Adipose, bone, cartilage, muscle	[24, 48, 50–60]
		PEG	Adipose, bone, cartilage, liver, heart	[61–72]
		PGA	Adipose, cartilage, heart, ligament, liver, vasculature	[73–86]
		PLA	Adipose, cartilage, heart. ligament, liver, skin, vasculature	[73, 80–83, 85, 87–90]
		PCL	Cartilage, heart, ligament, liver, vasculature	[83, 90–97]
		PU	Bone, cartilage, heart, liver, skin, vasculature	[84, 95, 98–103]
		POP	Heart, vasculature	[5, 104–107]
		PHBV	Bone, cartilage, ligament, liver. skin	[108–115]
	Peptide		Bone	[116–121]

Modified from [4, 5].

upon isolation from biological tissues from batch to batch, poor mechanical performance, and low machinability. Despite the ability of natural polymers to mimic the cellular milieu, these drawbacks are the reasons for developing synthetic-biomaterials. A significant advantage of synthetic polymers over natural polymers is the consistency in their properties, such as degradability, from patient to patient. Some of the commonly used synthetic biomaterials are poly(lactic acid-*co*-glycolic acid) (PLGA), poly(ethylene glycol) (PEG), and peptide-based materials. Apart from their poor mechanical properties and low machinability, natural polymers such as collagen and chitin also present difficulties during scaffold transplantation. Scaffolds produced from these materials cannot be easily melted by applying heat. They require special solvents during scaffold processing [2]. A class of synthetic polymers has been developed for overcoming this difficulty. For example, poly(α-hydroxy ester)s, polyanhydrides, polyorthoesters, and polyphosphazens are such resorbable synthetic polymers (Table 19.1).

19.2 Natural Biomaterials for Nerve Tissue Repair

The ability to impart the desired properties and the flexibility to produce a wide variety of synthetic biomaterials have resulted in various classes of such materials. Novel biomaterials are being produced for improved performance. Yet, natural biomaterials play a significant role in tissue engineering because of their inherent biocompatibility. These materials have the highest potential to be successful in NTE applications. The advancements in micromachining and the achievement of high-resolution micropatterning at the cellular and subcellular level are pushing the feasibility of using natural biomaterials to a higher level.

The adult mammalian nervous system is capable of undergoing an impressive degree of plasticity after an injury. The term *plasticity* refers to the changes taking place at the cellular and tissue level. Plasticity of the nervous system comprises the process of generation of new cells. This generation occurs from the stem cells from a few specific location of the nervous system. The dentate gyrus [122, 123] or the subependymal layers along the lateral ventricles [124, 125] are such locations that contain stem cells for new cell generation. The dentate gyrus is part of the hippocampal formation, which is a compound structure in the medial temporal lobes. Apart from the plasticity induced by the injury, the mammalian nervous system shows also non-injury-induced plasticity [122, 123, 126, 127].

The consequent damage due to an injury and its effect to the nervous system are very much event-specific and follows a spatial and temporal manner. The effects of the injury at the cellular level depend on the type and extent of the injury. In the case of a contusion injury to the spinal cord, the center of the lesion (the region suffering damage due to injury or disease) gets affected in the form of cell death at that location, Wallerian degeneration, activation of astrocytes, and breakdown of the native extra cellular matrix (ECM). Wallerian degeneration refers to the separation of the axon from the neuron cell body. During this process, the neuron can be cut or crushed depending on the nature

of the injury. Wallerian degeneration is also known as *anterograde* or *orthograde* degeneration. It occurs in injuries of both the central nervous system (CNS) and the peripheral nervous system (PNS). The degeneration starts after an axonal injury. At the stage preceding the onset of degeneration, the electrical excitability of the distal axon stump remains or tends to remain intact. During the degeneration process, disintegration of the axonal skeleton and breakdown of axonal membrane take place, and they are followed by degradation of the myelin sheath and infiltration by macrophages. Macrophages and the Schwann cells exculpate the injured region from the Wallerian degeneration debris [128]. The activation of astrocytes causes inhibition of axonal growth as it proliferates glial scar production. Astrocytes are the main components of the glial scar [129]. They build a dense web using their plasma membrane extensions, fill the cavity or empty space at the lesion caused by the loss of neuronal cells, and effect modification of the ECM. The latter takes place as a result of the secretion of laminin, fibronectin, tenscin C, and proteoglycans [130, 131]. The components of the secretion are believed to have inhibitory effects on neuroregeneration [131, 132].

In the previous section, we discussed the plasticity of the mammalian nervous system, which exhibits spontaneous growth in CNS, but neurons in CNS tissue do not regenerate to a significant extent. This inability to recover from damage results in permanent loss of function. The PNS, however, has a better ability for recovery and axonal regeneration [133], but this ability of PNS is also limited and not sufficient to restore complete functionality. The purpose of NTE is to promote the plasticity of the nervous system. The purpose of producing biomaterials for NTE is the augmentation and improvement of this limited ability of regeneration of the neural tissue.

Within the lesions of the nervous system (both CNS and PNS), there are various active processes such as tissue remodeling, cell death, astrocyte activation, glial scar formation, and more. Researchers have been using natural biomaterials to confront these processes for neuroregeneration. The following sections, we will describe some of the recent developments in natural materials for NTE. The general strategies for nervous system repair using natural biomaterials (such as drug delivery devices, cell carriers for cell replacement therapy, and as scaffold) for replacing native ECM and facilitating axonal growth will be discussed in the following sections. For this discussion, natural biomaterials have been classified into four categories: (i) intact tissue ECM or acellular, (ii) material derived from individual components of the ECM, (iii) wound-healing proteins, and (iv) others. An example of the first category is decellularized nerve tissue. The term *decellularization* refers to a process of isolating the ECM of a tissue from the cells within. This results in an ECM scaffold made of the original tissue. Collagen and hyaluronan are examples for the second category. Fibrin falls under the third category. Agarose, an example for the last category, is a non-ECM and non-native natural biomaterial [134].

19.2.1 Acellular Tissue Grafts

Each year, the number of people suffering from nerve injuries is increasing. Worldwide, no less than 2 million people fall victim to peripheral nerve injuries

(PNIs) annually. The cost associated with the treatment is quite significant, which excludes the estimated cost of paralysis of approximately $7 billion. The present standard for nervous system injury (NSI) treatment is autografting, especially for PNI. This technique is associated with high cost, possible loss of sensation at the donor site, and the requirement of two surgeries. One of the most feasible alternatives is allografts, which, however, require intensive immunosuppressive treatments for the prevention of host rejection. For this reason, significant efforts have been made to remove cellular material from allografts [135]. The decellularized tissue is one of the most preferred natural biomaterial for the purpose of building a neural tissue scaffold for neuroregeneration. This is also considered one of the optimal natural biomaterials for NTE. These decellularized tissues are produced with a top-down approach. First, a native tissue is selected, and then the undesirable cellular components are removed. This results in a completely natural material for neural tissue repair that can mimic the native milieu. These native tissues for decellularization come from either some donor or extracted from a cadaver. The decellularized ECM scaffolds are recellularized with the receptor's own cells, more specifically with potent stem or progenitor cells. Stem cells then differentiate into the neural cells, thus effecting neuroregeneration. There are different kinds of processes available for decellularizing tissues. They are (i) physical treatment, (ii) chemical treatment, and (iii) enzymatic treatment.

19.2.1.1 Chemical Decellularization

For performing chemical decellularization, a combination of reagents is chosen based on parameters such as thickness, ECM composition, and the intended use of the tissue or organ. The chemicals used to decellularize tissues include alkaline and acid compositions, ionic detergents, non-ionic detergents, zwitterionic detergents, hypotonic and hypertonic reagents, tri(*n*-butyl)phosphate, and chelating agents [136].

19.2.1.1.1 Alkaline and Acid Treatments

Alkaline and acid treatments result in the dissolution of the cytoplasmic components of the cells. They also wash away the nucleic acids (RNA and DNA). Commonly used reagents are acetic acid, hydrochloric acid, peracetic acid (PAA), sulfuric acid, and ammonium hydroxide. They are quite effective for dissolving the cytoplasmic components by disrupting cell membranes and intracellular organelles. These reagents are also quite capable of dissolving important and desired cellular components in the process. One good example is the alkaline and acid treatment of collagen. During the treatment, important components such as GAGs can also be deliquesced. GAGs are carbohydrate polymers that form proteoglycans by attaching to ECM proteins. These proteoglycans help trap and store growth factors within the ECM. A successful and efficient application of alkaline and acid treatment of thin ECM is the decellularization of porcine tissues of different types, such as urinary bladder submucosal (UBS) layer and the basement membrane plus tunica propria (UBM, Urinary bladder matrix) with poly-amic acid (PAA) (~0.10–0.15% (w/v)) [136]. PAA also disinfects the tissue, as during processing microorganisms and oxidizing microbial enzymes enter the tissue.

These components serve as disinfectants [137, 138]. Through PAA treatment of these tissues, the ECM is able to retain several native GAGs, such as chondroitin sulfate A, heparin, heparin sulfate, hyaluronic acid, and dermatan [139], as well as laminin and fibronectin [140, 141]. Moreover, PAA treatment does not destroy the functionality and structure of many growth factors. This includes the basic fibroblast growth factor, transforming growth factor-β, and vascular endothelial growth factor [142, 143]. PAA treatment also somewhat preserves the mechanical properties of the decellularized scaffold [144].

19.2.1.1.2 Non-Ionic Detergent Treatment

Compared to methods such as alkaline and acid treatment, non-ionic detergent treatments are less adverse. This is one of the reasons for the extensive use of non-ionic detergents for decellularizing tissues. Such detergents do not interrupt protein–protein interactions. They only interrupt lipid–lipid and lipid–protein interactions. For not destroying the protein–protein interaction within the tissue, these treatments [145]. For decellularization, Triton X-100 is one of the most popular and much preferred non-ionic detergents (Table 19.2). During decellularization, the required time of exposure to Triton X-100 varies over a wide range depending on the tissue to be treated, which can be from a few hours to 2 weeks [153, 155, 157, 160–163]. The success of non-ionic detergents also varies with the tissue type. For example, with regard to the ECM components, no difference in the sulfated GAG content in the anterior cruciate ligament (ACL) was found after 4 days of Triton X-100 treatment, whereas for the heart valve all the GAGs were destroyed after 24 h of Triton X-100 treatment [136]. This contrast, as shown in the example, implies that for some tissues non-ionic detergents does not provide satisfactory results, whereas for some cases they are quite successful and regarded as a preferred method of decellularization.

19.2.1.1.3 Ionic Detergent Treatments

Ionic detergents functionally differ from the non-ionic detergents with regard to their interference with protein–protein interaction. Their advantage is that they effectively dissolve the cytoplasmic and nuclear cellular membranes. Table 19.2 lists some of the widely used ionic detergents. The advantage of sodium dodecyl sulfate (SDS) over other ionic detergents is that it has better removal success with nuclear and cytoplasmic components [162]; but this comes with the disadvantage of disruption of tissue structure and loss of GAGs. Sodium deoxycholate is also quite similar in success with regard to removal cellular components but it is more aggressive and has more adverse effects on the native tissue structure. It causes more disruption of the tissue structure, and hence it is not advisable to execute decellularization with it alone. It is usually used with zwitterionic detergents to decellularize nerve tissue. Studies have shown that the most effective method for the complete removal of neuronal cellular components and complete decellularization of the nerve ECM is through a combination of zwitterionic detergents with Triton X-200 [166, 168].

Table 19.2 Decellularization methods and chaotropic agents commonly used.

Types	Method name	Mode of action	Effects on ECM	References
Physical	Snap-freezing	Intracellular ice crystals causes disruption of cell membrane	ECM can be fractured or disrupted while freezing rapidly	[146–152]
	Mechanical force	Cells burst due to applied pressure	The force acting on the ECM can cause damage	[144, 153]
	Mechanical agitation	Can cause cell lysis by itself but can expedite the removal of cellular component when used with chemical exposure.	If aggressive agitation or sonication is used for removing cellular material, it can disrupt the ECM	[144, 153–155]
Chemical	Alkaline acid	Solubilizes cytoplasmic components of cells; causes disruption to nucleic acids	Removes GAGs	[144, 156–159]
	Non-ionic detergents			
	Triton X-100	Disrupts lipid–lipid and lipid–protein interactions, while leaving protein–protein interactions intact	Result and efficiency dependent on tissue type being decellularized; removes GAGs	[153, 155, 157, 160–163]
	Ionic detergents			
	Sodium dodecyl sulfate (SDS)	Solubilizes cytoplasmic and nuclear cellular membranes; more likely to denature proteins	Cytoplasmic proteins and nuclear remnants are removed; more likely to disrupt native tissue structure; removes GAGs; causes damage to collagen	[153, 162, 164–168]
	Sodium deoxycholate		Causes more disruption compared to SDS	[153, 162, 164–168]
	Triton X-200		More efficient while used with zwitterionic detergents in terms of removing cellular contents	[153, 162, 164–168]
	Zwitterionic detergents			
	CHAPS	Exhibit properties of non-ionic and ionic detergents	Effectiveness is similar to that of Triton X-100 in terms of cell removal and ECM disruption	[155]

(Continued)

Table 19.2 (Continued)

Types	Method name	Mode of action	Effects on ECM	References
	Sulfobetaine-10 and -16 (SB-10, SB-16)		Mild disruption of ECM when used with Triton X-200	[153, 162, 164–168]
	Tri(*n*-butyl)-phosphate	Disrupts protein–protein interactions	Removes different types of cells; retains the mechanical properties with minimum alteration but causes loss of collagen	[161, 162]
	Hypotonic and hypertonic solutions	Osmotic shock causes cell lysis	Efficient for cell lysis, but not effective while removing the cellular remnants	[155, 162, 169–171]
	EDTA, EGTA	Chelating agents that bind divalent metallic ions, thereby disrupting cell adhesion to ECM	No isolated exposure, typically used with enzymatic methods (e.g., trypsin)	[172–175]
Enzymatic	Trypsin	Cleaves peptide bonds on the C-side of Arg and Lys	ECM structure can be disrupted with extended exposure; fibronectin, laminin, elastin, and GAGs are removed	[172–175]
	Endonucleases	Catalyze the hydrolysis of the interior bonds of ribonucleotide and deoxyribonucleotide chains	Difficult to remove from the tissue and could invoke an immune response	[155, 162, 164, 176]
	Exonucleases	Catalyze the hydrolysis of the terminal bonds of ribonucleotide and deoxyribonucleotide chains		

Adapted from [136].

19.2.1.1.4 Zwitterionic Detergent Treatments

Zwitterionic detergents possess the characteristics of both ionic and non-ionic detergents. Their tendency to interact with proteins is in between those of ionic and non-ionic detergents; that is they tend to denature the cellular proteins more than non-ionic detergents but less than ionic detergents. CHAPS is one of the most widely used zwitterionic detergents. It is the acronym of 3-((3-cholamidopropyl)dimethylammonio)-1-propane sulfonate. It is mostly used for decellularizing blood vessels [155], whereas the sulfobetaine-10 (SB-10) and sulfobetaine-16 (SB-16) are the long-established zwitterionic detergents for the decellularization of nerve tissues. SB-10 and SB-16 are combined with Triton X-200 for the complete decellularization of peripheral nerve tissue. This

combination is more effective in terms of functionality and less destructive compared to the combination of Triton X-200 and sodium deoxycholate [166, 168].

19.2.1.1.5 Tri (n-Butyl) Phosphate Treatments

Tri(*n*-butyl)phosphate (TBP) is a colorless and odorless organic solvent and is an organophosphorus compound. Organophosphorus compounds are organic compounds consisting of carbon–phosphorus bonds, and these compounds are degradable. They are used as chaotropic agents for the decellularization of ligaments and tendons. [136]. TBP has been studied for its toxicity on living nerve tissue [177], but it is not used for nerve tissue decellularization. Therefore, this detergent will not be discussed in detail in this chapter.

19.2.1.1.6 Hypotonic and Hypertonic Treatments

For this kind of treatment, the lysing of the cells within tissues is done by applying an osmotic shock with a hypotonic or hypertonic solution. Examples of such solutions are deionized water or low-ionic-strength solutions. Hypertonic saline is capable of dissociating DNA from proteins [178], whereas hypotonic solutions cause cell lysis by means of osmotic effect [179]. Cell lysis results in the least possible modification of ECM and its structure [180]. A general trend for this class of treatments is to immerse the target tissue alternatively in a hypotonic and a hypertonic solution for several cycles. This cyclic alteration helps to achieve the maximum amount of osmotic effect for rapid decellularization.

19.2.1.2 Physical Decellularization

Physical decellularization of tissue is carried out by applying physical processes, such as a change in temperature, applying force or pressure, electrical disruption, sonication, agitation, and so on. One example of the thermal methods is rapid freezing and thawing. In this method, the lysing of cells and plasma membrane is done by the microscopic ice crystals formed by the rapid freezing [152, 181]. Rapid freeze–thaw thermal decellularization is followed by chemical washes to remove the undesired debris. This method is preferred for application to strong, thick tissues. In case of pressure-based physical decellularization, hydrostatic pressure is applied on the tissue, resulting in the disruption of the ECM structure. This method is performed at an elevated temperature to prevent the unwanted ice crystal formation. The electrical disruption method uses an electrical pulse to create micro-pores at the plasma membrane, thus causing cell death.

19.2.1.2.1 Thermal Decellularization

The freeze–thaw method is effective for cell lysis, but for proper decellularization this must be followed by some subsequent processes. The purpose of these processes is to cleanse the remnants produced by the freeze–thaw method. These remnants consist of membranous and intracellular components. A single freeze–thaw cycle can reduce adverse immune response after reconstructive surgery on the host. An example of such immune response is leukocyte infiltration into the vascular ECM scaffolds [180]. Multiple freeze–thaw cycles are sometimes used [152, 181, 182] without causing significant protein loss from the target tissue [183].

19.2.1.2.2 Force and Pressure Decellularization

In this type of treatment to decellularize tissues, hydrostatic pressure is applied, and this method is more efficient in terms of process time. Hydrostatic-pressure-based decellularization is more effective than detergent and enzyme decellularization of blood vessels and corneal tissue [180]. A consequence of this process is the formation of ice crystals during the process [184, 185], which causes damage to the ECM structure. One way of overcoming this issue is the use of heat during the process, but this remedy comes with an inherent deleterious effect on the ECM. The use of heat, though prevents ice crystal formation, also disrupts the ECM, introducing additional entropy [186].

19.2.1.2.3 Nonthermal, Irreversible Electroporation

Electroporation, also known as electropermeabilization, is a technique used in molecular biology to increase the permeability of the cell membrane [187]. The process is performed by applying an electrical field, more specifically microsecond electrical pulses, resulting in an increase in the permeability of the cell membrane. This increase in permeability occurs as a result of the formation of micro-pores. The microsecond pulses destabilize the electrical potential across the membrane and produce micro-pores on the membrane [188, 189]. Nonthermal, irreversible electroporation (NTIRE) is a method for tissue decellularization by damaging the tissue selectively by destroying only the cell membrane and leaving other components intact. This method is limited by the size of the probe used for the process, which restricts their applicability to smaller sized tissues. So far, the NTIRE method has been used for intracranial surgical application [190], but there appear to be no studies using this method for neural tissue decellularization. Studies have been reported using NTIRE for blood vessels and vascular smooth muscle cells (VSMCs) [191–193].

19.2.1.3 Enzymatic Decellularization

The mechanism of enzymatic decellularization is the breaking down of the connection between and interaction among the nucleic acids and other cellular components. These kinds of decellularization methods use protease digestion, calcium chelating agents, and nucleases for decellularizing tissues [172, 173]. The most used enzymes include lipases, thermolysin, galactosidase, nucleases, and trypsin, among others. The use of enzymes for decellularization is strictly material-dependent. For example, trypsin is aggressive toward collagen and elastin, and dispase is more effective for thin tissues such as lungs. Table 19.2 summarizes the methods available for decellularization [136].

There are numerous successful uses of decellularized ECM or acellular tissue for nerve regeneration, such as nerve grafts created by different types of chemical decellularization [194, 195], thermal decellularization such as the freeze–thaw technique [196, 197], and the cold-preserve method [198]. ECM grafts extracted from tissues have successfully served for defect repair in PNS [168, 199–203]. For axonal regeneration, chemical processes of decellularization have demonstrated superiority [166]. As shown in Figure 19.1, chemical decellularization has had remarkable success in preserving the intricate basal lamina structure of peripheral nerves [168], and scientists consider this as an important component

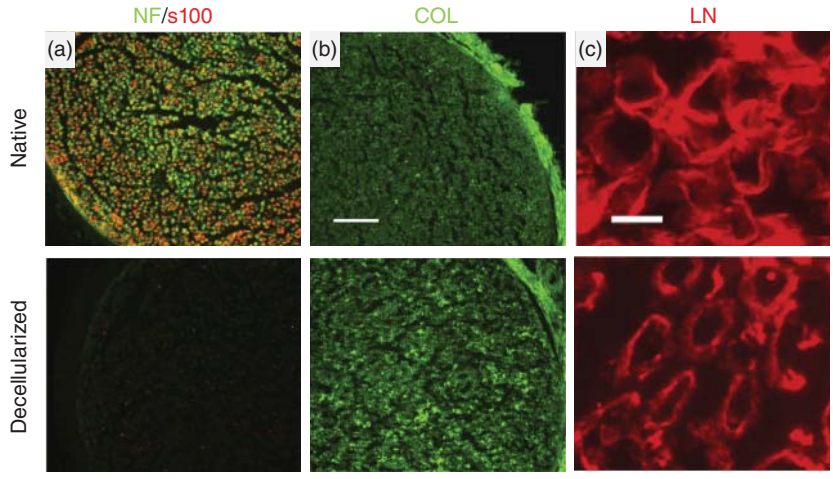

Figure 19.1 Cross sections of native and decellularized peripheral nerve grafts. Both native and decellularized nerve grafts were immunostained with (a) anti-neurofilament (NF, green), anti-100 (red), (b) anti-collagen (Col, green), and (c) anti-laminin (LN, red). The comparison between native (top) and decellularized (bottom) nerve grafts shows that, after the decellularization process, the nerve graft loses most cellular components (NF and s100). The ECM components (collagen and laminin) within the nerve remain intact after the decellularization process. Scale bar = 100 μm. (Khaing 2012 [134]. Reproduced with permission of Khaing.)

for axonal regeneration. Chemical decellularization is commercially available now and being used as a clinical tool for PNS injury repair. This technology is licensed for AxoGen® Inc. and is known as "Avance® nerve graft". There are other FDA-approved, commercially available tools for similar applications, such as "NeuraGen® grafts," which are type I collagen conduits. Studies show that acellular nerve grafts perform better than these conduits types, though the best results are obtained using isografts [204]. Isografts are tissue grafts between two genetically identical individuals such as monozygotic twins. Isografts have high success rates, as there are only rare cases of their rejection.

Fresh peripheral nerve grafts have been successfully used for the treatment of spinal cord injury (SCI) [205–208]. Researchers have established acellular peripheral nerve grafts as scaffolds for the regeneration of axons of the CNS. Recent studies have reported success of such nerve grafts, and they claim to have no inflammatory responses and provide equivalent performance for supporting axonal growth as with fresh nerve graft [134].

The major advantages of acellular nerve grafts, as described before, are as follows: (i) they consist of proper concentrations and combinations of ECM components, especially proteins, indigenous to the tissue, and (ii) they retain proper tissue microarchitecture for the host tissue. These advantages may be limited to the CNS. Defects caused by SCI and traumatic brain injury are irregular in case of humans. This can make the use of the "stiff" acellular peripheral nerve less effective. Moreover, the axonal growth in PNS is said to be sensitive to the topography [209], but for CNS it is not considered a necessity. There are clear evidences

to show that SCs transplanted into injured spinal cord are capable of orienting themselves longitudinally even though there is no additional scaffolds present [210].

19.2.2 Collagen-Based Biomaterials

Collagen-based biomaterials belong to a class of materials that have had long success in supporting both CNS and PNS axonal growth. The reason behind this is their biologically relevant features such as topography and growth factors, as well as the ease of incorporation of the cells into collagen-based materials. This high flexibility has made them one of the most reliable and successful classes of biomaterials for NTE.

Collagen-based biomaterials have been studied extensively over the years. One reason for this is the availability of various collagen sources and the ease of their extraction. Collagen also serves as an excellent cell attachment site. It is non-immunogenic after *in vivo* implantation, in general [211, 212]. These characteristics have established collagen as a highly desirable base material for tissue engineering. Collagen is found naturally in various forms (as many as 28 or more), but type I collagen is the most abundant one [213].

Peripheral nerve regeneration is one of the challenging topics in regenerative medicine, which has led to extensive studies on biomaterials for the regeneration of PNS [214–216]. There are numerous collagen-based biomaterials that have been developed for the purpose of repairing PNS injuries [217–219]. There are FDA-approved, commercially available conduits made of type-I collagen, for example, NeuraGen®, Neuroflex™, and NeuroMarix™. Type I collagen made of two nerve cuffs/wraps constitutes NeuroWrap™ and NeuroMend™ [219]. There are aligned collagen conduits that have been successfully used for axonal regeneration [212, 220, 221]. There are ongoing researches to improve collagen-based materials by controlling the pore orientation [222, 223], neurotropic factor addition [224, 225], and cell delivery [226, 227]. Collagen-based biomaterials also promote CNS axonal growth. Researchers have used conduits made of collagen filaments for a spinal cord transection model [228] and achieved remarkable success for axonal outgrowth. Even without any addition of topological features, collagen gels have been used for SCI in rats. The gels exhibited biocompatibility, axonal growth support, and occasional limited functional recovery [229]. Figure 19.2 shows a detailed schematic of collagen-based engineered neural tissue (EngNT) fabrication and the neurite outgrowth. Some are results of *in vitro* neurite outgrowth using aligned collagen gels in combination with astrocytes. Collagen-based hydrogels are also used for differentiating neural progenitor cells (NPCs) into neurons and glial cells [17]. These hydrogels also support *in vitro* neurite outgrowth of CNS neurons [231].

Electrospun collagen nanofibers are very popular for serving as an excellent model for understanding the mechanism of cell–substrate interaction in terms of the call response with surface morphology variation. Studies have shown that for NPCs, there is a significant escalation of cell proliferation on fibrous surfaces in contrast to smooth surfaces [232]. Also, from a comparison between aligned and disoriented collagen, it was found that cell proliferation is more likely to increase

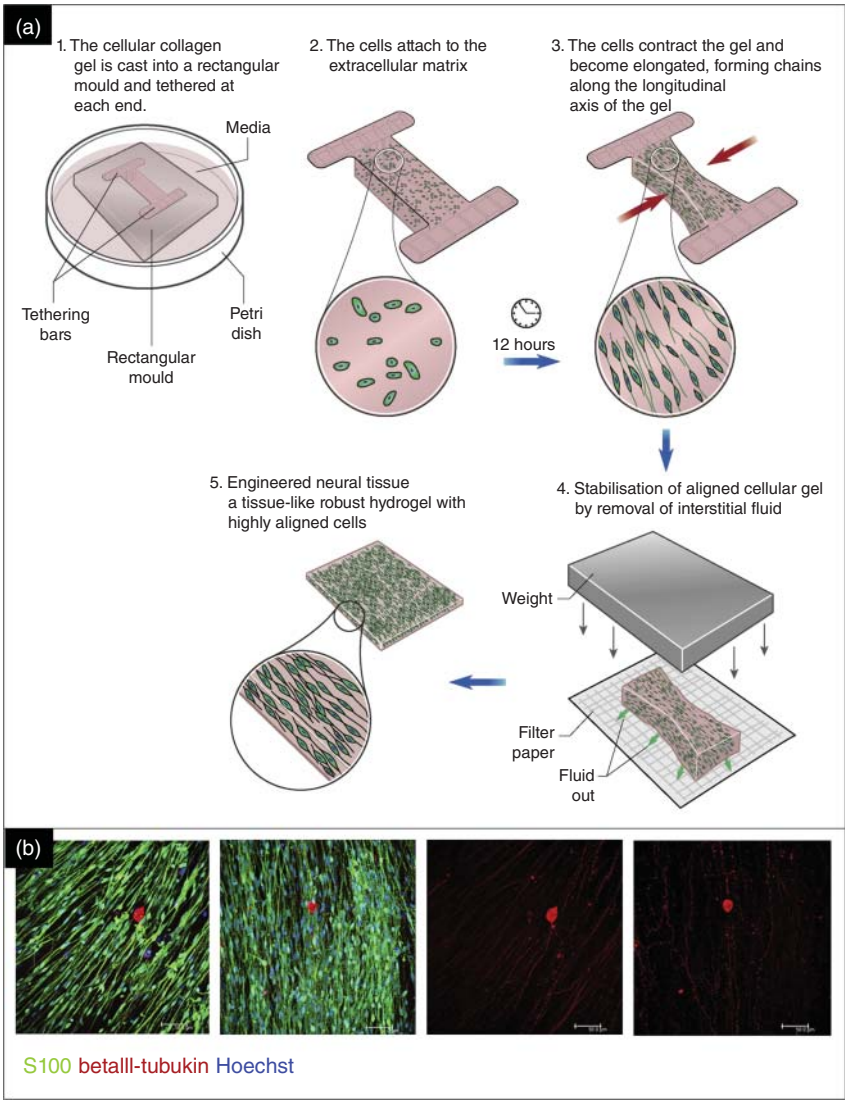

Figure 19.2 (a) Stages in the fabrication of engineered neural tissue (EngNT). Schwann cells are set within a tethered collagen gel (1), the cells attach to the matrix (2) and self-align parallel to the longitudinal axis (3), then the aligned cellular hydrogel is stabilized using plastic compression to remove interstitial fluid (4), and yield a robust engineered neural tissue (5). (b) EngNT containing live Schwann cells supports and guides the growth of neurites *in vitro*. Confocal micrographs show Schwann cells (green) supporting aligned neurite growth (red) after 3 days in co-culture (*z*-dimension 20 μm, step size 1 μm). Scale Bar = 50 μm. (Georgiou 2013 [230]. Reproduced with permission from Elsevier.)

in aligned collagen-based material. Despite their successful use as biomaterials, collagen-based materials still possess some unresolved issues while being used for CNS reparation. Collagen is more likely to interact and bind with integrin, a cell surface receptor. Therefore, the collagen-based materials are more prone to higher rates of interaction with the surrounding cells when placed *in vivo*. As described previously, there are numerous sources of collagen in the body, but the ECM of CNS has the characteristic of having a lower amount of fibrous collagen. Instead, there is a high amount of liner polysaccharides and GAGs, such as heparin sulfate, hyaluronan, and chondroitin sulfate [233]. The concern arises from the dissimilarity of the amount of fibrous collagen in the collagen-based material used for CNS reparation and the native concentration of fibrous collagen. This forces us to carefully consider the possible interaction of the implanted collagen-based biomaterial in the host tissue for the repair of CNS.

19.2.3 Hyaluronan-Based Biomaterials

Hyaluronan (HA) is another collagen-like ECM component of adult mammalian CNS and PNS. It is also commonly known as hyaluronic acid or hyaluronate. HA is commonly used as base biomaterial for nerve regeneration. Like collagen-based materials, HA is also non-immunogenic. The conserved sequence of the HA makes it more biocompatible (which is the reason for its non-immunogenic behavior), and hence it is preferred as a base biomaterial. Another reason for its wide use is the ease of modification of its properties such as stiffness and regeneration rate. The percentage of HA varies during the life-span, resulting in a fall of HA as much as 75% from fetus to adulthood [234]. HA is possibly a crucial and fundamental component of the stem cell niche in adult CNS [235], which makes HA-based materials highly convenient for fundamental studies of NPC development and its differentiation [236–241] as well as *in vitro* 3D culture systems. HA hydrogels possess another useful feature, namely optical transparency. This feature enables photo-cross-linking and other advanced fabrication technique, resulting in further enhancement of the development of *in vitro* culture systems. Some examples are the fabrication of HA scaffolds of various geometries with variable pore sizes and pore shapes and controlled generation rate [242]. It is possible to make HA-based scaffold with this wide variety by adopting freeform fabrication techniques. It is also possible to create directional gradients of molecules within the micro-fabricated nerve conduit. An example of such a conduit is shown in Figure 19.3. These kinds of freeform fabrication of scaffolds can be performed by additive manufacturing with stereolithography (SLA) [244, 245]. Such fabrications can produce highly complicated scaffolds with exceptional precision. It is one of the most studied solid freeform fabrication (SFF) methods. In this method, object or structures are built with a bottom up approach (Figure 19.4). For SLA fabrication, a 3D model is first developed (Figure 19.4a) and then sliced into 2D planar images (along the z-direction). These 2D slices are then projected via a digital micromirror device (DMD) on to a photocurable substance and photocured. Each slice is built on the previous one, thus resulting in the desired 3D structure.

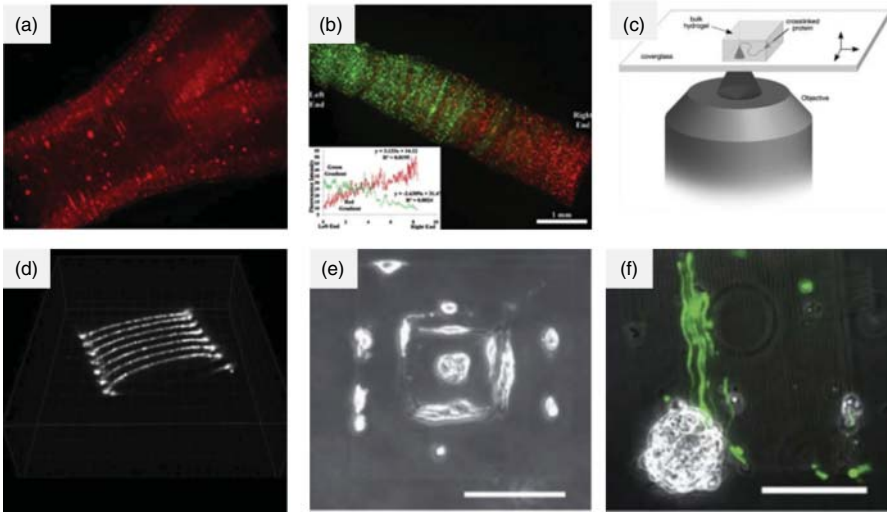

Figure 19.3 Advanced fabrication techniques using HA-based biomaterials. (a) Fluorescence micrographs of HA scaffold. The scaffold was fabricated with a digital micromirror-array device (DMD) (a freeform fabrication technique). (b) Two-gradient HA scaffold fabricated with DMD microfabrication system. The gradients are running in opposite directions (the inset plot indicates the microparticle concentration in terms of intensity profile of the fluorescence). The fluorescent microparticles (red and green) were mixed in a pre-polymer solution. Scale bar = 1 mm. (c) Schematic of multiphoton excitation (MPE) photo-cross-linking of proteins within 3D hydrogels. (d) Confocal microscopy images of a BSA helix (12 turns, each spaced 10 μm apart) inside an HA-based hydrogel. The helix was fabricated with a piezoelectric transducer (PZT) stage. (e) DRGs seeded on IKVAV-modified patterns of concentric squares on the surface of HA hydrogels. The outer square has a side length of 200 μm. (There are in total 49 squares with an increment of 2 μm). Scale bar = 100 μm. (f) Hippocampal progenitors (E16) seeded onto protein structures (fabricated on an HA hydrogel surface) which were modified with YIGSR peptides (pattern length = 200 μm and spacing = 2 μm). The figure shows an overlay of (i) a phase-contrast image before fixation and (ii) immunofluorescence of the neuronal marker b-III tubulin (green) after fixation. Scale bar = 100 μm. (Panels (a) and (b): Suri 2011 [242]. Reproduced with permission of Springer. Panels (c)–(f): Seidlits 2010 [243]. Reproduced with permission of John Wiley and Sons.)

The photo-cross-linkability of HA materials facilitates ease of SLA fabrication of complex scaffolds with precise spatiotemporal resolution. Recent advances in the lithographic process have enabled the fabrication of micrometer- sized free-standing structures at a very small level. Multiphoton lithography enables ease of fabrication with high resolution for submicrometer features while obviating the necessity of a photomask. Figure 19.4b shows the principle of the two-photon fabrication system. Another state-of-the-art freefrom fabrication method widely used in tissue engineering is femtosecond laser fabrication (Figure 19.4c). Using multiphoton lithography, free-standing protein structures inside HA hydrogels at the sub-micrometer level have been fabricated. For example, researchers have fabricated dense bovine serum albumin (BSA) guidance structures inside HA hydrogels using this technique [243]. HA-based

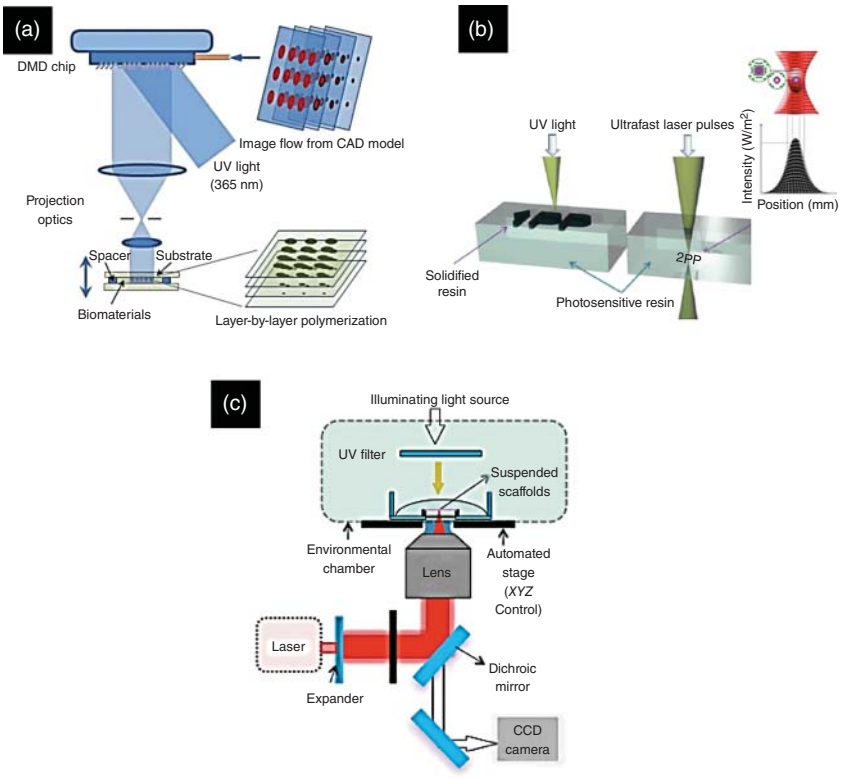

Figure 19.4 Additive manufacturing for tissue engineering. (a) Schematic of a projection printing setup called dynamic optical projection stereolithography (DOPsL). UV light illuminates the DMD mirror system, which generates an optical pattern according to the image flow from the control computer. The optical pattern is projected through an optical lens onto the photosensitive biomaterial to fabricate a 3D scaffold in a layer-by-layer manner. (b) Principle of operation for single- and two-photon polymerization. (c) Schematic of femtosecond laser fabrication set-up. (Hribar 2014 [246]. Reproduced with permission from Royal Society of Chemistry.)

materials are an excellent choice for the delivery of NPCs, as the NPCs have a tendency to bind HA [134]. There have been numerous studies investigating HA materials for NPC delivery [238, 241, 247, 248] as well as the delivery of growth factors [249], bioactive oligonucleotides [250], and other bioactive molecules [251, 252]. In general, the HA does not possess good cell adhesion property. The use of cell-adhesive peptides or other natural ECM components enables HA-based materials to support *in vitro* axonal growth [253] and to limit the inflammatory/scar response *in vivo* [254, 255].

Studies have found that the presence of HA with high molecular weight, specially preformed gels made from high molecular weight HA in the case of transected spinal cord, is linked to scar-free wound healing in fetal and early postnatal stages [256, 257]. Figure 19.5 shows the reduced glial scar response for preformed gels in transected spinal cord, which was made from HA with high molecular

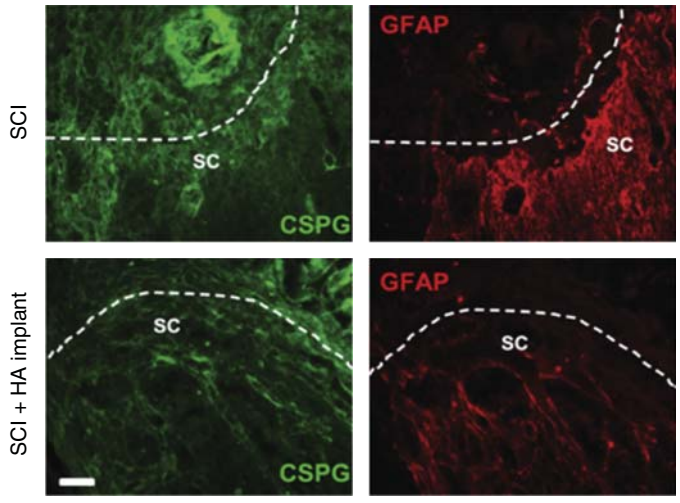

Figure 19.5 Central nervous system (CNS) repair with HA-based biomaterials. The images are of a rat spinal cord injury (SCI) where photo-cross-linked HA of high molecular weight was placed into the SCI lasion. The images were taken on day 10 (post injury) during examination of the inhibitory scar component (CSPG, green) and astroglia cell response (GFAP, red) in rats with SCI alone or with SCI and high molecular weight HA implants. For the animals with HA implant, both astrocyte response (GFAP expression) and inhibitory scar component (CSPG expression) were reduced. Scale bar = 100 μm. Dotted line represents lesion edge. sc = spinal cord. (Khaing 2011 [258]. Reproduced with permission from IOP Publishing.)

weight. The implantation of such gels results in the alteration of the extracellular environment of the lesion of the injury, which is responsible for this reduced glial scar response. The use of HA-based gels is also believed to give rise to less inflammatory response for SCI repair. The challenge in developing HA-based materials is the determination of the optimum combination of components to produce a successful biomaterial. As mentioned previously, in general, HA lacks cell adhesion attribute. This drawback of HA is overcome by combining HA with additional ECM components, and so the optimization of the component combination arises.

19.2.4 Fibrin-Based Biomaterials

Fibrin is a component of blood and is formed by the polymerization of fibrinogen caused by thrombin. Fibrin is a fibrous, nonglobular protein. It is involved in natural blood coating in the case of bleeding wounds. This fibrous protein is a long-used natural biomaterial for tissue engineering. The gelation and degradation rates of fibrin glue (or gel) can be controlled by controlling the composition of its components, namely fibrinogen and thrombin [259, 260]. The source of extraction of this widely used material has great influence on the host immune response, possible contaminants, and the degradation rate. Because of its natural function, fibrin is mostly considered for wound healing and as a sealant. But, there are applications of fibrin also as a material for scaffolds.

The use of fibrin for repairing PNIs can be classified as follows: (i) as a filler biomaterial for hollow tubes made from other materials [261], and (ii) as conduits made out of it [262–264]. Regardless of their class of application, use of fibrin as a biomaterial for peripheral nerve injury is characterized by short-length ($\leq$15 mm) nerve regeneration, that is, axonal growth [261, 263], and limited functional recovery [264]. For longer lengths (>15 mm) of axonal regeneration, use of fibrin alone is not sufficient [264], and additional components or factors (cells or growth factors) would be necessary for functional recovery.

There are experimental models of CNS repair with fibrin-based materials, and they have been under study for years. Fibrin-based material has been used for drug delivery in a SCI in rodents [265]. When used as scaffold, fibrin-based materials increase the delay in neurite sprouting and also astrocyte accumulation at the lesioned area [266]. Figure 19.6 shows an application of fibrin-based biomaterials for the repair of a CNS injury. Fibrin-based hydrogels are a decade old means for stem cell delivery, and fibrin-based systems are being used as experimental models not only for nervous system repairs [267–271] but also for repairs of bone tissue [272–274] and heart tissue [275–277].

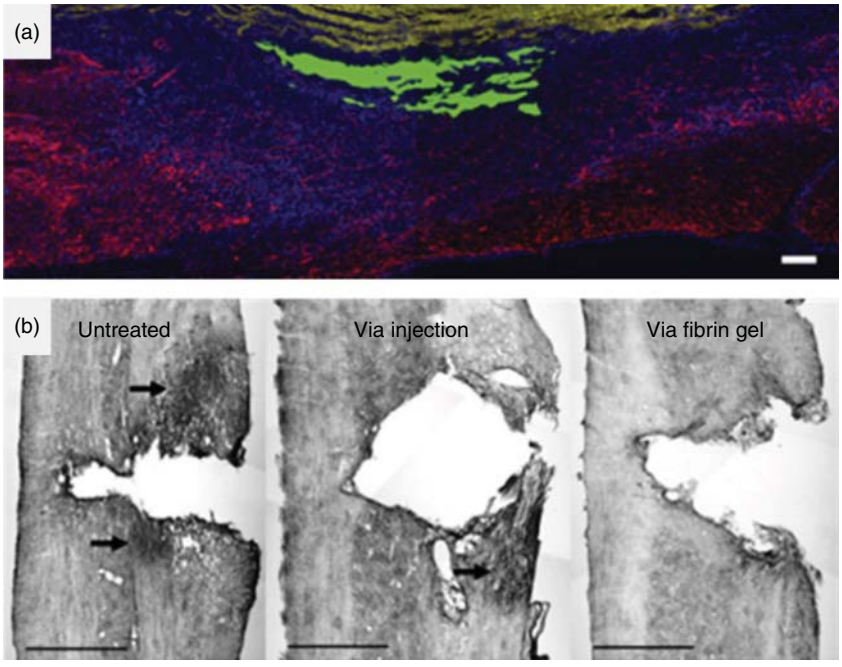

Figure 19.6 CNS repair with fibrin-based biomaterials. (a) Fibrin scaffold (shown in green) implanted a week after a dorsal hemisection lesion. The fluorescent image also shows artificial dura (yellow), neural fibers (red) and nuclei (blue). Scale bar = 200 μm. (b) Fibrin-based drug delivery system (the delivered drug was choindrotinase ABC, 250 mU). Undigested GAG chains (CS-56 immunolabeling, indicated with black arrow) serve as a proof of the effectiveness of the fibrin-based drug delivery system for CNS repair. Scale bar = 500 μm. (Khaing 2012 [134]. Reproduced with permission from Elsevier.)

The advantages of the fibrin-based materials are their versatility and their FDA approval, which make them really a good choice for developing biomaterials for neural regeneration. But, the feasibility of fibrin-based materials for CNS and PNS repair is limited by their fast degradation rate. The time required for the repair of PNS and CNS ranges from weeks to months. To enable fibrin-based material to match the required degradation profile, PEGylated fibrin gels (fibrin modified with PEG) have been used successfully [21].

19.2.4.1 Agarose-Based Materials

Agarose is a polysaccharide polymer material, generally extracted from seaweed and more specifically from agar. Agar is found in red algae. Agarose is a straight-chain polymer formed by agarobiose. Agarobiose (4-*O*-β-D-galactopyranosyl-3,6-anhydro-l-galactose) is a disaccharide composed of D-galactose and 3,6-anhydro-L-galactopyranose. Agar has been used as a food ingredient for a long time (discovered in the late 1650s or early 1660s). For scientific or biological use, agarose is widely used in gel electrophoresis. It is a commercially available product and can be found with a wide range of melting and gelling temperatures. Apart from these, agarose has many reported uses as a biomaterial for tissue engineering. Because of high biocompatibility and non-immunogenic characteristics, it is a preferred material for biological application [278, 279]. Like the HA-based materials, agarose-based materials, by nature, also lacks the ability to adhere to cells. Hence, modification is needed for functionalizing agarose-based materials to impart the cell adhesion characteristic to these materials. Such modified agarose-based materials are used as (i) anisotropic scaffolds, (ii) templated scaffolds, and (iii) patterned biomaterials. Advanced functionalization techniques are needed to enable these agarose-based materials to be able to serve the purposes mentioned earlier.

There have been efforts to modify agarose by introducing biologically active functional groups [280–282]. The addition of laminin-1 (a growth-promoting glycoprotein) in the agarose-based hydrogel can enable it to support axonal growth at PNS. Both *in vitro* [281] and *in vivo* [280] analysis of such hydrogel scaffolds with an anisotropic gradient of laminin-1 showed substantial increase in the axonal growth rate. Figure 19.7 illustrates the use of agarose-based materials for PNS repair.

There are remarkable successes in CNS repair using agarose-based materials at the experimental level. Linear pore scaffolds have been made out of agarose-based material for SCI repair using freeze-drying [283] and templating [279]. The results obtained from such scaffolds show that they can enhance axonal growth and that this enhancement can be further improved by adding growth-promoting brain-derived neurotrophic factors [284]. These linear agarose scaffolds promote the occurrence of regenerating axons exiting the lesion site and reconnecting with the host spinal cord distally to mediate functional recovery. For example, an animal model that received linear templated scaffolds, after dorsal column injury at the cervical level 3, showed a significant increase in the number of axons that reached the distal end of the lesion compared to an animal with a nonoriented scaffold [285].

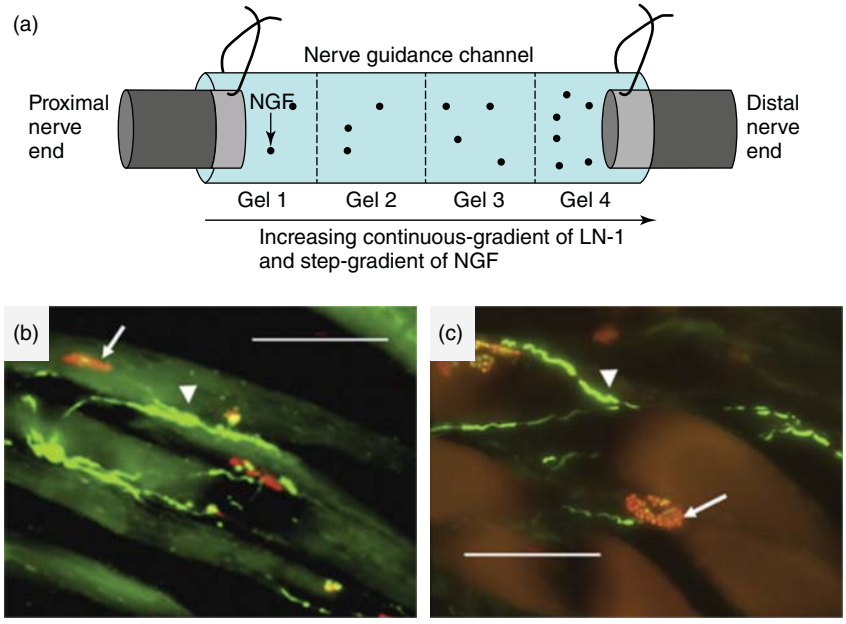

Figure 19.7 Peripheral nervous system (PNS) repair using agarose-based biomaterials. (a) Schematic of a nerve guidance channel for peripheral nerve repair. The channel consists four layers of agarose hydrogels. A step gradient of NGF and a continuous-gradient LN1 were incorporated into the gels. The following staining was used for both step-gradient scaffolds (b) and continuous-gradient scaffolds (c), acetylcholine receptors (AChR, red, indicated by arrows) synaptic vesicles (SV2, green), and neurofilament (green, arrow head). The fluorescent images show that regeneration occurred through both types of grafts after transection of the sciatic nerve in rats. Scale bar = 100 μm. (Dodla 2008 [280]. Reproduced with permission from Elsevier.)

This emphasizes the guidance feature of linear scaffolds. Figure 19.8 shows a case of CNS repair with a linear agarose scaffold. Though the results show that the well-designed scaffold could enhance the axons exiting the scaffold, it is also necessary to apply growth-promoting factors to improve this phenomenon to the desired level to achieve functional recovery. As mentioned previously, agarose is optically transparent. So, like HA-based hydrogels, advanced fabrication and patterning techniques are also used for agarose-based materials to fabricate scaffolds with complex structures. Figure 19.9 shows an advanced fabrication technique based on multiphoton chemistry. The figure shows the fabrication process of a 3D agarose hydrogel consisting of two spatially defined bioactive molecules [286]. These molecules are a transcription factor and a growth factor. A major advantage of agarose-based materials is that they are less expensive than other alternatives such as HA-based materials. Moreover, compared to the latter, agarose is not a native ECM component, thereby making it more bio-inert *in vivo*. But like HA-based materials, to develop biomaterials based on agarose, a cell adhesive factor needs to be added.

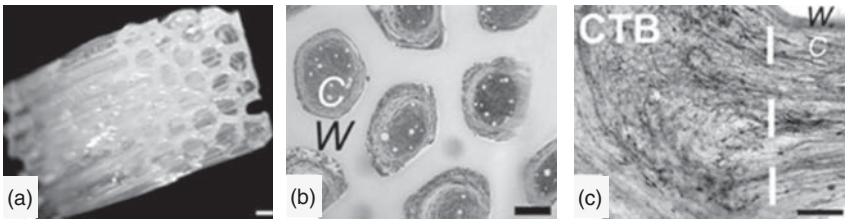

Figure 19.8 CNS repair using agarose-based biomaterials. (a) Macroscopic architecture of a scaffold fabricated from aligned agarose. The channels in the scaffold were produced by casting ultrapure agarose (30 mg ml^{-1}). Agaorse is spun into templated fiber bundles (polystyrene fiber made of multicomponent fiber bundle) following the dissolution of the fiber, which yields the channels on the scaffold. Scale bar = 200 μm. (b) Toluidine blue stain of scaffold. The stained images were taken at 4 weeks after implantation (dorsal column lesion site). "W" stands for wall of scaffold and "C" stands for channel of scaffold. Scale bar = 250 μm. (c) Axonal regeneration: cholera toxin B (CTB)-labeled regenerated sensory axons exit from the scaffold. (Figures adapted from [285].)

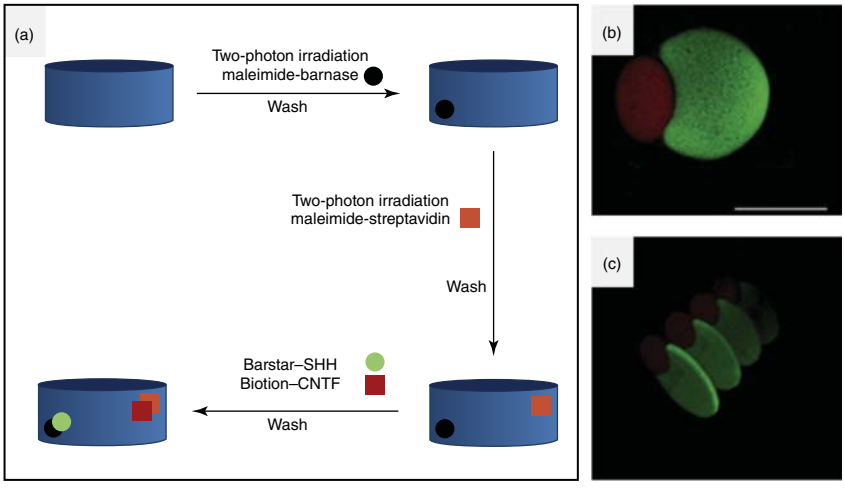

Figure 19.9 An example of use of agarose-based biomaterials in advanced fabrication techniques where two protein structures were simultaneously incorporated into agarose hydrogel using advanced microfabrication. (a) Schematic of the fabrication process. (b) A confocal micrograph demonstrating the localization of barstar-SHH (green) and CNTF (red) to the volumes patterned (for a layer at 400 μm). Scale bar = 100 μm. (c) A 3D reconstructed stack (software: Image J 3D viewer). (Khaing 2012 [134]. Reproduced with permission from Elsevier.)

19.3 Synthetic Biomaterials for Nerve Tissue Repair

In the previous section we discussed a wide range of uses of natural biomaterials and their success in regenerating nerve tissue. Despite the wide variety of natural biomaterials available, there have been significant efforts to develop synthetic materials for tissue engineering. These biomaterials provide considerable flexibility in imparting the desired mechanical properties, degradation rate, and shape to the developed material. A significant impact on stem cell differentiation can be

brought about by controlling the mechanical properties and conforming to the specifications of the transplantation site through shape control and cell growth via the degradation rate [287, 288]. This flexibility to tailor the desired properties has encouraged researchers to develop synthetic biomaterials. One of the major drawbacks of the vast majority of the synthetic biomaterials is their lack of ability to adhere to the cell.. Addition of functional groups is needed to functionalize the synthetic biomaterials for enabling them to adhere and culture stem cells. Other major concerns for synthetic biomaterials include their biocompatibility, suitability for transplantation, and immune response.

19.3.1 Polymer-Based Materials

The following sections will focus on polymers that are utilized to regenerate nerve tissue. The application of polymers in tissue regeneration is done in conjunction with stem cells. The custom-defined properties imparted to synthetic biomaterial scaffolds manifest the desired mechanical properties for stem cell differentiation, but they usually lack the cell adhesion ability and the required degree of biocompatibility. The concern for biocompatibility arises from the by-products from the degraded synthetic-polymer-based material. Some of the widely used polymer-based materials for nerve regeneration are discussed in the following sections.

19.3.1.1 Poly(Lactic-*co*-Glycolic Acid)-Based Materials

Poly(lactic-*co*-glycolic acid) or PLGA is a copolymer. Monomeric glycolic acid and monomeric lactic acid are connected through ester bonds to form PLGA. Because of its high degree of biocompatibility and biodegradability, it is one of the polymers approved by the FDA for use in therapeutic devices. The reason behind the wide use of PLGA for tissue engineering is its degradation to its monomers in the presence of cells. These degraded constituents are natural metabolites, which is an advantage of using PLGA as the scaffold material for tissue engineering. But, it also may have some disadvantages [4]. These remnants are acidic by nature, which can have a negative impact on their metabolism. PLGA scaffold is extensively used for NTE to seed stem cells in the case of nervous system repair. There have been promising and successful applications of PLGA-based scaffolds for SCI in terms of functional recovery [58]. It has also been found that human embryonic stem (ES) cells seeded within PLGA-based scaffolds can differentiate into neurons [55]. The differentiation is dependent on the appropriate clues. Based on such clues, these cells can also differentiate in to cartilage and hepatic tissues.

The use of PLGA as a biomaterial for tissue engineering poses a challenge because of its lack of cell adhesion ability. To overcome this drawback, different available techniques are used such as hydrolysis, aminolysis, blending, and covalent attachment of adhesive peptides [289]. Studies show that the mixing of poly-L-lysine (PLL) in the PLGA polymer matrix can significantly improve the latter's biological properties.

19.3.1.2 Poly(Ethylene Glycol)-Based Materials

PEG is another biomaterial that has been extensively used for tissue engineering. It is a highly preferred synthetic biomaterial because it possesses the ability

to resist protein absorption. PEG-based scaffolds are fabricated by photopolymerization. This process requires the addition of a photoinitiator. The properties of the produced scaffold are sensitive to the amount of photoinitiator used during the process. Chemical modification enables these scaffolds to accommodate bioactive molecules such as peptides and heparin [4].

PEG-based materials are very much suitable for NTE for CNS system repair, such as SCI. It has been shown that NPCs can be cultured within PEG-based scaffolds for NTE [66,290]. PEG-based materials can be functionalized using PLL, resulting in increased cell adhesion [291]. There have been studies showing the co-culture of NPCs and ECs using macroporous PEG scaffolds. The addition of ECs assisted the *in vivo* formation of a microvasculature inside the nerve tissue [64].

19.3.2 Peptide-Based Biomaterials

Peptides are naturally occurring biological molecules made up of short sequences of amino acid monomers, and can produce self-assembling scaffolds. Using naturally occurring, protein-derived motifs, peptide-based scaffolds can incorporate the functionality of protein-based scaffolds. Various peptide-based materials are able to self-assemble themselves into 3D scaffolds.

Peptide-based self-assembling scaffolds have been used for stimulating the differentiation of murine NPCs into neurons [121]. These scaffolds consist of the IKVAV (isoleucine-lysine-valine-alanine-valine) peptide sequence. This sequence has been proven to be a promoter of neurite outgrowth [292]. This study illustrates the significance of peptide sequence selection for peptide-based material to be useful for NTE.

19.4 Development of Nanofibrous Scaffolds

Nanomaterials are now one of the most, if not the most, promising materials for tissue engineering. The development and use of nanomaterials has received much attention in recent years. The most striking feature of nanomaterial-based tissue engineering is that it has overturned the traditional top-down approach and established the bottom-up approach. Nanomaterial-based scaffolds facilitate molecular design, investigations of biomaterials at the nano, micro, and meso scales, and molecular and mechanical interactions with the biological component of the scaffolds and/or with host tissues [293]. The development of such nanostructed scaffolds requires intense interdisciplinary research, combining fields such as computational chemistry, biochemistry, rheology, phage display, stem cell biology, and neurology. Figure 19.10a presents a schematic of the systematic development approach for nanostructured scaffolds. For such developments, some methodological approach should be followed. Figure 19.10b shows a generalized summary of the steps that should be adopted for effective design and development of nanostructured scaffolds. These steps comprise (i) the choice of material, (ii) design of the scaffold (iii) identification of one or more bioactive motifs, (iv) synthesis and purification of the material,

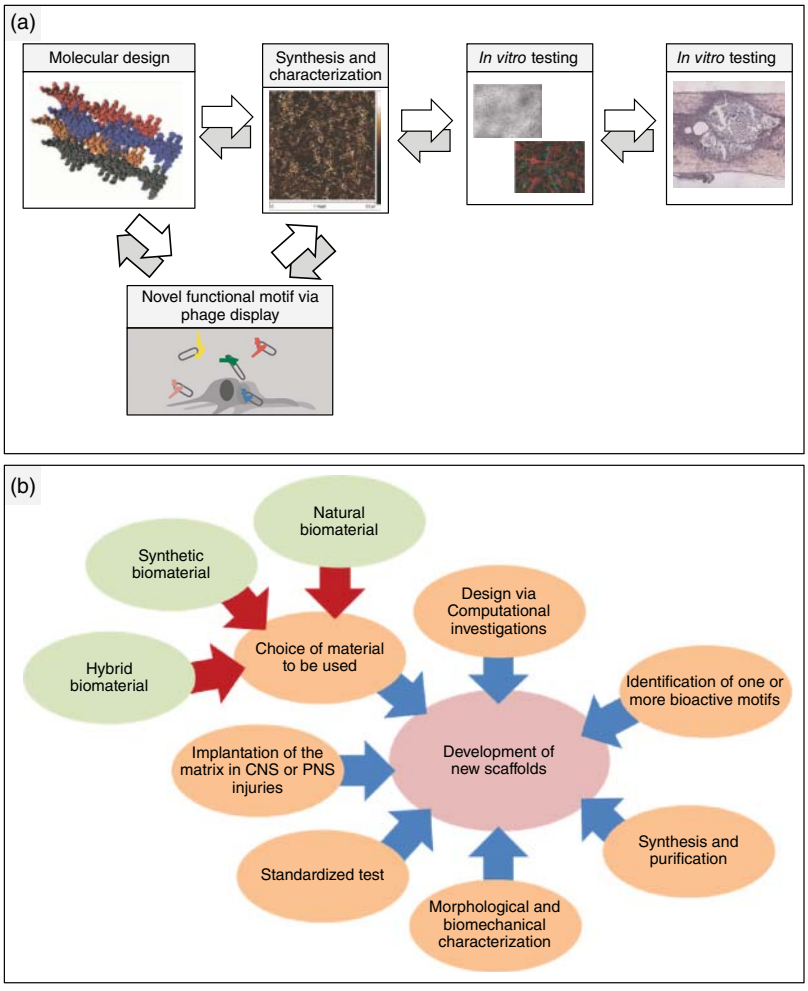

Figure 19.10 (a) Schematic illustration of the generalized approach for developing nanostructured scaffolds. The presented approach shows a continuous feedback among the different steps, which results a synergic integration of the different disciplines. (b) Schematic of the required steps for nanostructured scaffold development. (Saracino 2013 [293]. Reproduced with permission from Royal Society of Chemistry.)

(v) morphometrical and biomechanical characterization of the scaffold, (vi) standardized tests in two- and three-dimensional cell cultures or organotypic culture systems, and (vii) implantation of the matrix in animal models [293]. The choice of material is the first logical step, as the inherent properties of base material would shape the rest of the steps, to achieve the desired properties for the scaffold material. There are computational models for designing the scaffolds that would predict the required motif for the engineered scaffold. Such prediction is followed by the identification of the motifs that are required to be added. The synthesis and characterization will provide a good confirmation

to the developed computational model. The standardized test will evaluate the biological properties *in vitro* and the optimization needed for promoting cell survival and differentiation. The implantation of the designed scaffolds into an animal model will provide *in vivo* performance and feasibility study of the developed material. We will discuss some of these steps in the following sections.

19.4.1 Biodegradation of the Scaffold

For neural tissue engineering, the biomaterial should have a desirable rate of biodegradation to make space for the regenerated tissue. At the same time, it should last long to support axonal growth. The material should perform two different roles throughout its life: (i) initially, it should act as a barrier to protect the engineered microenvironment of the implant from the host environment, and (ii) it should degrade at a desired rate to support the proliferation of the transplanted cells into the host tissue [66]. The degradation must yield only nontoxic remnants [294].

When implanted in a host, the biodegradation takes place in two steps: (i) bulk degradation via hydrolysis, and (ii) proteolytic degradation by enzymes. The infiltration of water and free radicals and the secretion of esterases by immune cells regulate the surface erosion of a hydrogel, initially. With time, the modulus of the hydrogel decreases and glial and immune cells infiltrate the scaffold. These factors promote the proteolytic degradation of the hydrogel core [295].

There are various methods available for controlling the degradation of the natural biomaterial used for scaffold in neural tissue engineering, such as acetylation [296, 297], increasing the oxidation degree [298], and tuning the molecular weight distribution (MWD) of oxidized alginates [299]. Changing the range of acetylation can increase the degradation rate of chitosan. But at high degradation rates, the scaffolds lose their stiffness, compromising their structural integrity. Controlling of the degree of oxidation, the degradation rate, and the elastic modulus can be done independently. But a higher rate of oxidation results in the loss of scaffold stiffness. The tuning of molecular weight distribution (MWD) of oxidized alginates enables the modulation of the degradation rate of gels and limiting the changes in elastic modulus and swelling ratio [299].

For controlling the biodegradability and biocompatibility of synthetic biomaterials, there are several methods available. Such materials are designed in a way that provides the desired level of structural integrity, biocompatibility, and biodegradability. For example, the degradability and biocompatibility of PEG hydrogels are dependent on the length of hydrolytically degradable lactic acid units within the polymer cross-link, and thus their degradability and biocompatibility can easily be regulated by controlling the length of lactic acid units. The cellular response and interaction with the scaffold also change with the variation of the length of this hydrolytically degradable lactic acid [299]. Enzymatic-cleavage-sensitive oligopeptides can affect the degradation rate, but these oligopeptides do not cause any alteration to the mechanical properties of the scaffold material. These hydrogels can be degraded by targeted proteases

such as collagenases and plasmin [293]. For example, RGD- and MMP-2-specific cleavable substrates were incorporated into SAPs (RADA16) [300] and PA [301] in genetically engineered protein polymers. The result of this was an increase in the degradation rate.

19.4.2 Computational Modeling of Self-assembling Nanomaterials

Self-assembly is a process of spontaneous orientation or organization of a pre-existing disordered system. Such phenomena are pervasive in nature and omnipresent at all scales. Self-assembly is the consequence of local interactions among the components of the pre-existing system and occurs in the absence of any external influence. We see this phenomenon in a variety of highly performing and functional materials such as collagen, cellular organelles, bones, and teeth. Researchers have been encouraged by this natural phenomenon to develop self-assembling systems: for example, engineered materials based on bi and triblock copolymers and complex phospholipids [302], DNA [303], protein, and peptide-based materials [293].

Computational modeling and simulation have become increasingly useful for the design and development of systems and understanding the underlying mechanism of physical phenomena. The evolution of modern computing has provided us with high-performance computers. With the help of parallelization, this high-speed computing enables us to perform multiscale modeling, which is necessary for the computational investigation of self-assembling nanomaterials. Computational modeling also helps us to implement and improve the existing theoretical models available for self-assembly and to simulate this self-assembling process not only at the building block level but also their hierarchical organization [293].

Figure 19.11 shows a schematic of the aggregation mechanism of self-assembly. The figure depicts the three stages of the polypeptide aggregation pathway: (i) lag phase, (ii) growth phase, and (iii) condensation phase. The lag phase is the stage at which nucleation occurs. Nucleation is followed by oligomer formation and relaxation into stabilized nuclei. Relaxation occurs after the newly formed oligomer reaches a critical size. In the growth phase, the stabilized nuclei grow and elongate, resulting in formation of protofibrils and fibrils. In the condensation stage, the formation of organized fibers and scaffolds takes place. There may also be formation of amorphous aggregates along with the fibers and scaffolds. Figure 19.11 also refers to the computational models available to simulate the different stages of this aggregation. Table 19.3 summarizes some available techniques for sampling algorithms and structural coarsening models. These models are widely used for computer simulation of the self-assembly of peptides.

19.4.3 Characterization

The knowledge gathered from the literature and from computational modeling can provide us with the necessary information to synthesize the biomaterials and scaffolds. These biomaterials can then be used for *in vitro* and *in vivo* testing. But prior to *in vitro* and *in vivo* testing, proper characterization of the newly developed material and the study of its morphometric and biomechanical properties

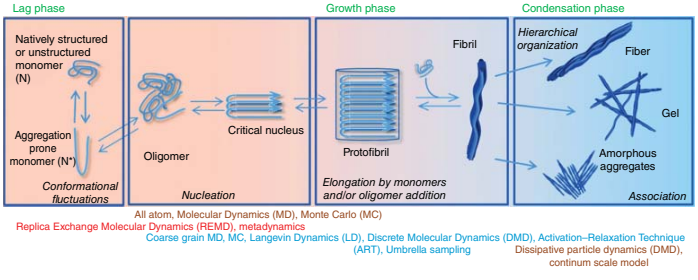

Figure 19.11 Schematic illustration of the self-assembly mechanism. There are various computational techniques that are suitable for investigating different stages. These techniques are shown in the figure just below their applicable stages. (Saracino 2013 [293]. Reproduced with permission from Royal Society of Chemistry.)

Table 19.3 List of sampling algorithm and structural coarsening models.

Sampling algorithm		
Name	**Structural unit**	**Description**
Monte Carlo (MC)		Monte Carlo simulation is a nondeterministic simulation in which properties are determined from a sample population (generated by random numbers). System conformations are generated randomly by the movements of the structural units, one at a time, which follows the Metropolis algorithm to generate conformation according to the probability.
Molecular dynamics (MD)		Atomic or molecular trajectories are determined by Newton's equations of motion. A potential function (force field/interatomic potential) is essential to describe the particle interaction. The system evolves with time.
Replica exchange molecular dynamics (REMD) and replica exchange Monte Carlo (REMC)		A number of copies of a system are run at different temperatures with random initialization. The configurations at different temperatures are exchanged based on the Metropolis criterion. It is also known as parallel tempering.
Metadynamics		The simulation is driven by free energy. It is an artificial dynamics-based technique and these dynamics are defined by the number of collective variables (CVs). These simulations are biased by a history-dependent potential along the trajectories of the CVs. It is usually applied within molecular dynamics.
Discrete molecular dynamics (DMD)		The simulation uses discontinuous step-function potentials instead of a continuous potential. The simulation technique solves the ballistic equation for collision instead of the Newton's equation of motion (which is the case for traditional MD).
Langevin dynamics (LD)		Langevin dynamics extends the MD to allow friction, high-velocity collision, and jostling of solvent to mimic the viscous aspect of solvent.
Umbrella sampling and steered molecular dynamics (SMD)		Molecular dynamics in which bias potentials are introduced to drive the system from a thermodynamic state to another one along a reaction coordinate
Activation–relaxation technique (ART)		ART samples the energy landscape of a system to find its global energy minimum. It overcomes the issue of short timescale for simulation of MD. The configurational space becomes a network of local energy minimum (or equilibrium). An event in ART is described as a movement from one equilibrium to another following two step: an activation step and a relaxation step.

(Continued)

Table 19.3 (Continued)

Sampling algorithm Name	Structural unit	Description
Structural coarsening models		
All-atom	Atom	Each atom is explicitly considered.
Caflisch model	Backbone atoms and side chains	Four spherical backbone beads, four spherical hydrophobic side chain beads and two spherical hydrophilic side chain beads represents the monomer model of peptide [304].
Shea model	Sub-residue interaction centers	Two interaction centers per residue along backbone and one for side chains [305].
PRIME model	Backbone atoms and side chains	It is an off-lattice, mid-resolution, unbiased protein model with three backbone spheres and one sphere for the side chain [306].
OPEP force field	Back bone atoms and side chains	One bead for each backbone atom and a specific bead for all side chains.
Stedall model	Monomer	The peptide sequences are considered as a rigid rod of unit length. Each half peptide is defined by a linear array of n interaction sites, resulting $2n$ interaction sites with even distribution along the length [307].
Tridimensional lattice	Monomer	Each peptide chain comprises a number of beads (hydrophobic, hydrophilic, or neutral) and these beads are confined to the vertices of a cube [308].
Amphiphilic model	Monomer	Hydrophobic, peptide, and epitope; these three kinds of monomers are considered to connect the coarse-grained model with the atomistic model [309].
Tube model	Polypeptide chain	The representation of polypeptide chain is done by a tube. This model is based on the intrinsic symmetry of polypeptide chains, which allows the free energy landscapes to be obtained within a unified framework (for folding and aggregation) [310].
Cuboid model	One or more peptides	Monomers are expressed as cuboid.
DPD	Group of atoms or volume of fluid	Polymer chains are modeled by beads composed of groups of atoms

Adapted from [293].

are necessary. These characterizations are crucial, as the results from these characterizations validate the molecular modeling and the performance predictions, made based on the modeling.

There are various characterization techniques available, such as chromatographic analysis, mass spectrometry, bonding force analysis, morphological

analysis, and viscoelastic characterization. Chromatographic and mass spectrometry analyses determine the synthesis quality as well as the material purity. Mass spectrometry is performed after chromatographic analysis. For morphological analysis, there are various microscopy techniques available. Figure 19.12 summarizes an ideal scheme for the characterization of scaffolds used in neural tissue engineering.

X-ray diffraction (XDR) can detect the secondary structures and also determine the interatomic distances of the developed biomaterial (Figure 19.12a). These XDR patterns are radially oriented. The diffraction pattern and the radial integrated diffracted intensity plot are used to verify the presence of secondary structures. Using XDR, it is also possible to detect the van der Waals distances of packed amino acid side chains and aromatic–aromatic (π–π) interactions. This distance is a significant factor for the self-assembly of peptides and proteins. Another method, which is also a preferred one, for characterizing the secondary structure of the peptides, is Fourier transform infrared spectroscopy (FTIR). FTIR is less light sensitive than the circular dichroism (CD) and enables the detection of the presence of tertiary and quaternary structures of proteins [312, 313].

For measuring the shape and dimensions of nanofibers, atomic force microscopy (AFM), scanning electron microscopy (SEM), and transmission electron microscopy (TEM) are most widely used. AFM is less preferred for jellified hydrogels and electrospun scaffolds because of its size limit for scanning. There are other disadvantages of AFM for topographic analysis, such as its probe dependence [314]. Apart from these, AFM is a very powerful imaging technique and is extensively used for analyzing static dry samples and dynamic processes in liquid buffers [315]. The AFM tips are chemically modified to detect the molecular targets and to interact with them, as suggested by its name "atomic force microscopy". But these tips are not limited to detecting the targeted molecule only. They can also determine biomechanical properties such as the elastic modulus of the nanofibers [316]. The other microscopy methods (SEM and TEM) have higher resolution than AFM, and between TEM and SEM, TEM provides higher resolution, which, of course, is sample dependent [293]. Again, compared to TEM, SEM has a larger depth of view, so it can analyze larger amounts of the sample. SEM is also a useful tool for measuring surface porosity, which is a very important factor, as structural integrity as well as mechanical properties such as resistance to the applied strain, the quasi-static modulus, and stress are inversely related to the amount of porosity [317]. Mechanical properties are usually determined by the tensile test, but for electrospun scaffolds, traction tests are performed. Along with surface porosity testing, swelling test is also very important because it provides a quantification of real porosity. The viscoelastic behavior is determined by a rheometer. Among all the different types of rheometers, the laminar flow rheometer is the most preferred one for soft materials. Rheometric analysis provides us with the material properties at the meso scale and characterizes the viscoelastic response of the developed material. It also provides information about the shear storage modulus (G'), shear loss modulus (G''), and loss factor ($\tan\delta$) (Figure 19.12e). The storage modulus (G') is the energy stored during sear deformation and the loss modulus (G'') is the dissipation of the energy, which are analogous to solid-like and liquid-like

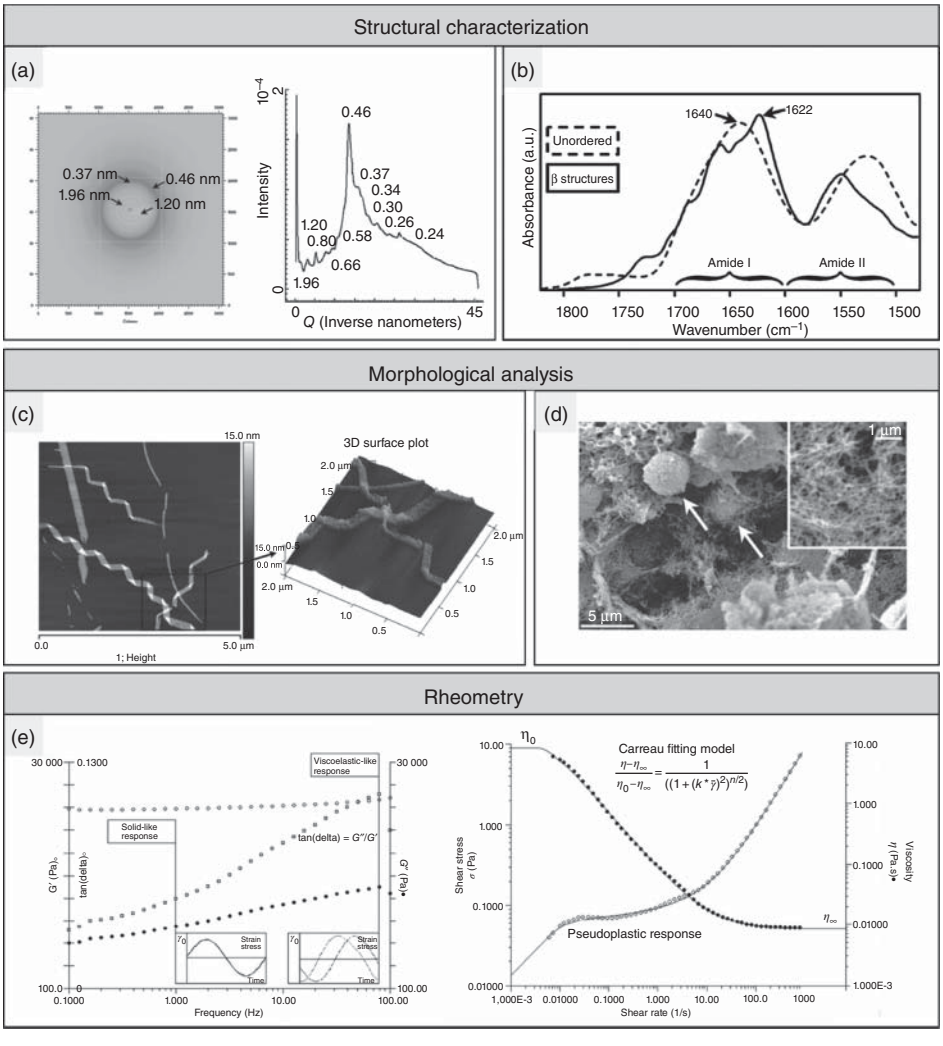

Figure 19.12 Characterization process of the scaffolds used in neural tissue engineering. Structural characterization of nanomaterials. (a) X-ray diffraction pattern with a typical β-sheet main ring at 4.6 Å distance and its radial integrated diffraction pattern distance. (b) FT-IR spectra in the region of amide I. This is used for monitoring the assembling kinetics and the presence of recurring β-structures [311]. (c) Morphological analysis of nanostructured scaffolds: 2D AFM image (left) and 3D zoom plot (right, enlarged box) of twisted ribbons of BMHP1-derived SAP. (d) Scanned electron microscopy image of neural stem cells (showed with arrows) embedded in a 3D self-assembled scaffold. Inset: high-magnified field of the nanostructured scaffold. (e) Rheological characterization of a hydrogel scaffold (left): oscillatory frequency sweep test of an assembled hydrogel, shear storage modulus (G'), shear loss modulus (G'') and the loss tangent (tan δ) are represented. Insets: (left) In-phase solid-like response at low frequency and out-of-phase viscoelastic-like responses at high frequency of the tested scaffold. Graphical correlation of viscosity and shear stress as a function of the shear rate. (right) Mathematical model for extrapolating of viscosity at zero shear rate (η^0) and viscosity at infinite shear rate (η^∞). (Saracino 2013 [293]. Reproduced with permission.)

behavior, respectively. The loss factor (tan δ) is the ratio of the loss modulus (G'') to the storage modulus (G'). So, if the loss factor is greater than 1, the material behaves like a viscous liquid and if it is less than 1, it refers to an elastic solid. In the case of hydrogels, the magnitude of the loss factor is less than 1 [318]. Rheological parameters such as shear thinning and stiffness are important parameters for material design for neural tissue engineering. Stiffness is necessary for supporting neuronal regeneration, and shear thinning is important for easier delivery via injection and quick recovery after transplantation [319]. Another characterization tool for nanostructured scaffolds is thermogravimetric analysis (TGA). This is a characterization process that measures the shift of the physical and chemical properties with the change of temperature: more specifically, with increase of temperature. This characterization method is used for determining the thermal stability of synthetic biomaterials such as hydrogels [320–323].

19.5 Summary and Future Direction

In this chapter, we presented a broad picture of the widely used natural biomaterials and some of the commonly used synthetic biomaterials. We also provided a comprehensive overview of the applications of these materials for CNS and PNS repair, as well as a summary of a generalized methodology to develop nanostructured scaffolds; and elaborated some of the steps to provide an insight to the readers. The ongoing developments in micro-fabrication, and micro-patterning techniques will open new possibilities for both the natural and synthetic biomaterials in neural tissue engineering. The current stage of neural tissue engineering demands the development of smart materials that are capable of assimilating the features of the native tissue and can provide better mechanical stiffness for neural outgrowth with improved cell attachment sites for enhanced repair of the neural tissue. Tissue engineering is a highly interdisciplinary research field. The design and engineering of biomaterials and scaffolds requires expertise ranging from materials science to computational analysis. Such as, the selection of motifs for synthetic biomaterials requires the knowledge of biology and materials science. Newly emerging fields such as "materiomics" will enable us to investigate material structures, properties, and function at multiple scales ranging from nano to macro. The development of the tools for materiomics, such as molecular dynamics and continuum methods, and various multiscale experimental and characterization tools and techniques will enable us to synthesize more complex biomimicking materials and scaffolds, which would result in enhanced regeneration of neural tissues with higher degree of functional recovery.

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20

Neural-Tissue Engineering Interventions for Traumatic Brain Injury

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20.1 Introduction

Neuro-tissue engineering, a continuously growing subfield of tissue engineering, combines two disciplines, namely engineering and life sciences, to develop biological substitutes that ideally aim to replace, regenerate, and repair damaged tissues, as well as to integrate them with the host tissue in an attempt to imitate nature [1]. Because its potential application in neuro-therapeutics and translation to rehabilitation efforts, neuro-tissue engineering research in the field of neurotrauma is urgently needed. This field addresses the inability of the central nervous system (CNS) tissue to regenerate after an injury [2]. In addition, neuro-tissue engineering not only holds promise in clinical settings such as traumatic brain injury (TBI) but also in scientific settings where biological substitutes may act as *in vitro* real tissue-like test systems. This can greatly expand our knowledge on the effect of drugs or stressed pathological conditions on neural cells and theirs microenvironment.

TBI occurs when a sufficient force is transferred to the brain, such as a blow to the head as in a car accident, a fall, or even a bullet wound accident causing an injury to the CNS tissue. Upon injury, the CNS and peripheral nervous system (PNS) react differently. PNS has a higher propensity to regenerate compared to the CNS; this fact has different implications for future neurotherapy and rehabilitation [3].

Around 1.7 million individuals sustain traumatic brain injuries each year in the United States. This results in death and significant socioeconomic and psychological burden, along with long-term cognitive and physical disabilities [4]. The rates of TBI highly affect different age groups, children (up to 4 years) and adults, among them young adults (15–24 years) and the elderly (>65 years). These injuries consist of mostly falls, motor vehicle accidents, violence, and military combat. According to the Center for Disease Control and Prevention (CDC), TBI-related hospitalization and emergency department (ED) visit rates increased by 11% and 70%, respectively, from 2001 to 2010 because of increased in TBI cases [5].

Of interest, it has been shown that TBI-related death rates have decreased by 7%, which may be attributed to the increasing preventive measures [6]. TBI can

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be classified as mild, moderate, or severe using the Glasgow Coma Scale (GCS), which is a neurological assessment method involving the assessment of the eye, verbal, and motor responses providing a total GCS score ranging from 3 to 15 [6]. A GCS score that falls in range 13–15, 9–12, or 3–8 is classified as mild, moderate, and severe, respectively. While mild insults may result in temporary disruption in mental status, severe insults result in permanent motor and cognitive impairments [7].

The pathophysiology of TBI can be divided into two interrelated subcategories: primary and secondary injuries. Primary injury includes neural cell death, which is an immediate consequence of the insult itself and cannot be therapeutically treated. On the other hand, the secondary damage is not caused by the traumatic insult *per se*, but is associated with the secondary pathophysiological cascades initiated by the primary injury. These mechanisms are deemed the principal target for potential interventions.

Primary brain injury takes place at the time of trauma in the form of direct impact, penetrating injury, or blast waves that exert direct mechanical forces which are transmitted to intracranial contents [7]. These insults result in significant different forms of damage manifested as extra- and intra-parenchymal hematomas or focal contusions in the outermost layer consisting of the cranial vault and superficial cerebral layers of the gray matter composed of neuronal cell bodies [7]. The shear forces further reach deep into the white matter, resulting in diffuse axonal injury, often associated with poor neurological outcomes [7, 8].

Primary brain injury acts as the initiating factor, triggering a cascade of molecular and systemic events that make up the latent second brain injury. This phase is an ongoing dynamic phase consisting of metabolic and electrolyte imbalances, generation of reactive oxygen species (ROS), neurotransmitter-mediated excitotoxicity, apoptosis, inflammatory responses, and secondary ischemia [7, 8]. Recent evidence suggests that inflammation plays a dual role in traumatic injuries. While it acutely promotes cell death and causes a break in the blood–brain barrier (BBB) and spinal cord barriers, it also induces – at chronic time points – the synthesis of neurotropic factors and proliferation of oligodendrocyte precursor cells to aid in re-myelination [9]. Lee *et al*. showed that attenuating the leukocyte recruitment to the site of injury, using ghrelin treatment, correlated with a better histological outcome [10].

The response to TBI is a balance between reparative processes and impediments to regeneration, with the balance tilting toward the latter. Some studies implicate endogenous reparative matrix proteins in TBI, including fibronectin and laminin; however, more research is needed to assess their impact [11]. Alleviating impediments to axonal regeneration, on the other hand, have shown more promise [12]. In general, the CNS contains different population of neural cells including neurons and glial cells (non-neuronal cells). In particular, there are four types of glial cells surrounding the neurons, namely astrocytes, oligodendrocytes, microglia, and ependymal cells [13]. Astrocytes act as the supporting, detoxifying, and reacting cells that play a role in inflammation, induction of cells, exchange of electrolytes, control of extracellular pH, and uptake of metabolites and neurotransmitters. Oligodendrocytes are responsible for myelin deposition in the CNS. Microglial cells are specialized macrophages, responding to injury,

and ependymal cells are cerebrospinal fluid (CSF)-producing cells and contribute to the formation of the blood–CSF barrier [14].

Following TBI, the microglial cells are the first glial cells to be activated at the site of injury and defend against pathogens and neurotoxins. The inflammatory mediators of microglia further activate astrocytes, which rapidly proliferate, contributing to the formation of a reactive glial scar, which has a dual role. First, it has been shown to wall off the injury, and, second, it repairs the BBB and maintains the chemical and physical integrity of the CNS. Molecules such as myelin inhibitors that hinder axonal regeneration and prevent the recruitment of neural stem cell progenitors are also produced [15]. The glial scar or gliosis is unique to the CNS and consists predominately of reactive astrocytes and proteoglycans, which are heavily glycosylated proteins. Several studies have shown that enzymatic degradation of chondroitin sulfate proteoglycans at the injury site enhances regeneration of neurons and recovery of function *in vivo* [2, 16]. Therefore, the success of neural regeneration strategies is partly a function of controlling glial scar formation and diminishing the effect of secreted inhibitory molecules.

In light of the pathophysiology and consequences of TBI, this chapter aims to review different current neuro-tissue engineering approaches that focus on minimizing tissue degeneration and promoting tissue regeneration. First, in order to fully understand cell-based therapies, we examine the process of neurogenesis, which covers the relationship between developmental and neural stem cell biology as well as the natural endogenous potential of neural repair by resident neural stem cells. After laying down the foundation of neural repair, we present the most recent therapeutic advances in line with the four categories (cell-based therapies, biomaterials-based therapies, application to living system, and transition to the clinic), as discussed previously.

Cell-based therapies in the third section look into the use of different cell types, ranging from adult to embryonic cell sources, to obtain the optimal cellular milieu for regeneration process. The milieu is further enhanced using biological and synthetic scaffold materials that can be used as a delivery material for drugs and cells to replace lost endogenous cells, allowing for a suitable microenvironment for cell survival, tissue regeneration, and host tissue integration. By combining the principles of cell- and biomaterial-based therapies, a 3D biological construct becomes more within reach.

20.2 Neurogenesis in CNS: Resident Neural Stem Cells

The complex and close relationship between developmental and neural stem cell biology necessitates further discussion. Typically, primary neural stem cells, known as neuroepithelial cells, reside in the wall of the neural tube, which results from the fusion of lateral folds of the neural plate. These cells collectively make the neuroepithelium, which essentially gives rise to other cell types of the brain [16]. Currently, there is an ongoing debate investigating the lineage relationship between neurons and glia and questioning the developmental origin of neural stem cells. For some time, it was assumed that neurons and glia arise

from two different pools. However, several studies have showed that radial glia might divide to produce cortical neurons with their known role in guiding neural migration and producing cortical astrocytes [17–19]. Several studies using immunohistochemical, anatomic, and electrophysiological techniques have shown that the majority of the neural stem cells in the neurogenic region have morphological characteristics strikingly similar to those present in radial glial cells. Indeed, with the help of retroviral vector techniques, it was shown that precursor cells expressed markers of radial glial cells, reinforcing the theory that radial glial cells give rise to neural stem cells in the neurogenic areas [18, 19].

For the most part of the twentieth century, the dogma that new neurons in the adult mammalian brain cannot be replaced after their death was accepted by the scientific community [20]. With an increasing number of studies, the dogma gradually subsided and the concept grew that new neurons can be generated in the adult mammalian brain (reviewed in [20]). Today, neurogenesis is known to be functional and persistent even in the absence of trauma in two neurogenic areas, namely the subventricular zone (SVZ)/olfactory bulb and the dentate gyrus (DG) of the hippocampus. The SVZ, situated near the ependyma, lining the ventricles of the forebrain, is made up of four different layers embodying ependymal cells, cell bodies, and branches of astrocytes, oligodendrocytes, and myelin [20]. It is known that new neurons generated in this region migrate in a rostral stream to the olfactory bulb, wherein they differentiate into olfactory interneurons [21].

As for the DG of the hippocampus, it is composed of three major layers: the granular cell layer, the acellular molecular layer, and the polymorphic cell layer (hilus). The neural precursor cells exist in the subgranular layer, which is the interface between the hilus and the granular layer [13]. They migrate laterally to the granular cell layer to differentiate into granule neurons [21]. Other studies have reported finding neural stem-like cells or transient neurogenesis in non-neurogenic regions like amygdala or the spinal cord, which normally do not generate neurons (reviewed in [22]).

Overall, the resident neural stem cells discussed above can migrate to the injury lesion in an attempt to repair the injured tissue, but it is not robust enough to fully neurorepair the damage. Hence, to achieve a better therapeutic result, it is fundamental to build a permissive microenvironment that will guide both endogenous and exogenous neural stem cells to differentiate into functional neural cells. This brings about the need and application of neuro-tissue injuring concepts that can help in blocking the process of injury-induced scar formation while activating neural regeneration of normal functional and structural neural tissues.

20.3 Cell-Based and Neuroprotection Therapeutic Strategies

This section aims to evaluate the neuroregeneration strategies at a cellular level involving cellular replacement, neurotropic factor delivery, blockage of growth inhibitory proteins, and manipulation of intracellular signaling. The starting

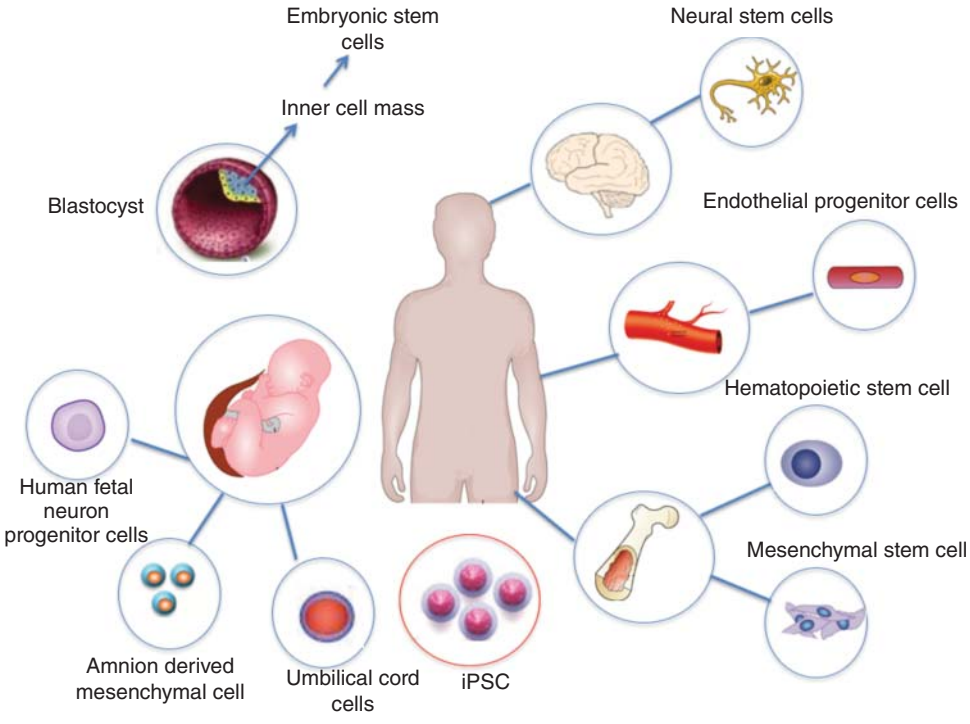

Figure 20.1 Different cell sources used: (i) Adult cells including resident neural stem cells, endothelial progenitor cells from peripheral blood, and bone-marrow-derived stem cells, (ii) embryonic stem cells derived from inner cell mass of blastocyst, (iii) umbilical cord and amniotic-sac-derived cells, (iv) human fetal neuron progenitor cells, (v) iPSC, a hybrid between adult and embryonic stem cell.

point for any attempt to engineer a neural tissue or organ substitute is the consideration that the cells can be employed to replace and compensate for the inherent tissue lost. One aspect of regenerative medical strategies involves the implantation of cells, whether endogenous or otherwise [23]. There are generally two important elements to be considered when choosing the most suitable cell types for neural regeneration: (i) the degree of immune tolerance, and (ii) whether they are available in large quantities. In particular, the latter point is critical for the translation to the clinical setting. Figure 20.1 represents an overview of the different types of cells used from different sources for neuroregeneration.

Donor neural cells can be derived from two source pools: adult and embryonic cell sources. Adult stem cells include the resident neuronal stem cells (neural stem/progenitor cells: NSPCs), and bone-marrow-derived adult stem cells include both hematopoietic and mesenchymal stem cells (MSCs) [24]. As for the embryonic stem cells (ESCs), which arise from the inner cell mass of the blastocysts, they can develop into three embryonic germ layers (i.e., mesoderm, endoderm, and ectoderm) that represent different cell populations. Also, a hybrid between adult stem cells and ESCs introduces another line of cells called

induced pluripotent stem cells (iPSCs) [25]. Two problems arise from using such cells, namely their immunogenicity and their potential to form tumors [26]. The use of cocultures of the above-mentioned cell types increases the efficiency of neuro-tissue engineering and mimics the *in vivo* cell cross talk [1].

Primarily, neural stem cells (NSCs) can be directly implanted at the site of injury from a cell donor, or can be used to stimulate their endogenous potential for neurogenesis via growth factors [27]. In TBI, the use of stem cells holds great promise in reversing neurological deficits by replacing lost neurons and rebuilding damaged networks. A study by Mahmood *et al*. evaluated the effects of transplanting bone marrow mesenchymal cells (BMSCs) implanted within a collagenous scaffold on the expression of a growth inhibitory molecule known as neurocan as well as on axonal plasticity in TBI rats [28]. Cells were transplanted into the lesion cavity 7 days after TBI with controlled cortical impact (CCI) [28]. The rats receiving cell therapy showed reduced expression of TBI-induced neurocan as well as upregulation of growth-associated protein 43 (GAP-43) compared to rats treated with saline only [28]. This was shown through immunohistochemical studies, western blot analysis, laser-capture microdissections, and quantitative real-time polymerase chain reaction [28]. To further help reestablishment of functional networks, Yu *et al*. proposed that GAP-43 might participate in the beneficial effects of NSCs by promoting the communication between grafted NSCs and the host cells [29].

Interestingly, endothelial progenitor cells (EPCs), acquired from adult peripheral blood, have been suggested to have a therapeutic potential in cerebral white matter injury by aiding in vascular repair and axonal survival, particularly via paracrine trophic effects [30]. Specifically, targeting the neurovascular unit collectively using EPC therapy may potentially improve the functional outcome by suppressing the secondary effects after TBI. Similarly, Nichols *et al*. investigated the potential for development of autologous-cell-based therapeutic regimen using human peripheral blood-derived MSC population [31]. This study showed a decrease in the levels of apoptosis and increase in the production of neurotrophic factor [31].

Human-amnion-derived mesenchymal cells (AMSCs) have been proposed as promising sources of stem cells. Yan *et al*. induced AMSCs *in vitro* into NSCs whereby they intriguingly expressed high levels of the ciliary neutrophil factor neurotrophin 3 and glial-cell-derived neurotrophic factor [32]. Moreover, human fetal neuron progenitor cells have also been shown to improve long-term functional outcome by prolonging the survival of neurons, diminishing the size of the lesion, enhancing angiogenesis, and, most importantly, reducing astroglial reaction [33]. This was studied by transplanting human fetal neuron progenitor cells 24 h after TBI in rats for 12 weeks. To further understand functional recovery, long-term assessment of cell therapies is needed [33].

However, questions about the safety and efficacy were raised in regard to tumor formation and graft differentiation [33, 34]. Generally, the use of gene transfection and administered cytokines carries a greater risk of tumor formation and immune rejection, despite improving the capability of cell transplantation [26]. Similarly, the use of ESCs or iPSCs carries the same risk because of the reactivation of pluripotency genes, including *c-myc*, resulting in overproliferation

of cells [35]. One way of reducing these possible risks is by using electrical stimulation during neural differentiation. This novel method was associated with enhanced differentiation of mouse BMSCs into neural cells *in vitro* and *in vivo*, and expression of neurogin 2 (Ngn2), which in turn improved the cells' capability of transplantation by partly inhibiting astrocytic differentiation during cell growth. This was shown by transplanting these electrically stimulated cells into a mouse TBI model [35].

Cramer *et al*. investigated the natural regeneration potential of resident neural cells [36]. Neuroprogeniter cells were collected from the neocortex of embryonic green-fluorescent protein (GFP)-expressing mice and implanted in TBI region. They were shown to generate electrophysiologically mature neurons forming the different cortical layers [36]. On another note, a study investigated the effects of combining BMSCs with olfactory ensheathing cells, which ultimately showed significant improvement of the neurological function of TBI rats [37]. Taken together, there is no ideal single cell type for transplantation.

Mechanisms behind improved functional recovery after cell-based therapies have been investigated in a series of studies [34, 38–40]. One can argue that the functional improvement is directly related to cell transplantation itself, but it may be attributed to the neuroprotective and modulatory influences as well [38]. When NSCs were identified in the adult forebrain, much interest was shown in identifying possible mitogens that positively impact neuroregeneration by making the local environment more hospitable for growth [40]. One way in which this occurs is by providing trophic support in the following forms: neurotrophic factors such as nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), ciliary neurotrophic factor (CNTF), glial cell-derived neurotrophic factor (GDNF), and leukemia inhibitory factor (LIF), and other ECM proteins such as laminin, fibronectin, and collagen I/III and IV [41, 42]. A summary of the neurotrophic factors and their importance in neuronal growth, repair, and development is depicted in Table 20.1. As described, many benefits of cell-based therapies can be attributed to the secretion of neurotrophic factors and their effects on neuroprotection and neurogenesis (Table 20.1).

Yan *et al*. observed that MSC treatment downregulated inflammatory cytokines and microglia in brain parenchyma, while, on the other hand, upregulated anti-inflammatory cytokines [32]. The anti-inflammatory mechanism was shown to be related to tumor necrosis factor (TNF)-stimulated gene 6 protein (TSG-6) secretion, which works by reducing pro-inflammatory cytokines [40]. Furthermore, cells were genetically modified to encode the BDNF gene, which plays a role in the survival and differentiation of neural cells [40]. Transplantation of these cells caused an overexpression of BDNF, which in turn led to better neurite outgrowth and overexpression of synaptic proteins. Fundamentally, protective effects of BDNF-modified NSC transplantation seemed greater than those of unaltered NSCs [40].

Of interest, inhibition of the glial scar formation may be useful for neuroregeneration [14]. Thus, appropriate control of glial cell behavior may improve the microenvironment and consequently may contribute to the survival and functional differentiation of neuronal stem cells [2]. On the other hand, the glial

Table 20.1 Neurotrophic factors involved: A summary of neurotrophic factors, and their importance for neuronal growth, repair, and development as well as their characteristics.

Neurotrophic factors	Characteristics	References
Nerve growth factor (NGF)	Member of family of neurotrophins that induce the survival and proliferation of neurons.	[43, 44]
	Induces the formation of neurite projections in cell culture, and *in vivo* may stimulate the innervations of tissues.	
Brain-derived neurotrophic Factor (BDNF)	Preservation of neurons by affecting growth, differentiation, and upkeep of these cells.	[44, 45]
	Located at neuronal synapses, where cell-to-cell communication occurs.	
Neurotrophin-3 (NT-3)	Controls survival and differentiation of mammalian neurons.	[44]
	Important in early postnatal development.	
Ciliary neurotrophic factor (CNTF)	Influences cell development and functioning by triggering intracellular signaling cascade, which in turn develops and maintains the nervous system.	[46]
	Promotes survival of motor neurons.	
Glial-cell-derived neurotrophic factor (GDNF)	*In vitro*, promotes the survival and differentiation of dopaminergic neurons. Prevents apoptosis when axons are out of motor neurons.	[47]
Leukemia inhibitory factor (LIF)	Involved in the maintenance and development of the nervous system. Stimulates neurons, particularly motor neurons.	[48]

scar represents an attempt at wound healing, limiting inflammation and protecting healthy tissue [2, 14]. Furthermore, it has been suggested that post-lesion activation of nuclear factor kappa B (NF-κB) contributes to the scar formation and expression of axonal growth inhibitors [49]. Interestingly, by blocking this protein by genetic ablation or overexpression of the dominant negative mutant, functional recovery and axonal regeneration in the mature CNS was significantly improved (reviewed in [50]). As such, the NF-κB signaling pathway is a possible target for pharmacological intervention for functional restoration following TBI [49]. Direct intrathecal implantation of mesenchymal stromal cells leads to enhanced neuroprotection via an NF-κB-mediated increase of interleukin-6 (IL-6) production [50]. Overall, modulating inflammation, releasing neurotrophic factors, and inhibition cell apoptosis have all been proposed as possible underlying mechanisms [51].

The addition of stem cells peripherally post TBI is not without shortcomings. The route of administration of stem cells is instrumental in defining the disadvantages of using cell-based therapy. For example, intravenous injection of stem cells has ample potential to cause systemic side effects [52]. In addition, the efficacy of introducing cells peripherally is inefficient and wasteful [52]. In one study, MSCs were injected into the carotid of a rat 2 h after middle cerebral artery

occlusion [53]. The distribution and tissue location of the majority of BMSCs (95%) within the first 24 h were in the spleen [53]. Even when transplanted into the lesion of interest, stem cells have low survival because of the harsh environment that is unsuitable for stem cells to enroot [53]. Only ~0.2–10% of cells injected directly into the lesion on interest survive [54]. Consequently, as discussed later in the "use of bioactive scaffolds" Section 20.4.1, proper means and modes to deliver replacement cells are needed.

Besides the limited cell viability, another challenge of cell transplantation is the ability to tightly control neural subtyping. Indeed, transplanting stem cells without control may cause random axonal sprouting, resulting in possible unproductive neural networks or hypersensitivity reactions [55]. One study reported the production of only γ-aminobutyric acid (GABA) and glutamatergic neurons, since the precursors capable of generating dopaminergic neurons are lost [55]. One way to properly guide differentiation is by the use of elements such as neurotrophic factors [55]. Conversely, another study investigated the use of pre-differentiated embryonic cells into GABAergic neuron astrocytes to improve functional outcome [52]. Nonetheless, these challenges limit the use of NSCs. In general, combining enriched environments, embryonic neural stem cells, and growth factors resulted in improved functional recovery following TBI to a greater magnitude compared to a single therapy such as cell transplantation alone.

As such, developing safe and efficient sources of stem cells, whether autologous or allogeneic, capable of effective differentiation into functional neurons *in vivo* remains a challenge in the field of regenerative medicine. A major drawback associated with the administration of stem cells into the CNS directly is the lack of supporting microenvironment. Fundamentally, regeneration of the CNS requires not only endogenous and exogenous NSCs but also a suitable microenvironment.

20.4 Construct Technology: Biomaterials Approach

The term *construct technology* addresses the design and engineering of tissue-like constructs and their use as a delivery method for factors that aid in tissue regeneration [56]. Endogenous impediments to both axonal and neuronal regeneration and recovery post injury necessitate innovative therapeutic approaches, the most compelling of which are biomaterial-based strategies. As discussed later, the introduction of active biomaterial substances to augment or replace the natural function of a tissue shows promise as a therapeutic intervention for TBI. The biomaterial substance not only shows promise for self therapeutic benefits but also can be used as a delivery material for growth factors and extracellular matrix proteins. As a result, the construct represents the lost endogenous healthy tissue environment. Biomedically active materials can allow recruitment of host cells or enhance axonal regrowth within the damaged areas.

Successful therapeutic strategies like temperature regulation and increased oxygen supply to the damaged brain tissue have been difficult and less efficacious than interventions in other organ systems [57]. This may be due to the complexity

of the CNS and the inhospitable environment in and around the injured site for repair and recovery. Current therapies for improving clinical outcomes of TBI include limiting inflammation and its repercussions, preventing secondary cell death, and enhancing the plasticity of spared circuits [57].

20.4.1 Scaffold-Based Treatment

Clinical efficacy of the cell transplantation techniques has been limited by poor cell survival, uncontrolled differentiation, and ineffective integration into the host tissue, primarily due to an inhospitable environment at and around the injury site [58]. Additionally, growth factors, hormones, and short synthetic peptide sequence are either inadequately concentrated on the area of interest or easily diffuse and degrade unless bound in place [27]. As a result, a scaffold to provide a suitable environment for cell regeneration and repair is deemed important to maximize efficacy. Supporting these considerations, studies using scaffold show better regeneration and growth than those employing cell suspension alone [59, 60]. Enhanced cell survival observed during transplantations of intact tissue might be attributed to a 3D architecture with higher accessibility of extracellular adhesive proteins, allowing for a cellular environment that is more closely resembling of the *in vivo* milieu.

A scaffold in tissue engineering is defined as a provisional biodegradable 3D material that supports cell integration prior to *in vivo* inoculation [61]. Scaffolds can be derived from biological sources, synthetic constructs, or a combination of both to maximize benefits of both sources. Several groups have demonstrated that cell transplantation within biomaterials is better than controls without biomaterials in terms of cell survival and behavioral recovery [62]. Some biological and synthetic polymers used are illustrated in Figure 20.2. Hyaluronan and methyl cellulose, ultrafoam collagen I scaffold, and Matrigel have been studied for cell transport. These are made from mouse extracellular matrix extracts comprised of ECM proteins, such as collagen and laminin, in addition to growth factors including fibroblast growth factor 2 (FGF-2) and epidermal growth factor

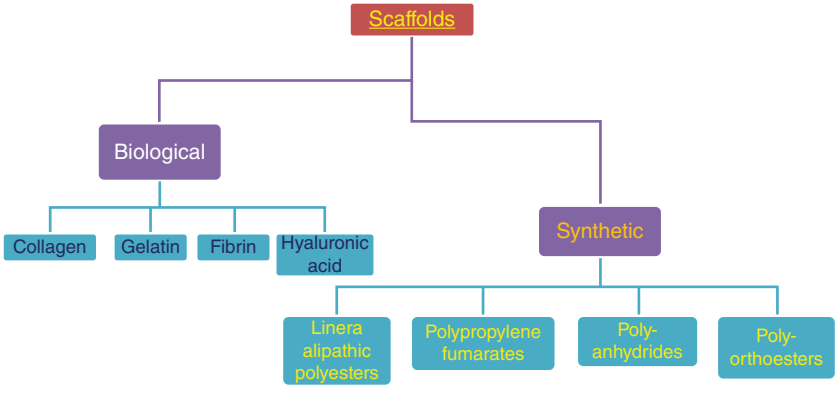

Figure 20.2 Different biological and synthetic polymers. These are studied not only in tissue engineering but also as neural-tissue engineering constructs for TBI [56, 63].

Table 20.2 *ECM factors identified:* Three important ECM proteins for the use of neural-tissue engineering constructs with their respective characteristics.

ECM factor	Characteristic	References
Laminin	Basement membrane glycoprotein that is involved in the following: cell adhesion, cell movement, development, and differentiation.	[64]
Fibronectin	Involved in the following: cell adhesion, development, migration, and control of actin molecules. Additionally involved in wound healing and embryonic development.	[64]
Collagen I/III and IV	Collagen is a main constituent of connective tissue, and is the most abundant protein found in mammals.	[65]

(EGF) [54]. Individual ECM proteins that are also studied for the use of neuro tissue engineering constructs are included in Table 20.2. [54].

Different constructs of scaffolds are tailored in a tissue-specific manner by influencing the 3D architecture: the porous spaces within a scaffold, the physical characteristics like stiffness of the scaffold, and the cell-binding ability of the scaffold. Examples of the scaffold stiffness and their effect on neural-tissue engineering are highlighted in Table 20.3. Essentially, the different stiffness of these scaffolds preferentially gear differentiation into different cell tissue types. The ultimate scaffold for CNS tissue engineering has yet to be found; therefore, ongoing research is comparing different models and combinations of scaffolds

Table 20.3 *Scaffold stiffness characteristics*: Different scaffold constructs with characteristic stiffness and their respective particular effects on neuro-tissue differentiation.

Stiffness characteristics of scaffolds		References
NSPC on methacrylamide chitosan hydrogel		[66]
Soft (<1 kPa)	Differentiated into astrocytes and neurons	
Moderate (3.5 kPa)	Maximum amount of proliferation	
Stiffest (>7 kPa)	Differentiated into oligodendrocytes	
Dissociated hippocampal cells on amphiphile hydrogels		[67]
7 kPa–25 kPa	Increased neuronal differentiation maturation and synapse density	
MSCs		[68]
0.1–1 kPa	Differentiated to neural like cells	
Intermediate	Myogenic cells	
Bone like stiffness (25–40 kPa)	Osteogenic cells	
Human ESCs		[69]
Stiff polyacrylamide hydrogels	Increased viability	

to maximize the differentiation and formation of engineered neural tissue. One major advantage of using scaffolds is the creation of 3D geometric structures tailored to the size and shape of the injury.

On the other hand, there are drawbacks associated with the different types of scaffolds used. Porous or fibrous scaffolds limit the development of contractile force because of the inherent stiffness of scaffold, incomplete biodegradation, liberation of potentially toxic substances during degradation, and poor cell alignment and morphology. Hydrogel scaffolds exhibit no mechanical or spatial restrictions, while polymer scaffold needs to be molded into a particular shape prior to implantation. As in a TBI scenario, the injury site results in an irregularly shaped cavity that requires a scaffold that conforms to the shape of the injury while still having sufficient architecture to provide a suitable environment for repair and integration. Hence, the hydrogel approach shows more promise and as such can be considered among the most adequate model scaffolds to date.

Several studies have shown that combination treatments involving the use of cells, bioactive molecules, and biomaterials are more effective than a single-component strategy. Combination treatments enhance cell survival and integration after cell transplantation and achieve local delivery to the brain, circumventing the BBB, and systemic side effects. Examples of combination treatments and studies and their benefits are shown in Table 20.4. Biomaterials act as a delivery mode for therapeutic molecules (GFs, proteins, small molecules) in a sustained and tunable manner. Additionally, they act as a delivery mode for cells and ensure their retention and integration at the site of grafting.

Table 20.4 Different combination treatment studies: Examples of combination treatments between different scaffolds, cells, model types, and the respective outcomes of each study.

Scaffold	Cell	Type of model	Outcome	References
Collagen type 1	Neural stem cells (NSCs)	Rat with 2-h middle cerebral artery occlusion	Structural and functional recovery of neural tissue following ischemic injury	[70]
Collagen hydrogels with laminin-derived cell adhesive polypeptides	Neural stem cells (NSCs)	3D hydrogel matrix *in vitro* culture	Number of living NSCs was higher in the collagen hydrogels with laminin polypeptide	[71]
Elastin-like polypeptides modified with RGD	PC-12 cells (ATCC)	PC-12 cells, *in vitro* cell culture	Increase in PC-12 neurite extension	[72]
PLGA and PEG polymers with brain-derived neurotrophic factor (BDNF)	PC-12 cells engineered to express the trkB receptor	PC-12 cells, *in vitro* cell culture	Increased delivery of bioactive BDNF	[73]

20.4.1.1 Carbon Nanotubes Approach

Another modality that has gained significant interest in TBI regeneration is the use of carbon nanotubes (CNTs) – sheets of graphite rolled into a continuous nanoscale cylinder. CNTs have offered a new strategy in the attempt to regenerate and repair TBI, in part due to their unique mechanical, electrical, and structural properties. They display superior strength, flexibility, electrical conductivity, and diverse capacity for chemical functionalization using different biomolecules [74]. One of the biggest challenges in the use of CNTs is their toxicity, which is due to their solubility. Whether or not CNTs precipitate or aggregate out of solution, they pose potential toxicity to cells. Functional modification of CNTs – particularly by affecting their surface by the addition of chemical groups or polymers – allows for more bioactivity and more efficient internalization and secretion by cells [74]. Polymers such as polyethyleneimine (PEI) and poly-L-ornithine (PLO) have been reported to promote neural cell attachment and subsequent neurite outgrowth, and hydrophilic polymers such as polyethylene glycol (PEG) were found to increase CNT solubility in aqueous solution [74]. The use of PEG with CNTs has been shown to offer high-capacity antioxidant therapeutic effects, having a large role to play in the future solutions in neural injury [75]. In addition to the functionalization by rudimentary positively charged polymers, the use of biologically active molecules such as bio-factors, like many of those mentioned throughout this chapter, shows excellent promise not only for incorporation of CNTs but also to broaden their versatility [74].

20.4.2 Biologics and Drug-Based Treatment

In addition to cell-based therapies, where they are shown to secrete neuroprotective and neurogenic factors, the direct addition of neurotrophic hormones and other drugs, or biologics-based treatment, has shown promise. Studies have shown that intraventricular infusion factor hormones show better outcome for regeneration and recovery. One such study has shown that both epidermal growth factor (EGF) and erythropoietin (EPO) added together (but not alone) promote regeneration of damaged cerebral cortex in rats [63]. In another study, the absence of interferon gamma (IFNγ)-KO in mice with sciatic nerve axotomy showed increased proportional degeneration of neurons in the ventral horn as compared to controls [76]. Therefore, in addition to EGF and EPO, IFNγ may have neuroprotective effects. Lastly, glial-cell-line-derived neurotrophic factor (GDNF) enhanced the number and caliber of regenerated axons *in vivo* and increased neurite outgrowth of dorsal root ganglion neurons (DRGNs) *in vitro* [77]. One study showed that GDNF had a direct effect on neurons for axonal regeneration and spinal cord remyelination [77].

In addition to neurotrophic factors and hormones, substratum ECM materials are known to show direct neurodevelopmental effects. Fibronectin was shown to have an important role in the developing CNS as well as to promote axonal regeneration of adult neurons. In one study, plasma fibronectin, more specifically the α5β1 integrin-mediated binding to the recombinant fibronectin fragment, showed significant increase of neurite production, including axons, as compared

to laminin or merosin [78]. Understandably, the RGD (arginine-glycine-aspartate) sequence from fibronectin shows promise in stimulating connection and viability of neurons. Likewise, laminin and laminin-derived peptides have also promoted cell adhesion and viability of neurons. The laminin short synthetic peptide sequence YIGSR (tyrosine-isoleucine-glycine-serine-arginine) and IKVAV (isoleucine-lysine-valine-alanine-valine) are prime examples [79] [80]. Other intercellular adhesion molecule (ICAM) substrates have also been implicated in neural growth and development. A neural cell adhesion molecule (NCAM)-derived FGF-receptor agonist, named the FGL-peptide, was shown to induce neurite outgrowth and neuronal survival in primary rat neurons [81]. NCAM- and ICAM-mediated adhesion as well as other cell adhesion molecules have shown significant importance time and time again for neuronal survival and growth. Consequently, they might be crucial in any attempt at a regenerative biomedical engineering approach for the treatment of TBI. Interestingly enough, cell–substrate interactions are not only influenced by what is binding to what but also by the spatial orientation relative to each other.

Moreover, pharmacological treatments that are available to combat CNS diseases including TBIs are still being explored and need further studies. For example, progesterone use has shown promise in early phase trials; however, Phase III clinical trials demonstrated no clinical benefit, which stands in contrast to the preclinical, early single-center trials [82, 83]. Limited diffusion of drugs and pharmacologic molecules across the BBB further restricts the efficiency of today's therapeutic options and highlights the need for investment in other regenerative medical strategies [57]. That said, the BBB may be one reason behind the drawbacks met with in pharmacological treatment methods; thus, cell transplantation and endogenous cell repair stimulation are promising future therapeutic approaches, particularly in the use of biomaterials to promote recovery and repair post TBI.

20.5 Application to Living System: Translational Approaches

This section describes the most recent advances in developing *in vitro* and *in vivo* models that focus on creating neural constructs for eventual implantation. To date, this remains at the forefront of neural-tissue engineering. To begin with, Sasai is a stem cell biologist at the Riken Center for Developmental Biology, Kobe, Japan. His efforts led to cultivating optic cups, tissue layers of the cerebral cortex, pituitary gland, and cerebellum in a Petri dish. His experiments performed on mouse ESCs underscore the importance of achieving a delicate balance, controlling the cells' environment while allowing enough room for them to naturally float and form aggregates known as embryoid bodies [84]. In other words, maximizing external cues and tightly controlling the environment might counteract the attempt at regenerating neural tissues. The embryonic bodies, also known as brain balls, subsequently form a large number of cortical precursor cells, which assemble into characteristic layers of the cerebral cortex [84].

A technique known as *serum-free floating culture of embryoid body-like aggregate with quick reaggregation* (SFEBq) uses a 3D culture termed a *floating culture* where cells are able to communicate and interact with each another [84]. Cells are suspended in wells and form aggregates of ~3000 cells per well and are coaxed to differentiate into neural progenitor cells [84]. These cells signal each other to spontaneously organize into the neuroepithelium. Eventually, external cues induce neuroepithelial cells to differentiate into different neural subtypes, which give rise to specific brain structures [85]. Using this technique, Sasai constructed adenohypophysis process in which the mouse ESCs were stimulated to differentiate into non-neuronal head ectoderm and hypothalamic neuroectoderm [86]. When treated with hedgehog signaling, these cells formed layers and 3D structures like Rathke's-pouch. Corticotrophs and somatotrophs were subsequently produced and grafted *in vivo* in hypopituitary mice, resolving the glucocorticoid levels [86]. Indeed, deriving human adenohypophysis from human ESCs or induced PSCs is a definite future challenge [86]. Similarly, Sasai and his colleagues demonstrated the self-formation of stratified cerebral cortical tissues in culture using the SFEBq technique. By modifying the culture medium and adding basement membrane matrix components, they were also able to create a self-organizing optic cup and neural retina morphology [85]. This opens avenues for regenerative medicine in retinal degeneration and for transplantation of artificial retinal tissue instead of simple cell transplantation. Other studies have also come up with *in vitro* models to study neurodevelopment processes specific to human brain development. Lancaster *et al*. cultured brain tissues termed *cerebral organoids* from human PSCs [87]. Although much of the experiments done are in mouse ESCs, it provides researchers with models to explore the different neuropathologies and mechanisms underlying regeneration, ultimately leading to the possible development of drugs and gene therapy to reverse CNS injury.

20.6 Future Outlook: Transition to the Clinic

The field of tissue engineering is still young and offers much future potential. Huge strides are being made in neural-tissue engineering for the sole reason of necessity. TBI in particular is of special importance due to its increasing incidence rates and neuropsychological consequences; therefore, intervention to ameliorate TBI effects is of continual pursuit. Following injury, the typical physiological response consists of a complex inflammatory response, which subsequently results in scar tissue formation. While the scar represents an attempt at wound healing, it may very well limit regeneration. The main obstacle is to assist the damaged neural tissue to grow beyond the site of scar formation in both the brain and spinal cord.

The CNS carries an inherent potential to regenerate following an injury, but it is not robust enough to overcome the severe neurological deficits [88]. To aid this natural process of regeneration, neural-tissue solutions are at the forefront in the treatment of injured CNS, proposing appealing solutions that promise to preserve neural networks and enhance neuroplasticity. There is no single

treatment that will restore the original normal function of the injured brain, and neural-tissue engineering is a multidisciplinary field that incorporates key elements in regeneration, namely the use of stem cells, supporting scaffold, and external and internal cues [23].

Cell-based therapies that aim to reduce neurodegeneration and enhance neuroprotection post a CNS injury hold great promise in overcoming functional impairment. Yet, technical challenges, including limited cell survival, lack of control over differentiation and proliferation, and invasiveness of transplantation, put a brake in translating neural-tissue engineering solutions to the clinic. One way of overcoming these challenges is delivering cells on suitable biomaterials that considerably support and enhance their niche. These details are of paramount importance in understanding the brain regenerative mechanisms and cellular interactions between the implanted tissue and the host. One problem with such methods arises because of the inadequacy of comparison and universal controls. Different combinatorial methods offer different benefits and different disadvantages. When would the ratio of benefits to disadvantages be sufficient for neuro-tissue engineering?

Additionally, different methods cannot be compared objectively without a common basis for comparison. Before neural-tissue engineering can be implemented successfully in clinical settings, there are several obstacles that need to be surpassed including reproducibility, safety, and development of effective and controlled approaches. The success in bringing the treatments from the bench to the bedside requires rigorous preclinical studies and innovative clinical trials to ultimately develop off-the-shelf (allogeneic cells) or personalized (autologous cells) products that are cost effective and efficient for manufacturing in large amounts. In spite of all of these hurdles, the growing number of studies and advancements in neural-tissue engineering are yielding more and more promising solutions whether drug- or cell-based therapies in combination with biomaterials or enriched environments. These approaches hold great neurotherapeutic potential for CNS injuries in future.

Abbreviations

AMSCs	amnion-derived mesenchymal cells
BBB	blood–brain barrier
BMSCs	bone-marrow-derived mesenchymal cells
BDNF	brain-derived neurotrophic factor
CNTs	carbon nanotubes
CDC	Center for Disease Control and Prevention
CNS	central nervous system
CSF	cerebrospinal fluid
CNTF	ciliary neurotrophic factor
DG	dentate gyrus
DRGN	dorsal root ganglion neurons
ESCs	embryonic stem cells
ED	emergency department

EPCs	endothelial progenitor cells
EGF	epidermal growth factor
EPO	erythropoietin
FGF-2	fibroblast growth factor 2
GCS	Glasgow Coma Scale
GDNF	glial-cell-line-derived neurotrophic factor
GFP	green fluorescent protein
GAP-43	growth-associated protein 43
IFNγ	interferon gamma
IL-6	interleukin 6
LIF	leukemia inhibitory factor
MSCs	mesenchymal stem cells
NGF	nerve growth factor
NSCs	neural stem cells
NSPCs	neural stem/progenitor cells
Ngn2	neurogin 2
NT-3	neurotrophin-3
NF-κB	nuclear factor kappa B
PSCs	pluripotent stem cells
PLO	poly-L-ornithine
PEG	polyethylene glycol
PEI	polyethyleneimine
SVZ	subventricular zone
TBI	traumatic brain injury

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21

Bionics in Tissue Engineering

Thanh D. Nguyen and Brian P. Timko

21.1 Introduction

We review the current state of bionics – systems containing both biological tissues and electronic components. Historically, these two systems have been disparate, since electronic devices were rigid, potentially toxic, and not prone to forming useful interfaces with cells or tissue. Recent advances in materials science and related fields have ushered in new classes of devices that interact with biological systems in a far more exquisite manner. Those systems involve nanomaterials that interact with tissues on a subcellular level, substrates that are conformal, and in some cases bioresorbable, onboard electronics that enable telemetry, and chemical interfaces that are amenable to integration with biological systems. These bionic systems could serve to complement and enhance tissue function.

Tissue engineering represents one field in which bionics is poised to play a key role. That field has developed in part out of the demand to for synthetic constructs that could repair damaged, degenerated, or nonfunctioning organs; it could effectively circumvent the need for donors and therefore enhance the current standard of care [1]. Polymer tissue scaffolds have been developed to support 3D culture of many cell types – including human embryonic stem cells (hESCs) that could enable clinical translation [2–5] – but tissue engineering remains a nascent field.

Recent advances in bionics have enabled increasingly functional tissue. For example, nanomaterials dispersed throughout tissue scaffolds have been shown to present topographic and chemical cues that recapitulate the cells' native microenvironment and direct the formation of tissue. Tissues grown in those nanocomposite scaffolds exhibited superior electrical and contractile properties as a result of those cues. Cellular alignment – a prerequisite for developing cardiac or smooth muscle tissue – has been induced by mechanical guides such as microchannels [6]. Engineered tissue could moreover include integrated circuits, which might provide real-time feedback relating to electrical and/or chemical activity within the tissue.

Bionic interfaces could also be established with existing tissue. Advances in conformal device arrays have enabled materials that can be grafted onto myocardium or blood vessels. Those technologies could continuously monitor

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catastrophic events, such as a heart attack or blood clot, where early detection is crucial. Sensors coupled with stimulating electrodes could enable closed-loop systems that intervene immediately. High-resolution device arrays could also have substantial applications in prosthetics, enabling the patient to control artificial limbs with far greater fidelity than possible with current technology.

Integrated biocompatible systems containing electronic interfaces, power sources, and supporting electronics have been developed. Taken together, these could enable new classes of interfaces – "cyborg" tissue [7] – that address a wide range of unmet needs. We address recent advances in this chapter.

21.2 Electronics for Biointerfaces

Bionic tissues require the integration of electronic components with biological cells in order to monitor, repair, or enhance electrical function. The first step toward this goal is to create electronic devices that can effectively interface with cells or tissues and perform the desired tasks. Conventional silicon-based electronic devices are rigid and not suitable to directly interface with tissues, which are soft and curvilinear. This limitation has been addressed by recent advances in soft organic electronics, solid-state materials, and lithography techniques. Devices have been assembled on soft, biocompatible substrates, enabling tissue interfaces not achievable by other means. In this section, we discuss novel electronic materials that have been used to stimulate or record signals from tissue.

21.2.1 Pioneering Devices

21.2.1.1 Cochlear Implants

Clark has been a pioneer in the development of cochlear implants for treating severe hearing loss [8]. In a healthy individual, auditory signals are transduced through the inner hair cells on the cochlea; sounds cause those cells to vibrate and electrical impulses are transferred to the adjacent ganglion cells. Severe to profound deafness is characterized by a loss of hair cells, although the underlying nerve cells are typically intact [9].

An effective cochlear implant would need to address nerve cells in both a temporally and spatially selective fashion. Temporal coding refers to the frequency of elicited action potentials in the nerve fibers, which are in phase with the transduced sound waves. But each region of the cochlea is selectively sensitive to a different frequency; high-frequency waves are transduced by neurons at the basal end, while low-frequency waves are transduced at the distal end. An implant would need to stimulate in the same spatially selective manner to ensure proper encoding of pitch.

Clark developed a multichannel electrode implant that could encode speech into electrical voltage signals and directly stimulate the auditory nerves (Figure 21.1) [8]. The device, which was implanted directly onto the cochlea, consisted of a microphone, an auditory processor that converted sounds into electrical impulses, and an array of up to 22 metallic electrodes on a flexible

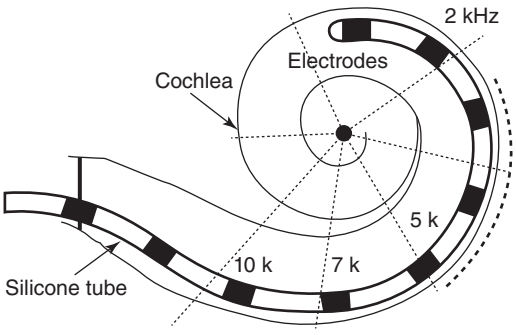

Figure 21.1 Bionic prostheses: Cochlea with implant consisting of a microelectrode array wrapped around a silicone tube. (Clark 2012 [8]. Reproduced with permission of Taylor & Francis.)

silicone substrate. The device was long and narrow, and with gradient stiffness that allowed effective insertion into the cochlea. The device was approved by the U.S. Food and Drug Administration (FDA) in 1985 and 1990 for adults and children, respectively. It has restored auditory function to many deaf patients.

21.2.1.2 Retinal Implants

Stimulating electrodes have also been used as retina prosthesis to recover eyesight in blind patients. Diseases such as retinitis pigmentosa or age-related macular degeneration cause patients to suffer from injured or degenerated photoreceptors that cannot receive light. However, as with the cochlea, the underlying neurons may remain functional. Devices containing photodiodes, transducers, and current-injecting electrode arrays have been developed and implanted either on or below the surface of the retina. Those devices were validated in clinical trials and have been commercialized [10].

21.2.1.3 Bionic Arms Employing Neuromuscular–Electrode Interface

Bionic prostheses incorporate joints with motors and signal transducers and mimic the functionality of natural limbs. Early versions were controlled using external electrical controllers. More recent versions incorporate sensors that measure stimuli produced by existing muscles or nerves, and transduce those stimuli to achieve the appropriate motion in the prosthesis.

Hiyama *et al*. developed concentric-ring electrodes that were placed on skin to measure forearm muscle motions by electromyography, where specific motions were correlated to the movements of individual fingers [11]. The signals were decoded and transduced to control individual fingers in the prosthetic. The concentric electrodes represented a substantial advance over conventional, bulky electrode pads, which could not achieve adequate spatial resolution to distinguish between the movements correlating to each finger. By placing a reference electrode (1 mm in diameter) at the center of a working ring-electrode (6 mm in diameter) in a tiny place, the concentric electrodes minimized interferences by the activities of proximal muscles, enabling a fine control of the prosthetic motions.

Despite those advances, electromyography measurements suffered from noises associated with skin motions, rendering the sensing and decoding of

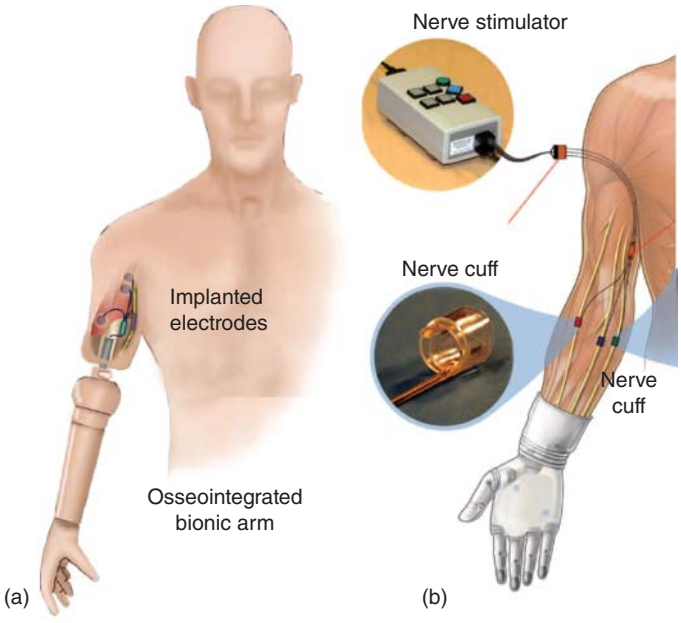

Figure 21.2 Mind-controlled bionic prosthetic arms using electrode/neuromuscular interfaces. (a) Osseointegrated bionic arm employing direct interfaces between implanted electrodes and epimysial muscles. (b) Bionic arm with nerve cuff-electrodes to stimulate real touch-sensing signals. (Adapted from [12, 13].)

muscular activities difficult. To solve this problem, electrodes were implanted subcutaneously and in direct connection with muscle fibers. That interface was established with osseointegrated bionic arms [12], which were connected directly to bones through a titanium screw. The electrodes were implanted and connected directly with epimysial muscles inside stumps of damaged arms (Figure 21.2a). Those interfaces enhanced sensitivity and resolution in recording, decoding, and controlling neuromuscular activities, enabling long-term stability of the prostheses. Further, the integration of the bionic arm with bones via a screw (i.e., one pivot point) mitigated restrictions of swing motions in conventional prostheses, which are connected to body via entire stump areas of injured arms.

Electrodes can also be connected directly to nerve fibers, enabling two-way communication with the prosthetic. Tan *et al*. reported a direct nerve–electrode interface that could enable touch sensation for bionic hands [13]. Nerve cuffs with different electrode channels were implanted on peripheral nerves in the forearm of patients with prosthetic arms. These nerve cuffs were connected to subcutaneous electrodes, leading to nerve stimulators (Figure 21.2b). When the bionic hand touched an object, an electrical signal from the stimulators excited the nerve cuff and provided sensory feedback to the brain, enabling the patients with natural sensations – natural tapping, constant pressure, light moving touch, and vibration – for at least 2 years.

21.2.2 Organic Electronics

Starting in the 1950s, serial achievements in conducting organic materials were reported. In 1954, Gutmann and Lyons reported the conducting polymer complex perylene–bromine [14]. In 1964, Little reported his prediction of a presentation of superconductivity in organic macromolecules even at room temperatures [15]. Following that, Greene and Street published their experimental works to verify the ability of polymer (SN)x to be both conducting and superconducting [16]. This work has led to further study and characterization of such conducting macromolecules by MacDiarmid and Heeger and their collaborative work with Shirakawa in acetylene polymerization, which formed a conductive film similar to aluminum foil [17, 18]. The research of those three scientists resulted in a Nobel Prize in 2000, a milestone in the development of conducting polymers.

During the 1990s, scientists turned their attention to semiconducting organics which have been useful in light-emitting diodes (LEDs), photovoltaics, and thin-film transistors. Petacene has been widely studied within this context because it has a relatively high charge carrier mobility [19]. Other materials that have received attention are tetracene, rubrene, oligoacenes, oligothiophenes, polyfluorenes, and thiophene [20].

21.2.2.1 Mechanism

The most common characteristics of conducting and semiconducting polymers is that they all have delocalized π electrons from the π-bonding in the chemical structure. For example, pentacene's chemical structure is comprised of five benzene molecules (Figure 21.3a) [19]. The π electrons in each of the benzene molecules interact with those in the adjacent molecules, thereby reducing the bandgap, which is usually large for organic materials and polymers. Pentacene can conduct electricity by holes (i.e., p-type) with a mobility of up to $5\,cm^2\,V^{-1}\,s^{-1}$, similar to amorphous silicon [21]. This moderately high mobility of pentacene is the reason why this material has been used most extensively for organic semiconductors. With the same mechanism, conducting polymers such as polypyrrole (Figure 21.3b), polyaniline, and poly(3,4-ethylenedioxythiophene)-polystyrene sulfonate (PEDOT-PSS) gain their high electrical conductivity from the delocalized π electron in each of their monomers [22].

21.2.2.2 Organic Device Applications in Bionic Tissues

Electronic skin, or e-skin, has been developed. That material contained strain gauges and electromyogram sensors designed to measure deformations and

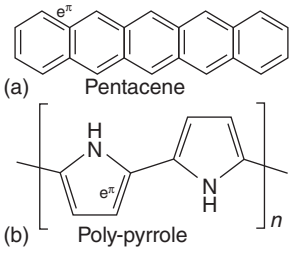

Figure 21.3 Chemical structures of (a) pentacene, an organic semiconductor (Kitamura 2008. Reproduced with permission of Institute of Physics Publishing.), and (b) polypyrrole, a conducting polymer. (Deng 2004. Reproduced with permission of the American Chemical Society.)

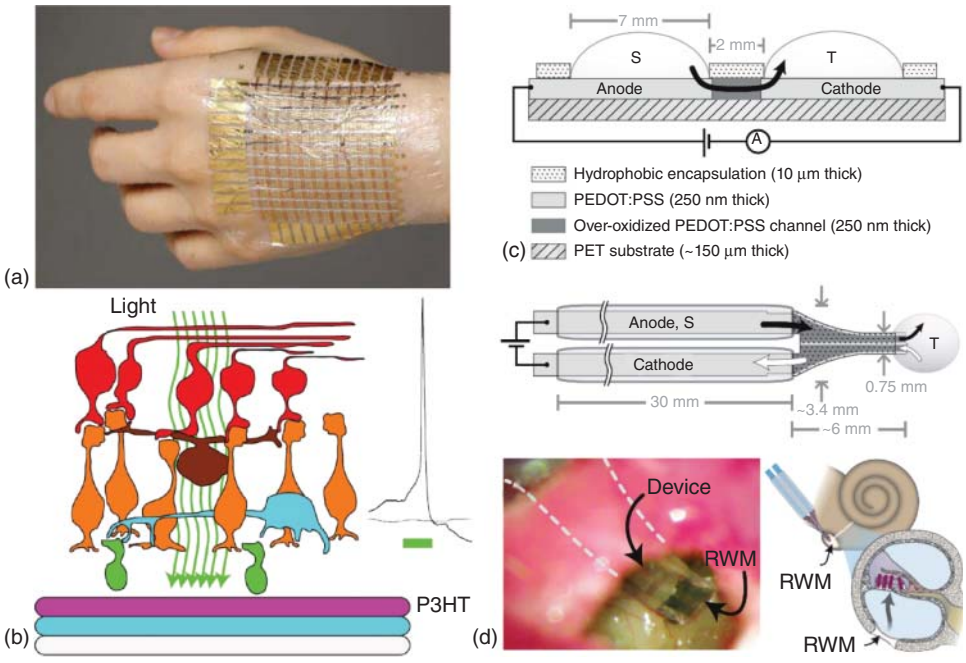

Figure 21.4 Organic electronic devices for biointerfaces. (a) e-Skin for monitoring health signals. (b) Photovoltaic film P3HT for optical retinal stimulation. (c) Schematic of an ionic pump that transfers cationic species from a source reservoir (S) into a target reservoir (T) in (top) planar and (bottom) syringe-like form factors. (d) (Left) Photograph and (right) scheme of the device depicted in (c) implanted near the round window membrane (RWM) of a guinea pig cochlea. (Adapted from [23–25].)

electrical activity on the surface of the skin (Figure 21.4a) [23]. The devices were fabricated a polyethylenenaphthalate (PEN) substrate with a total thickness of only 2 μm to ensure flexibility. The fabrication process used the air-stable semiconductor dinaphtho[2,3-*b*:2′,3′-*f*]thieno[3,2-*b*]thiophene (DNTT), self-assembled (SAM) monolayer gate dielectrics, and paralyene interlayer separators. Semiconducting and metal layers were created by vacuum deposition, while paralyene was deposited by chemical vapor deposition. The functional semiconducting layer was only 30 nm thick but could sustain a saturation mobility of up to $3\,cm^2\,V^{-1}\,s^{-1}$. Because of its thinness, the e-skin could be bent down to a radius of 5 μm, enabling conformal interface with the surface of the skin.

The soft nature of organic and polymeric electrodes enabled mechanical compatibility with soft and delicate nerve tissues [26]. For example, the organic compound paralyene was shown to be biocompatible and to support the growth of viable neuronal cells *in vitro* [27]. A film of poly(3-hexylthiophene) (P3HT) was interfaced with explanted rat retinas and was able to stimulate the firing activities of injured nerves [24]. In that work, photovoltaic P3HT was interfaced directly with a rat retina with degenerated photoreceptors but viable and functional ganglion nerve cells (Figure 21.4b). Under the photoexcitation, P3HT

generated localized potentials, which depolarized the ganglion cells and triggered electrical impulses. There was no difference in the firing patterns between normal retinas and the injured ones in P3HT under the same photostimulation, showing the possibility of using the photovoltaic polymer to excite visual nerve cells with damaged photoreceptor. The achievement could offer a new approach toward achieving retina prosthesis.

Organic electronics that function as ion pumps have also been developed; those devices mimicked neuronal synapses and could stimulate interfaced tissue by delivering neurotransmitters [25]. The device consisted of a PEDOT:PSS film that was divided into anode and cathode regions by an electronically insulating but still ionically conducting gap. An applied voltage established an electrochemical circuit that oxidized the anode, inducing the migration of a cationic species (M^+) from a source reservoir, through the anode, and into a target reservoir (Figure 21.4c, top). The device could deliver several neurotransmitters – glutamate (Glu), aspartate, and γ-amino butyric acid (GABA), all of which are cationic at sufficiently acidic pH. The device was configured into a syringe-like form factor (Figure 21.4c, bottom), enabling delivery to external electrolyte and individual cells. For example, glutamate delivered to astrocytes *in vitro* activated the receptors on those cells, triggering an influx of Ca^{2+}. *In vivo*, the device was validated by stimulating the auditory system in a guinea pig model. It was implanted near the round window membrane (RWM), an established entry port into the cochlea (Figure 21.4d). Glu delivered by the device passed through the RWM and stimulated the inner hair cells, eliciting an auditory response [25].

21.2.3 Solid-State Nanomaterials

Nanomaterials open new avenues for the development of bionic tissues. They have been synthesized with well-defined geometry and composition, and chemical routes toward surface functionalization have been well established [28, 29]. Since they are free-standing, nanomaterials can be readily integrated into 3D tissue constructs or can be used as 3D probes that interface with cells in ways not achievable by other means. In this section, we discuss recent advances in gold and silicon nanomaterials, which have been crucial in the areas of tissue regeneration and nanoelectronics, respectively.

21.2.3.1 Nanocomposites in Tissue Engineering

In the examples described thus far, devices were used as active interfaces capable of eliciting or recordings signals from cells. Nanomaterials can also behave passively to modulate or enhance cellular function. Lipid bilayers, which are chemically similar to cell membranes, form extraordinarily tight junctions with carbon nanotubes (CNTs) [30]. Those junctions, along with the high conductivity of CNTs, have been exploited to modulate the electrical behavior of interfaced cells. Neurons cultured on CNT mats exhibited enhanced network activity and significantly higher after-potential depolarization, potentially because the CNTs formed electrical shortcuts between proximal and distal regions of the neurons [31, 32]. Similar effects were observed with cultured

cardiomyocytes, which exhibited increased spontaneous electrical activity and larger domains of synchronously beating cells, compared to those cultured on planar surfaces [33]. CNT mats have also been interfaced with organotypic spinal cord explants; cells directly interfaced with the mat, as well as those adjacent up to five cells deep, expanded more neuronal fibers, displayed higher growth cone activity, and exhibited increased synaptic activity [34].

A key advantage of free-standing nanomaterials is that they can be readily dispersed throughout 3D constructs, and so are particularly amenable to tissue engineering applications. Cardiac tissue engineering in particular has benefited from nanomaterials. Cardiac tissue scaffolds should exhibit a sufficiently low Young's modulus so that they do not inhibit mechanical motion, but also should be electrically conductive to ensure efficient coupling between adjacent cardiomyocytes. Conventional cardiac tissue scaffolds – alginate, collagen, chitosan, or synthetic polymers such as poly(lactic-*co*-glycolic acid) – exhibit suitable mechanical properties but are inherently nonconductive [35, 36]. Gold nanospheres embedded within hydrogel [37] or gold nanowires (AuNWs) within alginate [38] imparted electrical conductivity but did not substantially alter the mechanical properties of those scaffolds (Figure 21.5a,b). Cardiomyocytes

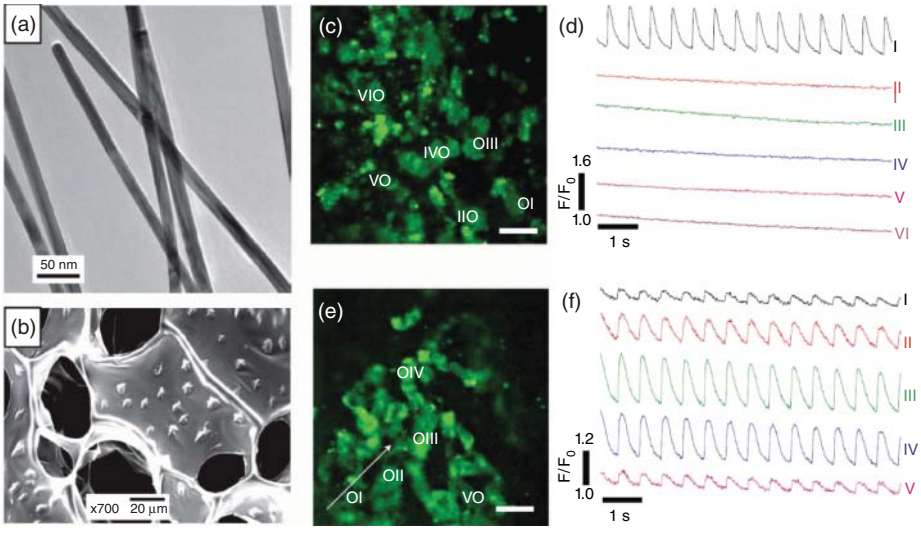

Figure 21.5 Alginate–AuNW nanocomposites. (a) TEM of AuNWs. (b) SEM of AuNWs embedded within lyophilized alginate scaffold. (c–f) Calcium transient was assessed at specified points (white circles) by monitoring calcium dye fluorescence (green). (c) Sites monitored in pristine scaffold, where site I is the stimulation point. (d) Calcium transients were observed only at the stimulation point in the unmodified scaffold. F/F_0 refers to measured fluorescence normalized to background fluorescence. (e) Sites monitored in an Alg–NW scaffold. The stimulation point was 2 mm diagonally to the lower left of point I (i.e., off the figure). The white arrow represents the direction of propagation. (f) Calcium transients were observed at all points. (Dvir 2011 [38]. Reproduced with permission of Nature Publishing Group.)

cultured within the scaffolds exhibited increased expression of Cx43, a transmembrane protein responsible for Ca^{2+} flux between adjacent cardiomyocytes, and actinin, responsible for mechanical integrity. Fluorescence microscopy revealed that cardiac tissue cultured in pristine alginate scaffolds beat in isolated clusters localized at the simulation point (Figure 21.5c,d); in contrast, tissue cultured in alginate–AuNW nanocomposite scaffolds beat synchronously over at least 2 mm (Figure 21.5e,f).

Nanomaterials embedded within tissue scaffolds serve to recapitulate the cells' native microenvironment by presenting both nanotopographic and chemical cues [36]. Gold nanomaterials are especially useful in this context, since they can be rationally synthesized with defined geometry and are readily functionalized with thiolated ligands. For example, electrospun gold–silk nanofibers were synthesized and functionalized with the integrin-binding peptide RGD (SNF_{Au}+RGD), which promotes cellular adhesion [39]. Human mesenchymal stem cells (hMSCs) cultured on those scaffolds exhibited higher cell density and increased cell area compared to those cultured on pristine fibers or nanocomposite fibers without RGD. SNF_{Au}+RGD also promoted the development of cytoskeletal structure within 24 h of culture, as was clear from evidence of well-organized actin filaments, compared to disordered actin filaments in cells grown under the same conditions on pristine scaffolds [39]. Gold nanoparticles have also been attached to decellularized omental matrices [40] and coiled polycaprolactone fibers [41], both of which are established matrices for cardiac tissue growth. Primary cardiac cells cultured on those matrices exhibited larger contraction amplitude, lower excitation threshold, higher aspect ratio, and faster signal transmission velocity compared to cells cultured on primary scaffolds.

21.2.3.2 Silicon Nanowire Nanoelectronics for Molecular-Scale Sensing

A new class of molecular-scale electronic interfaces can be formed with cells and tissue using chemically synthesized semiconductor nanowires (NWs) as functional elements. These NWs have received intense interest in recent years, leading to the development of structures with rationally controlled geometry, composition, and electronic properties [42]. The underlying biological detection using NWs is their configuration as field-effect transistors (NW-FETs), which exhibit a conductance change in response to variations in the charge or potential at the surface of the NW. Functionalized NW-FETs have been used as species-specific chemical or biological sensors with extremely high sensitivity because of their 1D nanoscale geometry. They could measure pH, femtomolar-level concentrations of proteins (e.g., prostate specific antigen), and single viruses [28].

NW-FETs can also be used to measure extracellular potentials generated by action potentials in electrically active cells, or field potentials in whole tissue [28]. NW-FETs are, in fact, ideal for that purpose since they exhibit the same size scale as subcellular features (e.g., individual ion channels), can be readily assembled into arrays with device pitch <2 μm, and can be fabricated on nonconventional substrates, including those that are flexible [43, 44]. When passivated with HfO_2 (a high-*k* dielectric), NW-FETs remained functional in physiological conditions for at least 1 year [45].

NW-FETs assembled on 2D substrates enabled extracellular electrical measurements at subcellular resolution. In an early work, devices were interfaced with individual axons and dendrites of cultured neurons, enabling multiplexed readouts of signal propagation throughout a single cell. They were also interfaced spontaneously with beating cardiomyocyte monolayers. In that study, the devices recorded signals with high signal-to-noise (>10), confirming the ability of NW-FET devices to form a good junction with cell membranes. Multiplexed NW-FETs were also used to measure activation sequences across the surface of *ex vivo* myocardium, and to probe network connectivity within brain slices from the lateral olfactory cortex [46].

Nanowire nanoelectronic scaffolds (nanoES) have been developed. They consisted of highly flexible, macroporous sheets containing NW-FETs and could be integrated with collagen, which has been commonly used as a substrate for growing 3D engineered tissue. That hybrid system supported the growth of neurons, cardiomyocytes, and smooth muscle cells. The NW-FETs within demonstrated integrated sensory capabilities: they could measure 3D electrical activity, the response of engineered neural and cardiac tissue to drugs, and distinct pH changes inside and outside of vascular smooth muscle constructs [47].

One distinct advantage of NW-FETs is that NWs are free-standing structures, and can therefore form functional elements in 3D probe-like devices. Because of their small size, they can readily penetrate cell membranes, enabling intracellular nanoelectronic measurements. NW-FETs containing kinked NW elements were fabricated and coated with a phospholipid layer to facilitate entry across the cell membrane (Figure 21.6a) [48]. As those devices approached and penetrated the membrane of spontaneously beating cardiomyocytes, a distinct signal transition was observed: submillisecond spikes, associated with fast Na^+ transients, were recorded in the extracellular space just outside the membrane, and ~200-ms signals were measured when the device entered the cell (Figure 21.6b) [48]. That experiment was the first example in which a nanoelectronic device was used to measure action potentials intracellularly.

Nanotubes have also been used as functional elements in intracellular sensors. Branched-nanotube intracellular field-effect transistors (BIT-FETs) were fabricated by grafting a silicon oxide nanotube onto a silicon NW backbone, which functioned as the channel [49, 50]. Active silicon nanotube transistors (ANTTs) were fabricated by defining source and drain electrodes directly onto the nanotube, which itself acted as the channel [51]. In either case, the nanotube could readily penetrate a cell membrane, causing the interior of the nanotube to match the intracellular potential (Figure 21.6c,d).

While the substrates supporting nanoelectronic devices can be soft, conformal, and biocompatible, surgical implantation is typically required for *in vivo* monitoring. This limitation was recently addressed with injectable scaffolds composed of microelectrode or NW sensors and interconnects fabricated onto a flexible polymer substrate [52]. Those devices, which were as much as 2 mm wide, could be loaded into a glass pipette and injected through an opening with diameter as small as 100 μm. After injection into either a man-made or biological cavity, the scaffold unfurled; the electrodes could then be attached to external electronics through an anisotropic conductive film, with device yield as high as 90%.

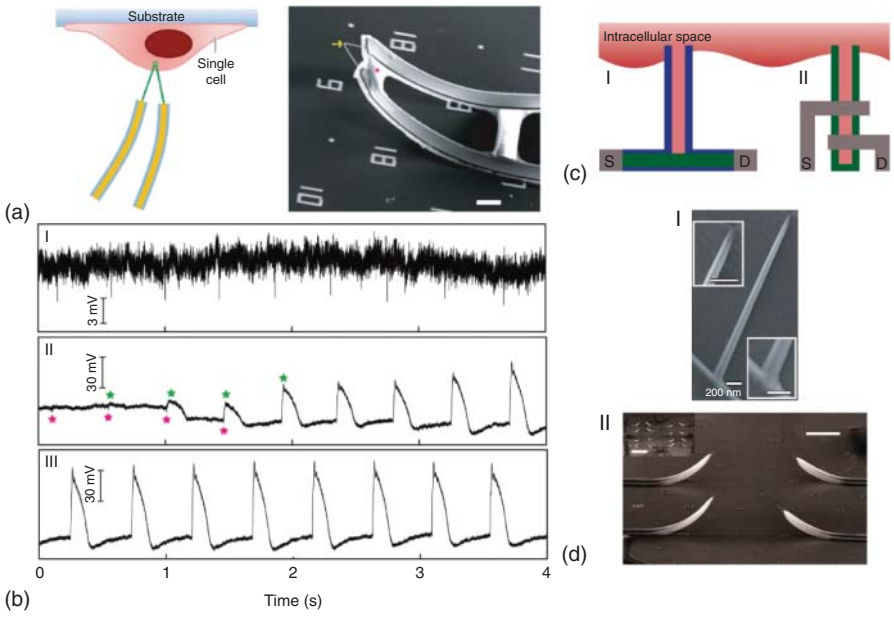

Figure 21.6 3D NW probes for intracellular measurements. (a) (Left) Schematic and (right) SEM image of a kinked NW-FET from which intracellular or extracellular potentials can be measured. (b) Electrical recording from beating cardiomyocytes: (I) extracellular recording, (II) transition from extracellular to intracellular recordings during cellular entrance, and (III) steady-state intracellular recording. Green and pink stars denote the peak positions of intracellular and extracellular signal components, respectively. (c) Schematic diagram of (I) BIT-FET and (II) ANTT. (d) SEM image of (I) BIT-FET and (II) array of ANTTs. (Adapted from [46, 48], and [49].)

Injectable scaffolds were injected into the lateral ventricle or hippocampus of live rodents. Because of the small size of the needle, the scaffolds could be injected through the skull with minimal invasiveness, highlighting a distinct advantage of the technology. Sixteen-electrode arrays injected into the hippocampus recorded local field potentials, with spatiotemporal mapping characteristic of that region of the brain [52]. Such scaffolds – which have been shown to exhibit excellent long-term stability and biocompatibility as well as mechanical properties approaching that of native tissue – could enable long-term interfaces with spatial resolution not achievable by other means.

21.2.4 Flexible Solid-State Microelectronics

Microelectronic devices have also been fabricated on soft, flexible, and stretchable electronics. Those devices are defined using conventional lithography, and so are readily prepared in large device arrays that can be integrated with other structures, such as onboard circuitry or battery sources (see Section 21.3). Flexible microelectronics have been interfaced with skin, heart, and brain [53–55].

A synthetic epidermis enabled with microelectronics has recently been developed [53]. As in the case of e-skin (Section 21.2.2.2), a key challenge was to fabricate a substrate and devices with thicknesses, elastic moduli, bending

modulus, and areal mass density matching those of the epidermis. Aside from ensuring a conformal interface, the highly flexible substrate would adhere to the surface of the skin based solely on van der Waals interactions. Multicomponent devices including temperature sensors, strain gauges, electrophysiological sensors (i.e., electroencephalography (EEG), electrocardiography (ECG)), LEDs, and radio frequency (RF) circuits were fabricated on ultrathin polymeric substrates. The electronic components were composed of thin films of silicon or gallium arsenide. Interconnects were fabricated in a serpentine design, enabling them to be stretched without breaking. The entire device patch had a substrate thickness of only ~30 μm, a bending stiffness <1 nNm, and an elastic modulus less than 150 kPa, matching well with the parameters of the epidermis; the patch exhibited a small bending radius and significant stretchability. The device patch was laminated onto the surface of the skin and was used to monitor cardiac and muscle potentials, electrocardiogram and electromyogram, respectively.

Conformal device patches have also been laminated onto the surface of cardiac tissue. Those patches measured electrophysiological signals with high temporal and spatial resolution [54]. The functional elements in those patches were doped crystalline silicon nanoribbons, which were patterned onto conventional silicon wafers and then transferred onto the polymer substrate. Conducting and dielectric layers were then deposited so that the nanoribbons could be individually addressed. The chip also contained onboard amplifiers and multiplexers, which were deposited in a layer-by-layer fashion with dielectric layers separating active components. Despite its complexity, the entire patch had a thickness of only about 25 μm, and so exhibited a small bending radius and could be readily conformed to the surface of the heart. A device containing 288 functional elements was laminated onto the surface of a porcine heart, and could measure activation sequences over the surfaces. Such a technology could offer a novel method to monitor cardiac diseases.

A similar system that could measure signals was interfaced with the brain. It included 720 silicon nanomembrane transistors. The patch was conformally laminated *in vivo* on top of the visual cortex of a cat brain and was able to map electrical impulses over a large area. It was used to record spatial properties of brain activity, including sleep spindles, visual evoked responses, and electrographic seizures [55].

The flexible and stretchable inorganic microelectronics described in this section pave a way for a new class of bionic tissues. They could eventually be integrated with synthetic tissue scaffolds (see Section 21.2.3.2), enabling continuous feedback on tissue viability, both during culture and after implantation.

21.3 Novel Power Sources

Active technologies such as the ones described throughout this chapter require a power source. Conventional lithium-ion batteries have been employed in clinical devices ranging from pacemakers [56] to remotely triggered drug delivery systems [57]. Those batteries, however, are bulky, potentially toxic, and,

in most cases, non-biodegradable. They are also inadequate for devices with long lifetimes; pacemaker batteries, for example, last on average only 6–7 years, necessitating periodic replacement of the entire device [58].

The development of new energy sources is particularly important within the context of the bionics, since, as described in previous sections, those systems are often designed to be small, biodegradable, and biologically benign. Here, we review new technologies that could be incorporated into future bionic systems.

21.3.1 Inductively Coupled Systems

Inductively coupled systems enable both power and data transmission using RF waves [56]. Those waves, which have frequencies ranging from 3 kHz to 300 GHz, couple with coiled wires (inductors) to generate a current. Inductor coils can be readily fabricated using standard lithographic techniques and so could be integrated into a variety of microelectronics for device implants.

Wireless blood pressure monitors have been reported. One type of device consisted of a circular cuff with an integrated capacitive pressure sensor and an inductive coil that could receive power from a 433-MHz RF wave (Figure 21.7a) [59]. Another type of sensor, designed to measure pressure at the pulmonary artery, was integrated onto a standard medical-grade stent [60]. That device also contained a pressure sensor, as well as an RF rectifier that could achieve both data transmission and remote powering (Figure 21.7b). The device was designed with substantially different powering and transmission frequencies (3.7 and 2.4 GHz, respectively) to prevent interference. It was validated in a pig, demonstrating its ability to operate while buried within thick tissue [60].

21.3.2 Degradable Batteries

Electrochemical cells are realized when two electrodes of differing electrochemical potential (the anode and cathode) are immersed in an electrolyte. When the circuit is completed, redox reactions between the two electrodes produce a potential difference that drives a current (Figure 21.8a).

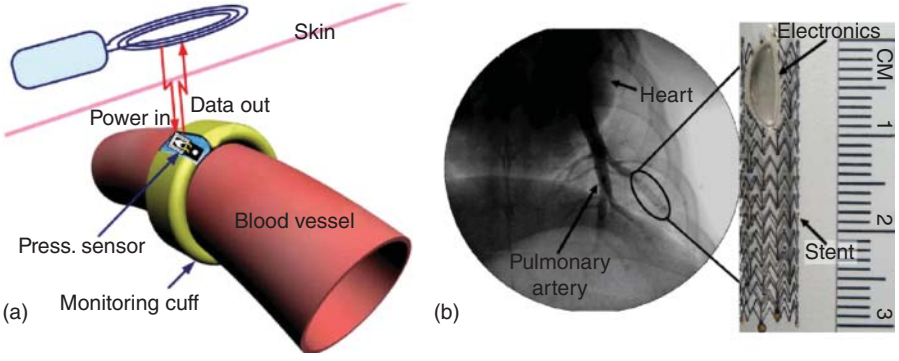

Figure 21.7 Wireless pressure sensors realized on a (a) cuff electrode and (b) stent. (Adapted from [57, 59, 60].)

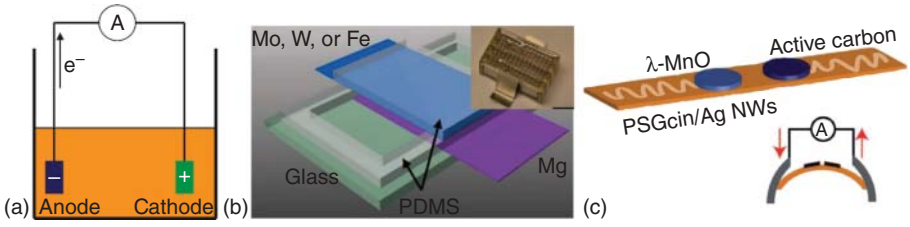

Figure 21.8 Dissolvable batteries. (a) Schematic of a simple electrochemical cell and examples of (b) a biodegradable and (c) an edible battery. (Adapted from [61, 62].)

Conventional batteries can be nondegradable, toxic, and/or hazardous to the environment. Recently, biodegradable and biologically benign alternatives have been investigated. One such device consisted of a magnesium foil anode and an iron, tungsten, or molybdenum cathode, each of which dissolved into nontoxic byproducts in physiological media (Figure 21.8b) [61]. (In fact, with the exception of tungsten, those elements are essential for biological function [63].) The device was packaged in a polyanhydride casing, which is also biodegradable, and the electrolyte was phosphate buffered saline (PBS). A single cell could produce a stable current as high as 0.1 mA cm^{-2} at up to 0.75 V, for at least 24 h. When stacked in series, those batteries achieved an output voltage of 1.5–1.6 V, sufficiently high to power a conventional LED [61]. Other transient electronic components have been also reported – an Mg/MgO inductor and capacitor, Si/MgO/Mg transistor, Si diode, Mg interconnects and resistors, and silk substrates – enabling the fabrication of fully integrated, dissolvable microelectronic devices [64].

Transient electronics have also been proposed as the basis for an edible battery [62]. That device consisted of flexible composite electrodes and a sodium ion electrochemical cell encased in a gelatin capsule. The gelatin capsule could be programmed to dissolve at a predefined time points, which would deploy the battery by causing it to become hydrated. In that device (Figure 21.8c), the anode and cathode were composed of l-manganese oxide (λ-MnO_2) and active carbon, respectively, and the electrodes were composed of a conductive mixture of poly(glycerol-*co*-sebacate)-cinnamate (PGScin) and sintered silver nanowires (Ag NWs). The polymer hydrolyzes into naturally occurring monomers, while the silver nanowires are well below the tolerable dose threshold for humans, and are expected to eventually oxidize into Ag^+ and resorbed.

21.3.3 Piezoelectrics for Capturing Mechanical Energy

Materials that harvest energy from the body's natural mechanical motion could be useful in long-lasting devices since they would obviate the need to periodically replace or manually charge batteries. One way to harvest energy is with piezoelectric materials. Those materials accumulate an electric charge in response to a mechanical stress, or, conversely, deform when an electric field is applied. They have been used extensively as mechanical sensors, actuators, and transducers. Macroscale piezoelectric transducers have also been used to harvest mechanical energy in living systems. Polyvinylidene fluoride, a piezoelectric polymer, was interfaced with a dog thorax and generated a voltage as high as 10 V from each

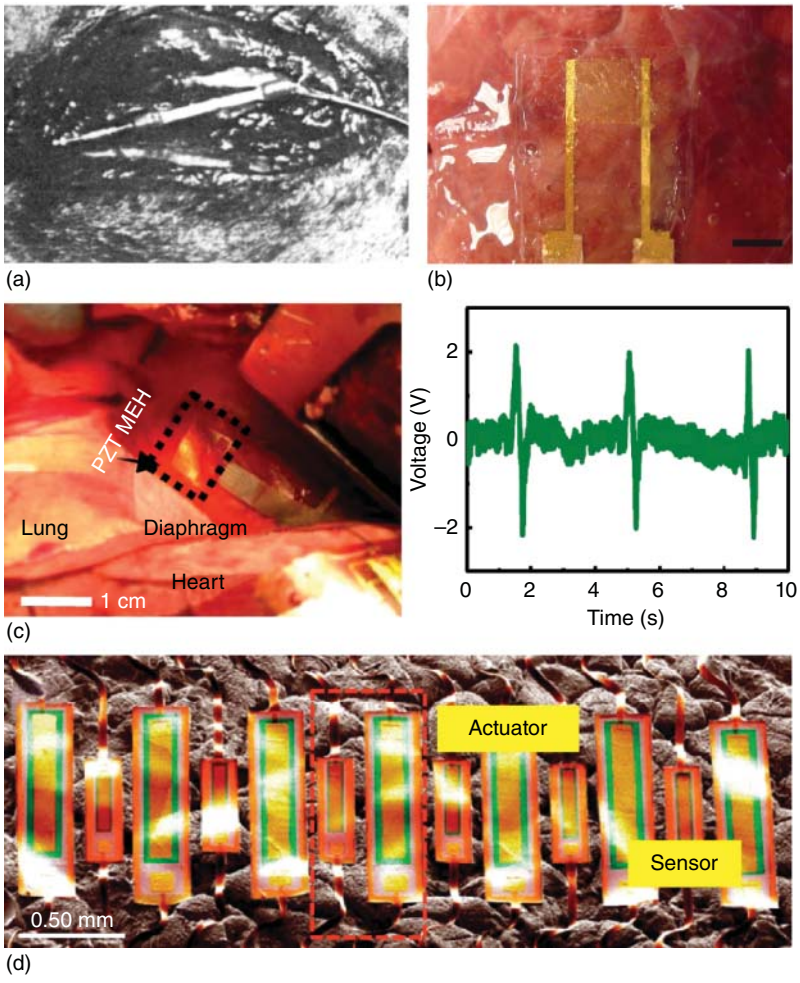

Figure 21.9 Piezoelectric devices for harvesting mechanical energy. (a) A polyvinylidene fluoride energy-harvesting device implanted on a dog's thorax. (b) A PZT energy-harvesting device interfaced with cow-lung tissue. (c) A PZT energy-harvesting device integrated on bovine diaphragm (left) and electrical signals (right) produced by the device from diaphragm motions. (Adapted from [65–67].) (d) An array of PZT sensors and actuators laminated onto skin to measure viscoelastic moduli. (Dagdeviren 2015 [68]. Reproduced with permission of Nature Publishing Group.)

deformation (Figure 21.9a) [65]. Flexible piezoelectric devices were also embedded into the sole of shoes to collect electricity from walking [69].

More recently, piezoelectric devices have been developed at the micro- or nanoscale to enable more intimate biological interfaces [66, 70–73]. One device was grafted directly onto the surface of an explanted cow lung (Figure 21.9b) [66, 71]. The functional element was lead zirconate titanate (PZT), which is one of the most efficient piezoelectric materials [73]. PZT nanoribbons were synthesized, assembled onto a flexible silicone sheet, and addressed with metal electrodes

that were deposited using soft lithography [66]. The entire chip was only a several micrometers thick, and so exhibited a small bending radius and could be readily grafted onto the surface of a curvilinear organ such as an explanted cow lung. The lung was artificially inflated at 3 Hz to mimic a natural breathing motion, and an open circuit (V_{oc}) voltage of 1 V (peak to peak) and a and short-circuit current (I_{sc}) of 30 nA were generated from the device. That power was moderate, but represents the first proof of concept that PZT nanomaterials can be used to harvest mechanical motions of biological organs.

PZT-based devices with far larger output powers were achieved by fabricating groups of PZT nanomaterials in parallel (to increase I_{sc}), and then by connecting many groups in series (to increase V_{oc}) [67]. Those devices, as seen in Figure 21.9c, also contained onboard rectifiers and chargeable batteries, and so were capable of both collecting and storing energy. Polyimide films functioned as both the substrate and encapsulant; they were highly flexible, but also served to protect the devices from physiological liquids. The devices were validated *in vivo* by implanting them onto the surface of a bovine or ovine heart, lung, or diaphragm. Substantially, devices implanted on the bovine heart produced energy to power a pacemaker, demonstrating the utility of piezoelectric devices for self-powered electronics [67].

Piezoelectric-based systems can also measure the mechanical properties of their local environment by recording and analyzing artificially induced perturbations. Such a system was recently developed to measure the visoelastic modulus of near-surface regions of the epidermis [68]. It consisted of a thin, conformal elastomeric membrane that supported PZT (lead zirconate titanate) nanoribbons and stretchable serpentine interconnects (Figure 21.9d). The nanoribbons were configured as either actuators or sensors; the sensors were modulated by an externally applied AC field to generate mechanical perturbations, while the sensors recorded those perturbations after they propagated through the skin. Visoelastic modulus could be calculated by comparing those two signals.

PZT sensor arrays could be used to measure the mechanical properties of human skin. Analysis of *ex vivo* samples revealed that modulus was a function of age or location of the body, and also changed upon application of a moisturizing cream (e.g., 1% acrylamidomethylpropane sulfonic acid). In human subjects, results obtained from dermatologic malignancies revealed that skin lesions in the breast or leg regions had lower moduli than their healthy counterparts. Such technology could eventually be used to diagnose skin disorders, including cancer, and could also be adapted and deployed to other regions of the body [68].

21.4 3D Printing

Engineered tissues have typically been prepared using scaffolds composed of hydrogels, porous lyophilized polymers, or electrospun fibers. Those materials have been well characterized, but control over their geometry, or the ability to modulate composition throughout a single construct, has been limited. Microlithographic techniques offer greater control, but are typically used to

prepare 2D scaffolds, which then must be assembled into 3D scaffolds in a layer-by-layer fashion, which is time consuming. 3D printing could open new avenues in tissue engineering not accessible by other techniques.

3D printing has been used to create vascular networks within engineered tissue, addressing a critical challenge within tissue engineering. In one approach, a 3D printer was used to prepare sacrificial microfibers that templated microchannels in a hydrogel matrix [74]. The microfibers were composed of agarose, a naturally occurring polysaccharide. They were printed at 80 °C, at which point the agarose was a viscous solution; when the fibers cooled below 32 °C, they formed a gel. A photo-cross-linkable hydrogel was then deposited around the microfibers and polymerized. The agarose microfibers were removed by aspiration, leaving behind microchannels with controllable diameter between 100 and 1000 μm (Figure 21.10a). Those channels were perfused with human umbilical vein endothelial cells (HUVECs), which are models for the endothelial cells that form the walls of blood vessels. The HUVECs formed a monolayer on the surface of the microchannels and expressed a high level of the CD31 marker, indicating the formation of cell–cell junctions. MC3T3 cells, which are osteoblast precursors, were encapsulated within the hydrogel. Cells encapsulated within the hydrogel with microchannels exhibited a far higher viability on culture days 1 and 7, compared to those encapsulated by a hydrogel without the channels. This technique could have far-reaching implications in tissue engineering, since a lack of vasculature, and therefore inadequate oxygen and nutrient diffusion, is a limiting factor in the formation of thick, dense tissue.

A 3D printer with multiple print heads has been used to create scaffolds composed of disparate polymers, including hydrogels laden with cells (e.g., "cell inks") [75]. That printer enabled vascularized tissue by printing a cross-linkable gelatin methacrylate (GelMA) matrix with Pluronic F127 polymer networks embedded within; the Pluronic was subsequently aspirated, leaving behind microchannels. HUVECs cultured within those microchannels formed confluent monolayers resembling blood vessels (Figure 21.10b). GelMA could also be

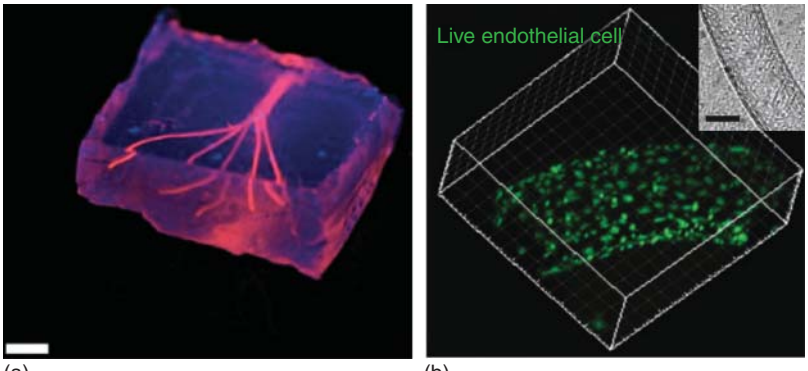

Figure 21.10 3D printed vascular network. (a) Dye-filled microchannels in a hydrogel matrix and (b) synthetic blood vessel composed of HUVECs. (Adapted from [74, 75].)

loaded with fibroblasts and printed without affecting the viability of the cells. All ink types could be printed simultaneously, enabling hybrid constructs containing extracellular matrix (i.e., GelMA without cells fibroblasts), patterned fibroblasts, and vessel-like structures lined with endothelial cells [75].

3D printers have also been used to print solid-state electronic components such as metals, sensors, and antennas [76–79]. They were generally achieved using visoelastic inks that stiffened after extrusion through the printer nozzle. A microscale lithium-ion battery with intercalated electrodes has also been realized in this fashion. The anode and cathode were composed of $Li_4Ti_5O_{12}$ (LTO) and $LiFePO_4$ (LFP), respectively [80]. They were as narrow as 30 μm, and showed an aspect ratio of up to 11 (Figure 21.11a). They were realized by suspending LTO and LFP nanoparticles in a mixture solution of water, ethylene glycol, glycerol, and a cellulose-based viscosifier; after printing onto a substrate, the LTO and LFP inks dried to form a solid structure. Multiple layers could be printed in a layer-by-layer fashion to create high-aspect-ratio structures. The cell was filled with $LiClO_4$ electrolyte and packaged inside a PMMA and glass cover. It could produce about 1.5 mAh cm^{-2} at a stable working voltage of 1.8 V when discharged below 5 °C [80].

Recently, solid-state electronics have been integrated with polymers and engineered tissue to create the first truly bionic devices. The seminal example was an artificial human ear with an integrated radio receiver [81]. The ear itself was printed using ink containing an alginate hydrogel and living chondrocytes, which compose cartilage. Structural elements were printed with silicone. The electronic element was printed using ink containing silver nanowires suspended in silicone. It had a coiled geometry, which could inductively couple with electromagnetic fields from an external transmitter (Figure 21.11b). Taken together, these inks enabled a bionic ear with integrated biological, structural, and electronic elements. It was cultured in chondrocyte medium for up to 70 days, over which time cells remained viable and the Young's modulus increased. The electrode could wirelessly receive radio waves with a frequency up to 5 GHz, higher than the normal human hearing range [81].

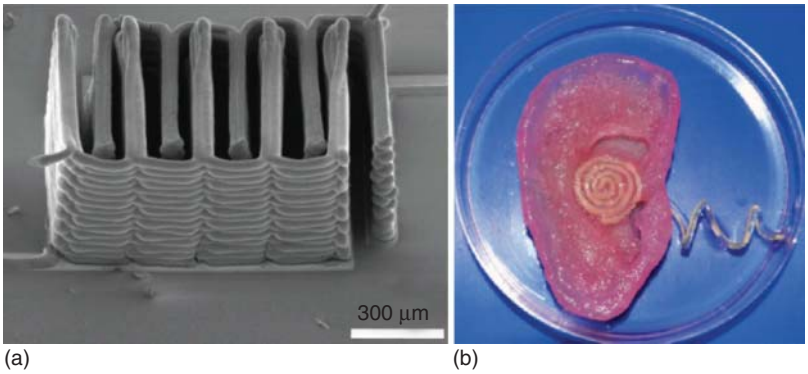

Figure 21.11 (a) 3D printed microscale battery with high-aspect-ratio interdigitated electrodes, and (b) 3D printed bionic ear with integrated radio receiver. (Adapted from [80, 81].)

21.5 Conclusions and Future Directions

This chapter describes early achievements toward the development of bionics – systems containing both biological tissues and electronic components. Advances in synthetic and fabrication techniques enable the creation of novel electronic materials and devices at both the nano- and micro-scales. Those devices exhibit structures that enable conformal and biocompatible interfaces with cells and tissues. Along with functional electronic devices, novel power sources such as degradable batteries, inductively coupled power sources, and materials that harvest human body motions have emerged. Techniques such as 3D printing have been also employed to create bionic tissues that can possess complex 3D geometries and heterogeneously patterned cells. Taken together, these achievements lay an important foundation for the development of bionic tissues with enhanced functions that offer new methods for monitoring and treating diseases.

Many challenges remain to be solved. First, acute and chronic inflammatory effects will need to be assessed for new devices. Bioresorbable materials, especially those that contain nanomaterials, will require special attention to determine the biodistribution and toxicity of each component [82]. Systematic studies relating to the size, composition, and surface chemistry of solid-state materials, as well as to the chemical structure of supporting biopolymers, will be crucial. Second, materials that integrate with host tissues – thin films and engineered tissue in particular – will need to be designed with vascular networks to ensure proper oxygen and nutrient transport [83]. Third, as with all electronic systems, lifetime is a critical concern and will need to be carefully assessed, particularly in the case of systems that require surgery for implantation and removal. Fourth, the effect of bionics on the physiology of surrounding cells will need to be carefully assessed. Considerations relating to the control of cellular growth, and in some cases to the differentiation of hESCs [6, 84], will need to be addressed to ensure that devices can be used clinically without rejection. Finally, looking further forward, the potentially massive amounts of data produced by bionic devices will need to be managed, with regard to processing, storage, and security [85].

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