The Handbook of Nanomedicine

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Preface

Nanomedicine is the application of nanobiotechnology to clinical medicine. However, new technologies do not always enter medical practice directly. Nanobiotechnologies are being used to research the pathomechanism of disease, refine molecular diagnostics, and help in the discovery, development, and delivery of drugs. In some cases, nanoparticles are the nanomedicines. The role is not confined to drugs before devices, and surgical procedures are refined by nanobiotechnology, referred to as nanosurgery.

The Handbook of Nanomedicine covers the broad scope of this field. Starting with the basics, the subject is developed to potential clinical applications, many of which are still at an experimental stage. The prefix *nano* is used liberally and indicates the nanodimension of existing scientific disciples and medical specialties. Two important components of nanomedicine are nanodiagnostics and nanopharmaceuticals, which constitute the largest chapters.

Keeping in mind that the readers of the book will include nonmedical scientists, pharmaceutical personnel, as well as physicians, technology descriptions and medical terminology are kept as simple as possible. As a single-author book, duplication is avoided. I hope that readers at all levels will find it a concise, comprehensive, and useful source of information.

There is voluminous literature relevant to nanomedicine. Selected references are quoted in the text.

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Contents

List of Figures

List of Tables

List of Abbreviations

AFM	atomic force microscopy
BBB	blood–brain barrier
BioMEMS	biological micro electromechanical systems
CNS	central nervous system
DNA	deoxyribonucleic acid
DPN	dip pen nanolithography
ELISA	enzyme-linked immunosorbent assay
FDA	Food and Drug Administration (USA)
FRET	fluorescence resonance energy transfer
LNS	Lipid nanosphere
MEMS	micro electromechanical systems
MRI	magnetic resonance imaging
NCI	National Cancer Institute (USA)
NIH	National Institutes of Health (USA)
NIR	near-infrared
NP	nanoparticle
ODN	oligodeoxynucleotide
PAMAM	polyamidoamine (dendrimers)
PCR	polymerase chain reaction
PEG	polyethylene glycol
PEI	polyethylenimine
POC	point-of-care
QD	quantum dot
RLS	resonance light scattering
RNA	ribonucleic acid
SNP	single nucleotide polymorphism
SPM	scanning probe microscope
SPR	surface plasmon resonance

Chapter 1
Introduction

Medicine is constantly evolving and new technologies are incorporated into the diagnosis and treatment of patients. This process is sometimes slow and there can be a gap of years before new technologies are integrated in medical practice. The reasons for the delay are as follows:

- Establishing the safety and efficacy of innovative treatments is a long process, particularly with clinical trials and regulatory reviews.
- The current generation of medical practitioners are still not well oriented toward biotechnology and conservative elements of the profession may be slow in accepting and learning about nanobiotechnology, which is at the cutting edge of biotechnology.
- The high cost of new technologies is a concern for healthcare providers. Cost–benefit studies are needed to convince the skeptics that some of the new technologies may actually reduce the overall cost of healthcare.

Molecular medicine is already a recognized term. It should not be considered a subspecialty of medicine as molecular technologies would have an overall impact on the evolution of medicine. Recognition of the usefulness of biotechnology has enabled progress in the concept of personalized medicine, which again is not a branch of medicine but simply indicates a trend in healthcare and simply means the prescription of specific treatments and therapeutics best suited for an individual (Jain 2007b). Various nanomachines and other nano-objects that are currently under investigation in medical research and diagnostics will soon find applications in the practice of medicine. Nanobiotechnologies are being used to create and study models of human disease, particularly immune disorders. Introduction of nanobiotechnologies in medicine will not create a separate branch of medicine but simply implies improvement in diagnosis as well as therapy and can be referred to as nanomedicine.

Nanomedicine

Nanomedicine is the application of nanotechnology to medicine and is based on three mutually overlapping and progressively more powerful molecular technologies (Freitas 2002):

K.K. Jain, *The Handbook of Nanomedicine*,

Table 1.1 Nanomedicine in the twenty-first century

Nanodiagnostics
Molecular diagnostics
Nanoendoscopy
Nanoimaging
Nanotechnology-based drugs
Drugs with improved methods of delivery
Regenerative medicine
Tissue engineering with nanotechnology
Transplantation medicine
Exosomes from donor dendritic cells for drug-free organ transplants
Nanorobotic treatments
Vascular surgery by nanorobots introduced into the vascular system
Nanorobots for detection and destruction of cancer
Implants
Bioimplantable sensors that bridge the gap between electronic and neurological circuitry
Durable rejection-resistant artificial tissues and organs
Implantations of nanocoated stents in coronary arteries to elute drugs and to prevent reocclusion
Implantation of nanopumps for drug delivery
Minimally invasive surgery using catheters
Miniaturized nanosensors implanted in catheters to provide real-time data to surgeons
NanoSurgery by integration of nanoparticles and external energy

Source: Jain PharmaBiotech.

1. Nanoscale-structured materials and devices, which hold great promise for advanced diagnostics biosensors, targeted drug delivery, and smart drugs
2. Benefits of molecular medicine via genomics, proteomics, and artificially engineered microorganisms
3. Molecular machine systems such as nanorobots that will allow instant diagnosis with destruction of cause of pathology, chromosome replacement and individual cell surgery in vivo, and the efficient augmentation and improvement in natural physiological function

Current research is exploring the fabrication of designed nanostructures, nanomotors, microscopic energy sources, and nanocomputers at the molecular scale, along with the means to assemble them into larger systems, economically and in great numbers. Some of the applications of nanobiotechnology in medicine are given in Table 1.1.

Basics of Nanobiotechnology in Relation to Nanomedicine

Nanotechnology (Greek word nano means dwarf) is the creation and utilization of materials, devices, and systems through the control of matter on the nanometer

length scale, i.e., at the level of atoms, molecules, and supramolecular structures. Nanotechnology, as defined by the National Nanotechnology Initiative (http://www.nano.gov/), is the understanding and control of matter at dimensions of roughly 1–100 nm, where unique phenomena enable novel applications. Encompassing nanoscale science, engineering, and technology, nanotechnology involves imaging, measuring, modeling, and manipulating matter at this length scale. It is the popular term for the construction and utilization of functional structures with at least one characteristic dimension measured in nanometers—a nanometer is one billionth of a meter (10^{-9} m). This is roughly four times the diameter of an individual atom and the bond between two individual atoms is 0.15 nm long. Proteins are 1–20 nm in size. The definition of "small," another term used in relation to nanotechnology, depends on the application but can range from 1 nm to 1 mm. Nano is not the smallest scale; further down the power of ten are angstrom (0.1 nm), pico, femto, atto, and zepto. By weight, the mass of a small virus is about 10 ag. An attogram is one thousandth of a femtogram, which is one thousandth of a picogram, which is one thousandth of a nanogram. The dimensions of various objects in nanoscale are given in Table 1.2.

Given the inherent nanoscale functional components of living cells, it was inevitable that nanotechnology will be applied in biotechnology, giving rise to the term nanobiotechnology. A brief introduction will be given to basic nanotechnologies from physics and chemistry, which are now being integrated into molecular biology to advance the field of nanobiotechnology. The aim is to understand the biological processes to improve diagnosis and treatment of diseases. Technical achievements

Table 1.2 Dimensions of various objects in nanoscale

Object	Dimension (nm)
Width of a hair	50,000
Red blood cell	2,500
Vesicle in a cell	200
Bacterium	1,000
Virus	100
Exosomes (nanovesicles shed by dendritic cells)	65–100
Width of DNA	2.5
Ribosome	2–4
A base pair in human genome	0.4
Proteins	1–20
Amino acid (e.g., tryptophan, the largest)	1.2 (longest measurement)
Aspirin molecule	1
An individual atom	0.25

Source: Jain PharmaBiotech.

in nanotechnology are being applied to improve drug discovery, drug delivery, and pharmaceutical manufacturing. A vast range of applications has spawned many new terms, which are defined as they are described in various chapters.

Relation of Nanobiotechnology to Nanomedicine

Nanobiotechnology already has an impact on healthcare. Research on biosystems at the nanoscale has created one of the most dynamic science and technology domains at the confluence of physical sciences, molecular engineering, biology, biotechnology, and medicine (Roco 2003). Numerous applications in the pharmaceutical industry such as drug discovery and drug delivery can be covered under the term "nanobiopharmaceuticals." The relationship of nanobiotechnology to nanomedicine and related technologies is depicted graphically in Fig 1.1.

Landmarks in the Evolution of Nanomedicine

Historical landmarks in the evolution of nanomedicine are listed in Table 1.3.

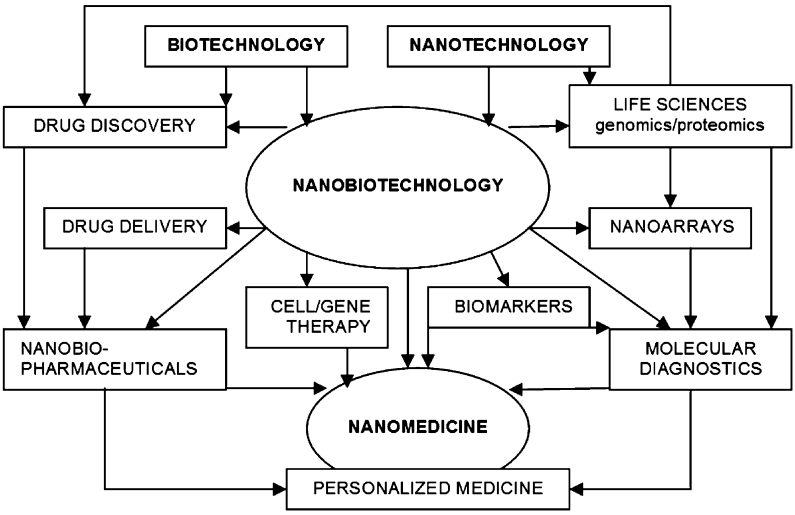

Fig. 1.1 Relationship of nanobiotechnology to nanomedicine
Source: Jain PharmaBiotech.

Table 1.3 Historical landmarks in the evolution of nanomedicine

Year	Landmark
1905	Einstein published a paper that estimated the diameter of a sugar molecular as about 1 nm
1931	Max Knoll and Ernst Ruska discovered the electron microscope—enables subnanomolar imaging
1959	Nobel Laureate Richard Feynman gave a lecture entitled "There's Plenty of Room at the Bottom," at the Annual Meeting of the American Physical Society. He outlined the principle of manipulating individual atoms using larger machines to manufacture increasingly smaller machines (Feynman 1992)
1974	Start of development of molecular electronics by Aviram and Rattner (Hush 2003)
1974	Norio Tanaguchi of Japan coined the word "nanotechnology"
1979	Colloidal gold nanoparticles used as electron-dense probes in electron microscopy and immunocytochemistry (Batten and Hopkins 1979)
1981	Conception of the idea of designing molecular machines analogous to enzymes and ribosomes (Drexler 1981)
1984	The first description the term dendrimer and the method of preparation of poly(amidoamine) dendrimers (Tomalia et al 1985)
1985	Discovery of buckyballs (fullerenes) by Robert Curl, Richard Smalley, and Harold Kroto, which led to the award of the 1996 Nobel Prize in Chemistry (Smalley 1985; Curl et al 1997)
1987	Cancer targeting with nanoparticles coated with monoclonal antibodies (Douglas et al 1987)
1987	Publication of the visionary book on nanotechnology potential *Engines of Creation* (Drexler 1987)
1988	Maturation of the field of supramolecular chemistry relevant to nanotechnology: construction of artificial molecules that interact with each other leading to award of the Nobel prize (Lehn 1988). Awarded the Nobel Prize
1990	Atoms visualized by the scanning tunneling microscope discovered in the 1980s at the IBM Zürich Laboratory (Zürich, Switzerland), which led to the award of a Nobel Prize (Eigler and Schweizer 1990)
1991	Discovery of carbon nanotubes (Iijima et al 1992)
1994	Nanoparticle-based drug delivery (Kreuter 1994)
1995	FDA approved Doxil, a liposomal formulation of doxorubicin, as an intravenous chemotherapy agent for Kaposi's sarcoma. Drug carried by nanosize liposomes is less toxic with targeted delivery
1997	Founding of the first molecular nanotechnology company—Zyvex Corporation
1998	First use of nanocrystals as biological labels, which were shown to be superior to existing fluorophores (Bruchez et al 1998)
1998	Use of DNA-gelatin nanospheres for controlled gene delivery (Truong-Le et al 1998)
1998	Use of the term "nanomedicine" in publications (Freitas 1998)
2003	National Nanotechnology Initiative announced in the United States (Roco 2003)
2000	First FDA approval of a product incorporating the NanoCrystal® technology (Elan, King of Prussia, PA, USA), a solid-dose formulation of the immunosuppressant sirolimus—Rapamune® (Wyeth)
2003	The US Senate passed the Nanotechnology Research and Development Act, making the National Nanotechnology Initiative a legal entity, and authorized
2005	FDA approved Abraxane™, a taxane based on nanotechnology, for the treatment of breast cancer. The nanoparticle form of the drug overcomes insolubility problems encountered with paclitaxel and avoids the use of toxic solvents

Source: Jain PharmaBiotech.

Chapter 2
Nanotechnologies

Introduction

This chapter will focus on nanobiotechnologies that are relevant to applications in biomedical research, diagnostics, and medicine. Invention of the microscope revolutionalized medicine by enabling the detection of microorganisms and study of histopathology of disease. Microsurgery was a considerable refinement over crude macrosurgery and opened up the possibility of using procedures that were not carried out previously or were associated with high mortality and morbidity. Nanotechnologies, by opening up the world beyond microscale, will have a similar impact on medicine and surgery. Various nanobiotechnologies are described in detail in a special report on this topic (Jain 2007e). Those relevant to understanding of diseases, diagnosis, development of new drugs, and management of diseases are described briefly in this chapter.

Classification of Nanobiotechnologies

It is not easy to classify the vast range of nanobiotechnologies. Some just represent motion on a nanoscale, but most of them are based on nanoscale structures, which come in a variety of shapes and sizes. A few occur in nature, but most are engineered. The word nano is prefixed to just about anything that deals with nanoscale. It is not just nanobiotechnology but many other disciplines such as nanophysics and nanobiology. A simplified classification of basic nanobiotechnologies is given in Table 2.1. Some technologies such as nanoarrays and nanochips are further developments.

Micro- and Nanoelectromechanical Systems

The rapid pace of miniaturization in the semiconductor industry has resulted in faster and more powerful computing and instrumentation, which have begun to revolutionize medical diagnosis and therapy. Some of the instrumentation used for nanotechnology is an extension of microelectromechanical systems (MEMS),

K.K. Jain, *The Handbook of Nanomedicine*,

Table 2.1 Classification of basic nanobiotechnologies

Nanoparticles
Quantum dots
Nanocrystals
Lipoparticles
Magnetic nanoparticles
Polymer nanoparticles
Nanofibers
Nanowires
Carbon nanofibers
Dendrimers
Polypropylenimine dendrimers
Composite nanostructures
Nanoemulsions
Nanoliposomes
Nanocapsules enclosing other substances
Nanoshells
Nanovesicles
Cochleates
Nanoconduits
Nanotubes
Nanopipettes
Nanoneedles
Nanochannels
Nanopores
Nanofluidics
Nanostructured silicon
Nanoscale motion
Cantilevers
Visualization and manipulation at nanoscale
Atomic force microscopy
Scanning probe microscopy
Nanomanipulation
Surface plasmon resonance
Femtosecond laser systems

Source: Jain PharmaBiotech.

which refers to a key enabling technology used to produce microfabricated sensors and systems. The potential mass application of MEMS lies in the fact that miniature components are batch-fabricated using the manufacturing techniques developed in the semiconductor microelectronics industry enabling miniaturized, low-cost, high-performance, and integrated electromechanical systems. The "science of miniaturization" is a much more appropriate name than MEMS as it involves a good understanding of scaling laws and different manufacturing methods and materials that are being applied in nanotechnology.

MEMS devices currently range in size from one to hundreds of micrometers and can be as simple as the singly supported cantilever beam used in atomic force microscopy (AFM) or as complicated as a video projector with thousands of electronically controllable microscopic mirrors. Nanoelectromechanical systems (NEMS) devices exist correspondingly in the nanometer realm. The concept of

using externally controllable MEMS devices to measure and manipulate biological matter (BioMEMS) on the cellular and subcellular levels has attracted much attention recently. This is because initial work has shown the ability to detect single-base-pair mismatches of DNA and to quantifiably detect antigens using cantilever systems. In addition is the ability to controllably grab and manipulate individual cells and subsequently release them unharmed.

A new approach at the California Institute of Technology, called surface nanomachining, combines the processing methods of MEMS with the tools of electron beam nanofabrication to create three-dimensional (3D) nanostructures that move (and thus can do new types of things). This opens exciting new avenues of exploration and investigations in physics and biology at very short length scales.

BioMEMS

Because BioMEMS involves the interface of MEMS with biological environments, the biological components are crucially important. To date, they have mainly been nucleic acids, antibodies, and receptors that are involved in passive aspects of detection and measurement. These molecules retain their biological activity following chemical attachment to the surfaces of MEMS structures (most commonly, thiol groups to gold) and their interactions are monitored through mechanical (deflection of a cantilever), electrical (change in voltage or current in the sensor), or optical (surface plasmon resonance [SPR]) measurements. The biological components are in the nanometer range or smaller; therefore, the size of these systems is limited by the minimum feature sizes achievable using the fabrication techniques of the inorganic structures, currently 100 nm to 1 μm. Commercially available products resulting from further miniaturization could be problematic because of the expanding cost and complexity of optical lithography equipment and the inherent slowness of electron beam techniques. In addition to size limitations, the effects of friction have plagued multiple moving parts in inorganic MEMS, limiting device speeds, and useful lifetimes (Schmidt and Montemagno 2002).

Microarrays and Nanoarrays

Arrays consist of orderly arrangements of samples, which, in the case of biochips, may be complementary DNAs (cDNAs), oligonucleotides (ODNs), or even proteins. Macroarraying (or gridding) is a macroscopic scheme of organizing colonies or DNA into arrays on large nylon filters ready for screening by hybridization. In microarrays, however, the sample spot sizes are usually $<200\,\mu m$ in diameter and require microscopic analysis. Microarrays have sample or ligand molecules (e.g., antibodies) at fixed locations on the chip, while microfluidics involves the transport of material, samples, and/or reagents on the chip.

Microarrays provide a powerful way to analyze the expression of thousands of genes simultaneously. Genomic arrays are an important tool in medical diagnostic

and pharmaceutical research. They have an impact on all phases of the drug discovery process from target identification through differential gene expression, identification and screening of small-molecule candidates, to toxicogenomic studies for drug safety. To meet the increasing needs, the density and information content of the microarrays is being improved. One approach is fabrication of chips with smaller, more closely packed features—ultrahigh-density arrays that will yield

- high information content by reduction of feature size from 200 μm to 50 nm;
- reduction in sample size; and
- improved assay sensitivity.

Nanoarrays are the next stage in the evolution of miniaturization of microarrays. Whereas microarrays are prepared by robotic spotting or optical lithography, limiting the smallest size to several microns, nanoarrays require further developments in lithography such as dip-pen nanolithography (DPN). Technical discussion of these is beyond the scope of this work.

Protein Nanoarrays

High-throughput protein arrays allow the miniaturized and parallel analysis of large numbers of diagnostic markers in complex samples. This capability can be enhanced by nanotechnology. DPN technique has been extended to protein arrays with features as small as 45 nm and immunoproteins as well as enzymes can be deposited. Selective binding of antibodies to protein nanoarrays can be detected without the use of labels by monitoring small (5–15 nm) topographical height increases in AFM images.

BioForce Nanosciences' Protein Nanoarrays contain up to 25 million spots per square centimeter. They can be used to detect protein–protein interactions. BioForce's NanoReader uses a customized AFM to decipher molecules on a NanoArray chip. The company was awarded a grant in 2002 by the US Department of Defense for breast cancer research titled, "Protein Nanoarrays for Studying Malignant Progression in Breast Cancer Cell Lines." The project aims to construct a nanoscale protein array platform and use it as a basic research tool to study alterations in cellular signaling pathways that accompany breast cancer disease progression.

Microfluidics and Nanofluidics

Microfluidics is the handling and dealing with small quantities (e.g., microliters, nanoliters, or even picoliters) of fluids flowing in channels the size of a human hair (~50 μ m thick) even narrower. Fluids in this environment show very different properties than in the macro world. This new field of technology was enabled by advances in microfabrication—the etching of silicon to create very small features. Microfluidics is one of the most important innovations of biochip technology. Typical

dimensions of microfluidic chips are 1–50 cm^2 and have channels 5–100 μm. Usual volumes are 0.01–10 μl but can be less. Microfluidics is the link between microarrays and nanoarrays as we reduce the dimensions and volumes.

Microfluidics is the underlying principle of lab-on-a-chip devices, which carry out complex analyses, while reducing sample and chemical consumption, decreasing waste, and improving precision and efficiency. The idea is to be able to squirt a very small sample into the chip, push a button and the chip will do all the work, delivering a report at the end. Microfluidics allows the reduction in size with a corresponding increase in the throughput of handling, processing, and analyzing the sample. Other advantages of microfluidics include increased reaction rates, enhanced detection sensitivity, and control of adverse events.

Drawbacks and limitations of microfluidics and designing of microfluidic chips include the following:

- Difficulties in microfluidic connections
- Because of laminar flows, mixing can only be performed by diffusion
- Large capillary forces
- Clogging
- Possible evaporation and drying up of the sample

Applications of microfluidics include the following:

- DNA analysis
- Protein analysis
- Gene expression and differential display analysis
- Biochemical analysis

Nanotechnology on a Chip

Nanotechnology on a chip is a new paradigm for total chemical analysis systems. The ability to make chemical and biological information much cheaper and easier to obtain is expected to fundamentally change healthcare, food safety, and law enforcement. Lab-on-a-chip technology involves micro-total analysis systems that are distinguished from simple sensors because they conduct a complete analysis; a raw mixture of chemicals goes in and an answer comes out. Sandia National Laboratories is developing a handheld lab-on-a-chip that will analyze for airborne chemical warfare agents and liquid-based explosives agents. This development project brings together an interdisciplinary team with areas of expertise including microfabrication, chemical sensing, microfluidics, and bioinformatics. Although nanotechnology plays an important role in current efforts, miniaturized versions of conventional architecture and components such as valves, pipes, pumps, and separation columns are patterned after their macroscopic counterparts. Nanotechnology will provide the ability to build materials with switchable molecular functions that could provide completely new approaches to valves, pumps, chemical separations, and detection. For example, fluid streams could be directed by controlling surface energy

without the need for a predetermined architecture of physical channels. Switchable molecular membranes and the like could replace mechanical valves. By eliminating the need for complex fluidic networks and microscale components used in current approaches, a fundamentally new approach will allow greater function in much smaller, lower-power total chemical analysis systems.

A new scheme for the detection of molecular interactions based on optical readout of nanoparticle labels has been developed. Capture DNA probes were arrayed on a glass chip and incubated with nanoparticle-labeled target DNA probes, containing a complementary sequence. Binding events were monitored by optical means, using reflected and transmitted light for the detection of surface-bound nanoparticles. Control experiments exclude significant influence of nonspecific binding on the observed contrast. Scanning force microscopy revealed the distribution of nanoparticles on the chip surface.

Nanoarrays, ultraminiaturized versions of the traditional microarrays, can actually measure interactions between individual molecules down to resolutions of as little as 1 nm. Nanoarrays are the next evolutionary step in the miniaturization of bioaffinity tests for proteins and nucleic acids.

Use of Nanotechnology in Microfluidics

Construction of Nanofluidic Channels

Techniques such as nanoimprinting are used to construct large arrays of nanoscale grooves with efficiency and speed. Now such grooves can be sealed with similar ease, to form nanofluidic channels. Laser-assisted direct imprint techniques enable the construction of millions of enclosed nanofluidic channels side by side on a single substrate, which is ideal for such parallel processing. Other methods have been used to fabricate flexible tubes whose diameters are 100 nm, i.e., 10 times narrower than those used in current microfluidic systems. The tubes could be used to make stacked, interconnected fluidic networks designed to shunt fluids around biochips that sense and analyze chemicals.

Restriction mapping of DNA molecules has been performed using restriction endonucleases in nanochannels with diameters of 100–200 nm (Riehn et al 2005). Restriction mapping with endonucleases is a central method in molecular biology. It is based on the measurement of fragment lengths after digestion, while possibly maintaining the respective order. The location of the restriction reaction within the device is controlled by electrophoresis and diffusion of Mg^{2+} and EDTA. It is possible to measure the positions of restriction sites with precision using single DNA molecules with a resolution of 1.5 kbp within 1 min.

Most of the microscale flow visualization methods evolved from methods developed originally for macroscale flow. It is unlikely, however, that developed microscale flow visualization methods will be translated to nanoscale flows in a similar manner. Resolving nanoscale features with visible light presents a fundamental challenge. Although point-detection scanning methods have potential to increase

the flow measurement resolution on the microscale, spatial resolution is ultimately limited by the optical probe volume (length scale on the order of 100 nm), which, in turn, is limited by the wavelength of light employed (Sinton 2004). Optical spatially resolved flow measurements in nanochannels are difficult to visualize. There is a need for refinement of microscale flow visualization methods and the development of direct flow measurement methods for nanoflows.

Moving (Levitation) of Nanofluidic Drops with Physical Forces

The manipulation of droplets/particles that are isolated (levitated in gas/vacuum) from laboratory samples containing chemicals, cells, bacteria, or viruses is important both for basic research in biology and biochemistry, as well as for application in nanodiagnostics.

Microfluidic drops can be moved with light—the lotus effect (Rosario et al 2004). On a super-rough surface, when light shines on one side of a drop, the surface changes, the molecules switch and the drop moves. Called digital microfluidics, this approach enables one to quickly move small drops around by shining light on them. This technology has potential applications in drug screening as it can be used for quickly analyzing and screening small amounts of biological materials. Hundreds of screens could be done on only one particular surface. The molecules, e.g. protein traces, do not interfere with movements of the drops because the surfaces are hydrophobic and the molecules have little contact with the surface.

The size of diamagnetic levitation devices has been reduced by using micron-scale permanent magnets to create a magnetic micromanipulation chip, which operates with femtodroplets levitated in air (Lyuksyutov et al 2004). The droplets used are 1 billion times smaller in volume than has been demonstrated by conventional methods. The levitated particles can be positioned with up to 300 nm accuracy and precisely rotated and assembled. Using this lab-on-a-chip, it might be possible to do the same thing with a large number of fluids, chemicals, and even red blood cells (RBCs), bacteria, and viruses.

Nano-Interface in a Microfluidic Chip

There are emerging experimental and conceptual platforms for probing living cells with nanotechnology-based tools in a microfluidic chip. Considerable advances have been made in measuring nanoscale mechanical, biochemical, and electrical interactions at the interface between biomaterials and living cells. By merging the fields of microfluidics, electrokinetics, and cell biology, microchips are capable of creating tiny, mobile laboratories. The challenge for the future of designing a nano-interface in a microfluidic chip to probe a living cell lies in seamlessly integrating techniques into a robust and versatile, yet reliable, platform (Helmke and Minerick 2006). Potential benefits of nanosystems on a microchip result from real-time detection of numerous events in parallel. In addition to early detection of cell-level dysfunctions, these systems will enable broad screening that encompasses not just a large number of toxic stimuli and disease processes but also population subgroups. This

will facilitate the development of personalized medicine. To reach this goal requires advancing the knowledge base of cellular and subcellular functions, perhaps by designing nanosystems that operate in the tissue milieu.

Visualization and Manipulation on Nanoscale

Atomic Force Microscopy

Basic AFM Operation

In its most basic form, atomic force microscope (AFM) images topography by precisely scanning a probe across the sample to "feel" the contours of the surface. The interaction between the needle and the surface is measured and an image is reconstructed from the data collected in this manner. With AFM, it is possible it is possible to reach an extremely high resolution. Because it can be applied under standard conditions in an aqueous environment, any significant perturbation of the sample can be avoided. In contrast to light microscopy and scanning electron microscopy (SEM), AFM provides the most optimal means to investigate the surface structures in three dimensions, with resolutions as high as 0.1–0.2 nm.

A key element of the AFM is its microscopic force sensor, or cantilever. The cantilever is usually formed by one or more beams of silicon or silicon nitride that is 100–500 μm long and about 0.5–5 μm thick. Mounted on the end of the cantilever is a sharp tip that is used to sense a force between the sample and the tip. For normal topographic imaging, the probe tip is brought into continuous or intermittent contact with the sample and raster-scanned over the surface.

Advantages of AFM

In addition to its superior resolution and routine 3D measurement capability, AFM offers several other clear advantages over traditional microscopy techniques. For example, scanning and transmission electron microscopy (SEM, TEM) image biologically inactive, dehydrated samples and generally require extensive sample preparation such as staining or metal coating. AFM eliminates these requirements and, in many cases, allows direct observation of native specimens and ongoing processes under native or near-native conditions.

Further adding to its uniqueness, the AFM can directly measure nanoscale interactive forces, e.g., ligand–receptor binding. Samples can be examined in ambient air or biological fluids without the cost and inconvenience of vacuum equipment. Sample preparation is minimal and allows the use of standard techniques for optical microscopy. The MultiMode AFM provides maximal resolution, while the BioScope AFM integrates the best of optical and AFM to help life scientists explore new frontiers.The ability of the ADM to create 3D micrographs with resolution down to the nanometer and angstrom scales has made it an essential tool for imaging surfaces in applications ranging from semiconductor processing to cell biology. In addition

to this topographical imaging, however, the AFM can also probe nanomechanical and other fundamental properties of sample surfaces, including their local adhesive or elastic (compliance) properties.

Microscopic adhesion affects a huge variety of events, from the behavior of paints and glues, ceramics, and composite materials, to DNA replication and the action of drugs in the human body. Elastic properties are similarly important, often affecting the structural and dynamic behavior of systems from composite materials to blood cells. AFM offers a new tool to study these important parameters on the micron to nanometer scale using a technique that measures forces on the AFM probe tip as it approaches and retracts from a surface.

Force-Sensing Integrated Readout and Active Tip

Researchers at the Georgia Institute of Technology (Atlanta, GA) have created force-sensing integrated readout and active tip (FIRAT), an extremely sensitive AFM technology that is capable of high-speed imaging 100 times faster than current AFM technology. Current AFM scans surfaces with a thin cantilever with a sharp tip at the end. An optical beam is bounced off the cantilever tip to measure the deflection of the cantilever as the sharp tip moves over the surface and interacts with the material being analyzed. FIRAT works a bit like a cross between a pogo stick and a microphone. In one version of the probe, the membrane with a sharp tip moves toward the sample, and just before it touches, it is pulled by attractive forces. Much like a microphone diaphragm picks up sound vibrations, the FIRAT membrane starts taking sensory readings well before it touches the sample. And when the tip hits the surface, the elasticity and stiffness of the surface determines how hard the material pushes back against the tip. So rather than just capturing a topography scan of the sample, FIRAT can pick up a wide variety of other material properties.

FIRAT can capture additional measurements not possible before with AFM, including parallel molecular assays for drug screening and discovery, as well as material property imaging. This research breakthrough could prove invaluable for many types of nano research, including translating into movies of molecular interactions in real time. FIRAT might eventually replace AFM.

Magnetic Resonance Force Microscopy

IBM has been working over a decade to develop nanoscale magnetic resonance imaging (MRI) technology called magnetic resonance force microscopy (MRFM). The company claimed a breakthrough in nanoscale MRI by directly detecting for the first time a faint magnetic signal from single electrons buried inside solid samples. The development represents a major milestone in the creation of a microscope that can make 3D images of molecules with atomic resolution. Such a device could have a major impact on the study of materials, ranging from proteins and pharmaceuticals

to integrated circuits for which a detailed understanding of the atomic structure is essential.

Knowing the exact location of specific atoms within tiny nanoelectronic structures, for example, could enhance circuit and chip designers' insight into their manufacture and performance, according to IBM. The ability to image the detailed atomic structure of proteins directly would also aid the development of new drugs. This new capability should ultimately lead to fundamental advances in nanotechnology and biology.

The central feature of MRFM is a silicon "microcantilever" that looks like a miniature diving board and is 1,000 times thinner than a human hair. It vibrates at a frequency of ~5,000 times a second, and a tiny but powerful magnetic particle attached to the tip attracts or repels individual electrons. Such technology aims to boost MRI sensitivity by some 10 million times when compared with the medical devices currently used to visualize organs in the human body.

An ultrahigh magnetic force microscopy is provided by SwissProbe developed at the Institute of Physics of the University of Basel, Switzerland. The magnetic sensors are crucial for high-resolution magnetic force microscopy. They are coated with suitably layered materials.

Scanning Probe Microscopy

The scanning probe microscope (SPM) system is emerging as an increasingly important tool for nonintrusive interrogation of biomolecular systems in vitro. Its particular merit is that it retains complete functionality in a biocompatible fluid environment and can track the dynamics of cellular and molecular processes in real time and real space at nanometer resolution, as an imaging tool, and with piconewton force-sensing/imposing resolution, as an interaction tool (Myhra 2004). The capability may have relevance as a test bed for monitoring cellular response to environmental stimuli and pharmaceutical intervention. The better-known recent contributions of SPM are toward explanatory and predictive descriptions of biomolecular interactions at surfaces and interfaces, and there are some recent attempts to reconfigure the SPM platform for demonstration of novel biodevice applications.

SPM enables high resolution without any of the drawbacks of electron microscopy, which can damage sensitive molecules by electrons. SPM enables investigation of biomolecules in fluid environments under physiological conditions and is useful for the study of biology at the nanoscale level.

Near-Field Scanning Optical Microscopy

Near-field scanning optical microscopy (NSOM) was the first technique that has overcome the limits of light microscopy by about 1 order of magnitude. Typically, the resolution range <100 nm is accessed for biological applications. Using

appropriately designed scanning probes allows for obtaining an extremely small near-field light excitation volume (some tens of nanometers in diameter). Because of the reduction of background illumination, high-contrast imaging becomes feasible for light transmission and fluorescence microscopy. The height of the scanning probe is controlled by atomic force interactions between the specimen surface and the probe tip. The control signal can be used for the production of a topographic (nonoptical) image that can be acquired simultaneously. The principle of NSOM with respect to biological applications, particularly study of the chromosomes, are described elsewhere (Hausmann et al 2006).

Scattering-type scanning near-field optical microscopy (s-SNOM) can determine infrared "fingerprint" spectra of individual poly(methyl methacrylate) nanobeads and viruses as small as 18 nm (Brehm et al 2006). Amplitude and phase spectra are found surprisingly strong, even at a probed volume of only 10^{-20} l, and robust in regard to particle size and substrate. This makes infrared spectroscopic s-SNOM a versatile tool for chemical and protein secondary structure identification.

Multiple Single-Molecule Fluorescence Microscopy

Fitting the image of a single molecule to the point spread function of an optical system greatly improves the precision with which single molecules can be located. In nanometer-localized multiple single-molecule (NALMS) fluorescence microscopy, short duplex DNA strands are used as nanoscale "rulers" for validation (Qu et al 2004). Nanometer accuracy was demonstrated for two to five single molecules within a diffraction-limited area. NALMS microscopy will greatly facilitate single-molecule study of biological systems because it covers the gap between fluorescence resonance energy transfer (FRET)-based (<10 nm) and diffraction-limited microscopy (>100 nm) measurements of the distance between two fluorophores. NALMS microscopy has been applied to DNA mapping with <10-nm resolution.

Nanoparticle Characterization by Halo^{TM}LM10 Technology

Halo^{TM} LM10 (NanoSight Ltd) is based on the laser illumination of a specially designed optical element on to which sample is simply placed manually or allowed to flow across the surface. This is the first nanoparticle characterization tool, specifically designed for liquid phase sizing of individual nanoparticles, with the use of a conventional light microscope. Particles as small as 20 nm have been successfully visualized by this method, each particle being seen as an individual point of light moving under Brownian motion within the liquid. The intensity of light scattered by a particle varies as the sixth power of its radius. By doubling the diameter of the particle, 64-fold more light is scattered by the particle. This has significant implications for the early and simple detection of aggregation, flocculation, and dimerization of particulates at the nanometer scale.

Use of a shorter-wavelength laser source capable of exciting fluorescent labels enables specific components within the sample to be distinguished from nonspecific background particles. The image can be analyzed by suitable software allowing changes in individual particle position to be followed, furnishing real-time information about particle diffusion and particle–particle interactions. In the fluorescence mode, correlation techniques can be used to derive information by use of the technique known as fluorescence correlation spectroscopy. Halo^{TM} LM10 is supported by Halo^{TM} GS10 software.

The laser source need only be a few milliwatts in power and can be delivered to the optical device via fiber-optic connection or the laser diode can be coupled directly to the edge of the optical element. The optical element can be manufactured in optical-quality plastic or in glass or silica. The optical element need only be a few millimeters square and 2–5 mm in depth.

Larger volumes of sample containing dilute concentrations of particles of interest can be analyzed by being configured within a flow cell. Fabrication of the optical element is by industry standard metal coating techniques such as those found in the electronics and optical devices industries. Applications relevant to nanobiotechnology are listed in Table 2.2.

Nanoscale Scanning Electron Microscopy

Pharmaceutical enterprises require a range of imaging products that provide high-quality information, allowing them to reach their own targets on technology, productivity, and ultimately profitability. With the increasing expectations upon drug delivery systems for efficient and controlled delivery of the active material, there is a matching need for accurate information on these mechanisms. One of the most effective instruments in this area is a scanning electron microscope (SEM)

Table 2.2 Applications of optical nanoscopy

Molecular diagnostics
Detection of viral particles
DNA analysis
Mycoplasma detection in animal cell culture
Contaminant monitoring
Drug delivery
Drug carriers
Monitoring drug efficacy in body fluids
Biofilm production and implants
Nanoparticles
Environmental
Biodefense
Airborne contaminants such as asbestos particles
Medical
Clinical diagnosis of viral diseases, e.g., cerebrospinal fluid
Cancer cell detection, e.g., metastases

Source: Nanosight Ltd.

from Carl Zeiss (Oberkochen, Germany) that has a unique ability to provide high-resolution images of a specimen under investigation. One example of the application of the EVO® EP instrument (Carl Zeiss) is in the manufacture of aspirin. The interaction of water with soluble aspirin demonstrates the mechanisms by which tablets lose mechanical strength and stability and hence release the active material. This process can be observed in real time in the SEM by introducing water vapor into the chamber at sufficiently high pressures that liquid water is condensed onto the specimen. During the wetting phase the particle absorbs water and fragments. During the drying phase the reverse processes can be followed in detail.

The latest product from Carl Zeiss, ULTRA 55 field emission SEM features a totally new "Complete Detection System," which enables simultaneous surface, compositional, and crystallographic imaging down to the nanometer level with high signal contrast and unsurpassed clarity.

Use of SEM to Reconstruct Three-Dimensional Tissue Nanostructure

Three-dimensional structural information is important in biological research. Excellent methods are available to obtain structures of molecules at atomic, organelles at electron microscopic, and of tissues at light-microscopic resolution. However, there is a need to reconstruct 3D tissue structure with a nanoscale resolution to identify small organelles such as synaptic vesicles. Such 3D data are essential to understand cellular networks that need to be completely reconstructed throughout a substantial spatial volume, particularly in the nervous system. Datasets meeting these requirements can be obtained by automated blockface imaging combined with serial sectioning inside the chamber of an SEM (Denk and Horstmann 2004). Backscattering contrast is used to visualize the heavy-metal staining of tissue prepared using techniques that are routine for TEM. The resolution is sufficient to trace even the thinnest axons and to identify synapses. Stacks of several hundred sections, 50–70 nm thick, have been obtained at a lateral position jitter of typically under 10 nm. This opens up the possibility of automatically obtaining the electron-microscope-level 3D datasets needed to completely reconstruct the neuronal circuits.

Visualizing Atoms with Scanning Electron Microscopy

Despite the use of electrons with wavelengths of just a few picometers, spatial resolution in a transmission electron microscope (TEM) has been limited by spherical aberration to typically $\sim$0.15 nm. Individual atomic columns in a crystalline lattice can therefore only be imaged for a few low-order orientations, limiting the range of defects that can be imaged at atomic resolution. A new technology, called aberration correction, fixes imperfections on the microscope's electron lenses. A 300-kV state-of-the-art electron microscope was aided by new computerized imaging technology developed by Nion Co. (Kirkland, WA). Oak Ridge National Laboratory researchers have presented direct images from this aberration-corrected scanning TEM that resolves a lattice in which the atomic columns are separated by $<$0.1 nm (1 Å) and can distinguish the individual, dumbbell-shaped atoms of a silicon crystal (Nellist et al

2004). To be able to see how materials bond together at an atomic level could prove a significant benefit to the semiconductor industry and chemistry. The next frontier will be seeing atoms in three dimensions.

Photoactivated Localization Microscopy

Photoactivated localization microscopy (PALM), a new light microscope developed at Howard Hughes Medical Institute in collaboration with researchers at the NIH and Florida State University, is so powerful that it allows scientists peering inside cells to discern individual protein at ~2 to 25 nm resolution (Betzig et al 2006). The basic concepts behind their new technique are simple. The researchers label the molecules they want to study with a photoactivatable probe, and then expose those molecules to a small amount of violet light. The light activates fluorescence in a small percentage of molecules, and the microscope captures an image of those that are turned on until they bleach. The process is repeated ~10,000 times, with each repetition capturing the position of a different subset of molecules. When a final image is created that includes the center of each individual molecule, it has a resolution previously only achievable with an electron microscope. Unlike electron microscopy, however, the new technique allows for more flexibility in labeling molecules of interest. The method is demonstrated in thin sections by imaging specific target proteins in lysosomes and mitochondria, and in fixed, whole cells by imaging vinculin at focal adhesions, actin within a lamellipodium, and the distribution of the retroviral protein Gag at the plasma membrane. A great feature of PALM is that it can readily be used with electron microscopy, which produces a detailed image of very small structures, but not proteins, in cells. By correlating a PALM image showing protein distribution with an electron microscope image showing cell structure of the same sample, it becomes possible to understand how molecules are individually distributed in a cellular structure at the molecular scale. Correlative PALM unites the advantages of light and electron microscopy, producing a revolutionary new approach for looking at the cell in molecular detail. As the PALM technology advances, it may prove to be a key factor in unlocking at the molecular level secrets of intracellular dynamics that are unattainable by other methods. However, the time needed to collect the thousands of single-molecule images that go into each PALM picture is cumbersome. With the camera snapping one to two pictures each second, it can take 2–12 h to image a single sample. Activating more molecules per frame would reduce the number images that must be collected, and making the molecules brighter would reduce the time needed to take each image. Either would help to speed up the PALM process. The technique is still undergoing refinements with an aim of developing a practical tool for use by biologists.

Optical Imaging with a Silver Superlens

Scientists at the University of California (Berkeley, CA) have created a superlens that can overcome a limitation in physics that has historically constrained the

resolution of optical images (Fang et al 2005).Using a thin film of silver as the lens and ultraviolet (UV) light, the researchers recorded the images of an array of nanowires at a resolution of ~60 nm, whereas current optical microscopes can only make out details down to 400 nm. This work has a far-reaching impact on the development of detailed biomedical imaging. With current optical microscopes, scientists can only make out relatively large structures within a cell, such as its nucleus and mitochondria. With a superlens, optical microscopes could reveal the movements of individual proteins traveling along the microtubules that make up a cell's skeleton. SEM and AFM are now used to capture detail down to a few nanometers. However, such microscopes create images by scanning objects point by point, which means they are typically limited to nonliving samples, and image capture times can take up to several minutes. Optical microscopes can capture an entire frame with a single snapshot in a fraction of a second, opening up nanoscale imaging to living materials, which can help biologists better understand cell structure and function in real time, and ultimately help in the development of new drugs to treat human diseases.

Fluorescence Resonance Energy Transfer

FRET is a process by which energy that would normally be emitted as a photon from an excited fluorophore can be directly transferred to a second fluorophore to excite one of its electrons. This, on decay, then generates an even longer wavelength photon. The extent of FRET is critically dependent on the distance between the two fluorophores as well as their spectral overlap. Thus FRET is a powerful reporter of the separation of the two fluorophores. FRET is a simple but effective tool for measurements of protein–protein interactions. It is one of the few techniques that are capable of giving dynamic information about the nanometer-range proximity between molecules, as opposed to simply the subcellular colocalization that is provided by fluorescence microscopy.

4Pi Microscope

The most prominent restrictions of fluorescence microscopy are the limited resolution and the finite signal. Established conventional, confocal, and multiphoton microscopes resolve at best ~200 nm in the focal plane and only 500 nm in depth.

4Pi microscope (Leica Microsystems, Wetzlar, Germany) uses a special phase- and wavefront-corrected double-objective imaging system linked to a confocal scanner to enable 4- to 7-fold increased axial resolution over confocal and two-photon microscope. Even in living specimens, axial sections of ~100 nm are obtained. The system maintains all advantages of fast scanning, Acousto-Optical Beam Splitting (AOBS®), and Spectral Detection of the Leica TCS SP2 AOBS for routine operation. The first marked leap in resolution in commercial 3D fluorescence

microscopy opens up new dimensions for research in cell and developmental biology. Colocalization studies of immunolabeled microtubules and mitochondria demonstrate the feasibility of 4Pi microscopy for routine biological measurements, in particular, to visualize the 3D entanglement of the two networks with unprecedented detail (Medda et al 2006).

Principle and Applications of Cantilevers

Cantilevers (Concentris, Basel, Switzerland) transform a chemical reaction into a mechanical motion on the nanometer scale. Measurements of a cantilever are as follows: length 500 μm, width 100 μm, thickness 25–500 μm, and deflection 10 nm. This motion can be measured directly by deflecting a light beam from the cantilever surface. Concentris uses an array of parallel vertical cavity surface-emitting lasers (VCSELs) as stable, robust, and proven light source. A state-of-the-art position-sensitive detector is employed as a detection device.

The static mode is used to obtain information regarding the presence of certain target molecules in the sample substance. The surface stress caused by the adsorption of these molecules results in minute deflections of the cantilever. This deflection directly correlates with the concentration of the target substance. The dynamic mode allows quantitative analysis of mass loads in the subpicogram area. As molecules get adsorbed, minimal shifts in the resonance frequency of an oscillating cantilever can be measured and associated to reference data of the target substance. Both modes can also be operated simultaneously.

The controlled deposition of functional layers is the key to converting nanomechanical cantilevers into chemical or biochemical sensors. Inkjet printing is a rapid and general method to coat cantilever arrays efficiently with various sensor layers (Bietsch et al 2004). Self-assembled monolayers of alkanethiols are deposited on selected Au-coated cantilevers and rendered sensitive to ion concentrations or pH in liquids. The detection of gene fragments is achieved with cantilever sensors coated with thiol-linked single-stranded DNA oligomers on Au. A selective etch protocol proves the uniformity of the monolayer coatings at a microscopic level. A chemical gas sensor is fabricated by printing thin layers of different polymers from dilute solutions onto cantilevers. The inkjet method is easy to use, faster, and more versatile than coating via microcapillaries or the use of pipettes. In addition, it is scalable to large arrays and can coat arbitrary structures in noncontact.

The applications of cantilever technology, Cantosens (Concentris), are listed in Table 2.3 and discussed further in Chapter 3.

Surface Plasmon Resonance

SPR is an optical–electrical phenomenon involving the interaction of light with the electrons of a metal. The optical–electronic basis of SPR is the transfer of the energy

Table 2.3 Applications of cantilever technology

Basic research
Study of chemical reactions or host–guest interactions on surfaces
Nanocalorimetry
Medical diagnostics
Parallel and label-free detection of disease markers, e.g., serum proteins or autoantibodies
Fast, label-free recognition of specific DNA sequences (SNPs, oncogenes, genotyping)
Detection of microorganisms and antimicrobial susceptibility
Drug discovery and life sciences research
Label-free biochemical assays and investigation of biomolecular interactions
Multiplexed assays
Process and quality control
Process monitoring
Purity analysis
Food analysis
Detection of trace contaminations, e.g., antibiotics, hormones, pesticides
Detection of microorganisms
Identification and quality control
Environmental monitoring
Detection of heavy-metal ions, pesticides, air pollutants
Water analysis
Fragrance and flavor analysis
Using neural networks to analyze cantilever sensor array signals can identify and characterize complex chemical mixtures (“electronic nose” or “tongue”)
Security devices
Detection of potentially hazardous chemicals and microorganisms
Workplace security

Source: Concentris GmbH.

carried by photons of light to a group of electrons (a plasmon) at the surface of a metal. Light is coupled into the surface plasmon by means of either a prism or a grating on the metal surface. Depending on the thickness of a molecular layer at the metal surface, the SPR phenomenon results in a graded reduction in the intensity of the reflected light. Biomedical applications take advantage of the exquisite sensitivity of SPR to the refractive index of the medium next to the metal surface, which makes it possible to measure accurately the adsorption of molecules on the metal surface and their eventual interactions with specific ligands. Applications of this technique include the following:

- Measurement in real time of the kinetics of ligand–receptor interactions
- Screening of lead compounds in the pharmaceutical industry
- Measurement of DNA hybridization
- Enzyme–substrate interactions
- Polyclonal antibody characterization

- Protein conformation studies
- Label-free immunoassays

Nanoparticles

Nanoparticles form the bulk of nanomaterials. Various types of nanoparticles were listed in the classification in Table 2.1. They can be made of different materials, e.g., gold. A nanoparticle contains tens to thousands of atoms and exists in a realm that straddles the quantum and the Newtonian. At those sizes every particle has new properties that change depending on its size. As matter is shrunk to nanoscale, electronic and other properties change radically. Nanoparticles may contain unusual forms of structural disorder that can significantly modify material properties and thus cannot solely be considered as small pieces of bulk material. Two nanoparticles, both made of pure gold, can exhibit markedly different behavior—different melting temperature, different electrical conductivity, different color—if one is larger than the other. That creates a new way to control the properties of materials. Instead of changing composition, one can change size. Some applications of nanoparticles take advantage of the fact that more surface area is exposed when material is broken down to smaller sizes. For magnetic nanoparticles, the lack of blemishes produces magnetic fields remarkably strong considering the size of the particles. Nanoparticles are also so small that in most of them the atoms line up in perfect crystals without a single blemish.

Zinc sulfide nanoparticles a mere 10 atoms across have a disordered crystal structure that puts them under constant strain, increasing the stiffness of the particles and probably affecting other properties, such as strength and elasticity. In similar semiconducting nanoparticles, such as those made of cadmium selenide, slight differences in size lead to absorption and emission of different wavelengths of light, making them useful as fluorescent tracers. The dominant cause of such properties is quantum mechanical confinement of the electrons in a small package. But the disordered crystal structure now found in nanoparticles could affect light absorption and emission also. X-ray diffraction of single nanoparticles is not yet possible and other methods are used to analyze x-ray diffraction images of nanoparticles so as to separate the effects of size from those of disordered structure.

Further description of nanoparticles will be given along with their applications in various chapters. The best known of the nanoparticles are quantum dots (QDs).

Quantum Dots

QDs are nanoscale crystals of semiconductor material that glow, or fluoresce when excited by a light source such as a laser. QD nanocrystals of cadmium selenide 200–10,000 atoms wide, coated with zinc sulfide. The size of the QD determines the frequency of light emitted when irradiated with low energy light. The QDs

were initially found to be unstable and difficult to use in solution. Work at Indiana University (Bloomington, IN) showed that embedding the dots in pores of a latex bead made them more stable. Multicolor optical coding for biological assays has been achieved by embedding different-sized QDs into polymeric microbeads at precisely controlled ratios. Their novel optical properties (e.g., size-tunable emission and simultaneous excitation) render these highly luminescent QDs ideal fluorophores for wavelength-and-intensity multiplexing. The use of 10 intensity levels and 6 colors could theoretically code 1 million nucleic acid or protein sequences. Imaging and spectroscopic measurements indicate that the QD-tagged beads are highly uniform and reproducible, yielding bead identification accuracies as high as 99.99% under favorable conditions. DNA hybridization studies demonstrate that the coding and target signals can be simultaneously read at the single-bead level. This spectral coding technology is expected to open new opportunities in gene expression studies, high-throughput screening, and medical diagnostics.

A scalable method has been reported for controlled synthesis of luminescent QDs using microemulsion-gas contacting at room temperature (Karanikolos et al 2004). The technique exploits the dispersed phase of a microemulsion to form numerous identical nanoreactors. In this approach, ZnSe QDs are synthesized by reacting hydrogen selenide gas with diethylzinc dissolved in the heptane nanodroplets of a microemulsion formed by self-assembly of a poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide) amphiphilic block copolymer in formamide. A single nanocrystal is grown in each nanodroplet, thus allowing good control of particle size by manipulation of the initial diethylzinc concentration in the heptane. The ZnSe nanocrystals exhibit size-dependent luminescence and excellent photostability. The particle-loaded emulsions are very stable in storage for months.

QD materials are costly and inconvenient for biomedical laboratories, as synthesis requires high-temperature techniques. A new synthesis developed at Indiana University–Purdue University makes use of room-temperature sonochemistry to generate QDs that span the full range of colors in the visible spectrum. This new, low-temperature procedure may also facilitate the large-scale synthesis of QDs and allow inclusion of temperature-sensitive materials in the synthesis procedure itself.

Latex beads filled with several colors of nanoscale semiconductor QDs can serve as unique labels for any number of different probes. When exposed to light, the beads identify themselves and their linked probes by emitting light in a distinct spectrum of colors—a sort of spectral barcode. The shape and size of QDs can be tailored to fluoresce specific colors. Current dyes used for lighting up protein and DNA fade quickly, but QDs could allow tracking of biological reactions in living cells for days or longer.

QDs can also be placed in a strong magnetic field, which gives an electron on the dot two allowed energy states separated by an energy gap that depends on the strength of the field. The electron can jump the gap by absorbing a photon of precisely that energy, which can be tuned, by altering the field, to correspond with the energy of a far-infrared photon. Once it is excited by absorption of a photon, the electron can leap onto the terminal of a single-electron transistor, where it “throws the switch” and is detected.

Due to their sheer brightness and high photostability, QDs have the ability to act as molecular beacons. When attached to compounds or proteins of interest, QDs enable researchers to track movements within biological media or whole organisms, significantly impacting the way medical professionals study, diagnose, and treat diseases. Applications of QDs include the following:

- Life sciences research—tracking proteins in living cells
- Fluorescence detection—microscopy, biosensors, multicolor flow cytometry
- Molecular diagnostics
- Ex vivo live cell imaging
- In vivo targeting of cells, tissues, and tumors with monitoring by PET and MRI
- High-throughput screening

Recent examples of their experimental use include the observation of diffusion of individual glycine receptors in living neurons and the identification of lymph nodes in live animals by near-infrared (NIR) emission during surgery. The new generations of QDs have far-reaching potential for the study of intracellular processes at the single-molecule level, high-resolution cellular imaging, long-term in vivo observation of cell trafficking, tumor targeting, and diagnostics. Best-known commercial preparations is Qdot^{TM} (Invitrogen, Carlsbad, CA, USA).

Gold Nanoparticles

DNA molecules are attached to gold nanoparticles, which tangle with other specially designed pieces of DNA into clumps that appear blue. The presence of lead causes the connecting DNA to fall apart. That cuts loose the individual gold nanoparticles and changes the color from blue to red. Gold nanoparticles are also used as a connecting point to build biosensors for detection of disease. A common technique for a diagnostic test consists of an antibody attached to a fluorescent molecule. When the antibody attaches to a protein associated with the disease, the fluorescent molecule lights up under UV light. Instead of a fluorescent molecule, a gold nanoparticle can be attached to the antibody and other molecules such as DNA can be added to the nanoparticle to produce barcodes. Because many copies of the antibodies and DNA can be attached to a single nanoparticle, this approach is much more sensitive and accurate than the fluorescent-molecule tests used currently.

Silica Nanoparticles

In the case of silica, the formation of diatom shell or sponge spicule has attracted much attention in the last decade since it could provide key information to elaborate new hierarchically structured materials and nanodevices. The mineral phase is thought to be formed by the controlled assembly of nanoparticles generated in vivo from diluted precursor solutions, in the presence of biomolecular templates.

Biomolecules present in silicifying organisms have been extracted and identified (Lopez et al 2005). Silicon particles vary in size from 25 to 1,000 nm. Biomimetic approaches have led to the identification of several natural or synthetic molecules that are able to activate silica formation in conditions that closely resemble those found in the intracellular compartments of living organisms. Additionally, several of these systems are able to form silica nanoparticles whose size range and limited polydispersity reproduce colloidal biosilica. Extraction and characterization of biosilicifying molecules from living organisms, however, is still limited. Silicon nanoparticles have been used in drug delivery and gene therapy.

Lipoparticles

Lipoparticles (Integral Molecular Inc, Philadelphia, PA, USA) are nanometer-sized spheres surrounded by a lipid bilayer and embedded with conformationally intact integral membrane proteins. Integral membrane proteins are a family of biological molecules that comprise >50% of existing drug targets. Characteristic features of these particles are as follows:

- Nanometer particles are surrounded by a lipid bilayer
- They are embedded with integral membrane proteins
- They retain native structural conformations
- Proteins spanning the membrane up to 14 times have been incorporated, including G protein-coupled receptors (GPCRs)
- They are soluble and stable target storage system
- They enable existing detection platforms to work with complex integral membrane proteins

Interactions with integral membrane proteins have been particularly difficult to study because the proteins cannot be removed from the lipid membrane of a cell without disrupting the structure and function of the protein. The ability to solubilize integral membrane proteins has applications for microfluidics, biosensors, high-throughput screening, antibody development, and structural studies of complex receptors. They are used in drug discovery.

Assembly of Nanoparticles into Micelles

Chemists at Rice University have discovered how to assemble gold and silver nanoparticle building blocks into larger structures based on a novel method that goes back to one of nature's oldest known chemical innovations, i.e., the self-assembly of lipid membranes that surround every living cell (Zubarev et al 2006). The method makes use of the hydrophobic effect, a biochemical phenomenon that all living creatures use to create membranes, ultrathin barriers of fatty acids that form a strong, yet dynamic, sack around the cell, sealing it from the outside world. Cell membranes are one example of a micelle, a strong bilayer covering that is made of two sheets of

lipid-based amphiphiles, molecules that have a hydrophilic end and a hydrophobic end. Like two pieces of cellophane tape being brought together, the hydrophobic sides of the amphiphilic sheets stick to one another, forming the bilayered micelle. The scientists synthesized V-shaped amphiphiles of polystyrene-*b*-poly(ethylene oxide) and attached 2-nm-diameter gold particles at the focal point of the V. Upon adding water and inducing micelle formation, the team found it could create tightly packed cylinders of gold nanoparticles measuring just 18 nm in diameter. All micelles form in three shapes: spheres, cylinders, and sack-like vesicles. By varying the length of the polystyrene arm, the solvents used, and the size of the gold particles, they were able to form spheres, vesicles, and vary the diameter of their cylinders, some of which grew to well over 1,000 nm in length. The new method may enable them to create a wide variety of useful materials, including potent cancer drugs and more efficient catalysts for the chemical industry.

Biomedical Applications of Self-Assembly of Nanoparticles

Researchers at Sandia National Laboratories and the University of New Mexico have perfected a commercially feasible way for orderly arrays of nanoparticles to self-assemble, each insulated from the others by silicon dioxide. The technique will not only enable new devices but could also solve one of the longest-standing problems with nanoparticles: forming orderly connections between the microscale and the nanoscale. The self-assembly technique prevents nanoparticles from clumping and insulates them from each other with silicon dioxide. By spin-coating precisely controlled thicknesses of silicon dioxide with embedded nanoparticles, the researchers hope to reduce to nanoscale the applications that until now have resisted downsizing. For example, the nanoparticles could be formed into thin films for nanoscale lasers, whose frequency depends on the nanoparticles' size.

The patented process created at Sandia uses an organic surfactant layer that ordinarily makes it difficult to process nanoparticles. Acting like a kind of grease, the patented approach scrubs the surfactants off the nanoparticles with an ozone compound and instead embeds them in oxide. In the two-step process, first a detergent solution is mixed with the nanoparticles, scrubbing off the grease and thereby making them water-soluble. In the second step, silica is introduced into the solution causing the nanoparticles to embed themselves into a silicon dioxide lattice when the compound solidifies. The 3D films and solids created with the process are stable indefinitely and can have application-specific ligands attached for biomedical devices. Even nanoparticles of different types could be combined to create specialized nanomolecules.

This approach should ease the transition from the micron-sized connections on currently available commercial chips to the orders-of-magnitude denser nanoscale structures. By using self-assembly techniques compatible with standard microelectronic processing, huge gaps in scale can be bridged by integrating nanocrystal arrays into standard silicon chips. The nanoparticles embedded in silicon dioxide

could become a massive number of stored charge cells. In the test material, the researchers demonstrated a kind of choreographed transmission among nanoparticles called a "Coulomb blockade." At low voltages no current passes, because each nanoparticle is separated from adjacent ones by a layer of silicon dioxide several nanometers thick. But at high voltages, current jumped by the cube of the voltage.

In addition, because nanoparticles typically range from 1 to 10 nm in diameter, their electrical properties are dominated by quantum confinement effects. Coulomb interactions in nanoparticles form excitons (electron–hole pairs) when they are pumped with optical energy from a laser. The distance between the electron and the hole is called the Bohr radius of the exciton and the resultant energized nanoparticle is called a QD. By merely changing the size of a QD, one can get different frequencies of emission upon pumping by a laser. For example, one can get them to emit light and could make them useful adjuncts to molecules that are being created to bind to cancer cells. Sandia National Laboratories has applied for a patent for identifying cancer cells early with such fluorescent markers.

Paramagnetic and Superparamagnetic Nanoparticles

Paramagnetic particles are important tools for cell sorting, protein separation, and single-molecule measurements. The particles used in these applications must meet the following requirements: uniform in size, highly paramagnetic, stable in physiological salt buffer, functionizable, and 100–1,000 nm in size. They have been used for the detection of model pathogens. Paramagnetic nanoparticles, which are linked to antibodies, enable highly specific biological cell separations.

Superparamagnetic iron oxide nanoparticles (SPIONs) with appropriate surface chemistry have been widely used experimentally for numerous in vivo applications such as MRI contrast enhancement, tissue repair, immunoassay, detoxification of biological fluids, hyperthermia, drug delivery, and in cell separation. These applications require that these nanoparticles have high magnetization values and size smaller than 100 nm with overall narrow particle size distribution, so that the particles have uniform physical and chemical properties. In addition, these applications need special surface coating of the magnetic particles, which not only has to be nontoxic and biocompatible but also has to allow a targetable delivery with particle localization in a specific area. Nature of surface coatings of the nanoparticles not only determines the overall size of the colloid but also plays a significant role in biokinetics and biodistribution of nanoparticles in the body. Magnetic nanoparticles can bind to drugs, proteins, enzymes, antibodies, or nucleotides and can be directed to an organ, tissue, or tumor using an external magnetic field or can be heated in alternating magnetic fields for use in hyperthermia (Gupta and Gupta 2005).

Magnetic labeling of cells provides the ability to monitor their temporal–spatial migration in vivo by MRI. Various methods have been used to magnetically label cells using SPIONs. Magnetic tagging of stem cells and other mammalian cells has the potential for guiding future cell-based therapies in humans and for the evaluation of cellular-based treatment effects in disease models.

Fluorescent Nanoparticles

Fluorescent nanoparticles can be used as labels for immunometric assay of C-reactive protein (CRP) using two-photon excitation assay technology (Koskinen et al 2004). This new assay technique enables multiplexed, separation-free bioaffinity assays from microvolumes with high sensitivity. The assay of CRP was optimized for assessment of CRP baseline levels using a nanoparticulate fluorescent reporter, 75 nm in diameter, and the assay performance was compared with that of CRP assay based on a molecular reporter of the same fluorophore core. The results show that using fluorescent nanoparticles as the reporter provides 2 orders of magnitude better sensitivity than using the molecular label, while no difference between precision profiles of the different assay types was found. The new assay method was applied for assessment of baseline levels of CRP in sera of apparently healthy individuals. These are being investigated for applications in molecular diagnostics.

Bacterial Structures Relevant to Nanobiotechnology

Bacterial Spores

Bacterial spores are robust and dormant life forms with formidable resistance properties, in part, attributable to the multiple layers of protein that encase the spore in a protective and flexible shield. The coat has a number of features pertinent to the emerging field of nanobiotechnology including self-assembling protomers and the capacity for engineering and delivery of foreign molecules (Ricca and Cutting 2003). The spore coat as a potential vehicle for heterologous antigen presentation and protective immunization (vaccination). It may also be exploited for drug and enzyme delivery as well as a source of novel self-assembling proteins.

Nanostructures Based on Bacterial Cell Surface Layers

Among the most commonly observed bacterial cell surface structures are monomolecular crystalline arrays of proteinaceous subunits termed S-layers, which are the simplest type of biological membrane developed during evolution. S-layer plays an important part in interactions of a microbial cell with the environment (Debabov 2004). S-layers are generally 5–10 mm thick and pores in the protein lattices are of identical size and morphology in the 2–8 nm range. S-layers have nanobiotechnological applications as given in Table 2.4.

Bacterial Magnetic Particles

Magnetic bacteria synthesize intracellular magnetosomes that impart a cellular swimming behavior referred to as magnetotaxis. The magnetic structures, magnetosomes, aligned in chains are postulated to function as biological compass needles allowing the bacterium to migrate along redox gradients through the Earth's

Table 2.4 Nanobiotechnological applications of S-layers

As a matrix for controlled immobilization of functional molecules
Binding of enzymes for bioanalytical biosensors
Immobilizing monoclonal antibodies for dipstick-style immunoassays
Immobilizing antibodies for preparation of microparticles for ELISA
S-layers as carriers for conjugated vaccines
S-layer-coated liposomes
Immobilization of functional molecules on S-layer-coated liposomes
Entrapping of functional molecules for drug delivery
S-layer-coated liposomes with immobilized antigens and haptens for vaccines
Vehicles for producing fusion proteins
Vaccines
Biosensors
Diagnostics

Source: Modified from Sleytr et al 2003.

geomagnetic field lines. Despite the discovery of this unique group of microorganisms several years ago, the mechanisms of magnetic crystal biomineralization have yet to be fully elucidated. A lipid bilayer membrane of approximately 2–4 nm in thickness encapsulates individual magnetosomes (50–100 nm in diameter). Magnetosomes are also referred to as bacterial magnetic particles (BMPs) to distinguish them from artificial magnetic particles (AMPs). The aggregation of BMPs can be easily dispersed in aqueous solutions compared with AMPs because of the enclosing organic membrane.

BMPs have potential applications in the interdisciplinary fields of nanobiotechnology, medicine, and environmental management (Matsunaga and Okamura 2003). The use of BMPs in immunoassays enables the separation of bound and free analytes by applying a magnetic field. Proteins can be attached covalently to solid supports such as BMPs that prevent desorption of antibodies during an assay. Large-scale production of functionally active antibodies or enzymes expressed on BMP membranes can be accomplished.

Cubosomes

Cubosomes (cubic phase nanoparticles) are self-assembled liquid crystalline nanoparticles. Due to their unique structure, which intermingles oil and water molecules, they are nanoporous. Their disintegration process in plasma has been investigated by in vitro and in vivo studies (Leesajakul et al 2004). Cubosomes were incubated with whole plasma or plasma components such as cholesterol and albumin. The lypolysis study indicated lipolytic activity of whole plasma toward cubosomes. Gel filtration chromatography revealed that high-density cholesterol (HDL)

affected cubosomes' integrity and gave rise to smaller particles that contained the components of both cubosomes and HDL. Upon incubation with low-density cholesterol (LDL), cubosomes fused with LDL. Albumin was shown to take up monoolein out of the particles. Cubosomes were disintegrated by whole plasma as a result of the interaction with plasma components. It was concluded that in vivo observation of a long circulation time of a hydrophobic substance in cubosomes was due to the sustained behavior of cubosome remnant particles.

Methods and compositions for producing lipid-based cubic phase nanoparticles were first discovered in the 1990s. Since then a number of studies have described properties such as particle size, morphology, and stability of cubic phase dispersions, which can be tuned by composition and processing conditions (Barauskas et al 2005). Stable particle dispersions with consistent size and structure can be produced by a simple processing scheme comprising a homogenization and heat treatment step. Cubosomes could be a core building block for several nano-based research projects. Procter & Gamble is exploring the manufacture of cubosomes to create new skin treatments for premature infants.

Dendrimers

Dendrimers (dendri means tree, mer means branch) are a novel class of 3D nanoscale, core–shell structures that can be precisely synthesized for a wide range of applications. Specialized chemistry techniques allow for precise control over the physical and chemical properties of the dendrimers. They are constructed generation by generation in a series of controlled steps that increase the number of small branching molecules around a central core molecule. Up to 10 generations can be incorporated into a single dendrimer molecule. The final generation of molecules added to the growing structure makes up the polyvalent surface of the dendrimer (see Fig 2.2). The core, branching, and surface molecules are chosen to give desired properties and functions (Fig 2.1).

As a result of their unique architecture and construction, dendrimers possess inherently valuable physical, chemical, and biological properties. These are as follows:

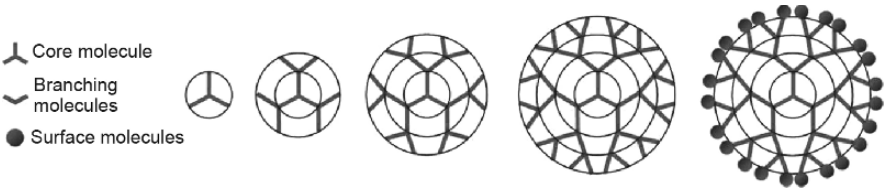

Fig. 2.1 The core, branching, and surface molecules of dendrimers
Source: Starpharma Holding Ltd, by permission.

- *Precise architecture, size, and shape control*—Dendrimers branch out in a highly predictable fashion to form amplified 3D structures with highly ordered architectures.
- *High uniformity and purity*—The proprietary stepwise synthetic process used produces dendrimers with highly uniform sizes (monodispersity) possessing precisely defined surface functionality and very low impurity levels.
- *High loading capacity*—Internal cavities intrinsic to dendrimer structures can be used to carry and store a wide range of metals, organic, or inorganic molecules.
- *High shear resistance*—Through their 3D structure dendrimers have a high resistance to shear forces and solution conditions.
- *Low toxicity*—Most dendrimer systems display very low cytotoxicity levels.
- *Low immunogenicity when injected or used topically*.

Properties

The surface properties of dendrimers may be manipulated by the use of appropriate "capping" reagents on the outermost generation. In this way dendrimers can be readily decorated to yield a novel range of functional properties. These are as follows:

- *Polyvalency*—The outer shell of each dendrimer can be manipulated to contain a large number of reactive groups. Each of these reactive sites has the potential to interact with a target entity, often resulting in polyvalent interactions.
- *Flexible charge and solubility properties*—Through use of appropriate capping groups on the dendrimer exterior, the charge and solubility of dendrimers can be readily manipulated.
- *Flexible binding properties*—By using appropriate capping groups on the dendrimer exterior, dendrimers can be designed to exhibit strong affinity for specific targets.
- *Transfection*—Dendrimers are able to move through cell boundaries and transport genetic materials into cell interiors.

Applications

Dendrimers, with their highly customizable properties, are basic building blocks with the promise of enabling specific nanostructures to be built to meet existing needs and solve evolving problems. Dendrimer research and development is currently making an impact on a broad range of fields as shown by exponential growth in the number of dendrimer-based publications. While the potential applications of dendrimers are unlimited, some of their current uses relevant to nanomedicine are summarized in Table 2.5.

Advances in understanding of the role of molecular weight and architecture on the in vivo behavior of dendrimers, together with recent progress in the design of biodegradable chemistries, have enabled the application of dendrimers as antiviral drugs, tissue repair scaffolds, targeted carriers of chemotherapeutics, and optical

Table 2.5 Potential applications of dendrimers in nanomedicine

Diagnostics
Sensors
Imaging contrast agents
Drug delivery
Improved delivery of existing drugs
Improved solubility of existing drugs
Drug development
Polyvalent dendrimers interacting simultaneously with multiple drug targets
Development of new pharmaceuticals with novel activities
Improving pharmacological activity of existing drugs
Improving bioavailability of existing drugs
Medicine and surgery
Prevention of scar tissue formation after surgery

Source: Jain PharmaBiotech.

oxygen sensors (Lee et al 2005). Before such products can reach the market, however, the field must not only address the cost of manufacture and quality control of pharmaceutical-grade materials, but also assess the long-term human and environmental health consequences of dendrimer exposure in vivo.

DNA–Nanoparticle Conjugates

DNA–DNA hybridization has been exploited in the assembly of nanostructures including biosensors and DNA scaffolds. Many of these applications involve the use of DNA ODNs tethered to gold nanoparticles or nanoparticles may be hybridized with one another. Two types of DNA–nanoparticle conjugates have been developed for these purposes. Both types entail the coupling of ODNs through terminal thiol groups to colloidal gold particles. In one case, the ODNs form the entire monolayer coating the particles, whereas in the other case the ODNs are incorporated in a phosphine monolayer, and particles containing discrete numbers of ODNs are separated by gel electrophoresis. A minimal length of 50 residues is required, both for separation by electrophoresis and for hybridization with cDNA sequences. These limitations of shorter ODNs are attributed to interaction between the DNA and the gold. In a new technique, glutathione monolayer-protected gold clusters were reacted with 19- or 20-residue thiolated ODNs and the resulting DNA-nanoparticle conjugates could be separated on the basis of the number of bound ODNs by gel electrophoresis and assembled with one another by DNA-DNA hybridization (Ackerson et al 2005). This approach overcomes previous limitations of DNA-nanoparticle synthesis and yields conjugates that are precisely defined with respect to both gold and nucleic acid content.

DNA Octahedron

A group of scientists at The Scripps Research Institute (San Diego, CA) has designed, constructed, and imaged a single strand of DNA that spontaneously folds into a highly rigid, nanoscale octahedron that is several million times smaller than the length of a standard ruler and about the size of several other common biological structures, such as a small virus or a cellular ribosome (Shih et al 2004). The octahedron consists of 12 edges, 6 vertices, and 8 triangular faces. The structure is ~22 nm in diameter. Making the octahedron from a single strand was a breakthrough. Because of this, the structure can be amplified with the standard tools of molecular biology and can easily be cloned, replicated, amplified, evolved, and adapted for various applications. This process also has the potential to be scaled up so that large amounts of uniform DNA nanomaterials can be produced. These octahedra are potential building blocks for new tools for basic biomedical science. With these we have biological control, and not just synthetic chemical control, over the production of rigid, wireframe DNA objects.

Potential Applications

Because all 12 edges of the octahedral structures have unique sequences, they are versatile molecular building blocks that could potentially be used to self-assemble complex higher-order structures. Possible applications include using these octahedra as artificial compartments into which proteins or other molecules could be inserted, something like a virus in reverse—DNA is on the outside and proteins on the inside. In nature, viruses are self-assembling nanostructures that typically have proteins on the outside and DNA or RNA on the inside. The DNA octahedra could possibly form scaffolds that host proteins for the purposes of x-ray crystallography, which depends on growing well-ordered crystals, composed of arrays of molecules.

Fullerenes

Fullerene technology derives from the discovery in 1985 of carbon-60 (C60), a molecule of 60 carbon atoms that form a hollow sphere 1 nm in diameter. The molecule was named buckyball or fullerene. Subsequent studies have shown that fullerenes actually represent a family of related structures containing 20, 40, 60, 70, or 84 carbons. C60, however, is the most abundant member of this family. Fullerenes are entirely insoluble in water, but suitable functionalization makes the molecules soluble. Initial studies on water-soluble fullerene derivatives led to the discovery of the interaction of organic fullerenes with DNA, proteins, and living cells. Subsequent studies have revealed interesting biological activity aspects of organic fullerenes owing to their photochemistry, radical quenching, and hydrophobicity to form 1D to 3D supramolecular complexes (Nakamura and Isobe 2003). In these areas of research, synthetic organic chemistry has played an important role in the creation of tailor-made molecules.

Upon contact with water, under a variety of conditions, C60 spontaneously forms a stable aggregate with nanoscale dimensions (25–500 nm), termed nano-C60 that are both soluble and toxic to bacteria (Fortner et al 2005). This finding challenges conventional wisdom because buckyballs are notoriously insoluble by themselves and most scientists had assumed they would remain insoluble in nature. The findings also raise questions about how the aggregates will interact with other particles and living things in natural ecosystems.

A new method of application of C60 to cultured cells has been described that does not require water solubilization techniques (Levi et al 2006). Normal and malignant cells take up C60 and the inherent photoluminescence of C60 is detected within multiple cell lines. Treatment of cells with up to 200 mg/ml (200 ppm) of C60 does not alter morphology, cytoskeletal organization, or cell cycle dynamics, nor does it inhibit cell proliferation. This study shows that pristine C60 is nontoxic to the cells and suggests that fullerene-based nanocarriers may be used for biomedical applications.

Tego BioSciences Corporations (Pasadena, CA, USA) is developing fullerenes as pharmaceuticals. Another company, Fullerene International Corporation, combines the capabilities of partners Mitsubishi Corporation, Materials and Electrochemical Research Corporation, and Research Corporation Technologies in a joint venture to commercialize fullerene materials.

Nanoshells

Nanoshells are ball-shaped, about the size of a virus or 1/20th of an RBC, and consist of a core of nonconducting glass that is covered by a metallic shell, typically either gold or silver. Nanoshells possess highly favorable optical and chemical properties for biomedical imaging and therapeutic applications. These particles are also effective substrates for surface-enhanced Raman scattering (SERS) and are easily conjugated to antibodies and other biomolecules. By varying the relative dimensions of the core and the shell, the optical resonance of these nanoparticles can be precisely and systematically varied over a broad region ranging from the near-UV to the mid-infrared. This range includes the NIR wavelength region where tissue transmissibility peaks, which forms the basis of absorbing nanoshells in NIR thermal therapy of tumors (Loo et al 2004). In addition to spectral tunability, nanoshells offer other advantages over conventional organic dyes including improved optical properties and reduced susceptibility to chemical/thermal denaturation. Furthermore, the same conjugation protocols used to bind biomolecules to gold colloid are easily modified for nanoshells. The core/shell ratio and overall size of a gold nanoshell influence its scattering and absorption properties.

Gold Nanoshells (Spectra Biosciences, Columbia, MD, USA) possess physical properties similar to gold colloid, in particular a strong optical absorption due to the collective electronic response of the metal to light. The optical absorption of gold colloid yields a brilliant red color, which has been of considerable utility in

consumer-related medical products such as home pregnancy tests. In contrast, the optical response of Gold Nanoshells depends dramatically on the relative sizes of the nanoparticle core and the thickness of the gold shell. By varying the relative core and shell thickness, the color of Gold Nanoshells can be varied across a broad range of the optical spectrum that spans the visible and the NIR spectral regions. Gold Nanoshells can be made to either absorb or scatter light preferentially by varying the size of the particle relative to the wavelength of the light at their optical resonance.

Several potential biomedical applications of nanoshells are under development, including immunoassays, modulated drug delivery, photothermal cancer therapy, and imaging contrast agents (Hirsch et al 2006).

Carbon Nanotubes

Carbon nanotubes are rolled-up sheets of carbon atoms that appear naturally in soot and are central to many nanotechnology projects. These nanotubes can go down to 1 nm in diameter, are stronger than any material in the universe, and can be of any length. These can be used as probes for AFMs that can image individual molecules in both wet and dry environments. This has enormous opportunities for application as conventional structure-based pharmaceutical design is hampered by the lack of high-resolution structural information for most protein-coupled receptors. It is possible to insert DNA into a carbon nanotube. Devices based on the DNA–nanotube combination could eventually be used to make electronics, molecular sensors, devices that sequence DNA electronically, and even gene delivery systems.

Medical Applications of Nanotubes

- Cyclic peptide nanotubes can act as a new type of antibiotic against bacterial pathogens.
- Nanoscale electromechanical systems (nanotweezers) based on carbon nanotubes have been developed for manipulation and interrogation of nanostructures within a cell.
- Carbon nanotubes can be used as tips for AFM.
- Lumen of a nanotube can carry payloads of drugs.
- Nanotubes can be used in biosensors.
- Blood-compatible carbon nanotubes, with heparin immobilized on the surface, are building blocks for in vivo nanodevices. Activated partial thromboplastin time and thromboelastography studies prove that heparinization can significantly enhance the blood compatibility of nanomaterials (Murugesan et al 2006b).

A study of the electrophoretic transport of single-stranded RNA molecules through 1.5-nm-wide pores of carbon nanotube membranes has revealed that RNA entry into the nanotube pores is controlled by conformational dynamics, and exit by hydrophobic attachment of RNA bases to the pores (Yeh and Hummer 2004). Differences in

RNA conformational flexibility and hydrophobicity result in sequence-dependent rates of translocation, a prerequisite for nanoscale separation devices.

The uptake of single-walled carbon nanotubes (SWNTs) into macrophage-like cells has been studied using the nanotubes' intrinsic NIR fluorescence (Cherukuri et al 2004). Nanotube uptake appears to occur through phagocytosis. There were no adverse effects on the cells and the nanotubes retained their unique optical properties. The new findings suggest that SWNTs might be valuable biological imaging agents, in part because SWNTs fluoresce in the NIR portion of the spectrum, at wavelengths not normally emitted by biological tissues. This may allow light from even a handful of nanotubes to be selectively detected from within the body. Although long-term studies on toxicity and biodistributions must be completed before nanotubes can be used in medical tests, the new findings indicate nanotubes could soon be useful as imaging markers in laboratory in vitro studies, particularly in cases where the bleaching, toxicity, and degradation of more traditional markers are problematic.

Nanopores

Nanopores are tiny structures that occur in the cell in nature for specific functions. At the molecular level, specific shapes are created that enable specific chemical tasks to be completed. For examples, some toxic proteins such as alpha-hemolysin can embed themselves into cell membranes and induce lethal permeability changes there due to its central pore. This protein consists of seven subunits that join together to form a tunnel through the cell membrane with a well-defined pore that narrows from 26 to $\sim$15—just larger than a single-stranded DNA molecule (van de Goor 2004). The first proposed application was DNA sequencing by measuring the size of a nanopore, application of an electric potential across the membrane, and waiting for DNA to migrate through the pore to enable one to measure the difference between bases in the sequence. This is tricky as the pore is very small with a diameter of $<$1/100th of the wavelength of visible light.

Protein engineering has applied to ion channels and pores, and protein as well as nonprotein nanopores have been constructed (Bayley and Jayasinghe 2004). Potential applications of engineered nanopores are as follows:

- Tools in basic cell biology
- Molecular diagnostics
- Drug delivery
- Nanomedicine

Agilent Technologies Inc (Palo Alto, CA) is collaborating with Harvard University (Cambridge, MA) to develop nanopore technology for analysis of nucleic acids that convert strings of nucleotides directly into electronic signatures. A membrane with nanometer-diameter channels called nanopores separates two solutions. When a current is applied across the membrane, charged biomolecules migrate through

the pores. As each nucleotide passes through the nanopore, an electric signature is produced that characterizes it because the size of the nanopore allows only a single nucleic acid strand to pass through it at one time. The concept of nanopore-based sequencing is shown in Fig 2.2.

Nanostructured Silicon

Silicon has been used for implants in the human body for several years. Following nanostructuring, silicon can be rendered biocompatible and biodegradable. BioSilicon^{TM} (pSiMedica Ltd) contains nanosized pores measuring 100 nm. The "silicon skeleton" between the pores comprises tens of silicon atoms in width. Initial applications are in drug delivery. The kinetics of drug release from BioSilicon^{TM} can be controlled by adjusting the physical properties of the matrix, including modifying the pore size. Other potential applications include nanospheres for targeted systemic and pulmonary drug delivery. Nanostructured silicon, as multilayered mirrors, can be used for subcutaneous implants for diagnostics. Nanostructures can be used as prostheses to improve adhesion to bone tissue.

Networks of Gold Nanoparticles and Bacteriophage

Biological molecular assemblies are excellent models for the development of nanoengineered systems with desirable biomedical properties. A spontaneous biologically active molecular network has been fabricated that consists of bacteriophage (phage) directly assembled with gold (Au) nanoparticles and termed Au-phage (Souza et al 2006). When the phage is engineered so that each phage particle displays a peptide, the networks preserve the cell surface receptor binding and internalization attributes of the displayed peptide. The spontaneous organization of these targeted networks can be manipulated further by incorporation of imidazole (Au-phage-imid), which induces changes in fractal structure and NIR

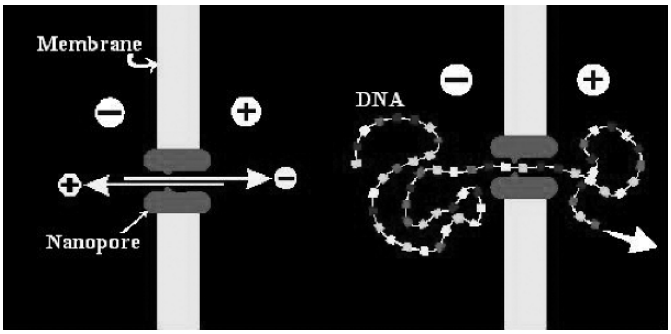

Fig. 2.2 Concept of nanopore-based sequencing
Source: Courtesy of Agilent Technologies Inc.

optical properties. The networks can be used as labels for enhanced fluorescence and dark-field microscopy, SERS detection, and NIR photon-to-heat conversion. Together, the physical and biological features within these targeted networks offer convenient multifunctional integration within a single entity with potential for nanotechnology-based biomedical applications such as biological sensors and cell-targeting agents. This genetically programmable nanoparticle with a biologically compatible metal acts as a nanoshuttle that can target specific locations in the body. For example, it could potentially locate damaged areas on arteries that have been caused by heart disease, and then deliver stem cells to the site that can build new blood vessels. It may be able to locate specific tumors, which could then be treated by either heating the gold particles with laser light and/or using the nanoparticles to selectively deliver a drug to destroy the cancer.

Nanotechnologies for Basic Research Relevant to Medicine

NanoSystems Biology

Systems biology is defined as the biology of dynamic interacting networks (Weston and Hood 2004). It is also referred to as pathway, network, or integrative biology. An analysis of the structure and dynamics of network of interacting elements provides insights that are not obvious from analysis of the isolated components of the system. Proteomics plays an important role in systems biology because most biological systems involve proteins. Systems biology is providing new challenges for advancing science and technology. Analyses of pathways may provide new insight into the understanding of disease processes, developing more efficient biomarkers, and understanding mechanisms of action of drugs.

NanoSystems Biology is the application of nanobiotechnology, microfluidics, and molecular imaging methods, in the study of systems biology (Heath et al 2003). It will play an important role in understanding biology of disease:

- It provides refined tools for the study of proteomics.
- Nanotechnology provides real-time single-particle tracing in living cells.
- Nanotechnology will facilitate dissecting of signaling pathways.

A cross-disciplinary project in NanoSystems Biology is being headed by three institutions: California Institute of Technology (Caltech, Pasadena, CA), the Institute for Systems Biology in Seattle, and the UCLA Geffen School of Medicine (Los Angeles, CA). The goal is to develop a suite of nanotechnology tools—ranging from integrated microfluidics to nanoelectronics to nanomechanical devices—that will enable a large-scale, systems biology-driven, multiparameter analysis within a clinical setting (i.e., every patient, every visit). Such an analysis constitutes an "informative diagnosis" of disease. Nanobiosensors are currently being developed for cancer biomarkers for early detection of the disease.

Molecular Motors

A molecular-level machine may be defined as an assembly of a number of molecules that are designed to perform movements. Molecular self-assembly is an important route toward the construction of artificial molecular-level devices. These devices are characterized by the energy source, the nature of the movement, the way it may be controlled, its repeatability, its purpose, and the time scale of the nanometer-scaled conformational changes. They play an important role in cell function.

Scientists at the California Institute of Technology are studying a remarkable little rotary motor located at the base of every flagellum of *Escherichia coli* bacteria, which spins to propel the organism. This remarkable device is driven by proton gradients, has its own shaft seals through the cell membrane, can achieve ~50% efficiency, and can spin bidirectionally up to a couple thousand revolutions per minute. A microrotary motor has been described, which is composed of a 20-μm-diameter silicon dioxide rotor driven on a silicon track by the gliding bacterium *Mycoplasma mobile* (Hiratsuka et al 2006). This motor is fueled by glucose and inherits some of the properties normally attributed to living systems.

Every cell in the body has "a dynamic city plan" comprised of molecular highways, construction crews, street signs, cars, fuel, and exhaust. Maintenance of this highly organized structure is fundamental to the development and function of all cells and much of it can be understood by figuring out how the functional units in the cell, molecular motors or biological nanomachines, do the work to keep cells orderly. Their function depends on catalytic activity of their constituent proteins. The miniature motor that drives our cells is the enzyme ATP synthase that converts food or light into ATP, the energy currency of the cell. Spinning at several thousand revolutions per minute, the detailed internal workings of the tiny motor are tough to decipher; high-speed imaging has been used to snap freeze-frames of the spinning shaft. Linear micromotors that move the cell, voltage-gated ion channels, DNA replication complexes, and countless other structures are quite complex and their functions are still not well understood. Lasers, detectors, and optics have been used to study how two protein machines, myosin and kinesin, move about in vitro. The results show how multiple motors compete for activity on their protein scaffolds. Kinesin and another motor, dynein, operate in large groups to produce >10 times the speed that is expected from a single molecule. The use of a technique called fluorescence imaging with one nanometer accuracy (FIONA), which has a spatial resolution of 1.5 nm, showed that myosin V, kinesin, and myosin VI all move in hand-over-hand fashion (Yildiz et al 2003). Further studies with FIONA have shown that average step size is ~8 nm for both dynein and kinesin and that these work together rather than against each other in vivo producing up to 10 times the in vitro speed (Kural et al 2005).

One of the unknowns about dynein was that the molecular site where chemical energy is initially released from ATP is very far away from where the mechanical force occurs and how the mechanical force was transmitted over a large distance. Using a variety of modeling techniques that allowed resolution at the level of atoms, scientists have identified a flexible, spring-like "coiled-coil" region within dynein,

which couples the motor protein to the distant ATP site (Serohijos et al 2006). It allows a very rapid transduction of chemical energy into mechanical energy. Conversion to mechanical energy allows dynein to transport cellular structures such as mitochondria that perform specific jobs such as energy generation, protein production, and cell maintenance. Dynein also helps force apart chromosomes during cell division. Although the research offers no immediate application to human disease, the authors noted that mutations of dynein have been implicated in some neurodegenerative and kidney disorders. Disruption of dynein's interaction with a particular regulator protein causes defects in nerve cell transmission and mimics the symptoms of patients with amyotrophic lateral sclerosis.

Scientists at the Pennsylvania Muscle Institute of the University of Pennsylvania School of Medicine (Philadelphia, PA) have shown that proteins that function as molecular motors are surprisingly flexible and agile, able to navigate obstacles within the cell (Ross et al 2006). These observations could lead to better ways to treat motor neuron diseases such as amyotrophic lateral sclerosis. Using a specially constructed microscope that allows researchers to observe the action of one macromolecule at a time, the team found that a protein motor is able to move back and forth along a microtubule—a molecular track—rather than in one direction, as previously thought. The proteins in this motor, dynein and dynactin, are the "long-distance truckers" of the cell: working together, they are responsible for transporting cellular cargo from the periphery of a cell toward its nucleus. Mutation in dynactin leads to degeneration of motor neurons, the hallmark of motor neuron disease. They found that a mutation decreases the efficiency of the dynein–dynactin motor in "taking out the trash" of the cell and thus leads to the accumulation of misfolded proteins in the cell, which may in turn lead to the degeneration of the neuron.

Another type of molecular motor provides the rigidity needed by the tiny sensors in the inner ear in order to respond to sound. This motor creates the proper amount of tension in the sensors and anchors itself to maintain that tension. A motor able to create structural changes by taking up slack in proteins and clamping down so that they remain in a rigid position may help explain many intricacies of cellular organization, such as how chromosomes line up and separate during cell division. Molecular motors called myosins are proteins that carry out cellular motion by attaching to and "walking" along fibers of actin. The interaction of actin and myosin is the mechanism behind cell actions such as muscle contractions, the pinching off of two daughter cells from a mother cell during division, and the hauling of cargo molecules around in a cell (Altman et al 2004). Of the 18 types of myosin molecules, the reported study examines myosin VI, thought to be responsible for setting the tension for stereocilia, actin-filled rods on the sound-sensing hair cells of the inner ear. A defect in myosin VI results in deafness. Although it was known that myosin moves along actin fibers, it had never previously been demonstrated how myosin could function as an anchor or a clamp. It was enabled by optical tweezers, a focused laser that allows the manipulation of microscopic beads. This study involved looking at a single molecule and how it behaves, but there are very few proteins in biology that have been analyzed and understood down to this level. Such studies that provide an understanding of the biological nanostructures would be stimulating for

nanobiotechnology. Designers of nanomachines can learn much from nanobiology as many nanostructures in biology can be used as tools in nanobiotechnology.

Biological molecular motors have a number of unique advantages over artificial motors, including efficient conversion of chemical energy into mechanical work and the potential for self-assembly into larger structures, as is seen in muscle sarcomeres and bacterial and eukaryotic flagella. The development of an appropriate interface between such biological materials and synthetic devices should enable us to realize useful hybrid micromachines. When the scientists discover how to design and mass-produce molecular motors artificially, it will be a major stride in the era of nanotechnology.

Nanomotor Made of Nucleic Acids

Although protein machines are abundant in biology, it has recently been proposed that nucleic acids could also act as nanomolecular machines in model systems. Several types of movements have been described with DNA machines: rotation and "scissors-like" opening and closing. A nanomachine that is capable of an extension–contraction movement has also been devised (Alberti and Mergny 2003). The simple and robust device described is composed of a single 21-base oligonucleotide and relies on a duplex–quadruplex equilibrium that may be fueled by the sequential addition of DNA single strands, generating a DNA duplex as a by-product. The interconversion between two well-defined topological states induces a 5-nm two-stroke, linear motor-type movement, which is detected by FRET spectroscopy. This system could be used to obtain precise control of movements on the nanometer scale.

Nucleic acids are increasingly used to build nanometer-scale structures that may be used in future nanotechnology devices. There are two key goals in this area: to perform controlled mechanical movements and to produce complex structures from simple molecular building blocks. Several areas, such as error correction and scaled-up self-assembly, require particular attention if the potential of nucleic acid-based nanotechnology is to be fulfilled.

A DNA nanomechanical device enables the positional synthesis of products whose sequences are determined by the state of the device (Liao and Seeman 2004). This machine emulates the translational capabilities of the ribosome. Ribosomes are miniature biological machines that weld together amino acids to form the enzymes that modulate body chemistry and the structural materials, like collagen. DNA was twisted and bent to build a structure that is approximately 110 nm long, 30 nm wide, and 2 nm thick, roughly the same size as a ribosome, though not as complex. The DNA machine can swivel into four geometric positions and can be locked into any one of them by another fragment of DNA, which provides the instructions. Locking the machine into position dials in the sequence of very short DNA strands that it will recognize and position for welding. The welding itself is performed by an enzyme that links DNA molecules. In the absence of the machine, this enzyme would create many different combinations of DNA strands; in its presence, only a single pre-programmed combination results. The device has potential applications that include

designer polymer synthesis, encryption of information, and use as a variable-input device for DNA-based computation.

phi29 DNA Packaging Nanomotor

Researchers from Purdue University (West Lafayette, IN) have constructed a functional phi29 nanomotor derived from the biological motor of bacteriophage phi29, a virus that infects bacteria. The virus uses the motor to package DNA and move it into the capsid, a shell made of proteins, as part of the viral reproduction process. The controllable, 30-nm imitating DNA-packaging motor was constructed and is driven by six synthetic ATP-binding packaging RNA (pRNA) monomers (Guo 2005a). The formation of ordered structural arrays of the motor complex and its components, the retention of motor function after the 3'-end extension of the pRNA, and the ease of RNA dimer, trimer, and hexamer manipulation with desired shape and size make this RNA-containing motor a promising tool for drug and gene delivery and for use in nanodevices. Six pRNAs form a hexameric ring by hand-in-hand interaction. When fed a supply of ATP fuel, the RNA strands kick against the axle in succession, much like pistons in a combustion engine. This phenomenon explains how RNA powers viral assembly. The nanomoter can stuff DNA into a protein shell and can be turned on and off. The motor components form regular arrays, which have potential applications in medicine and nanotechnology. Fusing pRNA with receptor-binding RNA aptamer, folate, short interfering RNA (siRNA), ribozyme, or another chemical group does not disturb dimer formation or interfere with the function of the inserted moieties. The motor pRNA can deliver siRNA, ribozyme, or other therapeutic molecules to specific cells and destroy them. This has been demonstrated in various cancer cells as well as in hepatitis B virus-infected cells. The use of such a nanomotor/pRNA/siRNA complex could extend the short half-life of therapeutic small molecules in vivo and overcome the delivery problems of molecules >100 nm.

The phi29 motor is considered to be the most powerful biological motor constructed to date and is well characterized and understood. The nanoscale size range is ideal for delivery inside the body. Anything smaller would be filtered out through the kidneys too quickly to be effective, and larger molecules would not be able to enter cells. The team plans to attach the nanomotor to a lipid sheet, cell membrane, and liposome, which would take the place of the capsid into which the phi29 biological motor pumps DNA. The nanomotor, once embedded into the outer wall of the liposome or cell membrane, would pump DNA, drugs, or other therapeutic molecules into the liposome pocket's open space, or directly into the cell through a controlled mechanism. The long-term goal is to place thousands of nanomotors in an array assembled on a porous surface, such as silicon, and to have them function for use in biosensing. In addition to working toward the goals of creating therapeutic and diagnostic tools, the team will expand understanding of the structure and function of the lipid membrane-embedded artificial motor and other possible applications.

In December 2006, the NIH selected Purdue University's nanomotor program as one of eight national nanomedicine development centers to be supported by a $7 million grant over 5 years. The team will study nanomotors for potential use

in the diagnosis and treatment of diseases such as cancer, AIDS, hepatitis B, and influenza. Nanomotors will be used to package and deliver therapeutic DNA or RNA to disease-causing cells. The aim is to create a medical tool using a device that mimics a natural biological structure. This biomimetic tool will be a hybrid of natural biological structures and synthetic structures that will operate on the nanoscale.

Disguising Quantum Dots as Proteins for Cell Entry

A UCLA (University of California, Los Angeles)-led team of chemists has developed a unique new coating for inorganic particles at the nanoscale that may be able to disguise QDs as proteins—a process that allows particles to function as probes that can penetrate the cell and light up individual proteins inside, and create the potential for application in a wide range of drug development, diagnostic tools, and medications (Michalet et al 2005).

The organic coatings—short chains of peptides—can be used to disguise QDs, quantum rods, and quantum wires so effectively that the cells mistake them for proteins, even when the coatings are used on particles that are inorganic and possibly even toxic. These peptide coatings trick the live cell into thinking that the nanoparticles are benign, protein-like entities. Therefore, one can use these coated particles to track the proteins in a live cell and conduct a range of studies at the molecular level. Using the new coatings, the UCLA team has been able to solubilize and introduce into the cell different-color QDs that can all be excited by a single blue light source. The color encoding method is similar to the encoding of information that is sent down an optical fiber, called "wavelength division multiplexing (WDM)." The peptide coating technology could, in principle, color-encode biology itself, by painting different proteins in the cell with different-color QDs. The scientists are developing methods to attach QDs of specific colors to the different proteins on cells' surface and inside cells. By painting a subset of proteins in the cell with different-color QDs, one can follow the molecular circuitry, the dynamic rearrangement of circuit nodes, and the molecular interactions. In addition to the capacity to paint and observe many different proteins with separate colors, QDs can be used for the ultimate detection sensitivity: observing a single molecule. Until now, tracking and following a single protein in the cell has been extremely difficult.

By using the new methods developed at UCLA, and observing with a fluorescence microscope and high-sensitivity imaging cameras, researchers can track a single protein tagged with a fluorescent QD inside a living cell in three dimensions and within a few nanometers of accuracy. This process is, in some ways, the molecular equivalent of using the global positioning system to track a single person anywhere on earth. Researchers can use optical methods to track several different proteins tagged with different-color QDs, measure the distances between them, and use those findings to better understand the molecular interactions inside the cell.

Particles disguised with the peptide coatings can enter a cell without affecting its basic functioning. Since the peptide-coated QDs are small, they have easy and rapid

entry through the cell membrane. In addition, since multiple peptides of various lengths and functions could be deposited on the same single QD, it would be feasible to create smart probes with multiple functions.

This work on coatings was inspired by nature. Some plants and bacteria cells evolved unique capabilities to block toxic heavy-metal ions as a strategy to clean up the toxic environment in which they grow. These organisms synthesize peptides, called phytochelatins that reduce the amount of toxic-free ions by strongly binding to inorganic nanoparticles made of the sequestered toxic salts and other products.

The peptide coating bridges the inorganic chemistry world with the organic world on the nanometer scale. These coatings will be used to provide electrical contact between nanoscale inorganic electronic devices and functional proteins, which would lead to the evolution of novel and powerful "smart drugs," "smart enzymes," "smart catalysts," "protein switches," and many other functional hybrids of inorganic–organic substances. It might enable the creation of a hybrid nanoparticle that could be specifically targeted to identify and destroy cancer cells in the body.

Application of Nanolasers in Life Sciences

The nanolasers, which are based on research conducted by Nanosys, were developed by growing semiconductor nanowires. The linewidths, wavelengths, and power dependence of the nanowire emission characterize the nanowires as active optical cavities. Current leading solid-state lasers, often made of gallium arsenide or gallium nitride, are made of multilayer thin films and measure several micrometers in size. The nanowire laser is 1,000 times smaller, allowing localized optical illumination. It can be tuned to emit light of different wavelengths from the infrared to the deep UV by simply changing the diameter or composition of the nanowire.

One of the smallest lasers ever made, nanowire nanolaser, is too small to be seen even with the aid of the most powerful optical microscope. The nanowire nanolasers are pure crystals of zinc oxide that grow vertically in aligned arrays like the bristles on a brush. These crystal nanowires range from 2 to 10 μm in length, depending on how long the growth process was allowed to proceed. The nanowire nanolaser has been successfully tested by a team of researchers from Lawrence Berkeley National Laboratory and the University of California at Berkeley. This device, which emits flashes of UV light, measures just <100 nm. Self-organized dendritic crystal growth is explored at the Berkeley Laboratory to assemble uniform semiconductor nanowires into highly ordered 1D microscale arrays that resemble comb structures. The individual ZnO nanowires have uniform diameters ranging from 10 to 300 nm. Under optical excitation, each individual ZnO nanowire serves as a Fabry–Perot optical cavity, and together they form a highly ordered nanowire UV laser array (Yan et al 2003).

It is the small area of illumination that holds near-term potential for the nanolaser. Near-term products could include ultrahigh-resolution photolithography for

next-generation microchips, as well as laser-powered biochips. Other potential applications for the nanolaser include high-density information storage, high-definition displays, photonics, optocommunications, and chemical analysis on microchips. Nanolaser spectroscopy have also been used to study very small biological structures. This technology has also been applied for biphotonic detection of cancer in single cells.

Nanogenomics

The term "nanogenomics" simply means the application of nanobiotechnology to study the genome, i.e., the total genetic material of an organism. Several technologies are used to study the genome. A few examples are given here and others are described in Chapter 3.

DNA Nanotechnology

DNA is not only the secret of life but also an ideal molecule for building nanometer-scale structures. Strands of DNA interact in the most programmable way. Their enormous variability provides ample scope for designing molecules (Seeman 2004). DNA scaffolds could hold guest molecules in orderly arrays for crystallography. Nanometer-scale DNA machines can function by having part of their structure change from one DNA conformation to another. These movements can be controlled by chemical means or by the use of special DNA strands.

Structural DNA nanotechnology consists of combining unusual DNA motifs by specific structurally well-defined cohesive interactions (primarily sticky ends) to produce target materials with predictable 3D structures. This effort has generated DNA polyhedral catenanes, robust nanomechanical devices, and a variety of periodic arrays in two dimensions. The system has been used to produce specific patterns on the mesoscale through designing and combining specific DNA strands, which are then examined by AFM. The combination of these constructions with other chemical components is expected to contribute to the development of nanoelectronics, nanorobotics, and smart materials.

Many new tertiary interactions are being discovered and some of these are being used for the purpose of generating new nucleic acid-based materials. These may ultimately lead to a new generation of capabilities for structural nucleic acid nanotechnology. As more knowledge is gained about the metabolism of DNA, new motifs may be discovered that are currently exploited by living systems and that can be used by the materials sciences to generate new materials. Structural nucleic acid nanotechnology is in its infancy, but it seems to be capable of remarkable versatility in the organization of matter on the nanoscale (Seeman 2003).

Researchers in the Department of Physics at the University of Oxford, UK, have developed a one-step method for the self-assembly of stable DNA nano structures. Three-dimensional DNA nanotetrahedrons can further self-assemble into

macrostructures. This technology, which is available for licensing, has potential medicine-related applications in

- drug delivery;
- gene therapy;
- vaccine delivery; and
- biosensors (molecular sensors).

A future goal is incorporation of DNA devices into nanorobotics. Nanoelectronic components, such as metallic nanoparticles or carbon nanotubes, will need to be combined with DNA molecules in compatible systems. This will not be easy, given the diverse chemical nature of these molecules. An ideal nanomachine is one that can replicate. Unlike linear DNA, branched DNA used in these devices does not lend itself to self-replication. Some efforts are in progress to overcome these problems and it may be possible to have DNA-based replicating machines in a few decades.

RNA Nanotechnology

RNA has an important role in nanoscale fabrication due to its amazing diversity of function and structure. RNA molecules can be designed and manipulated with a level of simplicity characteristic of DNA while possessing versatility in structure and function similar to that of proteins. RNA helicases are a large family of molecular motors that utilize nucleoside triphosphates to unwind RNA duplexes and to remodel RNA–protein complexes. These enzymes have a potential application in controlling conformational changes in nanoassemblies that contain RNA (Jankowsky et al 2005).

Scientists at the University of California, Santa Barbara, are using assembly and folding principles of natural RNA to build potentially useful artificial structures at the nanoscale. Reliable prediction and design of the 3D structure of artificial RNA building blocks has been achieved to generate molecular jigsaw puzzle units called tectosquares (Chworos et al 2004). They can be programmed with control over their geometry, topology, directionality, and addressability to algorithmically self-assemble into a variety of complex nanoscopic fabrics with predefined periodic and aperiodic patterns and finite dimensions. This work emphasizes the modular and hierarchical characteristics of RNA by showing that small RNA structural motifs can code the precise topology of large molecular architectures. It demonstrates that fully addressable materials based on RNA can be synthesized and provides insights into self-assembly processes involving large populations of RNA molecules.

The ability of RNA to fold into a variety of rigid structural motifs can provide potential modules for supramolecular engineering. Healthcare applications include the development of medical implants, regeneration of organs, and nanodiagnostics. The

most recent development in the exploration of RNA nanoparticles is for pathogen detection, drug/gene delivery, and therapeutic application (Guo 2005b).

Role of Nanobiotechnology in Identifying Single-Nucleotide Polymorphisms

Genetic analysis based on single-nucleotide polymorphisms (SNPs) has the potential to enable identification of genes associated with disease susceptibility, to facilitate improved understanding and diagnosis of those diseases, and should ultimately contribute to the provision of new therapies. To achieve this end, new technology platforms are required that can increase genotyping throughput, while simultaneously reducing costs by as much as 2 orders of magnitude. Development of a variety of genotyping platforms with the potential to resolve this dilemma is already well advanced through research in the field of nanobiotechnology. Novel approaches to DNA extraction and amplification have reduced the times required for these processes to seconds. Microfluidic devices enable polymorphism detection through very rapid fragment separation using capillary electrophoresis and high-performance liquid chromatography (LC), together with mixing and transport of reagents and biomolecules in integrated systems. The potential for application of established microelectronic fabrication processes to genetic analysis systems has been demonstrated (e.g., photolithography-based in situ synthesis of ODNs on microarrays). Innovative application of state-of-the-art photonics and integrated circuitry are leading to improved detection capabilities. The diversity of genotyping applications envisaged in the future, ranging from the very high-throughput requirements for drug discovery through to rapid and cheap near-patient genotype analysis, suggests that several SNP genotyping platforms will be necessary to optimally address the different niches.

Clinical Nanoproteomics

Nanoproteomics—application of nanobiotechnology to proteomics—improves on most current protocols including protein purification/display and automated identification schemes that yield unacceptably low recoveries with reduced sensitivity and speed while requiring more starting material. Low-abundance proteins and proteins that can only be isolated from limited source material (e.g., biopsies) can be subjected to nanoscale protein analysis—nano-capture of specific proteins and complexes and optimization of all subsequent sample handling steps leading to mass analysis of peptide fragments. This is a focused approach, also termed targeted proteomics, and involves examination of subsets of the proteome, e.g., those proteins that are specifically modified, or bind to a particular DNA sequence, or exist as members of higher-order complexes, or any combination thereof. This approach is used at Memorial Sloan-Kettering Cancer Center and Cornell University,

New York, to identify how genetic determinants of cancer alter cellular physiology and response to agonists. Some nanoproteomic technologies are described here briefly.

Multiphoton Detection

A new detection technique called multiphoton detection (MPD) is in development at BioTrace Inc and enables quantitation of subzeptomole amounts of proteins. It will be used for diagnostic proteomics, particularly for cytokines and other low-abundance proteins. BioTrace is developing supersensitive protein biochips to detect as low as 5 fg/ml (0.2 amol/ml) concentration of proteins. Thus, this innovative type of the P-chips might permit ∼1,000-fold better sensitivity than current protein biochips.

Nanoflow Liquid Chromatography

The use of LC in analytical chemistry is well established, but relatively low sensitivity associated with conventional LC makes it unsuitable for the analysis of certain biological samples. Furthermore, the flow rates at which it is operated are not compatible with the use of specific detectors, such as electrospray ionization (ESI) mass spectrometers. Therefore, due to the analytical demands of biological samples, miniaturized LC techniques were developed to allow for the analysis of samples with greater sensitivity than that afforded by conventional LC. In nanoflow LC (nanoLC) chromatographic separations are performed using flow rates in the low nanoliter per minute range, which result in high analytical sensitivity due to the large concentration efficiency afforded by this type of chromatography. NanoLC, in combination with tandem mass spectrometry, was first used to analyze peptides and as an alternative to other mass spectrometric methods to identify gel-separated proteins. Gel-free analytical approaches based on LC and nanoLC separations have been developed, which are allowing proteomics to be performed in faster and more comprehensive manner than by using strategies based on the classical 2D gel electrophoresis approaches (Cutillas 2005).

Protein identification using nanoflow liquid chromatography–mass spectrometry (MS)–MS (LC–MS–MS) provides reliable sequencing information for low femtomole level of protein digests. However, this task is more challenging for subfemtomole peptide levels.

High-Field Asymmetric Waveform Ion Mobility Mass Spectrometry

An ion mobility technology—high-field asymmetric waveform ion mobility mass spectrometry (FAIMS)—has been introduced as online ion selection methods

compatible with ESI. FAIMS uses ion separation to improve detection limits of peptide ions when used in conjunction with electrospray and nanoelectrospray MS. This facilitates the identification of low-abundance peptide ions often present at parts-per-million levels in complex proteolytic digests and expands the sensitivity and selectivity of nanoLC–MS analyses in global and targeted proteomics approaches. This functionality likely will play an important role in drug discovery and biomarker programs for monitoring of disease progression and drug efficacy (Venne et al 2005).

Nanoproteomics for Study of Misfolded Proteins

Misfolding and self-assembly of proteins in nano-aggregates of different sizes and morphologies (nano-ensembles, primarily nanofilaments, and nanorings) is a complex phenomenon that can be facilitated, impeded, or prevented, by interactions with various intracellular metabolites, intracellular nanomachines controlling protein folding, and interactions with other proteins. A fundamental understanding of molecular processes leading to misfolding and self-aggregation of proteins involved in various neurodegenerative diseases will provide critical information to help identify appropriate therapeutic routes to control these processes. An elevated propensity of misfolded protein conformation in solution to aggregate with the formation of various morphologies impedes the use of traditional physicochemical approaches for studies of misfolded conformations of proteins. In an alternative approach, the protein molecules were tethered to surfaces to prevent aggregation and AFM was used to probe the interaction between protein molecules depending on their conformations (Kransnoslobodtsev et al 2005). It was shown that formation of filamentous aggregates is facilitated at pH values corresponding to the maximum of rupture forces. A novel surface chemistry was developed for anchoring of Aβ peptides at their N-terminal moieties. The use of the site-specific immobilization procedure allowed to measure the rupture of Aβ−Aβ contacts at the single-molecule level. The rupture of these contacts is accompanied by the extension of the peptide chain detected by a characteristic elastomechanical component of the force–distance curves. Nanomechanical studies have potential applications for an understanding of the mechanisms of development of protein misfolding diseases such as Alzheimer's disease (AD).

Use of Nanotube Electronic Biosensor in Proteomics

An SWNT as a platform has been used for investigating surface–protein and protein–protein binding and developing highly specific electronic biomolecule detectors (Chen et al 2003). Nonspecific binding on nanotubes, a phenomenon found with a wide range of proteins, is overcome by immobilization of polyethylene oxide

chains. A general approach is then advanced to enable the selective recognition and binding of target proteins by conjugation of their specific receptors to polyethylene oxide-functionalized nanotubes. These arrays are attractive because no labeling is required and all aspects of the assay can be carried out in solution phase. This scheme, combined with the sensitivity of nanotube electronic devices, enables highly specific electronic sensors for detecting clinically important biomolecules such as antibodies associated with human autoimmune diseases. These arrays are attractive because no labeling is required and all aspects of the assay can be carried out in solution phase. Interfacing novel nanomaterials with biological systems could therefore lead to important applications in disease diagnosis, proteomics, and nanobiotechnology in general.

Study of Protein Synthesis and Single-Molecule Processes

All life relies on the actions and reactions of single molecules within cells, but these molecules are so tiny that they have long eluded direct, real-time investigation using conventional light microscopes. Breakthrough technologies are enabling researchers to have an unprecedented view into the workings of individual molecules.

Single-molecule methods enable observation of the stepwise movement of aminoacyl-transfer RNA (aa-tRNA) into the ribosome during selection and kinetic proofreading using single-molecule FRET (smFRET). Intermediate states in the pathway of tRNA delivery were observed using antibiotics and nonhydrolyzable GTP analogs. Three unambiguous FRET states have been identified: initial codon recognition, GTPase-activated, and fully accommodated states (Blanchard et al 2004a). The antibiotic tetracycline blocks the progression of aa-tRNA from the initial codon recognition state, whereas the cleavage of the sarcin–ricin loop impedes progression from the GTPase-activated state. These data support a model in which ribosomal recognition of correct codon–anticodon pairs drives rotational movement of the incoming complex of EF-Tu-GTP-aa-tRNA toward peptidyl-tRNA during selection on the ribosome. This is the basis of a mechanistic model of initial selection and proofreading.

Subsequently, tRNA molecules fluctuate between two configurations assigned as classical and hybrid states. The lifetime of classical and hybrid states, measured for complexes carrying aa-tRNA and peptidyl-tRNA at the A site, shows that peptide bond formation decreases the lifetime of the classical-state tRNA configuration by approximately 6-fold. These data suggest that the growing peptide chain plays a role in modulating fluctuations between hybrid and classical states. smFRET was also used to observe aa-tRNA accommodation coupled with elongation factor G-mediated translocation (Blanchard et al 2004b). Dynamic rearrangements in tRNA configuration are also observed subsequent to the translocation reaction. This work underscores the importance of dynamics in ribosome

function and demonstrates single-particle enzymology in a system of more than two components.

Using these technologies, one can collect photons of light coming from a single molecule. This information reports on a biomolecule's location, its interaction with other molecules, and tiny motions within the molecule itself. These tools allow viewing of an enzymatic reaction from the very intuitive perspective of movements. Enzymes are molecular machines with moving parts, but these motions are on the order of a billionth of a centimeter. Nanoscale technologies will help understand the mechanism of the ribosome, which is an assembly of ~60 different molecules working together to read the instructions for making new proteins coded in DNA. These instructions are presented to the ribosome in the form of messenger RNA (mRNA). The process of translating mRNA instructions into protein involves the selection by the ribosome of adaptor RNA molecules, called tRNA. It is the selection of specific tRNA molecules that determines the relationship between the gene sequence and the sequence of the resulting protein. The reaction between tRNA and the ribosome is the basis of what is called the universal genetic code. Ribosome is important for cell function and human health. Because protein synthesis is crucial to the life cycle of all bacteria, ~50% of antibiotics used today target ribosomal function. Ribosomal function is also a key to the success or failure of deadly viral infections such as HCV and HIV. Cancer cells, too, rely on protein synthesis to survive and multiply, so drugs that block ribosomal function in a cancer-specific manner might prove safe and effective in chemotherapy. Genetic aberrations in the DNA–ribosome relationship can cause the enzyme to produce faulty proteins that trigger cystic fibrosis (CF) and other inherited illnesses. Understanding the mechanism of the ribosome may be a fundamental first step for developing antibiotics, cancer therapies, and antiviral drugs. The ribosome may one day even be a target for gene therapy.

Nanofilter Array Chip

Massachusetts Institute of Technology engineers have developed a microfabricated nanofilter array chip that can size-fractionate protein complexes and small DNA molecules based on the Ogston sieving mechanism (Fu et al 2006). Nanofilter arrays with a gap size of 40–180 nm were fabricated and characterized. Millions of pores can be spread across a microchip the size of a thumbnail. In the model, proteins move through deep and shallow regions that act together to form energy barriers. These barriers separate proteins by size. The smaller proteins go through quickly, followed by increasingly larger proteins, with the largest passing through last. To date, the Ogston sieving model has been used to explain gel electrophoresis, even though no one has been able to unequivocally confirm this model in gel-based experiments. The performance of the current 1D sieves matches the speed of 1D gels, but it can be improved greatly. The sieves could potentially be used to replace 2D gels in the process of discovering disease biomarkers.

Study of Single-Membrane Proteins at Subnanometer Resolution

High-resolution AFM enables observation of substructures of single-membrane proteins at subnanometer resolution as well as their conformational changes, oligomeric state, molecular dynamics, and assembly (Janovjak et al 2006). Complementary to AFM imaging, single-molecule force spectroscopy experiments allow detection of molecular interactions established within and between membrane proteins. The sensitivity of this method makes it possible to detect the interactions that stabilize secondary structures such as transmembrane $UP\alpha$-helices, polypeptide loops, and segments within. It has elucidated unfolding and refolding pathways of membrane proteins as well as their energy landscapes. These approaches will provide insights into membrane protein structure, function, and unfolding. They could help to answer key questions on the molecular basis of certain neuropathological disorders.

Nanoparticle–Protein Interactions

Due to their small size, nanoparticles have distinct properties compared with the bulk form of the same materials. In a biological fluid, proteins associate with nanoparticles, and the amount and presentation of the proteins on the surface of the particles leads to an in vivo response. Proteins compete for the nanoparticle "surface," leading to a protein "corona" that largely defines the biological identity of the particle. Thus, knowledge of rates, affinities, and stoichiometries of protein association with, and dissociation from, nanoparticles is important for understanding the nature of the particle surface seen by the functional machinery of cells. Approaches have been developed to study these parameters and apply them to plasma and simple model systems, albumin and fibrinogen (Cedervall et al 2007). A series of copolymer nanoparticles are used with variation in size and composition (hydrophobicity). Isothermal titration calorimetry is suitable for studying the affinity and stoichiometry of protein binding to nanoparticles. The rates of protein association and dissociation are determined using SPR technology with nanoparticles that are thiol-linked to gold, and through size exclusion chromatography of protein–nanoparticle mixtures. This method is less perturbing than centrifugation and is developed into a systematic methodology to isolate nanoparticle-associated proteins. The kinetic and equilibrium binding properties depend on protein identity as well as particle surface characteristics and size.

Self-Assembling Peptide Scaffold Technology for 3D Cell Culture

Biomaterial scaffolds are components of cell-laden artificial tissues and transplantable biosensors. Some of the most promising new synthetic biomaterial scaffolds are composed of self-assembling peptides that can be modified to contain

biologically active motifs. Peptide-based biomaterials can be fabricated to form 2D and 3D structures (Holmes 2002).

3DM Inc is commercializing the patented self-assembling peptide scaffold technology exclusively licensed from the Massachusetts Institute of Technology (Cambridge, MA) for cell culture, drug discovery, bioproduction, and other applications across the life and medical sciences. The hydrogels are composed of short strand of standard amino acids and 99.5% water, which then self-assemble into very fine fibers resembling a bare extracellular matrix (ECM). Researchers in the fields of cancer biology, stem cell biology, and tissue engineering have been the first to realize that ECM and a tuned 3D microenvironment are critical for the proper understanding required for drug discovery, cell biology, and cell therapy development.

Until recently, 3D cell culture has required either animal-derived materials, with their inherent reproducibility and cell signaling issues, or much larger synthetic scaffolds, which fail to approximate the physical nanometer-scale and chemical attributes of native ECM. PuraMatrix (3DM Inc) nanometer-sized fibers, very difficult and expensive to create without 3DM's patented molecular self-assembly, provide a scaffold encapsulating cells in three dimensions and allow defined cell culture conditions, cell migration, nutrient diffusion, and cell harvesting (Zhang and Semino 2003). For the first time, the cell biology and drug discovery communities now have a biocompatible bare ECM, which can be combined with relevant proteins and growth factors to more closely resemble the in vivo milieu, and which is compatible with cGMP requirements for cell therapy, medical device, and bioproduction applications. The PuraMatrix gels have undergone extensive in vivo toxicology safety testing.

There is a growing trend in 3D cell culture, moving cell biology research away from flat 2D cell cultures in traditional petri dishes. Some of the pioneers in 3D cell culture lend their observations that 3D microenvironments can radically alter cell behavior, enabling cells to mimic in vivo responses to drug targets and medical therapies much more accurately. Given the growing body of literature, drug discovery efforts at major pharmaceutical and biotechnology companies are beginning to adopt 3D culture techniques in their cell-based assays, especially in the context of high-content screening. Using products like PuraMatrix^{TM} in order to create synthetic ECM scaffolds and tuned 3D microenvironments has proven to yield better data while also reducing the number of animals used for expensive in vivo testing. The National Cancer Institute's program on the cellular microenvironment will spur research and adoption of 3D culture techniques and products in both academia and industry.

Nanobiotechnology for Study of Mitochondria

While autosomal nuclear DNA genes are confined to the nucleus, limited to two copies per cell, the mitochondrial DNA (mtDNA) genes are distributed throughout the cytoplasm and are present in numerous copies per cell. The mtDNA molecule

is relatively small containing 16,569 nucleotide pairs. Mutations of mtDNA are responsible for several human diseases, e.g., neurological diseases. Mitochondrial diseases are often underdiagnosed and therapies are inadequate. Nanobiotechnology has been applied to the study of mitochondria (Weissig et al 2007).

Nanomaterials for the Study of Mitochondria

Some of the nanomaterials for study of mitochondrial nanobiotechnology are summarized in Table 2.6.

Study of Mitochondria with Nanolaser Spectroscopy

Because mitochondria are so small, averaging a few hundred nanometers, scientists have been unable to study them in vitro with the necessary precision. A unique laser operating in the nanometer range at the Department of Energy's Sandia National Laboratories has been used for studying the reactions of mitochondria in their functioning state. The laser, using samples obtained from the University of New Mexico School of Medicine, has shown that it can obtain clear signals from individual mitochondria in vitro. Work to date has shown the biolaser is able to

Table 2.6 Nanomaterials for the study of mitochondria

Nanomaterial	Modification	Application	Reference
Biosomes	Self-assembly of mitochondriotropic bola-amphiphile (DQAsomes)	Selective delivery of DNA to mitochondria to enable mitochondrial gene therapy	Cheng et al 2005
Liposomes	Mitochondria-specific ligands with hydrophobic anchor to form mitochondria-targeted liposomes	Mitochondrial-specific drug delivery	Boddapati et al 2005
Nanoparticles	Mitochondria-specific ligands with linker to form mitochondria-targeted nanoparticles	Selective accumulation in mitochondria to probe or manipulate mitochondrial function	Ju-Nam et al 2006
Quantum dots	Mitochondria-specific ligands with linker to form mitochondria-targeted quantum dots	To study the function and morphology of mitochondria	Kaul et al 2006

measure mitochondrial size through unexpected bursts of light given up by each mitochondrion. The laser, using the same means, can also measure the swelling effect caused by the addition of calcium ions (the reaction is considered to be the agent of apoptosis for both mitochondria and their host cells). If this laser probe helps us to understand how mitochondria in nerve cells respond to various stimuli, we may be able to understand how all cells make life or death decisions, i.e., how mitochondria send out signals that kill cells as well as energize them. The researchers expect to introduce neuroprotectant drugs into experiments and be able to test hundreds of possible protective substances daily instead of two or three formerly possible.

Nanobiotechnology and Ion Channels

Ion channels provide the basis for the regulation of electrical excitability in the central and peripheral nervous systems. They are proteins that are equipped with a membrane-spanning ion-conducting pore. Disturbances in ion channels are cause of many neurological disorders. Ion channels were traditionally studied by the patch clamp technique, which was derived from the conventional voltage clamp method. This method is now supplemented by nanotechniques, which are being used to study cell membranes and their proteins. Nanoscopy is the characterization of the membrane channels by techniques that resolve their morphological and physical properties and dynamics in space and time in the nano range. These techniques make the study of structure and function of single-channel molecules in living cells possible and are currently being developed for automated and high-throughput measurements and fluorescence.

AFM has been combined directly with the patch clamp technique for the characterization of biological mechanoelectrical transduction channels in living inner and outer hair cells of the cochlea. Using an AFM stylus with a tip diameter of only a few nanometers, it was possible to displace individual stereocilia of cochlear hair cells, resulting in opening of single transduction channels. In contrast to the outside-out and the inside-out patch clamp configuration, this technique allows investigation of single mechanosensitive ion channels in entire cells (Lehmann-Horn and Jurkat-Rott 2003).

Aquaporin Water Channels

Peter Agre received the 2003 Nobel Prize in Chemistry for his discovery of the aquaporin (AQP) water channels. The function of many cells requires that water move rapidly into and out of them. There was only indirect evidence that proteinaceous channels provide this vital activity until Agre and colleagues purified AQP1 from human erythrocytes and reported its cDNA sequence. They proved that AQP1 is a specific water channel by cRNA expression studies in *Xenopus* oocytes and by

functional reconstitution of transport activity in liposomes after the incorporation of the purified protein (Knepper and Nielsen 2004).

The atomic structure of mammalian AQP1 illustrates how this family of proteins is freely permeated by water but not by protons (hydronium ions, H_3O^+). Definition of the subcellular sites of expression predicted their physiological functions and potential clinical disorders. Analysis of several human disease states has confirmed that AQPs are involved in multiple different illnesses including abnormalities of kidney function, loss of vision, onset of brain edema, starvation, and arsenic toxicity (Agre and Kozono 2003).

Research in this area requires nanoscale both in size and in time scales. In molecular dynamics simulations of water in short (0.8 nm) hydrophobic pores the water density in the pore fluctuates on a nanosecond time scale. In long simulations (460 ns in total) at pore radii ranging from 0.35 to 1.0 nm, researchers have quantified the kinetics of oscillations between a liquid-filled and a vapor-filled pore (Beckstein and Sansom 2003). One cannot assume that the behavior of water within complex biological pores may be determined by extrapolation from the knowledge of the bulk state or short simulations alone. Simulations aimed at collective phenomena such as hydrophobic effects may require simulation times >50 ns.

Nanotechnology and Bioinformatics

Bioinformatics, also referred to as computational biology, is the use of highly sophisticated computer databases to store, analyze, and share biological information. This is a discipline at the interface of computer science, mathematics, and biology. The tremendous amount of data generated by new biotechnologies requires bioinformatic tools for analysis. Analyzing how multiple genes function together can produce terabytes of data. But as nanotechnology enables greater sensing and collecting of data, the information flow could become measured in petabytes, or quadrillion bytes of information. Bioinformatics is essential for microarray data analysis and even more so for nanoarray data. In nanotechnology, the investigation of behavior and properties across a wide range of length scales is vital. Over the past two decades, computational techniques have evolved to the point that they now cover all length and time scales from the electronic to the macroscale, the realm of nanotechnology.

The National Science Foundation has funded a Network for Computational Nanotechnology based at Purdue University. Formed by seven universities, the network aims to give academia and industry access to advanced simulation tools for various disciplines including biotechnology. Accelrys is the leading company for the application of bioinformatic tools to nanotechnology. Its computational nanotechnology modeling using quantum mechanics, classical atomistic methods, and/or mesoscale simulations enable scientists to visualize and predict behavior emerging at length scales of up to 100 nm.

In the near future, it may be possible to fully model an individual cell's structure and function by computers connected to nanobiotechnology systems. Such a detailed virtual representation of how a cell functions might enable scientists to develop novel drugs with unprecedented speed and precision without any experiments in living animals. An example of application of this approach is the construction of 3D nanomap of synapse.

3D Nanomap of Synapse

Researchers have constructed a new detailed nanomap of the 3D terrain of a neuronal synapse, which shows the tiny spines and valleys resolved at nanometer scale (Coggan et al 2005). It is already changing the conventional views of the synaptic landscape. A biologically accurate computer simulation of synaptic function combines 3D electron microscope maps with physiological measurements from real neurons. The textbook view of the synapse describes it as a place where rifle-like volleys of neurotransmitter are launched from one defined region of the sending neuron to another defined target on the receiving neuron. In contrast, the new data suggest that synapse can act like a shotgun, firing buckshot-like bursts of neurotransmitter to reach receptors arrayed beyond the known receiving sites. This method was applied to study the chick ciliary ganglion, which is a cluster of neurons that connect the brain to the iris of the eye. It launches the neurotransmitter acetylcholine from sac-like vesicles across the synapse to two types of receptors, called $\alpha 7$ and $\alpha 3$ nicotinic receptors. The image of this ganglion is not one of a simple synapse with a single release site, but multiple release sites. And it shows $\alpha 3$ receptors within the postsynaptic region, but $\alpha 7$ receptors outside this region. This model showed that if the neurotransmitter were released only from vesicles in active zones, as previously believed, it would be a poor match to actual properties of the neuron. But according to the new model of neurotransmitter release, called ectopic release, the location of $\alpha 7$ receptors can match the actual properties of the synapse very accurately. The new 3D modeling technique could offer a powerful tool for understanding neurological disease, such as myasthenia gravis. The model can also be used as a tool for drug discovery. Drug discovery efforts can be focused on the site of the anomaly.

Nanomanipulation

AFM enables the imaging and manipulation of biological systems at the nanometer scale. Examples include the following:

- Extraction of chromosomal DNA for genetic analysis
- Disruption of antibody—-antigen bonds
- Dissection of biological membranes

- Nanodissection of protein complexes
- Controlled modulation of protein conformations

Nanomanipulation by Combination of AFM and Other Devices

A new instrument was constructed by combining an objective-type total internal reflection fluorescence microscope with an AFM. This instrument can detect and confirm the result of cellular level manipulations made with the AFM part through the detection system of the highly sensitive fluorescence microscope part. In this combination, manipulations are now possible from the nanometer to the micrometer scale and the fluorescence detection system is sensitive enough even for localizing single molecules. This system was applied as a precise intracellular injector—nanoplanter (Nishida et al 2002). Fluorescent beads were first chemically immobilized onto a ZnO whisker that was glued to an AFM tip and were injected into a living cell together with the whisker. It was demonstrated that the system could clearly show the result of injection, i.e., the presence of a small number of fluorescent beads in the cell.

Nanomanipulation and nanoextraction on a scale close to and beyond the resolution limit of light microscopy is needed for many modern applications in biological research. For the manipulation of biological specimens a combined microscope enabling UV microbeam laser manipulation together with manipulation by an AFM has been used (Stark et al 2003). In a one-step procedure, human metaphase chromosomes were dissected optically by the UV laser ablation and mechanically by AFM manipulation. With both methods, sub-400 nm cuts could be achieved routinely. Thus, the AFM is an indispensable tool for in situ quality control of nanomanipulation. However, already on this scale the dilation of the topographic AFM image due to the tip geometry can become significant. Therefore, the AFM images were restored using a tip geometry obtained by a blind tip reconstruction algorithm. Cross-sectional analysis of the restored image revealed a 380-nm-wide UV laser cut and AFM cuts between 70 and 280 nm.

Surgery on Living Cells Using AFM with Nanoneedles

Scientists at the Research Institute for Cell Engineering (Hyogo, Japan) have developed a tool for performing surgical operations on living cells at nanoscale resolution using AFM and a modified AFM tip (Obataya et al 2005). The AFM tips are sharpened to ultrathin needles of 200–300 nm in diameter using focused ion beam etching. Force–distance curves obtained by AFM using the needles indicated that the needles penetrated the cell membrane following indentation to a depth of 1–2 μm. The force increase during the indentation process was found to be consistent with application of the Hertz model. A 3D image generated by laser scanning confocal microscopy directly revealed that the needle penetrated both the cellular and nuclear

membranes to reach the nucleus. This technique enables the extended application of AFM to analyses and surgery of living cells.

Optoelectronic Tweezers

The ability to easily manipulate cells has many applications, e.g., isolation and study of circulating fetal cells in a mother's blood sample to sort out abnormally shaped organisms from healthy ones. This sorting process is usually painstakingly done by hand. A technician finds the cell of interest under a microscope and literally cuts out the piece of glass where the cell is located, taking care not to harm the sample. The conventional manipulation techniques—including optical tweezers, electrokinetic forces, magnetic tweezers, acoustic traps, and hydrodynamic flows—cannot achieve high resolution and high throughput at the same time. Optical tweezers offer high resolution for trapping single particles but have a limited manipulation area owing to tight focusing requirements; on the other hand, electrokinetic forces and other mechanisms provide high throughput but lack the flexibility or the spatial resolution necessary for controlling individual cells.

An optical image-driven dielectrophoresis technique permits high-resolution patterning of electric fields on a photoconductive surface for manipulating single particles (Chiou et al 2005). Such optoelectronic tweezers can produce instant microfluidic circuits without the need for sophisticated microfabrication techniques. With direct optical imaging control, multiple manipulation functions are combined to achieve complex, multistep manipulation protocols. Microscopic polystyrene particles suspended in a liquid are sandwiched between a piece of glass and the photoconductive material. Wherever light hits the photosensitive material, it behaves like a conducting electrode, while areas not exposed to light behave like a nonconducting insulator. Once a light source is removed, the photosensitive material returns to normal. Depending upon the properties of the particles or cells being studied, they are either attracted to or repelled by the electric field generated by the optoelectronic tweezer. It requires 100,000 times less optical intensity than do optical tweezers. Parallel manipulation of 15,000 particle traps can be done on a $1.3\,\text{mm} \times 1.0\,\text{mm}$ area. The researchers are now studying ways to combine this technology with computer pattern recognition so that the sorting process could be automated. A program could be designed to separate cells by size, luminescence, texture, fluorescent tags, and basically any characteristic that can be distinguished visually.

Chapter 3
Nanomolecular Diagnostics

Introduction

Clinical application of molecular technologies to elucidate, diagnose, and monitor human diseases is referred to as molecular diagnosis. It is a broader term than DNA diagnostics and refers to the use of nucleic acid technologies that employ DNA, RNA, genes, or proteins as bases for diagnostic tests. The term "biotechnology diagnostics" also includes the use of monoclonal antibodies (MAbs) and enzyme-linked immunosorbent assay (ELISA). The term "genomic diagnostics" is used for application of molecular diagnostic technologies in genomics, which is the study of all of the genes in an organism—their sequences, structure, regulation, interaction, and products. A more detailed description of molecular diagnostics is presented elsewhere (Jain 2007a).

Because of the small dimension of nanoparticles, most of the applications of nanobiotechnology in molecular diagnostics fall under the broad category of biochips/microarrays but are more correctly termed nanochips and nanoarrays. Nanotechnology-on-a-chip is a general description that can be applied to several methods. Some of these do not use nanotechnologies but merely have the capability to analyze nanoliter amounts of fluids.

Biochips, constructed with MEMS on a micron scale, are related to micromanipulation, whereas nanotechnology-based chips on a nanoscale are related to nanomanipulation. Even though microarray/biochip methods employing the detection of specific biomolecular interactions are now an indispensable tool for molecular diagnostics, there are some limitations. DNA microarrays and ELISA rely on the labeling of samples with a fluorescent or radioactive tag—a highly sensitive procedure that is time-consuming and expensive.

The chemical modification and global amplification of the nucleic acid samples are achieved by polymerase chain reaction (PCR), which can introduce artifacts caused by the preferential amplification of certain sequences. Alternative label-free methods include SPR and quartz crystal microbalance, which rely on mass detection. Nanotechnologies also provide label-free detection. Nanotechnology is thus being applied to overcome some of the limitations of biochip technology. This chapter focuses on the application of nanotechnologies to nucleic acid as well as protein diagnostics.

K.K. Jain, *The Handbook of Nanomedicine*,

Nanodiagnostics

Nanomolecular diagnostics is the use of nanobiotechnology in molecular diagnostics and can be termed "nanodiagnostics" (Jain 2003a). Numerous nanodevices and nanosystems for sequencing single molecules of DNA are feasible. It seems quite likely that there will be numerous applications of inorganic nanostructures in biology and medicine as biomarkers. Given the inherent nanoscale of receptors, pores, and other functional components of living cells, the detailed monitoring and analysis of these components will be made possible by the development of a new class of nanoscale probes. Biological tests measuring the presence or activity of selected substances become quicker, more sensitive, and more flexible when certain nanoscale particles are put to work as tags or labels. Nanotechnology will improve the sensitivity and integration of analytical methods to yield a more coherent evaluation of life processes.

It is difficult to classify such a wide variety of technologies, but various nanotechnologies with potential applications in molecular diagnostics are listed in Table 3.1. Nanotechnology-on-a-chip was described earlier in this Chapter 2. Some of the other technologies will be described briefly in the following text using examples of commercial products. A more detailed description is given in a special book on this topic (Jain 2006b).

Rationale of Nanotechnology for Molecular Diagnostics

Numerous nanodevices and nanosystems for sequencing single molecules of DNA are feasible. It is likely that there will be numerous applications of inorganic nanostructures in biology and medicine as markers:

- Nanoscale probes would be suitable for a detailed analysis of receptors, pores, and other components of living cells that are on a nanoscale.
- Nanoscale particles, used as tags or labels, increase the sensitivity, speed, and flexibility of biological tests measuring the presence or activity of selected substances.
- Nanotechnology will improve the sensitivity and integration of analytical methods to yield a more coherent evaluation of life processes.

Nanoarrays for Molecular Diagnostics

Several nanoarrays and nanobiochips are in development (Jain 2007a). Some of these will be reviewed here.

Table 3.1 Nanotechnologies with potential applications in molecular diagnostics

Nanotechnology to improve polymerase chain reaction (PCR)
Nanotechnology-on-a-chip
Microfluidic chips for nanoliter volumes: NanoChip
Optical readout of nanoparticle labels
NanoArrays
Protein nanoarrays
Nanotechnology-based cytogenetics
Study of chromosomes by atomic force microscopy
Quantum dot fluorescent in situ hybridization (FISH)
Nanoscale single-molecule identification
Nanoparticle technologies
Gold particles
Nanobarcodes
Magnetic nanoparticles: ferrofluids, supramagnetic particles combined with MRI
Quantum dot technology
Nanoparticle probes
Nanowires
Nanopore technology
Measuring length of DNA fragments in a high-throughput manner
DNA fingerprinting
Haplotyping
DNA nanomachines for molecular diagnostics
Nanoparticle-based immunoassays
DNA–protein and nanoparticle conjugates
Nanochip-based single-molecular interaction force assays
Resonance light scattering technology
Nanosensors
Cantilever arrays
Living spores as nanodetectors
Nanopore nanosensors
Quartz nanobalance DNA sensor
PEBBLE (Probes Encapsulated by Biologically Localized Embedding) nanosensors
Nanosensor glucose monitor
Photostimulated luminescence in nanoparticles
Optical biosensors: e.g., SPR technology

Source: Jain PharmaBiotech.

NanoPro™ System

The NanoPro™ System (BioForce Nanosciences Inc, Ames, IA, USA) consists of three separate components:

1. The NanoArrayer™ embodies proprietary instrumentation and methods for creating a broad spectrum of NanoArray™-based biological tests. This device places molecules at defined locations on a surface with nanometer spatial resolution. The arrays of molecules (NanoArrays™, see below) are unique to BioForce and can only be created with a NanoArrayer™.

2. NanoArrays™ are ultraminiaturized biological tests with applications in many areas. The company's first NanoArray™ products are presently being evaluated for commercial utilization by potential users and are targeted toward the proteomics/genomics and diagnostics markets. These products include a custom nucleic acid NanoArray™ for RNA expression profiling as well as virus detection NanoArray™ called the ViriChip™. NanoArray™ chips are for protein expression profiling and immunodiagnostics.
3. The NanoReader™ is a customized AFM optimized for reading NanoArray™ chips. Using the AFM as a readout method optimizes analysis, with the following advantages:

   - No need for secondary reporter systems such as fluorescence, radioactivity, or enzyme-linked detection schemes
   - Reductions in materials used as several thousand molecules can be covered with one test
   - Ultimately an increased sensitivity with single-molecule detection

Nanofluidic/Nanoarray Devices to Detect a Single Molecule of DNA

One of the more promising uses of nanofluidic devices is isolation and analysis of individual biomolecules, such as DNA, which could lead to new detection schemes for cancer. This is now closer to realization with the development of a simple nanofluidic device within a silicon nanotube for a relatively long time (Fan et al 2005). This procedure entails first constructing silicon nanowires (SiNWs) on a substrate, or chip, using standard photolithographic and etching techniques, followed by a chemical oxidation step that converts the NWs into hollow nanotubes. Using this process, the investigators can reliably create nanotubes with diameters as small as 10 nm, though for their biomolecule isolation device they used nanotubes with a diameter of 50 nm. To trap DNA molecules, the investigators built a device consisting of a silicon nanotube connecting two parallel microfluidic channels. Electrodes provide a source of current used to drive DNA into the nanotubes. Each time a single DNA molecule moves into the nanotube, the electrical current increases suddenly. The current returns to its baseline value when the DNA molecule exits the nanotube. On average, a DNA molecule remains within the nanotube for ~7.5 ms, which should be sufficient to make a variety of analytical measurements that could reveal cancer-associated mutations. The investigators are now adding optical and electrical circuitry to probe the trapped DNA molecules.

The nanochannel array technology (BioNanomatrix, Philadelphia, PA, USA) is designed to enable direct visualization and linear analysis of multimegabase genomic DNA at the single-molecule level with high feature resolution in massive parallel fashion. The platform is also anticipated to significantly reduce the cost and time needed for the extensive data and integrative analyses that have hindered widespread use of whole-genome studies to date. It is expected to have broad

application in systems biology, personalized medicine, pathogen detection, drug development, and clinical research.

A 2D method for MS in solution is based on the interaction between a nanometer-scale pore and analytes (Robertson et al 2007). The technique involves creating a lipid bilayer membrane similar to those in living cells, and "drilling" a pore in it with a protein (α-HL) produced by the *Staphylococcus aureus* bacteria specifically to penetrate cell membranes. Charged molecules (such as single-stranded DNA [ssDNA]) are forced one at a time into the nanopore, which is only 1.5 nm at its smallest point, by an applied electric current. As the molecules pass through the channel, the current flow is reduced in proportion to the size of each individual chain, allowing its mass to be easily derived. In this experiment, various-sized chains in solution of the uncharged polymer polyethylene glycol (PEG) were substituted for biomolecules. Each type of PEG molecule reduced the nanopore's electrical conductance differently as it moved through, allowing the researchers to distinguish one size of PEG chain from another. Because the dimensions of the lipid bilayer and the α-HL pore, as well as the required amount of electrical current, are at the nanoscale level, the single-molecule MS technology may one day be incorporated into "lab-on-a-chip" molecular analyzers and ssDNA sequencers. This single-molecule analysis technique could prove useful for the real-time characterization of biomarkers (i.e., nucleic acids, proteins, or other biopolymers). With automated, unsupervised analytical and statistical methods, this technique should prove viable as a generalized analytical technique with nanopore arrays containing nanopores both with specific affinities for single biomarkers and with nonspecific transducers. In situ monitoring of cellular metabolism with such arrays should provide the sensitivity to monitor subtle changes observed through the release of biomarkers.

Self-Assembling Protein Nanoarrays

Protein microarrays provide a powerful tool for the study of protein function. However, they are not widely used, in part because of the challenges in producing proteins to spot on the arrays. Protein microarrays can be generated by printing cDNAs onto glass slides and then translating target proteins with mammalian reticulocyte lysate (Ramachandran et al 2004). Epitope tags fused to the proteins allowed them to be immobilized in situ. This obviates the need to purify proteins, avoided protein stability problems during storage, and captured sufficient protein for functional studies. This technology has been used to map pairwise interactions among 29 human DNA replication initiation proteins, recapitulate the regulation of Cdt1 binding to select replication proteins, and map its geminin-binding domain.

Lumera's NanoCapture technology is now being combined with nucleic acid programmable protein array (NAPPA) technology to create high-density protein arrays with 10,000 spots. The 10,000-spot array is a very important step toward the ultimate goal of producing a whole-proteome biochip. The current protein arrays are

limited to ~800 spots. The NAPPA technology starts with a printed cDNA array and generates a self-assembled protein array using a cell-free expression mix to produce proteins from the printed genes.

Fullerene Photodetectors for Chemiluminescence Detection on Microfluidic Chips

Solution-processed thin-film organic photodiodes have been used for microscale chemiluminescence (Wang et al 2007). The active layer of the photodiodes comprised a blend of the conjugated polymer poly(3-hexylthiophene) and a soluble derivative of fullerene C60. The devices had an active area of 1 mm × 1 mm and a broadband response from 350 to 700 nm, with an external quantum efficiency of >50% between 450 and 550 nm. The photodiodes have a simple layered structure that allows integration with planar chip-based systems. To evaluate the suitability of the organic devices as integrated detectors for microscale chemiluminescence, a peroxyoxalate-based chemiluminescence reaction (PO-CL) was monitored within a poly(dimethylsiloxane) (PDMS) microfluidic device. Quantitation of hydrogen peroxide indicated excellent linearity and yielded a detection limit of 10 μM, comparable with previously reported results using micromachined silicon microfluidic chips with integrated silicon photodiodes. The combination of organic photodiodes with PDMS microfluidic chips offers a means of creating compact, sensitive, and potentially low-cost microscale CL devices with wide-ranging applications in chemical and biological analysis and clinical diagnostics.

Protein Microarray for Detection of Molecules with Nanoparticles

A sensitive technique is being developed for optical detection of nanogold particle-labeled molecules on a protein microarray by applying the SPR and specific molecular binding using rolling circle amplification (Hsu and Huang 2004). A new type of protein chip is being developed based on protein-binding silica nanoparticles at the Fraunhofer Institute for Interfacial Engineering and Biotechnology IGB (Stuttgart, Germany). The surface of this minute particle with a diameter of <100 nm can be configured with many different capture proteins. The particles configured in this way are then applied to silicon carriers in thin, even layers. After contact is made with a sample, the chips can be analyzed using state-of-the-art MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS). Knowing the masses of the bound proteins provides a direct indication of their identity.

Protein Nanobiochip

Nanotechnology Group of the NEC Corporation has developed a prototype protein analysis technology that can analyze samples ~20 times faster than conventional

techniques. The company's technology can complete an analysis of a blood sample in ~60 or 70 min, when compared with the day or so taken by conventional methods.

Biomarker proteins as early warning signs for diseases such as cancer can be identified for diagnostic purposes by finding their isoelectric points and their molecular weights. Isoelectric points are chemical features that refer to the electrical state of a molecule when it has no net charge. Conventional protein chips use a gel across which an electric current is applied to find the targeted protein's isoelectric points. In the new process, instead of being filtered through a block of gel, the protein molecules are separated by their isoelectric points by a capillary action as the proteins flow in a solution along channels in the chip. A test chip by NEC measures 21 mm^2 and contained four sets of tiny channels in which the capillary action takes place. The protein molecules are then dried and irradiated by a laser. Their molecular weights are then measured by a mass spectrometer. The laser helps the proteins leave the chip, and the mass spectrometer is used to judge the molecular weights of the protein molecules in the samples by measuring how early they reach a detector. In the mass spectrometer, light molecules fly faster than heavy ones in an electric field. The mass spectrometer judges the weight of the molecules by monitoring the timing of when each molecule reaches a detector. In addition to being faster than techniques that use gel blocks, the new method also needs blood samples of ~1 μl compared with those of ~20 μl or more that are needed for gel-based techniques. NEC is now planning clinical trials, which should last 2–3 years. If those trials go well, the company should commercialize the technology around or after 2008. When commercialized, the technique could be used for health checks that might cost as little as $100.

Nanoparticles for Molecular Diagnostics

Gold Nanoparticles

Scientists at Northwestern University's Institute for Nanotechnology (http://www.nsec.northwestern.edu/~mkngrp/dnasubgr.html) have attached bits of DNA and Raman-active dyes onto gold particles no larger than 13 nm in diameter. The gold nanoparticles assemble onto a sensor surface only in the presence of a complementary target. If a patterned sensor surface of multiple DNA strands is used, the technique can detect millions of different DNA sequences simultaneously (Cao et al 2002). The current nonoptimized detection limit of this method is 20 fM. Gold nanoparticles were found to be particularly good labels for sensors because a variety of analytical techniques can be used to detect them, including optical absorption, fluorescence, Raman scattering, atomic and magnetic force, and electrical conductivity. They have used gold nanoparticles and a Raman spectroscopy technique to detect life-threatening bacteria and viruses such as anthrax and HIV. Raman approach could replace PCR and fluorescent tags commonly used today. The detection system also relies on chips dotted with DNA. If the targeted disease exists in the sample,

its DNA will bind onto the cDNA strands on the chip and gold particle. The chip is treated with silver-based solution, which coats the nanoparticles. When exposed to a light scanner, the coating enhances the signal enough to detect minute amounts of DNA. Since the Raman band is narrower than the fluorescent band, it allows more dyes to detect more targets quickly. If the sequence of interest is present in the sample, it will bind to the DNA and cause the solution to change color. Labeling oligonucleotide targets with gold nanoparticle rather than fluorophore probes substantially alters the melting profiles of the targets from an array substrate. Northwestern University scientists have developed a nanoparticle-based DNA detection system with 10 times more sensitivity and 100,000 times more specificity than current genomic detection systems (Park et al 2002).

Researchers from the University of Dortmund in Germany have devised a way to coax DNA to aggregate and separate gold nanoparticles on demand (Hazarika et al 2004). They used two ssDNA sequences, each of which is attached to a gold nanoparticle, and a third single strand that has three sections. The first two sections of the third strand match up with each of the nanoparticle strands, gluing them together so that the gold nanoparticles they carry are positioned close together. The nanoparticles can be pulled apart again using a third type of DNA strand that matches up with the entire glue strand of DNA. This glue removal strand first attaches to the free third section of the glue strand, then continues until the entire glue strand is pulled free. The method could be used in sensors that detect biological substances. It could also be used in programmable materials whose properties can be changed by the addition of a piece of DNA.

Quantum Dots for Molecular Diagnostics

There is considerable interest in the use of QDs as inorganic fluorophores, owing to the fact that they offer significant advantages over conventionally used fluorescent markers. For example, QDs have fairly broad excitation spectra—from ultraviolet to red—that can be tuned depending on their size and composition. At the same time, QDs have narrow emission spectra, making it possible to resolve the emissions of different nanoparticles simultaneously and with minimal overlap. Finally, QDs are highly resistant to degradation, and their fluorescence is remarkably stable. Advantages of QD technology are as follows:

- Simple excitation—Lasers are not required
- Simple instrumentation
- Availability of red/infrared colors enables whole-blood assays
- High sensitivity

QDs have been used as possible alternatives to the dyes for tagging viruses and cancer cells. A major challenge is that QDs have an oily surface, whereas the cellular environment is water-based. Attempts are being made to modify the surface chemistry of QDs so that they interact with water-friendly molecules like proteins

and DNA. The current goal is to develop QDs that can target a disease site and light it up. This can someday lead to an integrated system that will also use the QDs to diagnose as well to deliver drug therapies to the disease site. QDs can be designed to emit light at any wavelength from the infrared to visible to ultraviolet. This enables the use of a large number of colors and thus multiplexed assays can be performed. Potential applications of QDs in molecular diagnostics can be summarized as follows:

- Cancer
- Genotyping
- Whole-blood assays
- Multiplexed diagnostics
- DNA mapping
- Immunoassays and antibody tagging
- Detection of pathogenic microorganisms

Quantum Dots for Detection of Pathogenic Microorganisms

QDs have been used as fluorescent labels in immunoassays for quantitative detection of foodborne pathogenic bacteria (Yang and Li 2005). *Salmonella typhimurium* cells were separated from chicken carcass wash water using anti-*Salmonella* antibody-coated magnetic beads and reacted to secondary biotin-labeled anti-*Salmonella* antibody. QDs coated with streptavidin were added to react with biotin on the secondary antibody. Measurement of the intensity of fluorescence produced by QDs provided a quantitative method for microbial detection.

QDs can be used for ultrasensitive viral detection of a small number of microorganisms. QD-based tests can detect as low as 100 copies of hepatitis and HIV RNA. Applications of tests based on QDs for clinical diagnosis of viral infections are described in Chapter 11.

Bioconjugated QDs for Multiplexed Profiling of Biomarkers

Bioconjugated QDs, collections of different-sized nanoparticles embedded in tiny polymer beads, provide a new class of biological labels for evaluating biomarkers on intact cells and tissue specimens. In particular, the use of multicolor QD probes in immunohistochemistry is considered one of the most important and clinically relevant applications. The medical use of QD-based immunohistochemistry has been limited by the lack of specific instructions, or protocols, for clinicians. Preliminary results and detailed protocols for QD–antibody conjugation, tissue specimen preparation, multicolor QD staining, image processing, and biomarker quantification have been published (Xing et al 2007). The results demonstrate that bioconjugated QDs can be used for multiplexed profiling of biomarkers, and ultimately for correlation with disease progression and response to therapy. This will increase the clinician's ability to predict the likely outcomes of drug therapy in a personalized approach to disease management. Bioinformatics and systems biology is used to link each

individual's molecule profile with disease diagnosis and treatment decisions. The usefulness of these protocols was demonstrated by simultaneously identifying multiple biomarkers in prostate cancer tissue. In general, QD bioconjugation is completed within 1 day, and multiplexed molecular profiling takes 1–3 days depending on the number of biomarkers and QD probes used.

Imaging of Living Tissue with Quantum Dots

Tiny blood vessels, viewed beneath a mouse's skin with a newly developed application of multiphoton microscopy, appear so bright and vivid in high-resolution images that researchers can see the vessel walls ripple with each heartbeat. Cornell researchers have shown that capillaries many hundreds of microns below the skin of living mice were illuminated in unprecedented detail using fluorescence imaging labels, QDs, circulating through the bloodstream (Larson et al 2003). This is a new approach to using QDs for biological studies of living animals. Although there are easier ways to take a mouse's pulse, this level of resolution and high signal-to-noise illustrate how useful multiphoton microscopy with QDs can become in biological research for tracking cells and visualizing tissue structures deep inside living animals. Monitoring of vascular changes in malignant tumors is a possible application. This research will pave the way for many new noninvasive in vivo imaging methods using QDs.

Carbohydrate-encapsulated QDs can be used for medical imaging. Certain carbohydrates, especially those included on tumor glycoproteins, are known to have affinity for certain cell types and this can be exploited for medical imaging. Conjugating luminescent QDs with target specific glycans permits efficient imaging of the tissue to which the glycans bind with high affinity. Accurate imaging of primary and metastatic tumors is of primary importance in disease management. Second-generation QDs contain the glycan ligands and PEG of varying chain lengths. The PEG modifications produce QDs that maintain high luminescence while reducing nonspecific cell binding.

Procedures have been developed for using QDs to label live cells and to demonstrate their use for long-term multicolor imaging (Jaiswal et al 2003). The two approaches are endocytic uptake of QDs and selective labeling of cell surface proteins with QDs conjugated to antibodies. These approaches should permit the simultaneous study of multiple cells over long periods of time as they proceed through growth and development. Use of avidin permits stable conjugation of the QDs to ligands, antibodies, or other molecules that can be biotinylated, whereas the use of proteins fused to a positively charged peptide or oligohistidine peptide obviates the need for biotinylating the target molecule. A procedure has been described for the bioconjugation of QDs and specific labeling of both intracellular and cell surface proteins (Jaiswal et al 2004). For generalized cellular labeling, QDs not conjugated to a specific biomolecule may be used.

Fluorescent semiconductor QDs hold great potential for molecular imaging in vivo. However, the utility of existing QDs for in vivo imaging is limited because they require excitation from external illumination sources to fluoresce, which

results in a strong autofluorescence background and a paucity of excitation light at nonsuperficial locations. QD conjugates that luminesce by bioluminescence resonance energy transfer in the absence of external excitation have been prepared by coupling carboxylate-presenting QDs to a mutant of the bioluminescent protein Renilla reniformis luciferase (So et al 2006). The conjugates emit long-wavelength (from red to NIR) bioluminescent light in cells and in animals, even in deep tissues, and are suitable for multiplexed in vivo imaging. Compared with existing QDs, self-illuminating QD conjugates have greatly enhanced sensitivity in small animal imaging, with an in vivo signal-to-background ratio of $>10^3$ for 5 pmol of conjugate.

Magnetic Nanoparticles

Magnetic Nanoparticles for Bioscreening

Iron nanoparticles, 15–20 nm in size, having saturation magnetization, have been synthesized, embedded in copolymer beads of styrene and glycidyl methacrylate (GMA), which were coated with poly-GMA by seed polymerization (Maeda et al 2006). The resultant Fe/St-GMA/GMA beads had diameters of 100–200 nm. By coating with poly-GMA, the zeta potential of the beads changed from −93.7 to −54.8 mV, as measured by an electrophoresis method. This facilitated nonspecific protein adsorption suppression, as revealed by gel electrophoresis method, which is a requisite for nanoparticles to be applied to carriers for bioscreening.

Superparamagnetic Nanoparticles for Cell Tracking

Magnetic nanoparticles are a powerful and versatile diagnostic tool in biology and medicine. It is possible to incorporate sufficient amounts of superparamagnetic iron oxide (SPIONs) into cells, enabling their detection in vivo using MRI (Bulte et al 2004). Because of their small size, they are easily incorporated into various cell types (stem cells, phagocytes, etc.), allowing the cells to be tracked in vivo—for example, to determine whether stem cells move to the correct target area of the body.

Superparamagnetic nanoparticles (CD34 microbeads), used clinically for specific magnetic sorting, can be used as a magnetic cell label for in vivo cell visualization. In one study, human cells from peripheral blood were selected by CliniMACS CD34 Selection Technology (Miltenyi, Bergisch Gladbach, Germany) and implanted into rats with a cortical photochemical lesion, contralaterally to the lesion (Jendelova et al 2005). Twenty-four hours after grafting, the implanted cells were detected in the contralateral hemisphere as a hypointense spot on T2-weighted images; the hypointensity of the implant decreased during the first week. Staining techniques confirmed the presence of magnetically labeled human cells in the lesion 4 weeks after grafting. Thus CD34 microbeads can be used as a magnetic cell label for in vivo cell visualization. The fact that microbeads coated with different commercially

available antibodies can bind to specific cell types opens extensive possibilities for cell tracking in vivo.

Superparamagnetic Iron Oxide Nanoparticles for Calcium Sensing

A family of calcium indicators for MRI is formed by combining a powerful superparamagnetic iron oxide nanoparticle-based contrast mechanism with the versatile calcium-sensing protein calmodulin and its targets (Atanasijevic et al 2006). Calcium-dependent protein–protein interactions drive particle clustering and produce up to 5-fold changes in T2 relaxivity, an indication of the sensor's potency. Robust MRI signal changes are achieved even at nanomolar particle concentrations that are unlikely to buffer calcium levels. When combined with technologies for cellular delivery of nanoparticulate agents, these sensors and their derivatives may be useful for functional molecular imaging of biological signaling networks in live, opaque specimens.

Magnetic Nanoparticles for Labeling Molecules

Bound to a suitable antibody, magnetic nanoparticles are used to label specific molecules, structures, or microorganisms. Magnetic immunoassay techniques have been developed in which the magnetic field generated by the magnetically labeled targets is detected directly with a sensitive magnetometer. Binding of antibody to target molecules or disease-causing organism is the basis of several tests. Antibodies labeled with magnetic nanoparticles generate magnetic signals on exposure to a magnetic field. Antibodies bound to targets can thus be identified as unbound antibodies disperse in all directions and produce no net magnetic signal.

A novel nanosensor based on magnetic nanoparticles has been developed for rapid screens of telomerase activity in biological samples (Grimm et al 2004). The technique utilizes nanoparticles that, on annealing with telomerase-synthesized TTAGGG repeats, switch their magnet state, a phenomenon readily detectable by magnetic readers. High-throughput adaptation of the technique by MRI allowed processing of hundreds of samples within tens of minutes at ultrahigh sensitivities. Together, these studies establish and validate a novel and powerful tool for rapidly sensing telomerase activity and provide the rationale for developing analogous magnetic nanoparticles for in vivo sensing. Since elevated telomerase levels are found in many malignancies, this technique provides access to an attractive target for therapeutic intervention and diagnostic or prognostic purposes.

Superparamagnetic iron oxide nanoparticles have been functionalized to identify *Mycobacterium avium* spp. *paratuberculosis* (MAP) through magnetic relaxation (Kaittanis et al 2007). The results indicate that the MAP nanoprobes bind specifically to MAP and can quantify the bacterial target quickly in milk and blood with high sensitivity. The advantage of this approach is that detection is not susceptible to interferences caused by other bacteria. The use of these magnetic nanosensors is anticipated in the identification and quantification of bacteria in clinical and environmental samples.

Ferrofluids

CellTracks^{TM}Technology (Immunicon, Huntington Valley, PA, USA) is based on patented magnetic nanoparticles called ferrofluids. Ferrofluids consist of a magnetic core surrounded by a polymeric layer coated with antibodies for capturing cells. Ferrofluid particles are colloidal, and when mixed with a sample containing the target cells, the antibodies conjugated to the magnetic core bind the antigen associated with the target cells. It is combined with proprietary technologies for cell separation, labeling, and analysis. This is in development to screen, diagnose, stage, and monitor cancer based on circulating cancer cells in the blood. Potential applications include isolation of endothelial cells, which may be useful in the management of cancer and cardiovascular disease, and isolation of fungus or bacteria, which may be useful in the management of patients with serious infections.

Superconducting Quantum Interference Device

Superconducting quantum interference device (SQUID), developed at the University of California (Berkeley, CA), is a technique for specific, sensitive, quantitative, and rapid detection of biological targets by using superparamagnetic nanoparticles and a "microscope" based on a high-transition temperature (Chemla et al 2000). In this technique, a mylar film to which the targets have been bound is placed on the microscope alongside SQUID. A suspension of magnetic nanoparticles carrying antibodies directed against the target is added to the mixture in the well, and 1-s pulses of magnetic field are applied parallel to the SQUID. In the presence of this aligning field the nanoparticles develop a net magnetization, which relaxes when the field is turned off. Unbound nanoparticles relax rapidly by Brownian rotation and contribute no measurable signal. Nanoparticles that are bound to the target on the film are immobilized and undergo a slowly decaying magnetic flux, which is detected by the SQUID. The ability to distinguish between bound and unbound labels allows one to run homogeneous assays, which do not require separation and removal of unbound magnetic particles.

Study of Living Cells by Superparamagnetic Nanoparticles

Technologies to assess the molecular targets of biomolecules in living cells are lacking. A new technology called magnetism-based interaction capture (MAGIC) has been developed that identifies molecular targets on the basis of induced movement of superparamagnetic nanoparticles (MNPs) inside living cells (Won et al 2005). The scientists painted intracellular proteins with fluorescent materials and inserted magnetic nanoparticles-embedded drugs into the cell. These nanoprobes captured the small molecule's labeled target protein and were translocated in a direction specified by the magnetic field. Use of MAGIC in genome-wide expression screening identified multiple protein targets of a drug. MAGIC was also used to monitor signal-dependent modification and multiple interactions of proteins. It was also shown that internalized MNPs could be moved inside cells by an external magnetic

field, using a luminescent nanocrystal (NC) QD, which does not exhibit magnetism, as a control. The MNPs not only responded to application of the magnetic field but also rapidly dispersed when the magnetic field was removed and reassembled on reapplication of the magnetic field. MAGIC can be useful in the development of diagnostics and biosensors. Its ultimate use would be for the analysis of interactions inside living cells of patients.

Use of Nanocrystals in Immunohistochemistry

A method has been described for simple convenient preparation of bright, negatively or positively charged, water-soluble CdSe/ZnS core/shell NCs and their stabilization in aqueous solution (Sukhanova et al 2004). Single NCs can be detected using a standard epifluorescent microscope, ensuring a detection limit of one molecule coupled with an NC. NC–antibody (Ab) conjugates were tested in dot blots and exhibited retention of binding capacity within several nanograms of antigen detected. The authors further demonstrated the advantages of NC–Ab conjugates in the immunofluorescent detection and 3D confocal analysis of P-glycoprotein (P-gp), one of the main mediators of the multidrug resistance (MDR) phenotype. The labeling of P-gp with NC–Ab conjugates was highly specific. Finally, the authors demonstrated the applicability of NC–Ab conjugates obtained by the method described to specific detection of antigens in paraffin-embedded formaldehyde-fixed cancer tissue specimens, using immunostaining of cytokeratin in skin basal carcinoma as an example. They concluded that the NC–Ab conjugates may serve as easy-to-do, highly sensitive, photostable labels for immunofluorescent analysis, immunohistochemical detection, and 3D confocal studies of membrane proteins and cells.

Imaging Applications of Nanoparticles

There is rapid growth in the use of MRI for molecular and cellular imaging. Much of this work relies on the high relaxivity of nanometer-sized, ultrasmall dextran-coated iron oxide particles. Chemical modifications to nanosized virus particles may improve MRI. Attachment of a large number of gadolinium chelates, the chemical compound used in MRI contrast agents, onto the surface of the viral particles resulted in the generation of a very intense signal in a clinical MRI scanner (Anderson et al 2006). Magnetic nanoparticles, QDs, and ferrofluids are examples of some of the nanoparticles that have been used along with imaging technologies.

Magnetic Nanoparticles Combined with MRI

Highly lymphotropic superparamagnetic nanoparticles measuring 2–3 nm on average, which gain access to lymph nodes by means of interstitial–lymphatic fluid transport, have been used in conjunction with high-resolution MRI to reveal small

and otherwise undetectable lymph node metastases (Harisinghani et al 2003). The lymphotropic superparamagnetic nanoparticle used in this study was a monocrystalline iron oxide (Combidex, Advanced Magnetics, Cambridge, MA, USA). In patients with prostate cancer who underwent surgical lymph node resection or biopsy, MRI with lymphotropic superparamagnetic nanoparticles correctly identified all patients with nodal metastases. This diagnosis was not possible with conventional MRI alone and has implications for the management. In men with metastatic prostate cancer, adjuvant androgen deprivation therapy with radiation is the mainstay of management.

The presence of lymph node metastases is an important factor in breast cancer patient prognosis. Therefore, the precise identification of sentinel lymph nodes in these patients is critical. Draining lymphatic ducts and lymph nodes were clearly visualized in the mammary tissue of normal mice and in spontaneous and xenografted breast tumor models after a direct mammary gland or peritumoral injection of nanosize paramagnetic molecule, G6. Micro-magnetic resonance lymphangiography using the G6 contrast agent revealed the absence of filling in the metastatic foci of affected lymph nodes (Kobayashi et al 2004). Gd-DTPA-dimeglumine, by contrast, failed to depict lymphatic flow from the mammary tissue in normal mice using the same method. The superior temporal and spatial resolution of micro-magnetic resonance lymphangiography using the contrast agent G6 may facilitate the study of tumor lymphatic drainage and lymphatic metastasis in both experimental animals and clinical medicine. In addition, this may be a powerful new method for sentinel lymph node localization in human breast cancer.

Nanoparticles as Contrast Agent for MRI

The determination of brain tumor margins both during the presurgical planning phase and during surgical resection has long been a challenging task in the therapy of brain tumor patients. Multimodal (NIR fluorescent and magnetic) nanoparticles were used as a preoperative MRI contrast agent and intraoperative optical probe in a model of gliosarcoma with stably green fluorescence protein-expressing 9L glioma cells (Kircher et al 2003). Key features of nanoparticle metabolism, namely, intracellular sequestration by microglia and the combined optical and magnetic properties of the probe, allowed delineation of brain tumors both by preoperative MRI and by intraoperative optical imaging. This prototypical multimodal nanoparticle has unique properties that may allow radiologists and neurosurgeons to see the same probe in the same cells and may offer a new approach for obtaining tumor margins.

Alphanubeta3-targeted paramagnetic nanoparticles have been employed to noninvasively detect very small regions of angiogenesis associated with nascent melanoma tumors (Schmieder et al 2005). Each particle was filled with thousands of molecules of the metal that is used to enhance contrast in conventional MRI scans. The surface of each particle was decorated with a substance that attaches to newly forming blood vessels, which are present at tumor sites. The goal was to create a high density of the glowing particles at the site of tumor growth, so they are easily visible. Molecular MRI results were corroborated by histology. This study

lowers the limit previously reported for detecting sparse biomarkers with molecular MRI in vivo when the growths are still invisible to conventional MRI. Earlier detection can potentially increase the effectiveness of treatment. This is especially true with melanoma, which begins as a highly curable disorder, then progresses into an aggressive and deadly disease. A second benefit of the approach is that the same nanoparticles used to find the tumors could potentially deliver stronger doses of anticancer drugs directly to the tumor site with fewer side effects. Targeting the drugs to the tumor site in this way would also allow stronger doses without systemic toxicity than would be possible if the drug were injected or delivered in some other systemic way. The nanoparticles might also allow physicians to more readily assess the effectiveness of the treatment by comparing MRI scans before and after treatment. Other cancer types might be accessible to this approach as well, because all tumors recruit new blood vessels as they grow.

Manganese Oxide Nanoparticles as Contrast Agent for Brain MRI

A new MRI contrast agent using manganese oxide nanoparticles produces images of the anatomic structures of mouse brain which are as clear as those obtained by histological examination (Na et al 2007). The new contrast agent will enable better research and diagnosis of neurological disorders such as Alzheimer's disease (AD), Parkinson's disease (PD), and stroke. Furthermore, antibodies can be attached to the manganese oxide nanoparticles, which recognize and specifically bind to receptors on the surface of breast cancer cells in mouse brains with breast cancer metastases. The tumors were clearly highlighted by the antibody-coupled contrast agent. The same principle should allow other disease-related changes or physiological systems to be visualized by using the appropriate antibodies.

Gadonanotubes for MRI

More than 25 million patients in the United States undergo MRI each year and contrast agents are used in ~30% of these procedures. Gadolinium agents are the most effective and the most commonly used MRI contrast agents. A new class of contrast agents, gadonanotubes, uses the same highly toxic metal gadolinium (Gd^{3+}) that is used in MRI currently, but the metal atoms are encased inside a carbon nanotube (Sitharaman et al 2005). The ultrashort nanotubes are only about 20–100 times longer than they are wide, and once inside the nanotubes, the gadolinium atoms naturally aggregate into tiny clusters of ~10 atoms each. Clustering causes the unexplained increases in magnetic and MRI effects. Gadonanotubes are at least 40–90 times more effective than Gd^{3+}-based MRI agents now in use. Shrouding the toxic metals inside the benign carbon is expected to significantly reduce or eliminate the metal's toxicity. Currently available methods of attaching disease-specific antibodies and peptides can be applied to gadonanotubes, so they can be targeted to cancerous and other diseased cells.

Quantum Dots for Biological Imaging

Targeted QDs, coated with paramagnetic and pegylated lipids, have been developed for detection by MRI (Mulder et al 2006). The QDs were functionalized by covalently linking v3-specific peptides, and the specificity was assessed and confirmed on cultured endothelial cells. The bimodal character, the high relaxivity, and the specificity of this nanoparticulate probe make it an excellent contrast agent for molecular imaging purposes. Among other applications, those in cancer are most important.

Accurate imaging of diseased cells (e.g., primary and metastatic tumors) is of primary importance in disease management. The NIH has developed carbohydrate-encapsulated QDs with detectable luminescent properties useful for imaging of cancer or other disease tissues. Certain carbohydrates, especially those included on tumor glycoproteins, are known to have affinity for certain cell types. One notable glycan used in this technology is the Thomsen–Friedenreich disaccharide (Galbeta1-3GalNAc) that is readily detectable in 90% of all primary human carcinomas and their metastases. These glycans can be exploited for medical imaging. Encapsulating luminescent QDs with target-specific glycans permits efficient imaging of the tissue to which the glycans bind with high affinity.

Multifunctional nanoparticle probes based on semiconductor QDs have been used for cancer targeting and imaging in living animals. The structural design involves encapsulating luminescent QDs with an ABC triblock copolymer and linking this amphiphilic polymer to tumor-targeting ligands and drug delivery functionalities. In vivo targeting studies of human prostate cancer growing in nude mice indicate that the QD probes accumulate at tumors both by the enhanced permeability and retention of tumor sites and by antibody binding to cancer-specific cell surface biomarkers (Gao et al 2004). Using both subcutaneous injection of QD-tagged cancer cells and systemic injection of multifunctional QD, sensitive and multicolor fluorescence imaging of cancer cells have been achieved under in vivo conditions. These results raise new possibilities for ultrasensitive and multiplexed imaging of molecular targets in vivo.

Gold Nanorods and Nanoparticles as Imaging Agents

Gold nanorods excited at 830 nm on a far-field laser scanning microscope produced strong two-photon luminescence (TPL) intensities, and the TPL excitation spectrum can be superimposed onto the longitudinal plasmon band (Wang et al 2005a). The TPL signal from a single nanorod is 58 times that of the two-photon fluorescence signal from a single rhodamine molecule. Gold nanorods can be used as imaging agents as demonstrated by in vivo imaging of single nanorods flowing in mouse ear blood vessels.

Nanoprobes has reported that 1.9-nm gold nanoparticles may overcome many limitations to traditional x-ray contrast agents. Gold has higher x-ray absorption than iodine with less bone and tissue interference, thus achieving better contrast with lower x-ray dose. Because nanoparticles clear the blood more slowly than iodine

agents, they permit longer imaging times. In studies in mice, a 5-mm tumor growing in one thigh was clearly evident from its increased vascularity and resultant higher gold content. The gold particles thus enable direct imaging, detection, and measurement of angiogenic and hypervascularized regions. The 1.9-nm gold nanoparticles were found to clear through the kidneys: a closer examination of the kidneys revealed a remarkably detailed anatomical and functional display, with blood vessels <100 μm in diameter delineated, thus enabling in vivo vascular casting. Toxicity was also low: mice intravenously injected with the gold nanoparticles survived over 1 year without signs of illness.

Nanoparticles Versus Microparticles for Cellular Imaging

Typically, millions of dextran-coated ultrasmall iron oxide particles (USIOPs) must be loaded into cells for efficient detection. A recent study shows that single, micrometer-sized iron oxide particles (MSIOPs) can be detected by MRI in vitro in agarose samples, in cultured cells, and in mouse embryos (Shapiro et al 2004). Experiments studying effects of MRI resolution and particle size from indicated that significant signal effects could be detected at resolutions as low as 200 μm. Cultured cells were labeled with fluorescent MSIOPs such that single particles were present in individual cells. These single particles in single cells could be detected both by MRI and by fluorescence microscopy. Finally, single particles injected into single-cell-stage mouse embryos could be detected at later embryonic stages, demonstrating that even after many cell divisions, daughter cells still carry individual particles. These results demonstrate that MRI can detect single particles and indicate that single-particle detection will be useful for cellular imaging for certain purposes and may be preferable to nanoparticles. MSIOPs will useful in following the division of stem cells and in vivo labeling of cells.

Intravital Vascular Imaging

A significant impediment to the widespread use of noninvasive in vivo vascular imaging techniques is the current lack of suitable intravital imaging probes. A new strategy is the use of viral nanoparticles as a platform for the multivalent display of fluorescent dyes to image tissues deep inside living organisms (Lewis et al 2006). The bioavailable cowpea mosaic virus (CPMV) can be fluorescently labeled to high densities with no measurable quenching, resulting in exceptionally bright particles with in vivo dispersion properties that allow high-resolution intravital imaging of vascular endothelium for periods of at least 72 h. CPMV nanoparticles can be used to visualize the vasculature and blood flow in living mouse and chick embryos to a depth of up to 500 μm. Intravital visualization of human fibrosarcoma-mediated tumor angiogenesis using fluorescent CPMV provides a means to identify arterial and venous vessels and to monitor the neovascularization of the tumor microenvironment.

Study of Chromosomes by Atomic Force Microscopy

A better knowledge of biochemical and structural properties of human chromosomes is important for cytogenetic investigations and diagnostics. Fluorescence in situ hybridization (FISH) is a commonly used technique for the visualization of chromosomal details. Localizing specific gene probes by FISH combined with conventional fluorescence microscopy has reached its limit. Also, microdissecting DNA from G-banded human metaphase chromosomes either by a glass tip or by laser capture needs further improvement. Both AFM and SNOM have been used to obtain local information from G-bands and chromosomal probes (Oberringer et al 2003). The final resolution allows a more precise localization when compared with standard techniques, and the extraction of very small amounts of chromosomal DNA by the scanning probe is possible. Besides new strategies toward a better G-band and fluorescent probe detection, this method is focused on the combination of biochemical and nanomanipulation techniques, which enable both nanodissection and nanoextraction of chromosomal DNA.

Applications of Nanopore Technology for Molecular Diagnostics

Nanopore technology can distinguish between and count a variety of molecules in a complex mixture. For example, it can distinguish between hybridized or unhybridized unknown RNA and DNA molecules that differ only by a single nucleotide.

Nanopore blockade can be used to measure polynucleotide length. With further improvements, the method could in principle provide direct, high-speed detection of the sequence of bases in single molecules of DNA or RNA. Biosensor elements that are capable of identifying individual DNA strands with single-base resolution have been described. Each biosensor element consists of an individual DNA oligonucleotide covalently attached within the lumen of the α-HL pore to form a "DNA nanopore." The binding of ssDNA molecules to the tethered DNA strand causes changes in the ionic current flowing through a nanopore. On the basis of DNA duplex lifetimes, the DNA nanopores are able to discriminate between individual DNA strands up to 30 nucleotides in length differing by a single-base substitution. This is exemplified by the detection of a drug resistance-conferring mutation in the reverse transcriptase (RT) gene of HIV. In addition, the approach was used to sequence a complete codon in an individual DNA strand tethered to a nanopore. Studies on single channels reconstituted into planar lipid bilayer membranes suggest that nanometer-scale pores can be used to detect, quantitate, and characterize a wide range of analytes that includes small ions and ssDNA (Kasianowicz 2002).

Nanopore biosensors can enable direct, microsecond-time scale nucleic acid characterization without the need for amplification, chemical modification, surface adsorption, or the binding of probes. However, routine DNA analysis and sequencing will require a robust nanopore. Solid-state nanopores could be ideal, but fabrication methods need to be improved to develop an electrically addressable array of

pores with reproducible diameters in the required 10^{-9} m range. A simple method that enables efficient, not too hasty, electrophoretic translocation of DNA strand through the nanopore remains to be devised. This will require a better understanding of the factors that regulate polymer translocation through nanopores.

This technology can also be applied to the analysis of proteins. Scientists at the National Institute of Standards and Technology (Gaithersburgh, MD) believe that nanopore technology for biomolecules can be applied to cancer diagnosis. The speed and simplicity of this technology will facilitate the development of molecular diagnosis and its application to personalized medicine.

Eagle Research and Development (Boulder, CO) platform comprises an array of nanopores with each nanopore containing embedded semiconductors or field-effect transistors (FETs). As single molecules are driven through a nanopore by a voltage differential, the 3D charge profile of a molecule is measured by the FETs, enabling each molecule in the sample to be uniquely identified and precisely quantified. It does not require fluorescent or other labels, thermal cycling, or optics. This technology offers the prospect to eventually correlate DNA and its expressed proteins with specific disease states using an inexpensive, disposable, and portable device. For example, the device has the potential to enable the development of exquisitely targeted treatments using sequencing data both from a patient and from the disease-causing pathogen. Compared with other nanopore-based technologies for measuring molecules using electronic signals, the Eagle approach achieves a 1,000-fold higher sensitivity as a result of the FETs embedded in the nanopores. Applied Biosciences is collaborating with Eagle for development support and feasibility testing for applications in protein identification and detection of protein-binding events. Provided the ability to electronically profile the individual four nucleotides in DNA is further developed, the Eagle technology could potentially be the first to enable the identification and measurement of both DNA and proteins in a single sample at the same time. The technology could have significant implications for advancing personalized medicine based on its potential for faster, more efficient, and less expensive protein and nucleic acid identification, protein–protein and protein–small-molecule interaction measurements, and DNA sequencing.

DNA–Protein and DNA–Nanoparticle Conjugates

Semisynthetic conjugates composed of nucleic acids, proteins, and inorganic nanoparticles have been synthesized and characterized (Niemeyer 2004). For example, self-assembled oligomeric networks consisting of streptavidin and double-stranded DNA (dsDNA) are applicable as reagents in immunoassays. Covalent conjugates of ssDNA and streptavidin are utilized as biomolecular adapters for the immobilization of biotinylated macromolecules at solid substrates via nucleic acid hybridization. This “DNA-directed immobilization” enables reversible and site-selective functionalization of solid substrates with metal and semiconductor nanoparticles or, vice versa, for the DNA-directed functionalization of gold

nanoparticles with proteins, such as immunoglobulins and enzymes. This approach is applicable for the detection of chip-immobilized antigens. Moreover, covalent DNA–protein conjugates allow for their selective positioning along single-stranded nucleic acids, and thus for the construction of nanometer-scale assemblies composed of proteins and/or nanoclusters. Examples include the fabrication of functional biometallic nanostructures from gold nanoparticles and antibodies, applicable as diagnostic tools in bioanalytics.

DNA-modified nanoparticles have been used for colorimetric SNP analysis (Ihara et al 2004). These nanospheres were prepared by anchoring amino-terminated oligodeoxynucleotides (ODNs) with carboxylates onto a colored polystyrene sphere surface through amido bonds. About 220 ODN molecules were immobilized onto a nanosphere 40 nm in diameter. Preliminary studies using the microspheres with 1 μm diameter reveal that the specificity of hybridization was retained after modification. Three kinds of differently colored (RGB, red/green/blue) nanospheres bearing unique ODNs on their surface were prepared for detecting the p53 gene. The study of FRET showed that spheres R and G directly contact each other in the aggregates with the wild type. The RGB color system gave aggregates with specific colors corresponding to the added ODN samples, wild type or mutant. In addition, in the presence of both samples, all of the spheres formed aggregates with white emission as a consequence of mixing three primary colors of light. This means that the technique should enable an allele analysis.

Resonance Light Scattering Technology

Resonance light scattering (RLS) technology, developed at Genicon Sciences Corporation (now acquired by Invitrogen), offers uniquely powerful signal generation and detection capabilities applicable to a wide variety of analytical bioassay formats (Yguerabide and Yguerabide 2001). RLS exploits submicroscopic metallic particles (e.g., gold and silver) of uniform diameter (in the nanometer range), which scatter incident white light to generate monochromatic colored light that appears as highly intense fluorescence. Each RLS particle produces intense light scattering that can be viewed with the naked eye. Under low-power microscope magnification, individual 80-nm gold particles can be readily observed. The scattering produced by these particles creates a “halo” with an apparent 1 μm diameter. As a result, one can conduct ultrasensitive assays to define location and relative frequency of target molecules. RLS signal generation technology is up to 1,000,000 times more sensitive than current fluorescence signaling technology. Other advantages of RLS technology are that RLS signals do not require computer-enhanced imaging of data as they are so intense. Research applications of RLS technology are as follows:

- *Gene expression*—Relative gene expression studies on slide-based cDNA microarrays.
- *DNA sequencing*—RLS-based DNA sequencing on sequence-by-hybridization biochips.

- *Microfluidics*—RLS particles for solution-based assays in nanofluidic flow-through microarrays.
- *Immunohistology*—Rapid in situ localization/quantitation of proteins in tissue sections using RLS-coupled antibodies.
- *Homogeneous*—RLS particles for bimolecular, microvolume studies in solution.

Clinical applications of RLS technology are as follows:

- RLS technology is being used to score SNPs for discrimination of therapeutically relevant alleles.
- RLS technology provides ultrahigh-sensitivity probes for in situ hybridizations to quantitate therapeutically important DNA and RNA molecules.
- Antibody-coupled RLS particles can deliver increased sensitivity for detection of rare analytes in diagnostic assays.
- Nanoparticle-labeled bacterial RNA generates reproducible RLS signals that are at least 50 times more intense than state-of-the-art confocal-based fluorescence signals for detection of bacterial pathogens (Francois et al 2003).

DNA Nanomachines for Molecular Diagnostics

Manipulation of DNA has been shown to perform computational operations. Scientists at the MIT Media Laboratory and the Center for Biomedical Engineering have managed to attach a tiny radio antenna to DNA (Hamad-Schifferli et al 2002). When a radio-frequency magnetic field is transmitted to the antenna, the DNA molecule is zapped with energy and responds. The antenna is a cluster of metal, <100 atoms in size and ~ 1 nm long. A radio signal sent to a piece of dsDNA has been shown to unwind the two strands—a process called "dehybridization." The switching is reversible and does not affect neighboring molecules. The technique should also work on proteins, peptides, and other large molecules. MIT licensed the technology to EngeneOS Inc (Waltham, MA, USA) in 2001, but the company is no longer in business. Applications of this technology relevant to molecular diagnostics include biomolecular detectors for homogeneous assays and direct electronic readout of biomolecular interactions.

Nanobarcodes Technology

Scientists at Oxonica Inc (Oxfordshire, UK) have produced submicrometer metallic barcodes with striping patterns prepared by sequential electrochemical deposition of metal ions. The differential reflectivity of adjacent stripes enables identification of the striping patterns by conventional light microscopy. This readout mechanism does not interfere with the use of fluorescence for detection of analytes bound to particles by affinity capture, as demonstrated by DNA and protein bioassays. Among other applications such as SNP mapping and multiplexed assays for proteomics,

nanobarcodes can be used for population diagnostics and in point-of-care (POC) handheld devices. SurroMed is using this technology in developing a state-of-the-art phenotyping platform in a clinical setting with access to a large clinical population. This will enable biomarker-based drug development as a basis for personalized medicines. Key performance advantages relative to existing encoded bead technologies include the following:

- The ability to use the widely installed base of optical microscopes for readout.
- The ability to use multiple colors of fluorophores for quantitation.
- The ability to generate hundreds to thousands of unique codes that can be distinguished at high speed.

Nanobarcodes, with various submicrometer striping patterns, may be readily distinguished in an optical microscope (Walton et al 2002). Results from a library of these particles, of which over 100 different striping patterns have been produced, reveal that >70 patterns may be identified with $>90\%$ accuracy. The ability to chemically modify the surface of these particles makes them useful for bioanalytical measurements. Improvements in manufacturing and identification processes will lead to both larger numbers of striping patterns and improved identification accuracy.

Nanobarcode Particle Technology for SNP Genotyping

Oxonica's nanobarcode particle technology has been used in universal array for high-throughput SNP genotyping (Sha et al 2006). The particles are encoded submicron metallic nanorods manufactured by electroplating inert metals such as gold and silver into templates and releasing the resulting striped nanoparticles. The power of this technology is that the particles are intrinsically encoded by virtue of the different reflectivity of adjacent metal stripes, enabling the generation of many thousands of unique encoded substrates. Using SNP found within the cytochrome *P*450 gene family, and a universal short oligonucleotide ligation strategy, simultaneous genotyping of 15 SNPs was demonstrated—a format requiring discrimination of 30 encoded NWs (one per allele). To demonstrate applicability in practice, 160 genotypes were determined from multiplex PCR products from 20 genomic DNA samples.

Qdot Nanobarcode for Multiplexed Gene Expression Profiling

Qdot nanobarcode-based microbead random array platform (Invitrogen) is now available for accurate and reproducible gene expression profiling in a high-throughput and multiplexed format (Eastman et al 2006). Four different sizes of Qdots, with emissions at 525, 545, 565, and 585 nm are mixed with a polymer and coated onto the magnetic microbeads (8 μm-diameter) to generate a nanobarcoded QBeads. Twelve intensity levels for each of the four colors are used. Gene-specific oligonucleotide probes are conjugated to the surface of each spectrally

nanobarcoded bead to create a multiplexed panel, and biotinylated cRNAs are generated from sample total RNA and hybridized to the gene probes on the microbeads. A fifth streptavidin Qdot (655 nm or infrared Qdot) binds to biotin on the cRNA, acting as a quantification reporter. The intensity of the 655-nm Qdot reflects the level of biotinylated cRNA captured on the beads and provides the quantification for the corresponding target gene. The system shows a level of sensitivity, which is better than that with a high-density microarray system, and approaches the level usually observed for quantitative PCR. The QBead nanobarcode system has a dynamic range of 3.5 logs, better than the 2–3 logs observed on various microarray platforms. The hybridization reaction is performed in liquid phase and completed in 1–2 h, at least 1 order of magnitude faster than microarray-based hybridizations. Detectable fold change is lower than 1.4-fold, showing high precision even at close to single copy per cell level. Reproducibility for this proof-of-concept study approaches that of Affymetrix GeneChip microarray. In addition, it provides increased flexibility, convenience, and cost-effectiveness in comparison with conventional gene expression profiling methods.

Biobarcode Assay for Proteins

Scientists in the laboratory of Prof. Chad Mirkin at the Northwestern University have developed an ultrasensitive method for detecting protein analytes (Nam et al 2003). The system relies on magnetic microparticle probes with antibodies that specifically bind a target of interest and nanoparticle probes that are encoded with DNA that is unique to the protein target of interest and antibodies. Magnetic separation of the complexed probes and target followed by dehybridization of the ODNs on the nanoparticle probe surface allows the determination of the presence of the target protein by identifying the oligonucleotide sequence released from the nanoparticle probe. Because the nanoparticle probe carries with it a large number of ODNs per protein-binding event, there is substantial amplification and prostate-specific antigen (PSA) can be detected at 30 aM concentration. Alternatively, a PCR on the oligonucleotide barcodes can boost the sensitivity to 3 aM. Comparable clinically accepted conventional assays for detecting the same target have sensitivity limits of 3 pM, 6 orders of magnitude less sensitive than what is observed with this method. Further development of this technology has resulted in a biobarcode assay with a 500 zM target DNA sensitivity limit (Nam et al 2004). Magnetic separation and subsequent release of barcode DNA from the gold nanoparticles leads to a number of barcode DNA strands for every target DNA (Fig. 3.1).

A nanoparticle-based biobarcode assay was used to measure the concentration of amyloid β (Aβ)-derived diffusible ligands (ADDLs) in the cerebrospinal fluid (CSF) as a biomarker for AD (Georganopoulou et al 2005). Commercial ELISAs can only detect ADDLs in brain tissue where the biomarker is most highly concentrated. Studies of ADDLs in the CSF have not been possible because of their low concentration. The biobarcode amplification technology, which is a million times

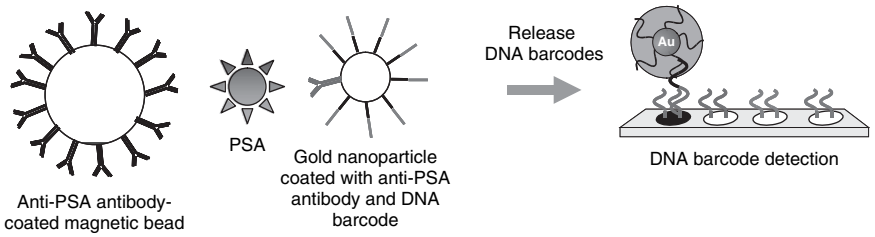

Fig. 3.1 Scheme of biobarcode assay for prostate-specific antigen (PSA). Reproduced by courtesy of Nanosphere Inc

more sensitive than ELISA, can detect ADDLs in the CSF where the biomarker is present in very low concentrations. This study is a step toward a diagnostic tool, based on soluble pathogenic markers for AD. The goal is to ultimately detect and validate the marker in blood.

Using the Verigene ID system (Nanosphere Inc, Northbrook, IL, USA), one can quantify the barcodes using the kind of technology found in a flatbed scanner, providing results as clear as an at-home pregnancy strip test. Biobarcode system is extremely sensitive for protein detection. At 30 aM, it is 5 orders of magnitude more sensitive than is ELISA (peak sensitivity of ~3 pM). The system has enormous potential for multiplexing. Hypothetically, it could simultaneously test for 415 different analytes by tagging the different gold beads with different barcode sequences. However, the fundamental issues with antibodies, such as crossreactivity, nonspecific binding, and lot-to-lot variability, remain. Antibodies can distort, fall apart, or cling to the wrong analyte. Verigene® Warfarin Metabolism Nucleic Acid test (Nanosphere Inc), which detects variants of genes, responsible for sensitivity to Warfarin has been approved by the FDA.

Single-Molecule Barcoding System for DNA Analysis

Molecular confinement offers new routes for arraying large DNA molecules, enabling single-molecule schemes aimed at the acquisition of sequence information. Such schemes can rapidly advance to become platforms capable of genome analysis if elements of a nascent system can be integrated at an early stage of development. Integrated strategies are needed for surmounting the stringent experimental requirements of nanoscale devices regarding fabrication, sample loading, biochemical labeling, and detection. Disposable devices featuring both micro- and nanoscale features have been shown to greatly elongate DNA molecules when buffer conditions are controlled for alteration of DNA stiffness (Jo et al 2007). Analytical calculations that describe this elongation were presented. A complementary enzymatic labeling scheme was developed that tags specific sequences (barcodes) on elongated molecules within described nanoslit devices that are imaged via FRET. Collectively, these developments enable scalable molecular confinement approaches for genome analysis.

Nanoparticle-Based Colorimetric DNA Detection Method

Nucleic acid diagnostics is dominated by fluorescence-based assays that use complex and expensive enzyme-based target or signal amplification procedures. Many clinical diagnostic applications will require simpler, inexpensive assays that can be performed in a screening mode. Scientists at Nanosphere Inc have developed a "spot-and-read" colorimetric detection method for identifying nucleic acid sequences based on the distance-dependent optical properties of gold nanoparticles without the need for conventional signal or target amplification (Storhoff et al 2004). In this assay, nucleic acid targets are recognized by DNA-modified gold probes, which undergo a color change that is visually detectable when the solutions are spotted onto an illuminated glass waveguide. They have improved the sensitivity of the spot test by developing method that monitors scattered light rather than reflected light from 40- to 50-nm-diameter gold particles. This scatter-based method enables detection of zeptomole quantities of nucleic acid targets without target or signal amplification when coupled to an improved hybridization method that facilitates probe–target binding in a homogeneous format. In comparison with a previously reported absorbance-based method, this method increases detection sensitivity by over 4 orders of magnitude and has been applied to the rapid detection of mecA in methicillin-resistant *S. aureus* genomic DNA samples.

Nanosphere Inc launched its Verigene^{TM} platform, an optical detection system, in 2003 for research environments. This device was later automated to enable one-step processing and the system includes sample preparation (for blood), microfluidics, and detection technologies in an integrated system, using simple disposable cartridges. The phase III system will be designed for medical professionals who do not typically use diagnostic systems.

Nanoparticle assemblies interconnected with DNA triple helixes can be used to colorimetrically screen for triplex DNA-binding molecules and simultaneously determine their relative binding affinities based on melting temperatures (Han et al 2006). Nanoparticles assemble only when DNA triple helixes form between DNA from two different particles and a third strand of free DNA. In addition, the triple helix structure is unstable at room temperature and only forms in the presence of triplex DNA-binding molecules that stabilize the triple helix. The resulting melting transition of the nanoparticle assembly is much sharper and at a significantly higher T_m than the analogous triplex structure without nanoparticles. Upon nanoparticle assembly, a concomitant red-to-blue color change occurs. The assembly process and color change do not occur in the presence of duplex DNA binders and therefore provide a significantly better screening process for triplex DNA-binding molecules when compared with standard methods.

SNP Genotyping with Gold Nanoparticle Probes

Nanosphere's ClearRead^{TM} nanoparticle technology enables a microarray-based method for multiplex SNP genotyping in total human genomic DNA without the

need for target amplification (Bao et al 2005). This direct SNP genotyping method requires no enzymes and relies on the high sensitivity of the gold nanoparticle probes. ClearRead^{TM} technology sandwiches a target DNA SNP segment between two oligonucleotide sequences to greatly increase detection specificity and sensitivity. One segment identifies any mutations in the DNA; the probe, a highly sensitive gold nanoparticle, creates a strong signal accurately, indicating the presence of a specific target SNP. Proof of principle, reproducibility, and the robust, simple, and rapid characteristics of this technology were demonstrated with unamplified DNA samples representing all possible forms of three genes implicated in hypercoagulation disorders. The assay format is simple, rapid, and robust pointing to its suitability for multiplex SNP profiling at the POC.

Nanoparticle-Based Up-Converting Phosphor Technology

Up-Converting Phosphor Technology (UPT^{TM}) is a proprietary label detection platform technology of OraSure Technologies Inc (Bethlehem, PA, USA) that can be applied to the detection of minute quantities of various substances such as antigens, proteins, and DNA. UPT particles are small ceramic nanospheres composed of rare earth metals and have been shown to be 1,000 times more sensitive than current fluorescent technologies. The use of OraSure's particle-based detection provides a stronger signal for each event detected and thereby enhances sensitivity in diagnostic assay systems. UPT has potential in a broad array of DNA testing applications including drug discovery, SNP analysis, and infectious disease testing. It is possible to detect nucleic acid targets in nonamplified DNA samples using easy, inexpensive, amplification-free hybridization-based assays and the ultrasensitive UPT reporters (Zuiderwijk et al 2003). Employment of UPT allows to bypass target amplification and therefore brings genetic-based testing a step closer to the POC environment. Detection of *S. pneumoniae* with only 1 ng of DNA indicates a potential for applications in the field of infectious diseases.

Surface-Enhanced Resonant Raman Spectroscopy

Surface-enhanced resonant Raman spectroscopy (SERRS)-Beads (Oxonica) brings various components of the technology into a single robust nanosized polymer-bead support with broad applications in molecular and immunodiagnostics. Compounds that show strong affinity for the silver enhancing surfaces and have good spectral resolution are selected experimentally, particularly organic fluorescent dyes, because of their strong excitation cross section. Initially using four dyes, tens to hundreds of unique labels are currently under development. The chosen dyes also have excitation peaks that overlap with the metal plasmon frequency, thereby adding the all-important resonant amplification to the signal intensity.

At the core of the bead is the Raman-active substrate, where silver colloid, with defined physical characteristics, provides the surface-enhancement substrate and is combined with the dye or dyes for specific bead encoding. Control of the various parameters surrounding dye:colloid aggregate permits SERRS response to be modulated as desired.

To protect the SERRS-active complex from degradation, the aggregate is encapsulated in a polymer coating, a process that incorporates a multitude of dye:colloid particles into the same bead. This leads to highly sensitive beads with responses in excess of that achieved using the conformation of single-dye molecules on an enhancing surface.

The polymer coating is treated further with a polymer shell to allow a variety of biologically relevant probe molecules (e.g., antibodies, antigens, nucleic acids) to be attached through standard bioconjugation techniques.

Oxonica is working closely with Avalon Instruments (Boston, MA, USA) to develop its RamanSpec plate reader with the SERRS-Beads configuration. While current development is focused on heterogeneous assays in a 96-well assay sample presentation, other designs include higher plate capacities (384-well) for higher-throughput screening and microarray slide reading for DNA and proteomic analysis.

Enhancement of Raman signal near silver colloidal nanoparticles is exploited to study the Raman spectrum of yeast cytochrome *c* (YCc) from *Saccharomyces cerevisiae* in a single molecule (Delfino et al 2005). The investigation is performed on proteins both in solution and immobilized onto a glass slide using a quasiresonant laser line as exciting source with low excitation intensity. In both cases, spectra acquired at different times exhibit dramatic temporal fluctuations in both the total spectrum and the specific line intensity, even though averaging of several individual spectra reproduces the main Raman features of bulk YCc. Analysis of the spectral intensity fluctuations from solutions reveals a multimodal distribution of some specific Raman lines, consistent with the approaching of single-molecule regime. Among other results, the statistical analysis of the spectra from immobilized samples seems to indicate dynamical processes involving the reorientation of the heme with respect to the metal surface.

Near-Infrared (NIR)-Emissive Polymersomes

A team of chemists, bioengineers, and medical researchers based at the University of Pennsylvania and the University of Minnesota has lodged fluorescent materials called porphyrins within the surface of a polymersome, a cell-like vesicle, to image a tumor within a living rodent (Ghoroghchian et al 2005). NIR-emissive polymersomes (50-nm- to 50-μm-diameter polymer vesicles) were generated through cooperative self-assembly of amphiphilic diblock copolymers and conjugated multi (porphyrin)-based NIR fluorophores (NIRFs). When compared with natural vesicles comprised of phospholipids, polymersomes were uniquely capable of incorporating and uniformly distributing numerous large hydrophobic NIRFs exclusively in

their lamellar membranes. Within these sequestered compartments, long polymer chains regulate the mean fluorophore–fluorophore interspatial separation as well as the fluorophore-localized electronic environment. Porphyrin-based NIRFs manifest photophysical properties within the polymersomal matrix akin to those established for these high-emission dipole strength fluorophores in organic solvents, thereby yielding uniquely emissive vesicles. Furthermore, the total fluorescence emanating from the assemblies gives rise to a localized optical signal of sufficient intensity to penetrate through the dense tumor tissue of a live animal. Robust NIR-emissive polymersomes thus define a soft-matter platform with exceptional potential to facilitate deep-tissue fluorescence-based imaging for in vivo diagnostic and drug delivery applications.

Nanobiotechnology for Detection of Proteins

Detection of proteins is an important part of molecular diagnostics. Uses of protein nanobiochips and nanobarcode technology for detection of proteins have been described in preceding sections. Other methods will be included in this section.

Captamers with Proximity Extension Assay for Proteins

Multivalent circular aptamers or "captamers" are formed through the merger of aptameric recognition functions with the DNA as a nanoscale scaffold. Aptamers are useful as protein-binding motifs for diagnostic applications, where their ease of discovery, thermal stability, and low cost make them ideal components for incorporation into targeted protein assays. Captamers are compatible with a highly sensitive protein detection method termed the "proximity extension" assay (Di Giusto et al 2005). The circular DNA architecture facilitates the integration of multiple functional elements into a single molecule: aptameric target recognition, nucleic acid hybridization specificity, and rolling circle amplification. Successful exploitation of these properties is demonstrated for the molecular analysis of thrombin, with the assay delivering a detection limit nearly 3 orders of magnitude below the dissociation constants of the two contributing aptamer–thrombin interactions. Real-time signal amplification, detection under isothermal conditions, specificity, and sensitivity would suggest potential application as a protein assay required for the further development of personalized medicine.

Nanobiosensors

Nanosensors are devices that employ nanomaterials, exploiting novel size-dependent properties, to detect gases, chemicals, biological agents, electric fields, light, heat, or other targets. The term "nanobiosensors" implies use of nanosensors for detection of

chemical or biological materials. Nanomaterials are exquisitely sensitive chemical and biological sensors (Jain 2003b).

The sensors can be electronically gated to respond to the binding of a single molecule. Prototype sensors have demonstrated detection of nucleic acids, proteins, and ions. These sensors can operate in the liquid or gas phase, opening up an enormous variety of downstream applications. The detection schemes use inexpensive low-voltage measurement schemes and detect binding events directly, so there is no need for costly, complicated, and time-consuming labeling chemistries such as fluorescent dyes or the use of bulky and expensive optical detection systems. As a result, these sensors are inexpensive to manufacture and portable. It may even be possible to develop implantable detection and monitoring devices based on these detectors.

Some of the technologies that can be incorporated in biosensing are already covered in earlier sections. An example is nanopore technology, which can form the basis of nanosensors. Some of the biosensor devices are described in the following sections.

Cantilevers as Biosensors for Molecular Diagnostics

Cantilevers (Concentris) are small beams similar to those used in AFM to screen biological samples for the presence of particular genetic sequences. The surface of each cantilever is coated with DNA that can bind to one particular target sequence. On exposure of the sample to beams, the surface stress bends the beams by $\sim$10 nm to indicate that the beams have found the target in the sample. This is considered biosensing.

Cantilever technology complements and extends current DNA and protein microarray methods because nanomechanical detection requires no labels, optical excitation, or external probes and is rapid, highly specific, sensitive, and portable. The nanomechanical response is sensitive to the concentration of ODNs in solution, and thus one can determine how much of a given biomolecule is present and active. In principle, cantilever arrays also could quantify gene expression levels of mRNA, protein–protein, drug-binding interactions, and other molecular recognition events in which physical steric factors are important. It can detect a single gene within a genome. Furthermore, fabricating thinner cantilevers will enhance the molecular sensitivity further, and integrating arrays into microfluidic channels will reduce the amount of sample required significantly. In contrast to SPR, cantilevers are not limited to metallic films, and other materials will be explored, e.g., cantilevers made from polymers. In addition to surface stress measurements, operating cantilevers in the dynamic mode will provide information on mass changes, and current investigations will determine the sensitivity of this approach. Currently, it is possible to monitor $>$1,000 cantilevers simultaneously with integrated piezoresistive readout, which in principle will allow high-throughput nanomechanical genomic analysis, proteomics, biodiagnostics, and combinatorial drug discovery.

Cantilevers in an array can be functionalized with a selection of biomolecules. Researchers at IBM, Zurich, Switzerland reported the specific transduction, via surface stress changes, of DNA hybridization and receptor–ligand binding into a direct nanomechanical response of microfabricated cantilevers (Fritz et al 2000). The differential deflection of the cantilevers was found to provide a true molecular recognition signal despite large nonspecific responses of individual cantilevers. Hybridization of complementary ODNs shows that a single-base mismatch between two 12-mer ODNs is clearly detectable. Similar experiments on protein A–immunoglobulin interactions demonstrate the wide-ranging applicability of nanomechanical transduction to detect biomolecular recognition. Microarrays of cantilevers have been used to detect multiple unlabeled biomolecules simultaneously at nanomolar concentrations within minutes (McKendry et al 2002).

A specific test that uses micrometer-scale beams or "microcantilever" can detect PSA. PSA antibodies are attached to the surface of the microcantilever, which is applied to a sample containing PSA. When PSA binds to the antibodies, a change in the surface stress on the microcantilever makes it bend enough to be detected by a laser beam. This system is able to detect clinically relevant concentrations of PSA in a background of other proteins. The technique is simpler and potentially more cost-effective than other diagnostic tests because it does not require labeling and can be performed in a single reaction. It is less prone to false positives, which are commonly caused by the nonspecific binding of other proteins to the microcantilever.

Potential applications in proteomics include devices comprising many cantilevers, each coated with a different antibody, which might be used to test a sample rapidly and simultaneously for the presence of several disease-related proteins. One application is for the detection of biomarkers of myocardial infarction such as creatine kinase at POC. Other future applications include detection of disease by breath analysis, e.g., by analyzing the presence of acetone and dimethylamine (uremia). Detection of a small number of *Salmonella enterica* bacteria is achieved by a change in the surface stress on the silicon nitride cantilever surface in situ upon binding of bacteria (Weeks et al 2003). SEMs indicate that <25 adsorbed are required for detection.

Antibody-Coated Nanocantilevers for Detection of Microorganisms

Researchers at Purdue University have made a discovery about the behavior of nanocantilevers that could be crucial in designing a new class of ultrasmall sensors for detecting viruses, bacteria, and other pathogens (Gupta et al 2006). The cantilevers, coated with antibodies to detect certain viruses, attract different densities or quantity of antibodies per area depending on the size of the cantilever. The devices are immersed into a liquid containing the antibodies to allow the proteins to stick to the cantilever surface. Instead of simply attracting more antibodies, the longer cantilevers also contained a greater density of antibodies. The density is greater toward the free end of the cantilevers. The cantilevers vibrate faster after the antibody attachment if the devices have about the same nanometer-range thickness ($\sim$20 nm) as the protein layer. Moreover, the longer the protein-coated nanocantilever, the

faster the vibration, which could only be explained if the density of antibodies were to increase with increasing lengths.

The cantilever's vibration frequency can be measured using an instrument called a laser Doppler vibrometer, which detects changes in the cantilever's velocity as it vibrates. This work may have broad impact on microscale and nanoscale biosensor design, especially when predicting the characteristics of bionanoelectromechanical sensors functionalized with biological capture molecules. The nanocantilevers could be used in future detectors because they vibrate at different frequencies when contaminants stick to them, revealing the presence of dangerous substances. Because of the nanocantilever's minute size, it is more sensitive than larger devices, promising the development of advanced sensors that detect minute quantities of a contaminant to provide an early warning that a dangerous pathogen is present.

Advantages of Cantilever Technology for Molecular Recognition

Cantilever technology has the following advantages over conventional molecular diagnostics:

- It circumvents the use of PCR.
- For DNA, it has physiological sensitivity and no labeling is required.
- In proteomics, it enables detection of multiple proteins and direct observation of proteins in diseases such as those involving the cardiovascular system.
- It enables the combination of genomics and proteomics assays.
- It is compatible with silicon technology.
- It can be integrated into microfluidic devices.

Cantilevers for Direct Detection of Active Genes

Researchers from the National Centre of Competence in Research at the Swiss Nanoscience Institute (Basel, Switzerland), in collaboration with Roche scientists, have developed an innovative method for the rapid and sensitive detection of disease- and treatment-relevant genes (Zhang et al 2006a). The new method detects active genes directly by measuring their transcripts (mRNA), which represent the intermediate step and link to protein synthesis. Short complementary nucleic acid segments (sensors) are attached to silicon cantilevers which are 450 nm thick and therefore react with extraordinary sensitivity. Binding of the targeted gene transcript to its matching counterpart on one of the cantilevers results in optically measurable mechanical bending.

Differential gene expression of the gene 1-8U, a potential marker for cancer progression or viral infections, could be observed in a complex background. The measurements provide results within minutes at the picomolar level without target amplification and are sensitive to base mismatches. An array of different gene transcripts can even be measured in parallel by aligning appropriately coated cantilevers alongside each other like the teeth of a comb. The new method complements current molecular diagnostic techniques such as the gene chip and real-time PCR. It could

be used as a real-time sensor for continuously monitoring various clinical parameters or for detecting rapidly replicating pathogens that require prompt diagnosis. These findings qualify the technology as a rapid method to validate biomarkers that reveal disease risk, disease progression, or therapy response. Cantilever arrays have potential as a tool to evaluate treatment–response efficacy for personalized medical diagnostics.

Portable Nanocantilever System for Diagnosis

BioFinger, a portable nano detection tool being funded by the EU, could be used as a cheap and fast method in the diagnosis of diseases such as cancer. It could also be used for chemical and food analysis. The BioFinger project is being funded by the European Commission's Information Society Technology Center. The machine detects and analyzes molecules in fluids using nano- and microcantilevers. During trials at Cork University Hospital in Ireland, the system will be used to detect a protein associated with prostate cancer, while the nanocantilever system, which can detect a single molecule, will be used to test blood samples for interleukin-6, a protein associated with inflammation. BioFinger incorporates the cantilevers on a disposable microchip, allowing it to be reconfigured with new on-chip cantilevers to detect different substances. The analysis, which can be performed anywhere, anytime, takes between 15 and 20 min. The system is likely to be considerably cheaper than traditional diagnosis techniques with each disposable chip expected to cost ~€8 ($10). It is extremely versatile and could be used to detect virtually any disease, as a pregnancy test, or even to determine blood types. Outside of the medical field, it could be used to analyze chemicals, detect bacteria in food, or test for water pollution. The new system, now in final development stages, is undergoing field testing and is expected to be in the market within 2–3 years.

Carbon Nanotube Biosensors

Over the years, researchers have sought to tailor carbon nanotubes to detect chemicals ranging from small gas molecules to large biomolecules. The tubes' small size and unique electronic properties make them especially adept at detecting minute changes in the environment. A new type of optical nanosensor uses SWNTs that modulate their emission in response to the adsorption of specific biomolecules (Barone et al 2005). It has two distinct mechanisms of signal transduction: fluorescence quenching and charge transfer. The nanotube-based chemical sensors developed so far generate an electric signal in the presence of a particular molecule. The basic design is widely applicable for such analytical tasks as detecting genes and proteins associated with diseases.

To test the feasibility of implanting the sensors in the body, oxidase- and ferricyanide-coated nanotubes were placed inside a sealed glass tube 1 cm long and 200 μm thick. The tube is riddled with pores large enough to let glucose enter but small enough to keep the nanotubes inside. The tube was then implanted in a sample

of human skin, and the sensor could be excited with infrared light and it could detect its fluorescence.

Carbon Nanotube Sensors Coated with ssDNA and Electronic Readout

A new class of nanoscale chemical sensors are based on ssDNA as the chemical recognition site and single-walled carbon nanotube FETs (SWNT-FETs) as the electronic readout component (Staii et al 2005). SWNT-FETs with a nanoscale coating of ssDNA respond to gas odors that do not cause a detectable conductivity change in bare devices. Responses of ssDNA/SWNT-FETs differ in sign and magnitude for different gases and can be tuned by choosing the base sequence of the ssDNA. ssDNA/SWNT-FET sensors detect a variety of odors, with rapid response and recovery times on the scale of seconds. The arrays of nanosensors could detect molecules on the order of 1 ppm. The sensor surface is self-regenerating: samples maintain a constant response with no need for sensor refreshing through at least 50 gas exposure cycles. The nanosensors could sniff molecules in the air or taste them in a liquid. This remarkable set of attributes makes sensors based on ssDNA-decorated nanotubes promising for "electronic nose" and "electronic tongue" applications ranging from homeland security to disease diagnosis.

Carbon Nanotube Sensors Wrapped with DNA and Optical Detection

SWNTs wrapped with DNA can be placed inside living cells and detect trace amounts of harmful contaminants using NIR light (Heller et al 2006). The sensor is constructed by wrapping the dsDNA around the surface of an SWNT, in much the same fashion as a telephone cord wraps around a pencil. The DNA starts out wrapping around the nanotube with a certain shape that is defined by the negative charges along its backbone. Subtle rearrangement of an adsorbed biomolecule can be directly detected by such a carbon nanotube. At the heart of the new detection system is the transition of DNA secondary structure from the native, right-handed "B" form to the alternate, left-handed "Z" form. The thermodynamics that drive the switching back and forth between these two forms of DNA structure would modulate the electronic structure and optical emission of the carbon nanotube. When the DNA is exposed to ions of certain atoms such as calcium or mercury, the negative charges become neutralized and the DNA changes shape in a similar manner to its natural shape-shift from the B to Z form. This reduces the surface area covered by the DNA, perturbing the electronic structure and shifting the nanotube's natural, NIR fluorescence to a lower energy. The change in emission energy indicates how many ions bind to the DNA. Removing the ions will return the emission energy to its initial value and flip the DNA back to the starting form, making the process reversible and reusable. The viability of this measurement technique was demonstrated by detecting low concentrations of mercury ions in whole blood, opaque solutions, and living mammalian cells and tissues where optical sensing is usually poor or ineffective. Because the signal is in the NIR, a property unique to only a handful of materials, it is not obscured by the natural fluorescence of polymers and

living tissues. The nanotube surface acts as the sensor by detecting the shape change of the DNA as it responds to the presence of target ions. This discovery opens the door to new types of optical sensors and biomarkers that exploit the unique properties of nanoparticles in living systems.

FRET-Based DNA Nanosensors

Rapid and highly sensitive detection of DNA is critical in diagnosing genetic diseases. Conventional approaches often rely on cumbersome, semiquantitative amplification of target DNA to improve detection sensitivity. In addition, most DNA detection systems (e.g., microarrays), regardless of their need for target amplification, require separation of unhybridized DNA strands from hybridized stands immobilized on a solid substrate and are thereby complicated by solution–surface binding kinetics. An ultrasensitive nanosensor is based on FRET capable of detecting low concentrations of DNA in a separation-free format. This system uses QDs linked to DNA probes to capture DNA targets (Zhang et al 2005b). The target strand binds to a dye-labeled reporter strand, thus forming a FRET donor–acceptor ensemble. The QD also functions as a concentrator that amplifies the target signal by confining several targets in a nanoscale domain. Unbound nanosensors produce near-zero background fluorescence, but on binding to even a small amount of target DNA (~50 copies or less), they generate a very distinct FRET signal. A nanosensor-based oligonucleotide ligation assay has been demonstrated to successfully detect a point mutation typical of some ovarian tumors in clinical samples.

Ion Channel Switch Biosensor Technology

The Ion Channel Switch (ICS^{TM}), developed by Ambri Ltd (Chatswood, Australia), is a novel biosensor technology based on a synthetic self-assembling membrane. The membrane acts like a biological switch and is capable of detecting the presence of specific molecules, and signaling their presence by triggering an electrical current (Cornell 2002). It has the ability to detect a change in ion flow upon binding with the target molecule, resulting in a more rapid result than that currently achievable using existing technologies. The Ambri ICS^{TM} biosensor is one of the first true nanobiosensor devices and is the basis of SensiDx^{TM} System that has been designed for POC testing in critical care environments in hospitals. By delivering precise, quantitative test results in an immediate timeframe, the SensiDx^{TM} System may assist in reducing the time of emergency diagnoses from hours down to minutes. This has a positive impact on both clinical decision-making and treatment costs.

Electronic Nanobiosensors

The Biodetect^{TM} system (Integrated Nano-Technologies, Henrietta, NY, USA) works by electronically detecting the binding of a target DNA molecule to sensors on a

microchip. The target molecules form a bridge between two electrically separated wires. In order to create a strong clear signal, the bound target molecules are chemically developed to form conductive DNA wires. These DNA wires "turn on" a sensor much like an on/off switch. Each chip contains multiple sensors, which can be independently addressed with capture probes for different target DNA molecules from the same or different organisms. Each sensor has hundreds of interdigitated wires that are electrically separated from its neighbors. A proprietary DNA Lithography™ process is used to attach capture probes to each of the sensors on the chip. These chips now have billions of capture probes per sensor, which greatly improves sensitivity. To form detectable DNA wires, target DNA molecules first must form a DNA bridge spanning the gap between the sensor wires. DNA bridge formation has been observed by fluorescent imaging techniques. The final step in the detection process is to metalize the DNA bridge to form a DNA wire. Various metalization chemistries have been developed, which enable metalization of the DNA bridges with very low levels of background deposition. After metalization, bridges can be readily detected by measuring the resistance or other electrical properties of the sensor. DNA wires can be seen using electron microscopy. This portable system is used for rapid detection of microorganisms. This technology will also form the basis of site-specific drug delivery and high-resolution image arrays using nanoscale electronic components.

A signal-on, electronic DNA biosensor has been described that is label-free and achieves a subpicomolar detection limit (Xiao et al 2006). The sensor, which is based on a target-induced strand displacement mechanism, is composed of a "capture probe" attached by its 5′ terminus to a gold electrode and a 5′ methylene blue-modified "signaling probe" that is complementary at both its 3′ and 5′ termini to the capture probe. In the absence of target, hybridization between the capture and signaling probes minimizes contact between the methylene blue and electrode surface, limiting the observed redox current. Target hybridization displaces the 5′ end of the signaling probe, generating a short, flexible ssDNA element and producing up to a 7-fold increase in redox current. The observed signal gain is sufficient to achieve a demonstrated (not extrapolated) detection limit of 400 fM, which is among the best reported for single-step electronic DNA detection. Moreover, because sensor fabrication is straightforward, the approach appears to provide a ready alternative to the more cumbersome femtomolar electrochemical assays described to date.

Capacitators are critical elements in electrical circuits and nanocapacitors are capacitors with electrodes spacing in the nano order. When used with ssDNA probes, target hybridization produces a measurable change in capacitance. When used in arrays, nanocapacitors can enable simultaneous detection of nucleic acids without labeling (Fortina et al 2005).

Electrochemical Nanobiosensors

An electrochemical biosensor combining microfluidics and nanotechnology has been developed by GeneFluidics (Monterey Park, CA, USA) with 16 sensors in

the array, each consisting of three single-layer gold electrodes: working, reference, and auxiliary. Each of the working electrodes contains one representative from a library of capture probes, which are specific for a clinically relevant bacterial urinary pathogen. The library included probes for *Escherichia coli*, *Proteus mirabilis*, *Pseudomonas aeruginosa*, *Enterocococcus* spp., and the *Klebsiella–Enterobacter* group. A bacterial 16S rRNA target derived from single-step bacterial lysis was hybridized both to the biotin-modified capture probe on the sensor surface and to a second, fluorescein-modified detector probe. Detection of the probe–target hybrids is achieved through binding of a horseradish peroxidase (HRP)-conjugated antifluorescein antibody to the detector probe. Amperometric measurement of the catalyzed HRP reaction is obtained at a fixed potential of –200 mV between the working and reference electrodes. Species-specific detection of as few as 2,600 pathogenic bacteria in culture, inoculated urine, and clinical urine samples can be achieved within 45 min from the beginning of sample processing. In a feasibility study of this amperometric detection system using blinded clinical urine specimens, the sensor array had 100% sensitivity for direct detection of gram-negative bacteria without nucleic acid purification or amplification (Liao et al 2006). Identification was demonstrated for 98% of gram-negative bacteria for which species-specific probes were available. When combined with a microfluidics-based sample preparation module, the integrated system could serve as a POC device for rapid diagnosis of urinary tract infections.

Quartz Nanobalance Biosensors

ssDNA-containing thin films are deposited onto quartz oscillators to construct a device capable of sensing the presence of the cDNA sequences, which hybridize with the immobilized ones. DNA, once complexed with aliphatic amines, appears as a monolayer in a single-stranded form by small angle x-ray scattering. A quartz nanobalance is then utilized to monitor mass increment related to specific hybridization with a cDNA probe. The crystal quartz nanobalance, capable of high sensitivity, indeed appears capable of obtaining a prototype of a device capable of sensing the occurrence of particular genes or sequences in the sample under investigation.

Viral Nanosensors

Virus particles are essentially biological nanoparticles. Scientists at the Massachusetts General Hospital (Boston, MA) have used herpes simplex virus (HSV) and adenovirus to trigger the assembly of magnetic nanobeads as a nanosensor for clinically relevant viruses (Perez et al 2003). The nanobeads had a superparamagnetic iron oxide core coated with dextran. Protein G was attached as a binding partner for antivirus antibodies. By conjugating anti-HSV antibodies directly to nanobeads using a bifunctional linker to avoid nonspecific interactions between medium components and protein G and using a magnetic field, the scientists

could detect as few as five viral particles in a 10-ml serum sample. This system is more sensitive than ELISA-based methods and is an improvement over PCR-based detection because it is cheaper, faster, and has fewer artifacts. Upon target binding, these nanosensors cause changes in the spin-spin relaxation times of neighboring water molecules, which can detect specific mRNA, proteins, and enzymatic activity by (NMR/MRI) techniques (Perez et al 2004).

PEBBLE Nanosensors

Scientists at the University of Michigan (Ann Arbor, MI) are developing PEBBLE (Probes Encapsulated by Biologically Localized Embedding) nanosensors, which consist of sensor molecules entrapped in a chemically inert matrix by a microemulsion polymerization process that produces spherical sensors in the size range of 20–200 nm (Sumner et al 2002). These sensors are capable of real-time inter- and intracellular imaging of ions and molecules and are insensitive to interference from proteins. PEBBLE can also be used for early detection of cancer.

PEBBLE nanosensors also show very good reversibility and stability to leaching and photobleaching, as well as very short response times and no perturbation by proteins. In human plasma they demonstrate a robust oxygen sensing capability, little affected by light scattering and autofluorescence (Cao et al 2004). PEBBLE has been developed further as a tool for diagnosis as well as treatment of cancer.

Microneedle-Mounted Biosensors

NanoSense (NanoPass Technologies Ltd, Haifa, Israel) is a MicroPyramid^{TM} chip that is integrated with biosensors for nanoliter-scale ion diagnostics for congestive heart failure and renal failure. This technology will be integrated with microneedles to provide reliable, inexpensive, and simple to operate transdermal device for ion diagnoses, in POC settings. This work is conducted in collaboration with MESA (Micro Electronics, Materials Engineering, Sensors and Actuators) Laboratories at the University of Twente, the Netherlands (http://www.mesaplus.utwente.nl/).

Optical Biosensors

Many biosensors that are currently marketed rely on the optical properties of lasers to monitor and quantify interactions of biomolecules that occur on specially derived surfaces or biochips. An integrated biosensor, based on phototransistor integrated circuits, has been developed for use in medical detection, DNA diagnostics, and gene mapping. The biochip device has sensors, amplifiers, discriminators, and logic circuitry on board. Integration of light-emitting diodes into the device is also possible. Measurements of fluorescent-labeled DNA probe microarrays and hybridization experiments with a sequence-specific DNA probe for HIV-1 on nitrocellulose substrates illustrate the usefulness and potential of this DNA biochip. A number

of variations of optical biosensors offer distinct methods of sample application and detection in addition to different types of sensor surface. SPR technology is the best-known example of this technology.

Laser Nanosensors

In a laser nanosensor, laser light is launched into the fiber, and the resulting evanescent field at the tip of the fiber is used to excite target molecules bound to the antibody molecules. A photometric detection system is used to detect the optical signal (e.g., fluorescence) originating from the analyte molecules or from the analyte–bioreceptor reaction (Vo-Dinh 2005). Laser nanosensors can be used for in vivo analysis of proteins and biomarkers in individual living cells.

Physicists at the University of Rochester have assembled a simple laser system to detect nanoparticles. They split a laser beam in two, sending one half to a sample. When the light hits a small particle, it is scattered back and recombines with the reserve half of the laser beam, producing a detectable interference pattern detectable only when a moving particle is present. This laser method works where others do not because it relies on the amplitude rather than the intensity of light. The amplitude is the square root of intensity, so it decays much less than intensity as the particles get smaller. Single particles as small as 7 nm in diameter have been detected.

Researchers at the University of Twente have developed an ultrasensitive sensor that could potentially be used in a handheld device to detect various viruses and measure their concentration within minutes. It requires only a tiny sample of saliva, blood, or other body fluid. The device uses a silicon substrate containing channels that guide laser light. Light enters into the substrate at one end and is split into four parallel beams. When these beams emerge at the other end, they spread out and overlap with one another, creating a pattern of bright and dark bands, known as an interference pattern, which are recorded. A commercial version of the biosensor is being developed in collaboration with Paradocs Group BV (The Netherlands). Although the sensor has been shown to detect only the HSV virus, it could be used to quickly screen people at hospitals and emergency clinics for control of outbreaks of diseases such as severe acute respiratory syndrome (SARS) and avian flu.

Nanoshell Biosensors

Nanoshells can enhance chemical sensing by as much as 10 billion times. That makes them ~10,000 times more effective at Raman scattering than traditional methods. When molecules and materials scatter light, a small fraction of the light interacts in such a way that it allows scientists to determine their detailed chemical makeup. This property, known as Raman scattering, is used by medical researchers, drug designers, chemists, and other scientists to determine what materials are made of. An enormous limitation in the use of Raman scattering has been its extremely weak sensitivity. Nanoshells can provide large, clean, reproducible enhancements of this effect, opening the door for new, all-optical sensing applications.

Scientists at the Rice University's Laboratory of Nanophotonics have found that nanoshells are extremely effective at magnifying the Raman signature of molecules, each individual nanoshell acting as an independent Raman enhancer. That creates an opportunity to design all-optical nanoscale sensors—essentially new molecular-level diagnostic instruments—that could detect as little as a few molecules of a target substance, which could be anything from a drug molecule or a key disease protein to a deadly chemical agent.

The metal cover of the nanoshell captures passing light and focuses it, a property that directly leads to the enormous Raman enhancements observed. Furthermore, nanoshells can be tuned to interact with specific wavelengths of light by varying the thickness of their shells. This tunability allows for the Raman enhancements to be optimized for specific wavelengths of light. The finding that individual nanoshells can vastly enhance the Raman effect opens the door for biosensor designs that use a single nanoshell, something that could prove useful for engineers who are trying to probe the chemical processes within small structures such as individual cells, or for the detection of very small amounts of a material, like a few molecules of a deadly biological or chemical agent. Nanoshells are already being developed for applications including cancer diagnosis, cancer therapy, testing for proteins associated with AD, drug delivery, and rapid whole-blood immunoassays.

Plasmonics and SERS Nanoprobes

Surface plasmons are collective oscillations of free electrons at metallic surfaces. These oscillations can give rise to the intense colors of solutions of plasmon resonance nanoparticles and/or very intense scattering. While the use of plasmonic particle absorption based bioaffinity sensing is now widespread throughout biological research, the use of their scattering properties is relatively less studied. Plasmon scatter can be used for long-range immunosensing and macromolecular conformation studies (Aslan et al 2005).

A variety of sensors, metallic nanostructured probes, metallic nanoshells and half-shells, and nanoarrays for SERS sensing have been developed at the Oak Ridge National Laboratory. The SERS technology can detect the chemical agents and biological species (e.g., spores, biomarkers of pathogenic agents) directly. A DNA-based technique based on surface-enhanced Raman gene (SERGen) probes can be also used to detect gene targets via hybridization to DNA sequences complementary to these probes. Advanced instrumental systems designed for spectral measurements and for multiarray imaging as well as for field monitoring (RAMiTS technology) have been constructed. Plasmonics and SERS nanoprobes are useful for biological sensing.

Novel Optical mRNA Biosensors

Drs. Phillipe Haas and A. Wild of the NCCR Nanoscale Science (Basel, Switzerland) have developed a novel optical mRNA biosensor for application in pathology. The

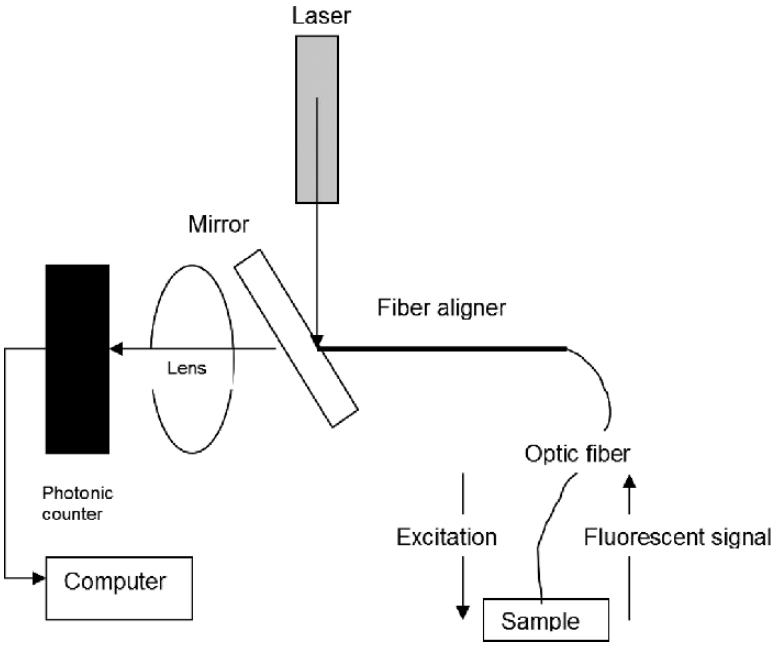

Fig. 3.2 Scheme of a novel optical mRNA biosensor

scheme of this biosensor is shown in Fig. 3.2. Molecular beacons that are highly sequence specific are used as molecular switches. This biosensor detects single molecules in fluids and can be used to search for molecular markers to predict the prognosis of disease.

Optonanogen Biosensors

Currently available commercial biosensing systems are large and designed to be used in laboratories. The Optonanogen program, coordinated by Centro Nacional de Microelectronica (CNM, Madrid, Spain), aims to apply the new micro- and nanotechnologies to DNA array production and analysis to develop a fully integrated biosensor system on a small scale. The aim is to achieve both miniaturization of the biochip format and an increase in the sensitivity of the assays performed. A prototype of the system will initially be used to detect mutations of the BRCA1 gene that are responsible for breast cancer in a small percentage of women. The final system, however, could be used to detect virtually any genetic anomaly as well as proteins linked to viruses, chemical contamination in food, or water pollution. The final device will be roughly the size of a human hand, allowing it to be used in physician's offices to determine the genetic predisposition of a patient to certain diseases in a matter of minutes. That compares to the hours or even days it can take to carry out the same analysis in a laboratory, which is generally only used to test high-risk groups such as women with a family history of breast cancer.

To detect genetic mutations, the Optonanogen system uses an array of 20 microcantilevers coated in nucleic acid that react when they come into contact with a DNA sample displaying the genetic anomaly. The sample is injected into the device via a microfluidic header and the deflection of the cantilevers—by as little as 0.1–0.5 nm—is picked up by a photodetector array based on the reflection of light off the cantilevers from VCSELs. The cantilever array and microfluidic header are low-cost components that would be disposable if used for medical analysis but which could be cleaned and reused for other applications. After evaluation trials later in 2005, a commercial version of the system is likely to be produced within 1 or 2 years by Sensia, a spin-off company from the CNM.

Surface Plasmon Resonance Technology

SPR is an optical–electrical phenomenon involving the interaction of light with the electrons of a metal. The optical–electronic basis of SPR is the transfer of the energy carried by photons of light to a group of electrons (a plasmon) at the surface of a metal. In Biacore systems, Uppsala, Sweden (part of GE Healthcare), SPR arises when light is reflected under certain conditions from a conducting film at the interface between two media of different refractive index. The media are the sample and the glass of the sensor chip, and the conducting film is a thin layer of gold on the chip surface. SPR causes a reduction in the intensity of reflected light at a specific angle of reflection (the SPR angle). When molecules in the sample bind to the sensor surface, the concentration and therefore the refractive index at the surface changes and an SPR response is detected. Plotting the response against time during the course of an interaction provides a quantitative measure of the progress of the interaction. This plot is called a sensorgram.

HTS Biosystems (Hopkinton, MA, USA) and Applied Biosystems Group (Foster City, CA, USA) are now codeveloping a next-generation microarray-based SPR system that is designed to help researchers profile and characterize biomolecular interactions in a parallel format. Applied Biosystems has introduced SPR in its 8500 Affinity Chip Analyzer. This instrument cannot match Biacore's variety of chip surface chemistries—Biacore offers nine different surfaces to Applied Biosystems' three—the new system targets the drug discovery market with its high-throughput format. The key strength of the system is that it can measure binding to all the different ligands under exactly the same conditions. The 8500 Affinity Chip Analyzer can simultaneously examine up to 400 binding interactions on a single chip during a 2-h run and can measure binding constants in the micromolar to picomolar range; the minimum analyte size for kinetic measurements is 8 kDa.

Miniature optical sensors that specifically identify low concentrations of environmental and biological substances are in high demand. Currently, there is no optical sensor that provides identification of the aforementioned species without amplification techniques at naturally occurring concentrations. Triangular silver nanoparticles have remarkable optical properties, and their enhanced sensitivity to their nanoenvironment has been used to develop a new class of optical sensors using localized SPR spectroscopy (Haes and Duyne 2004).

Surface-Enhanced Micro-Optical Fluidic Systems

The aim of the surface-enhanced micro-optical fluidic systems (SEMOFS) Europeans project is to develop a new concept for biosensors: a polymer-based card-type integrated "plasmon-enhanced SPR" sensor. The card will combine biologically active surfaces with integrated optics (light source, detection) and biocompatible multichannel microfluidics. The project aims to achieve a significant breakthrough, since all functions will be totally integrated on a single polymer-based chip. The final product shall be manufactured with large-scale, mass production techniques. The card will therefore be extremely low cost and disposable while providing increased sensitivity and diagnosis possibilities. The project will focus on the following:

- Increasing detection sensitivity and access to new information of the biological sample
- Microfluidics on polymer substrate enabling multichanneling (further enhancing sensitivity by parallel analysis) and integrated fluid actuators
- Integrated optical detection concept based on organic light emitting display (OLED)/waveguide/miniaturized spectrometer enabling card-type integrated solution and multichanneling
- Hybrid micromachining to ensure compatibility of the mastering and replication protocols with constraints of industrial-scale manufacturing
- Validation of expected applications and evaluation in clinical cancer diagnosis

Nanowire (NW) Biosensors

Since their surface properties are easily modified, NWs can be decorated with virtually any potential chemical or biological molecular recognition unit, making the wires themselves independent of the analyte. The nanomaterials transduce the chemical binding event on their surface into a change in conductance of the NW in an extremely sensitive, real-time, and quantitative fashion. Boron-doped SiNWs have been used to create highly sensitive, real-time electrically based sensors for biological and chemical species. Biotin-modified SiNWs were used to detect streptavidin down to at least a picomolar concentration range. The small size and capability of these semiconductor NWs for sensitive, label-free, real-time detection of a wide range of chemical and biological species could be exploited in array-based screening and in vivo diagnostics.

A novel approach to synthesizing NWs allows their direct integration with microelectronic systems for the first time, as well as their ability to act as highly sensitive biomolecule detectors that could revolutionize biological diagnostic applications. An interdisciplinary team of engineers in Yale University's Institute for Nanoscience and Quantum Engineering has overcome hurdles in NW synthesis by using a tried-and-true process of wet-etch lithography on commercially available silicon-on-insulator wafers. These NWs are structurally stable and demonstrate an

unprecedented sensitivity as sensors for detection of antibodies and other biologically important molecules. According to researchers, not only can the NWs detect extremely minute concentrations (as few as 1,000 individual molecules in a cubic millimeter), they can do it without the hazard or inconvenience of any added fluorescent or radioactive detection probes. The study demonstrated ability of the NWs to monitor antibody binding, and to sense real-time live cellular immune response using T-lymphocyte activation as a model. Within $\sim$10 s, the NW could register T-cell activation as the release acid to the device. The basis for the sensors is the detection of hydrogen ions or acidity, within the physiological range of reactions in the body. Traditional assays for the detection of immune system cells such as T cells or for antibodies usually take hours to complete.

When biological molecules bind to their receptors on the NW, they usually alter the current moving through the sensor and signal the presence of the substance of interest. This direct detection dispenses with the time-consuming labeling chemistry and speeds up the detection process considerably. NW biosensors are used for the detection of proteins, viruses, or DNA in a highly sensitive manner. They can be devised to test for a complex of proteins associated with a particular cancer and used for diagnosis as well as monitoring the progress of treatment.

Nanowires for Detection of Genetic Disorders

The surfaces of the SiNW devices have been modified with peptide nucleic acid (PNA) receptors designed to recognize wild type versus the F508 mutation site in the CF transmembrane receptor gene (Hahm and Lieber 2004). Conductance measurements made while sequentially introducing wild-type or mutant DNA samples exhibit a time-dependent conductance increase consistent with the PNA–DNA hybridization and enabled identification of fully complementary versus mismatched DNA samples. Concentration-dependent measurements show that detection can be carried out to at least the tens of femtomolar range. It provides more rapid results than do current methods of DNA detection. This NW-based approach represents a step forward for direct, label-free DNA detection with extreme sensitivity and good selectivity and could provide a pathway to integrated, high-throughput, multiplexed DNA detection for genetic screening.

NW Biosensors for Detecting Biowarfare Agents

Researchers at the Lawrence Livermore National Laboratory have developed a multistriped biosensing NW system for detecting biowarfare agents in the field (Tok et al 2006). It is constructed from submicrometer layers of different metals including gold, silver, and nickel that act as "barcodes" for detecting a variety of pathogens ranging from anthrax, smallpox, and ricin to botulinum. Antibodies of specific pathogens are attached to the NWs producing a small, reliable, sensitive detection system. The system could also be used during an outbreak of an infectious disease.

Concluding Remarks and Future Prospects of NW Biosensors

A review has shown that NW biosensors modified with specific SURFACE receptors represent a powerful nanotechnology-enabled diagnostic/detection platform for medicine and the life sciences (Patolsky et al 2006). Key features of these devices include direct, label-free, and real-time electrical signal transduction, ultrahigh sensitivity, exquisite selectivity, and potential for integration of addressable arrays on a massive scale, which set them apart from other sensor technologies that are currently available. NW biosensors have unique capabilities for multiplexed real-time detection of proteins, single viruses, DNA, enzymatic processes, and small organic molecule binding to proteins. Apart from their value as research tools, they have a significant impact on disease diagnosis, genetic screening, and drug discovery. They will facilitate the development of personalized medicine. Because these NW sensors transduce chemical/biological binding events into electronic/digital signals, they have the potential for a highly sophisticated interface between nanoelectronic and biological information processing systems in the future.

Nanoscale Erasable Biodetectors

Scientists at the Duke University's Pratt School of Engineering are designing biodetectors and structures scaled in nanoscale. The proposed erasable detectors are made of artificial elastin-like polypeptides (ELPs), which are short segments of proteins normally soluble in water. Crafted through genetic engineering with the aid of bacteria, such ELPs have the useful property of coming out of a solution to form a solid whenever a slight temperature increase or other alterations to the water induce a phase change. An ELP could also be chemically linked with another protein so that both "fusion proteins" leave solution together after such phase changes. This method could be used to create a "reversible" protein sensor on a glass slide. After dotting such a slide with microscopic amounts of surface-bound ELPs, the researchers discovered that dissolved fusion proteins would selectively attach to those microdots upon leaving the solution. They also found the "captured" fusion proteins could pull other select proteins from solution, so those could be chemically identified. Finally, they confirmed that microdot array could then be wiped clean of all attached proteins simply by "reversing the phase transition." In this case, the researchers added salt to the solution to induce the same kind of phase changes as does raising the water temperature. It is possible to create a surface for a sensor, do a binding reaction, detect a signal, then release everything. Then the same process can be repeated with the same fusion protein. An AFM is used that can deposit nanoscale amounts of material through a DPN. Instead of using a glass slide, a gold surface was fabricated on which to bind ELP nanodots because DPN works well on gold. A major reason for their improved success is that the gold surface was specially modified to prevent stray proteins from attaching to the experimental array. The Duke Group has also built a "nonfouling" platform by inducing methyl methacrylate molecules to grow into tall stalks from a gold surface through a self-assembly process known

as “atom transfer radical polymerization.” In the same process, molecules of PEG were also induced to form fuzzy branches extending from those stalks, creating the overall look of bottle brushes. In this case, the PEG branches formed a protective barrier that kept unwanted proteins from coming out of solution and sticking to the platform. The scientists are now exploring a method to build nanotowers of DNA block by block from the surface. They have described how the enzyme terminal deoxynucleotidyl transferase (TdTase) could be used to induce short DNA strands to form extensive chains. Those “polymerizing” chains, growing vertically from nanodots of gold patterned onto silicon, assembled into tower-like structures (Chow et al 2005). The process worked in a solution of enzyme and DNA building blocks, called nucleotides, with the TdTase grabbing floating nucleotides and pulling those into the extending structure. TdTase-catalyzed surface-initiated polymerization of DNA is expected be a useful tool for the fabrication of complex biomolecular structures with nanoscale resolution.

Future Issues in the Development of Nanobiosensors

New biosensors and biosensor arrays are being developed using new materials, nanomaterials, and microfabricated materials including new methods of patterning. Biosensor components will use nanofabrication technologies. Use of nanotubes, Buckminsterfullerenes (buckyballs), silica, and its derivatives can produce nanosized devices. Some of the challenges are listed below:

- Development of real-time noninvasive technologies that can be applied to the detection and quantitation of biological fluids without the need for multiple calibrations using clinical samples.
- Development of biosensors utilizing new technologies that offer improved sensitivity for detection with high specificity at the single-molecule level.
- Development of biosensor arrays that can successfully detect, quantify, and quickly identify individual components of mixed gases and liquid in an industrial environment.

It would be desirable to develop multiple integrated biosensor systems that utilize doped oxides, polymers, enzymes, or other components to give the system the required specificity. A system with all the sensor components, software, plumbing, reagents, and sample processing is an example of an integrated sensor. There is also a need for reliable fluid handling systems for “dirty” fluids and for relatively small quantities of fluids (nanoliter to attoliter quantities). These should be low cost, disposable, reliable, and easy to use as part of an integrated sensor system. Sensing in picoliter to attoliter volumes might create new problems in the development of microreactors for sensing and novel phenomenon in very small channels.

The University of Ulster (UU, Jordanstown, UK) has extensive experience and expertise in the design, fabrication, and characterization of a wide range of flexible electrode/substrate systems suitable for both 20 mA stimulation/sensing of nerve

bundles and in vivo biosensing. Requirements for such a thin-film sensing device include high substrate/metal adhesion, long-life durability, mechanical stability, and the ability to be patterned and also to exhibit full biocompatibility. UU's future objectives to research suitable thin-film coatings and processes with relevant characterization techniques that will permit the development of long-life in vivo sensor devices. This involves understanding the bioresponse of the body to various forms of thin-film and plasma surface modification processes. Also the thin-film sensing materials of interest such as platinum, gold, iridium/oxide, titanium, and various polymers require characterization. Cell and platelet growth studies will be correlated with surface science studies in order to develop optimal plasma modification- or deposition-based processes.

Applications of Nanodiagnostics

Applications of nanotechnologies in clinical diagnostics have been reviewed recently (Jain 2005b). Although applications of individual technologies are mentioned in the preceding section, some important areas of clinical application will be identified here.

Nanotechnology for Detection of Biomarkers

A biomarker is a characteristic that can be objectively measured and evaluated as an indicator of a physiological as well as a pathological process or pharmacological response to a therapeutic intervention. Classical biomarkers are measurable alterations in blood pressure, blood lactate levels following exercise, and blood glucose in diabetes mellitus. Any specific molecular alteration of a cell on DNA, RNA, metabolite, or protein level can be referred to as a molecular biomarker. From a practical point of view, the biomarker would specifically and sensitively reflect a disease state and could be used for diagnosis as well as for disease monitoring during and following therapy. Currently available molecular diagnostic technologies have been used to detect biomarkers of various diseases such as cancer, metabolic disorders, infections, and diseases of the central nervous system (CNS). Nanotechnology has further refined the detection of biomarkers. Some biomarkers also form the basis of innovative molecular diagnostic tests.

One project in this area draws together the expertise of a team of researchers from the Australian Institute for Bioengineering and Nanotechnology at The University of Queensland (UQ), the Fred Hutchinson Cancer Research Center (Seattle, WA), and the Seattle Biomedical Research Institute. This project is supported by a contribution of $2 million from the Queensland State Government through the National and International Research Alliances Program. In addition to Alliances funding, the project will receive support from the participating institutes and UQ spin-off company Nanomics Biosystems (Brisbane, Australia).

DNA Y-junctions have been used as fluorescent scaffolds for *Eco*RII methyltransferase–thioredoxin fusion proteins and covalent links were formed between the DNA scaffold and the methyltransferase at preselected sites on the scaffold containing 5FdC (Singer and Smith 2006). The resulting thioredoxin-targeted nanodevice was found to bind selectively to certain cell lines but not to others. The fusion protein was constructed so as to permit proteolytic cleavage of the thioredoxin peptide from the nanodevice. Proteolysis with thrombin or enterokinase effectively removed the thioredoxin peptide from the nanodevice and extinguished cell line-specific binding measured by fluorescence. Potential applications for devices of this type include the ability of the fused protein to selectively target the nanodevice to certain tumor cell lines, suggesting that this approach can be used to probe cell surface receptors as biomarkers of cancer and may serve as an adjunct to immunohistochemical methods in tumor classification.

Perfluorocarbon Nanoparticles to Track Therapeutic Cells In Vivo

Using perfluorocarbon nanoparticles 200 nm in size to label endothelial progenitor cells taken from human umbilical cord blood (UCB) enables their detection by MRI in vivo following administration (Partlow et al 2007). The MRI scanner can be tuned to the specific frequency of the fluorine compound in the nanoparticles, and only the nanoparticle-containing cells are visible in the scan. This eliminates any background signal, which often interferes with medical imaging. Moreover, the lack of interference means one can measure very low amounts of the labeled cells and closely estimate their number by the brightness of the image. Since several perfluorocarbon compounds are available, different types of cells potentially could be labeled with different compounds, injected, and then detected separately by tuning the MRI scanner to each one's individual frequency. This technology offers significant advantages over other cell-labeling technologies in development. Laboratory tests showed that the cells retained their usual surface markers and that they were still functional after the labeling process. The labeled cells were shown to migrate to and incorporate into blood vessels forming around tumors in mice. These nanoparticles could soon enable researchers and physicians to directly track cells used in medical treatments using unique signatures from the ingested nanoparticle beacons. They could prove useful for monitoring tumors and diagnosing as well as treating cardiovascular problems.

Monitoring of Implanted Neural Stem Cells Labeled with Nanoparticles

Noninvasive monitoring of stem cells, using high-resolution molecular imaging, will be important for improving clinical neural transplantation strategies. Labeling of human neural stem cells (NSCs) grown as neurospheres with magnetic nanoparticles

was shown to not adversely affect survival, migration, and differentiation or alter neuronal electrophysiological characteristics (Guzman et al 2007). Using MRI, the authors demonstrated that human NSCs transplanted to the neonatal, the adult, or the injured rodent brain respond to cues characteristic for the ambient microenvironment, resulting in distinct migration patterns. Nanoparticle-labeled human NSCs survive long term and differentiate in a site-specific manner identical to that seen for transplants of unlabeled cells. The impact of graft location on cell migration and MRI characteristics of graft cell death and subsequent clearance were also described. Knowledge of migration patterns and implementation of noninvasive stem cell tracking might help to improve the design of future clinical NSC transplantation.

Nanobiotechnologies for Single-Molecule Detection

Various nanobiotechnologies for single-molecule detection are listed in Table 3.2. These have been described in preceding sections.

Protease-Activated Quantum Dot Probes

QDs have been programmed to glow in presence of enzyme activity and give off NIR light only when activated by specific proteases (Chang et al 2005). Altered expression of particular proteases is a common hallmark of cancer, atherosclerosis, and many other diseases. NIR light also passes harmlessly through skin, muscle, and cartilage, so the new probes could detect tumors and other diseases at sites deep in

Table 3.2 Nanobiotechnologies for single-molecule detection

Visualization of biomolecules by near-nanoscale microscopy
Atomic force microscope
Scanning probe microscope
3D single-molecular imaging by nanotechnology
Near-field scanning optical microscope
Spectrally resolved fluorescence lifetime imaging microscopy
Nanolaser spectroscopy for detection of cancer in single cells
Nanoproteomics
Study of protein expression at the single-molecule level
Detection of a single molecule of protein
Erenna™ Bioassay System: digital single-molecule detection platform
Nanofluidic/nanoarray devices: detection of a single molecule of DNA
Carbon nanotube transistors for genetic screening
Nanopore technology
Portable nanocantilever system for diagnosis
Nanobiosensors
Quantum-dots-FRET nanosensors for single-molecule detection

Source: Jain PharmaBiotech.

the body without the need for a biopsy or invasive surgery. The probe's design makes use of a technique called "quenching" that involves tethering a gold nanoparticle to the QD to inhibit luminescence. The tether, a peptide sequence measuring only a few nanometers, holds the gold close enough to prevent the QD from giving off its light. The peptide tether used is one that is cleaved by the enzyme collagenase. The luminescence of the QDs is cut by $>70\%$ when they are attached to the gold particles. They remain dark until the nanostructures were exposed to collagenase after which the luminescence steadily returns. The ultimate aim of the research is to pair a series of QDs, each with a unique NIR optical signature, to an index of linker proteases. This probe would be important for understanding and monitoring the efficacy of therapeutic interventions, including the growing class of drugs that act as protease inhibitors. An important feature of the protease imaging probes described in this study is the combination of the contrast enhancement achievable through a probe that can be activated and is combined with the brightness, photostability, and tunability of QDs.

Nanotechnology for Point-of-Care Diagnostics

POC or near-patient testing means that diagnosis is performed in the doctor's office or at the bedside in case of hospitalized patients or in the field for several other indications including screening of populations for genetic disorders and cancer. POC involves analytical patient testing activities provided within the healthcare system, but performed outside the physical facilities of the clinical laboratories. POC does not require permanent dedicated space but includes kits and instruments, which are either hand-carried or transported to the vicinity of the patient for immediate testing at that site. The patients may even conduct the tests. After the laboratory and the emergency room, the most important application of molecular diagnostics is estimated to be at the POC. Nanotechnology would be another means of integrating diagnostics with therapeutics. Nanotechnology-based diagnostics provides the means to monitor drugs administered by nanoparticle carriers.

Nanoprobes for POC Diagnosis

A number of devices based on nanotechnology are among those with potential applications in POC testing. Researchers from Northwestern University's Institute for Nanotechnology (Evanston, IL) describe a new method of DNA detection that uses gold nanoparticle probes and microarrays of electrodes (Park et al 2002). It is 10 times more sensitive (causing fewer false negatives) and 100,000 times more selective (causing far fewer false positives) than current methods. The nanoprobes are coated with a synthesized string of nucleotides that complement one end of a target sequence in the sample being analyzed, so they can "grab" it if it is there. Another set of nucleotides, complementing the other end of the target, is attached to a surface between two electrodes. If the target sequence is present in the sample, it attaches to both the nanoprobes and the sequences on the surface between the electrodes,

so that the nanoprobes are anchored to the surface like a cluster of little balloons. When they are treated with a silver solution, they create a bridge between the electrodes and produce a charge. The technology could theoretically be used to detect any disease or condition with a unique genomic fingerprint. For example, it could differentiate between various antibiotic-resistant strains of streptococci, or detect cancerous cells, or quickly identify HIV or biological warfare agents like anthrax. A single chip could contain electrode pairs to test for thousands of biological targets at once. And because an electrical charge is either present or absent, there is no ambiguity in the results. Nanosphere Inc's Verigene™ platform will be suitable for the development of POC testing.

Carbon Nanotube Transistors for Genetic Screening

Carbon nanotube network FETs (NTNFETs) have been reported that function as selective detectors of DNA immobilization and hybridization (Star et al 2006). NTNFETs with immobilized synthetic ODNs have been shown to specifically recognize target DNA sequences, including H63D SNP discrimination in the HFE gene, responsible for hereditary hemochromatosis, a disease in which too much iron accumulates in body tissues. The electronic responses of NTNFETs upon ssDNA immobilization and subsequent DNA hybridization events were confirmed by using fluorescence-labeled ODNs and then were further explored for label-free DNA detection at picomolar to micromolar concentrations. A strong effect of DNA counterions on the electronic response was observed, suggesting a charge-based mechanism of DNA detection using NTNFET devices. Implementation of label-free electronic detection assays using NTNFETs constitutes an important step toward low-cost, low-complexity, highly sensitive, and accurate molecular diagnostics. Label-free electronic detection of DNA has several advantages over state-of-the-art optical techniques, including cost, time, and simplicity. The sensitivity of the new device is good enough to detect a single-base mutation in an amount of DNA present in 1 ml of blood. This technology can bring to market handheld POC devices for genetic screening, as opposed to laboratory methods using labor-intense labeling and sophisticated optical equipment. This device will be commercially developed by Nanomix Inc (Emeryville, CA, USA).

Nanocytometer

The nanocytometer is a pocket-sized device based on "pore-on-a-chip" technology that can rapidly identify diseases by testing a single drop of blood using an inexpensive disposable cartridge. The cartridges contain a silicon chip laden with artificial nanopores that mimic the filtration system of human cells. The nanocytometer enables work at the intersection of a number of disciplines, from biology and mechanical engineering to solid-state physics and chemical engineering (Carbonaro et al 2006). The tool has the potential to boost survival chances for leukemia, prostate cancer, or breast cancer patients, particularly in patients where

the cancer has recurred by offering early detection of rare, isolated cancer cells. The device is currently in the pipeline for commercial development.

Nanodiagnostics for the Battle Field

Researchers at MIT's Institute for Soldier Nanotechnologies have taken a major step toward making an existing miniature lab-on-a-chip fully portable, so the tiny device can perform hundreds of chemical experiments in any setting including the battlefield. This will make testing soldiers to see if they have been exposed to biological or chemical weapons much faster and easier. Neither of the previous approaches, mechanically force fluid through microchannels or capillary electro-osmosis, offers portability. Within the lab-on-a-chip, biological fluids such as blood are pumped through channels $\sim$ 10 μm wide. Each channel has its own pumps, which direct the fluids to certain areas of the chip, so they can be tested for the presence of specific molecules. In the new system, known as a 3D AC electro-osmotic pump, tiny electrodes with raised steps generate opposing slip velocities at different heights, which combine to push the fluid in one direction, like a conveyor belt. Simulations predict a dramatic improvement in flow rate, by almost a factor of 20, so that fast (mm/s) flows, comparable to that of pressure-driven systems, can be attained with battery voltages.

If exposure to biological or chemical weapons is suspected, the device can automatically and rapidly test a miniscule blood sample, rather than sending a large sample to a laboratory and waiting for the results. The chips are so small and cheap to make that they could be designed to be disposable, or they could be made implantable.

Nanodiagnostics for Integrating Diagnostics with Therapeutics

Molecular diagnostics is an important component of personalized medicine. Improvement of diagnostics by nanotechnology has a positive impact on personalized medicine. Nanotechnology has potential advantages in applications in POC diagnosis: on patient's bedside, self-diagnostics for use in the home and integration of diagnostics with therapeutics. All of these will facilitate the development of personalized medicines.

Scientists at the University of Texas Medical Branch (Galveston, TX) are developing nanoparticles with bound thioaptamers for diagnostics and therapeutics in biodefense. RNA and DNA ODNs can act as "aptamers," (i.e., as direct in vivo binders selected from large combinatorial libraries) for a number of proteins. Both in vitro enzymatic combinatorial selection and split-synthesis bead-based chemical combinatorial methods have been developed to identify phosphorothioate-modified oligonucleotide "thioaptamers" to a number of different infectious disease targets for detection, diagnostics, and therapeutics. Importantly, it has been noted that

sulfurization of the phosphoryl oxygens of ODNs often leads to their enhanced binding to numerous proteins. Monothiophosphate and dithiophosphate backbone-modified thioaptamers bind to proteins involved in the immune response, as well as to other proteins of the proteome. Bead-based high-throughput screening of thioaptamer bead libraries is used to select thioaptamers for the development of a thioaptamer as well as thioaptamer-gold nanoparticle-based proteomics arrays to identify and quantify toxins, viruses, proteins, and protein complexes relating to biodefense. Furthermore, selected thioaptamers delivered with liposomal nanoparticles can modulate the immune response and show promise as therapeutic agents targeting viruses such as West Nile virus and hemorrhagic fever arenaviruses. The lead thioaptamer, R12-2, shows specific binding to HIV-1 RT and inhibits the RNase H activity of intact HIV-1 RT. Suppression of virus was comparable with that seen with AZT (Somasunderam et al 2005).

Concluding Remarks About Nanodiagnostics

It is now obvious that direct analysis of DNA and protein could dramatically improve speed, accuracy, and sensitivity over conventional molecular diagnostic methods. Since DNA, RNA, protein, and their functional subcellular scaffolds and compartments are in the nanometer scale, the potential of single-molecule analysis approach would not be fully realized without the help of nanobiotechnology. Advances in nanotechnology are providing nanofabricated devices that are small, sensitive, and inexpensive enough to facilitate direct observation, manipulation, and analysis of single biological molecule from single cell. This opens new opportunities and provides powerful tools in the fields such as genomics, proteomics, molecular diagnostics, and high-throughput screening. A review of articles published over the past 10 years investigating the use of QDs, gold nanoparticles, cantilevers, and other nanotechnologies concluded that nanodiagnostics promise increased sensitivity, multiplexing capabilities, and reduced cost for many diagnostic applications as well as intracellular imaging (Azzazy et al 2006). Further work is needed to fully optimize these diagnostic nanotechnologies for clinical laboratory setting and to address the potential health and environmental risks related to QDs.

Various nanodiagnostics that have been reviewed will improve the sensitivity and extend the present limits of molecular diagnostics. Numerous nanodevices and nanosystems for sequencing single molecules of DNA are feasible. It seems quite likely that there will be numerous applications of inorganic nanostructures in biology and medicine as biomarkers. Given the inherent nanoscale of receptors, pores, and other functional components of living cells, the detailed monitoring and analysis of these components will be made possible by the development of a new class of nanoscale probes. Biological tests measuring the presence or activity of selected substances become quicker, more sensitive, and more flexible when certain nanoscale particles are put to work as tags or labels. Nanoparticles are the most versatile material for developing diagnostics.

Nanomaterials can be assembled into massively parallel arrays at much higher densities than is achievable with current sensor array platforms and in a format compatible with current microfluidic systems. Currently, QD technology is the most widely employed nanotechnology for diagnostic developments. Among the recently emerging technologies, cantilevers are the most promising. This technology complements and extends current DNA and protein microarray methods, because nanomechanical detection requires no labels, optical excitation, or external probes and is rapid, highly specific, sensitive, and portable. This will have applications in genomic analysis, proteomics, and molecular diagnostics. Nanosensors are promising for detection of bioterrorism agents that are not detectable with current molecular diagnostic technologies and some have already been developed.

Future Prospects of Nanodiagnostics

Within the next decade, measurement devices based on nanotechnology, which can make thousands of measurements very rapidly and very inexpensively, will become available. The most common clinical diagnostic application will be blood protein analysis. Blood in systemic circulation reflects the state of health or disease of most organs. Therefore, detection of blood molecular fingerprints will provide a sensitive assessment of health and disease. Another important area of application will be cancer diagnostics. Molecular diagnosis of cancer including genetic profiling would be widely used by the year 2015. Nanobiotechnology would play an important part, not only in cancer diagnosis but also in linking diagnosis with treatment.

In the near future, nanodiagnostics would reduce the waiting time for the test results. For example, the patients with sexually transmitted diseases could give the urine sample when they first arrive at the outpatient clinic or physician's practice; the results could then be ready by the time they go in to see the doctor. They could then be given the prescription immediately, reducing the length of time worrying for the patient and making the whole process cheaper.

Future trends in diagnostics will continue in miniaturization of biochip technology to nano range. The trend will be to build the diagnostic devices from bottom up starting with the smallest building blocks. Whether interest and application of nanomechanical detection will hold in the long range remains to be seen. Another trend is to move away from fluorescent labeling as miniaturization reduces the signal intensity, but there have been some improvements making fluorescent viable with nanoparticles.

Molecular electronics and nanoscale chemical sensors will enable the construction microscopic sensors capable of detecting patterns of chemicals in a fluid. Information from a large number of such devices flowing passively in the bloodstream allows estimates of the properties of tiny chemical sources in a macroscopic tissue volume. Estimates of plausible device capabilities have been used to evaluate their performance for typical chemicals released into the blood by tissues in response to localized injury or infection (Hogg and Kuekes 2006). These indicate that the

devices can readily discriminate a single cell-sized chemical source from the background chemical concentration in vivo, providing high-resolution sensing in both time and space. With currently used methods for blood analysis, such a chemical source would be difficult to distinguish from background when diluted throughout the blood volume and withdrawn as a blood sample.

Chapter 4
Nanopharmaceuticals

Introduction

The term "nanopharmaceuticals" covers discovery, development, and delivery of drugs. The postgenomic era is revolutionizing the drug discovery process. The new challenges in the identification of therapeutic targets require efficient and cost-effective tools. Label-free detection systems use proteins or ligands coupled to materials whose physical properties are measurably modified following specific interactions. Among the label-free systems currently available, the use of metal nanoparticles offers enhanced throughput and flexibility for real-time monitoring of biomolecular recognition at a reasonable cost. This chapter will deal with the use of nanobiotechnologies for drug discovery and development, an important part of nanobiopharmaceuticals. Some technologies will accelerate target identification, whereas others will evolve into therapeutics. The use of nanobiotechnologies for drug delivery is an important part of nanomedicine.

Nanobiotechnology for Drug Discovery

Current drug discovery process needs improvement in several areas. Although many targets are being discovered through genomics and proteomics, the efficiency of screening and validation processes need to be increased. Through further miniaturization, nanotechnology will improve the ability to fabricate massive arrays in small spaces using microfluidics and the time efficiency. This would enable direct reading of the signals from microfluidic circuits in a manner similar to a microelectronics circuit where one does not require massive instrumentation. This would increase the ability to do high-throughput drug screening. Basic nanotechnologies applicable to drug discovery have been reviewed elsewhere (Jain 2005c). Nanocrystals (QDs) and other nanoparticles (gold colloids, magnetic nanoparticles, nanobarcodes, nanobodies, dendrimers, fullerenes, and nanoshells) have received a considerable attention recently with their unique properties for potential use in drug discovery. Usefulness and limitations of some of these technologies for drug discovery will be described here.

K.K. Jain, *The Handbook of Nanomedicine*,

Gold Nanoparticles for Drug Discovery

Gold nanoparticles do not blink or burn out, even after hours of observation. These properties suggest that metal nanoparticles are a viable alternative to fluorophores or semiconductor nanoparticles for biological labeling and imaging. Other advantages are that the gold nanoparticles can be prepared easily, have very low toxicity, and can readily be attached to molecules of biological interest. In addition, the laser light used to visualize the particles is a wavelength that causes only minimal damage to most biological tissues. This technology could enable tracking of a single molecule of a drug in a cell or other biological samples.

SPR has also been successfully applied with colloidal gold particles in buffered solution. This application offers many advantages over conventional SPR. The support is cheap, easily synthesized, and can be coated with various proteins or protein–ligand complexes by charge adsorption. With colloidal gold, the SPR phenomenon can be monitored in any UV–vis spectrophotometer. For high-throughput applications, the technology has been adapted in an automated clinical chemistry analyzer. Among the label-free systems currently available, the use of metal nanocolloids offers enhanced throughput and flexibility for real-time biomolecular recognition monitoring at a reasonable cost.

Use of Quantum Dots for Drug Discovery

The use of QDs for drug discovery has been explored extensively. Both advantages and drawbacks have been described (Ozkan 2004). QDs can be used for tracking of single-molecule properties in living cells. Older imaging tools such as fluorescent dyes or polymer spheres are either too unstable or too big to effectively perform single-molecule tracking. QD conjugates are almost an order of magnitude brighter than fluorescent dyes and can be observed for as long as 40 min compared with ~ 5 s for the dyes. Length of observation time is critical for studying cellular processes that change rapidly over a span of several minutes. Cellular receptors are a critical target studied by scientists who develop new drug candidates for diseases including neurological disorders such as epilepsy and depression. More detailed understanding of the behavior of these receptors can open up new treatment options.

Nanolasers for Drug Discovery

Nanolasers could help discover drugs to halt the progression of neurodegenerative diseases like PD and AD as well as illnesses caused by radiation and chemical nerve agents. Mitochondria are involved in these diseases. Because mitochondria are so small, current techniques to find protective compounds are arduous and slow. Researchers at Sandia National Laboratory in New Mexico have combined the technology that makes lasers in compact disc players with the parts of living

cells to detect the death throes of minuscule mitochondria within cells. By flowing mitochondria through a solid-state microscopic laser-making cavity that is powered up to just below the onset of giving off laser light, the nanoscale mitochondria will do the "lasing" themselves. What's more, the laser light frequency given off by the mitochondria reveals their state of health.

Healthy mitochondria "lase" light at one frequency, while swollen and dying mitochondria lase at another. Using the nonlaser technique, laboratory researchers should be able to give large numbers of healthy mitochondria the "die" signal that they get from AD and PD diseases and then test to see whether there are any compounds that block the "die" signal and can save the mitochondria. The nanolaser technique would enable screening of thousands of compounds.

Among the more promising drugs known to protect mitochondria is cyclosporine, but it has proved less than ideal because it also weakens patients' immune systems. However, there are many variants of the compound that the nanolaser technique could quickly screen to see whether they might be effective as well. One of those variations might have few or no side effects.

Cells Targeting by Nanoparticles with Attached Small Molecules

Multivalent attachment of small molecules to nanoparticles can increase specific binding affinity and reveal new biological properties of such nanomaterials. Multivalent drug design has yielded antiviral and anti-inflammatory agents several orders of magnitude more potent than monovalent agents. Parallel synthesis of a library has been described, which is composed of nanoparticles decorated with different synthetic small molecules (Weissleder et al 2005). Screening of this library against different cell lines led to the discovery of a series of nanoparticles with high specificity for endothelial cells, activated human macrophages, or pancreatic cancer cells. This multivalent approach could facilitate development of functional nanomaterials for applications such as differentiating cell lines, detecting distinct cellular states, and targeting specific cell types. It has potential applications in high-throughput drug discovery, diagnostics, and human therapeutics.

Role of AFM for Study of Biomolecular Interactions for Drug Discovery

Scientists at the University of Linz in Austria use an approach called topography and recognition imaging (TREC). TREC uses any of a number of ligands such as antibodies, small organic molecules, and nucleotides bound to a carefully designed AFM tip-sensor that can, in a series of unbinding experiments, estimate affinity and structural data (Ebner et al 2005). If a ligand is attached to the end of an AFM probe, one can simulate various physiological conditions and look at the strength of the interaction between the ligand and receptor under a wide range of circumstances.

By functionalizing the tip, one can use it to probe biological systems and identify particular chemical entities on the surface of a biological sample. This opens the door to more effective use of AFM in drug discovery.

AFM has been used to study the molecular-scale processes underlying the formation of the insoluble plaques associated with AD. As one of a class of neurological diseases caused by changes in a protein's physical state, called "conformational" diseases, it is particularly well suited for study with AFM. Extensive data suggest that the conversion of the Aβ peptide from soluble to insoluble forms is a key factor in the pathogenesis of AD. In recent years, AFM has provided useful insights into the physicochemical processes involving Aβ morphology. AFM was the key in identifying the nanostructures that are now recognized as different stages of Aβ aggregation in AD and has revealed other forms of aggregation, which are observable at earlier stages and evolve to associate into mature fibrils. AFM can now be used to explore factors that either inhibit or promote fibrillogenesis. Using AFM enabled the comparison of two monoclonal antibodies (MAbs) being studied as potential treatments for AD to select the one that did a better job of inhibiting the formation of these protofibrils. M266.2, which binds to the central portion of the Aβ, completely inhibited the formation of protofibrils, while the other antibody, m3D6, slowed but did not totally stop their growth (Legleiter et al 2004). These results indicate that AFM can not only be reliably used to study the effect of different molecules on Aβ aggregation, but that it can provide additional information such as the role of epitope specificity of antibodies as potential inhibitors of fibril formation.

Nanoscale Devices for Drug Discovery

Researchers at Purdue University have built and demonstrated a prototype for a new class of miniature devices to study synthetic cell membranes in an effort to speed the discovery of new drugs for a variety of diseases, including cancer (Wang et al 2005a). The work is part of overall research being carried out by an interdisciplinary team of scientists and engineers who are members of a Center for Membrane Protein Biotechnology. The center was created at Purdue in 2003 through a grant from the Indiana 21st Century Research and Technology Fund, established by the state of Indiana to promote high-tech research and to help commercialize innovations.

The researchers have created a chip about 1 cm^2 that holds thousands of tiny vessels sitting on top of a material that contains numerous pores. The vessels are cylindrical cavities that are open at the top and sealed at the bottom with a material called alumina, which contains numerous pores measured in nanometers. This "nanoporous" material makes it possible to carry out reactions inside the vessels. The goal is to produce "laboratories-on-a-chip" less than a half-inch square that might contain up to a million test chambers, or "reactors," each capable of screening an individual drug.

Cell membranes contain a variety of proteins some of which act as tiny pumps that quickly remove chemotherapy drugs from tumor cells, making the treatment less effective. Cancer cells exposed to chemotherapy drugs produce a disproportionately large number of the pumps, causing the cells to become progressively more resistant to anticancer drugs. The aim of research is to find drugs that deactivate the pumps and thus make chemotherapy drugs more effective. The chips could dramatically increase the number of experiments that are possible with a small amount of protein.

Nanotechnology Enables Drug Design at Cellular Level

To create drugs capable of targeting some of the most devastating human diseases, scientists must first decode exactly how a cell or a group of cells communicates with other cells and reacts to a broad spectrum of complex biomolecules surrounding it. But even the most sophisticated tools currently used for studying cell communications suffer from significant deficiencies and typically can only detect a narrowly selected group of small molecules or, for a more sophisticated analysis, the cells must be destroyed for sample preparation. Researchers at the Georgia Tech (Atlanta, GA) have created a nanoscale probe, the scanning mass spectrometry (SMS) probe, which can capture both the biochemical makeup and topography of complex biological objects. The SMS probe can help map all those complex and intricate cellular communication pathways by probing cell activities in the natural cellular environment, which might lead to better disease diagnosis and drug design on the cellular level.

Nanobiotechnology-Based Drug Development

Dendrimers as Drugs

Dendrimers are a novel class of 3D nanoscale, core-shell structures that can be precisely synthesized for a wide range of applications. Specialized chemistry techniques allow for precise control over the physical and chemical properties of the dendrimers. They are most useful in drug delivery but can also be used for the development of new pharmaceuticals with novel activities. Polyvalent dendrimers interact simultaneously with multiple drug targets. They can be developed into novel targeted cancer therapeutics. Polymer–protein and polymer–drug conjugates can be developed as anticancer drugs. These have the following advantages:

- Tailor-made surface chemistry
- Nonimmunogenic
- Inherent body distribution enabling appropriate tissue targeting
- Possibly biodegradable

Fullerenes as Drug Candidates

A key attribute of the fullerene molecules is their numerous points of attachment, allowing for precise grafting of active chemical groups in 3D orientations. This attribute, the hallmark of rational drug design, allows for positional control in matching fullerene compounds to biological targets. In concert with other attributes, i.e., the size of the fullerene molecules, their redox potential, and its relative inertness in biological systems, it is possible to tailor requisite pharmacokinetic characteristics to fullerene-based compounds and optimize their therapeutic effect.

Fullerene antioxidants bind and inactivate multiple circulating intracellular free radicals, giving them unusual power to stop free radical injury and to halt the progression of diseases caused by excess free radical production. Fullerenes provide effective defense against all of the principal damaging forms of reactive oxygen species. C60 fullerene has 30 conjugated carbon–carbon double bonds, all of which can react with a radical species. In addition, the capture of radicals by fullerenes is too fast to measure and is referred to as "diffusion controlled," meaning the fullerene forms a bond with a radical every time it encounters one. Numerous studies demonstrate that fullerene antioxidants work significantly better as therapeutic antioxidants than other natural and synthetic antioxidants, at least for CNS degenerative diseases. In oxidative injury or disease, fullerene antioxidants can enter cells and modulate free radical levels, thereby substantially reducing or preventing permanent cell injury and cell death. Mechanisms of action of fullerene are as follows:

- Fullerenes can capture multiple electrons derived from oxygen free radicals in unoccupied orbitals.
- An attacking radical forms a bond with fullerene, creating a stable and relatively nonreactive fullerene radical.
- A tris-malonic acid derivative of the fullerene C60 molecule (C3) is capable of removing the biologically important superoxide radical.
- C3 localizes to mitochondria, suggesting that C3 functionally replaces manganese superoxide dismutase (SOD), acting as a biologically effective SOD mimetic (Ali et al 2004).

Fullerenes have potential applications in the treatment of diseases where oxidative stress plays a role in the pathogenesis. These include the following:

- Degenerative diseases of the CNS including PD, AD, and amyotrophic lateral sclerosis
- Multiple sclerosis
- Ischemic cardiovascular diseases
- Atherosclerosis
- Major long-term complications of diabetes
- Sun-induced skin damage and physical manifestations of aging

The first-generation antioxidant fullerenes are based on the C3 compound, produced by the precise grafting of three malonic acid groups to the C60 fullerene surface. C3 has shown significant activity against a spectrum of neurodegenerative

disorders in animal models. These animal models replicate many of the features of important human neurodegenerative diseases, including amyotrophic lateral sclerosis and PD.

The second-generation antioxidant fullerenes are based on DF-1, the dendrofullerene, produced by attaching a highly water-soluble conjugate to the C60 fullerene core. In preclinical testing, C60 has shown DF-1 to be highly soluble, nontoxic, and able to retain a high level of antioxidant activity in both cultured cells and animals.

A number of water-soluble C60 derivatives have been suggested for various medical applications. These applications include neuroprotective agents, HIV-1 protease inhibitors, bone-disorder drugs, transfection vectors, x-ray contrast agents, photodynamic therapy (PDT) agents, and a C60–paclitaxel chemotherapeutic.

Another possible application of fullerenes is to be found in nuclear medicine, in which they could be used as an alternative to chelating compounds that prevent the direct binding of toxic metal ions to serum components. This could increase the therapeutic potency of radiation treatments and decrease their adverse effect profile, because fullerenes are resistant to biochemical degradation within the body.

Nanobodies

Nanobodies, derived from naturally occurring single-chain antibodies, are the smallest fragments of naturally occurring heavy-chain antibodies that have evolved to be fully functional in the absence of a light chain. The Nanobody technology (Ablynx, Ghent, Belgium) was originally developed following the discovery that Camelidae (camels and llamas) possess a unique repertoire of fully functional antibodies that lack light chains (Conrath et al 2003). Like conventional antibodies, Nanobodies show high target specificity and low inherent toxicity; however, like small-molecule drugs they can inhibit enzymes and can access receptor clefts. Their unique structure consists of a single variable domain (VHH), a hinge region, and two constant domains (CH2 and CH3). The cloned and isolated VHH domain is a perfectly stable polypeptide harboring the full antigen-binding capacity of the original heavy chain. This newly discovered VHH domain is the basic component of Ablynx's Nanobodies. Ablynx's Nanobodies are naturally highly homologous to human antibodies. They can also be humanized to within 99% sequence homology of human VHH domains. Ablynx's Nanobody platform can quickly deliver therapeutic leads for a wide range of targets. Advantages of Nanobodies are as follows:

- They combine the advantages of conventional antibodies with important features of small-molecule drugs.
- Nanobodies can address therapeutic targets not easily recognized by conventional antibodies such as active sites of enzymes.
- Nanobodies are very stable.
- They can be administered by means other than injection.
- They can be produced cost-effectively on a large scale.

- Nanobodies have an extremely low immunogenic potential. In animal studies, the administration of Nanobodies does not yield any detectable humoral or cellular immune response.

The cloning and selection of antigen-specific nanobodies obviate the need for construction and screening of large libraries, and for lengthy and unpredictable in vitro affinity maturation steps. The unique and well-characterized properties enable nanobodies to excel conventional therapeutic antibodies in terms of recognizing uncommon or hidden epitopes, binding into cavities or active sites of protein targets, tailoring of half-life, drug format flexibility, low immunogenic potential, and ease of manufacture. Moreover, the favorable biophysical and pharmacological properties of nanobodies, together with the ease of formatting them into multifunctional protein therapeutics, leaves them ideally placed as a new generation of antibody-based therapeutics. They have a potential as cancer therapeutic agents (Revets et al 2005).

Another example of the use of nanobodies as novel drugs is nanobody-conjugated human trypanolytic factor for treatment of human African trypanosomiasis (HAT). Normal human serum (NHS) contains apolipoprotein L-I (apoL-I), which lyses African trypanosomes except resistant forms such as *Trypanosoma brucei rhodesiense*, which expresses the apoL-I-neutralizing serum resistance–associated (SRA) protein, endowing this parasite with the ability to infect humans and cause HAT. A truncated apoL-I (Tr-apoL-I) has been engineered by deleting its SRA-interacting domain, which makes it lytic for *T. b. rhodesiense*. Tr-apoL-I has been conjugated with a nanobody that efficiently targets conserved cryptic epitopes of the variant surface glycoprotein of trypanosomes to generate a new type of immunotoxin with potential for trypanosomiasis therapy (Baral et al 2006). Treatment with this engineered conjugate resulted in clear curative and alleviating effects on acute and chronic infections of mice with both NHS-resistant and NHS-sensitive trypanosomes.

Role of Nanobiotechnology in the Future of Drug Discovery

None of the nanoparticles available is ideal for all requirements of drug discovery. The choice may depend on the needs. QDs can be used for high-throughput cell-based studies with the advantage of multiplexing (i.e., multiple leads can be tested at the same time). However, as discussed earlier there are some limitations yet to be resolved for their use in the drug discovery studies, namely, toxicity, size variation, agglomeration, potential multiple drug attachment to a single QD, and blinking.

An increasing use of nanobiotechnology by the pharmaceutical and biotechnology industries is anticipated. Nanotechnology will be applied at all stages of drug development—from formulations for optimal delivery to diagnostic applications in clinical trials.

In another promising area of application, scientists at the Ohio State University are developing nonbiodegradable 3D scaffolds to hold stem cells for pharmaceutical

and biological research. These tissue constructs can be used to test new drugs. Since tissues grow in three dimensions and not two, 3D would be more suitable for early drug screening.

Nanobiotechnology in Drug Delivery

Drug delivery is one of the important considerations in drug development and therapeutics. New technologies are applied for constructing innovative formulations and delivering them. The focus is on targeted drug delivery. This is important for delivery of biopharmaceuticals and treatment of diseases such as cancer and neurological disorders. In the pharmaceutical industry, there is potential to provide new formulations and routes of drug delivery.

Nanoscale Delivery of Therapeutics

There are several requirements for developing a device small enough to efficiently leave the vasculature and enter cells to perform multiple, smart tasks. However, the major requirement involves size. Vascular pores limit egress of therapeutics to materials $\lesssim$50 nm in diameter, and cells will not internalize materials $>>$100 nm. As a result, the only currently available technology that fulfills these criteria consists of synthetic nanodevices. These are designed, synthetic materials with structures $<$100 nm in size. Unlike fictional mechanical nanomachines, based on machines that have been "shrunken" to nanometer dimensions, several true nanomolecular structures have now been synthesized and applied to drug delivery, gene transfer, antimicrobial therapeutics, and immunodiagnostics.

Nanobiotechnology Solutions to the Problems of Drug Delivery

One of the major problems with drugs is solubility, which is an essential factor for drug effectiveness, independent of administration route. It is also a major challenge for pharmaceutical companies developing new pharmaceutical products since nearly half of new chemically based drugs are insoluble, or poorly soluble, in water. Many, otherwise promising, compounds never reach the market. Others reach the market but in a suboptimal formulation, possibly providing low or unpredictable bioavailability, or posing an increased side-effect risk. Enhanced solubility technology can be used to reformulate such drugs and increase their commercial potential. Nanobiotechnology provides the following solutions to the problems of drug delivery:

- The particle size is reduced to nanometer size range to increase the surface area, thereby increasing the rate of dissolution, e.g., Nanoedge technology (Baxter, Round Lake, IL, USA).
- Improving solubilization of the drug.

- Using noninvasive routes of administration eliminates the need for administration of drugs by injection.
- Development of novel nanoparticle formulations with improved stabilities and shelf-lives.
- Development of nanoparticle formulations for improved absorption of insoluble compounds and macromolecules enables improved bioavailability and release rates, potentially reducing the amount of dose required and increasing safety through reduced side effects.
- Manufacture of nanoparticle formulations with controlled particle sizes, morphology, and surface properties would be more effective and less expensive than other technologies.
- Nanoparticle formulations that can provide sustained-release profiles up to 24 h can improve patient compliance with drug regimens.
- Direct coupling of drugs to targeting ligands, restricts the coupling capacity to a few drug molecules, but coupling of drug carrier nanosystems to ligands allows import of thousands of drug molecules by means of one receptor-targeted ligand (Vasir et al 2005). Nanosystems offer opportunities to achieve drug targeting with newly discovered disease-specific targets.

Nanosuspension Formulations

Nanosuspension formulations can be used to improve the solubility of poorly soluble drugs. A large number of new drug candidates emerging from drug discovery programs are water insoluble, and therefore poorly bioavailable, leading to abandoned development efforts. These can now be rescued by formulating them into crystalline nanosuspensions (Rabinow 2004). Techniques such as media milling and high-pressure homogenization have been used commercially for producing nanosuspensions (Patravale et al 2004). The unique features of nanosuspensions have enabled their use in various dosage forms, including specialized delivery systems such as mucoadhesive hydrogels. Nanosuspensions can be delivered by parenteral, peroral, ocular, and pulmonary routes. Currently, efforts are being directed to extending their applications in site-specific drug delivery.

Various particle sizes of spironolactone, a model low solubility drug, have been formulated to yield micro- and nanosuspensions of the type solid lipid nanoparticles and DissoCubes. The DissoCubes nanosuspension yielded highly significant improvements in bioavailability (Langguth et al 2005). Particle size minimization is not the major determining factor in the bioavailability improvement. Rather, the type of surfactant used as stabilizer in the formulations is of greater importance. Improvement in drug solubility in the intestine as well as in dissolution rate of spironolactone are the most likely mechanisms responsible for the observed effect, although additional mechanisms such as permeability enhancement may also be involved.

Nanotechnology for Solubilization of Water-Insoluble Drugs

The Ubisol-Aqua™ (Zymes LLC, Hasbrouck Heights, NJ, USA) delivery system uses nanotechnology to enable the solubilization and reformulation of water-insoluble drugs, nutrients, and cosmetic ingredients. The capacity of Ubisol-Aqua™ to expand the usefulness of such compounds has been scientifically demonstrated with water-soluble formulations of coenzyme Q10 (HQO™) and antifungal antibiotics. In March 2007, Zymes LLC reported that it has successfully solubilized fish oil and omega-3 fatty acids (DHA/EPA/ALA) with an average particle size of 34 nm. Zymes offers its delivery system technology to industry partners in need of more effective ways of making their ingredients water-soluble and thus more bioavailable.

Improved Absorption of Drugs in Nanoparticulate Form

In February 2007, a research team from the Federal Institute of Technology (Zurich, Switzerland) reported that reducing iron phosphate to the nanoscale could increase its absorption in the body. The team utilized the flame spray pyrolysis technique to assemble the iron nanoparticles. The researchers reported that when iron-deficient rats were given diets with the nano-iron phosphate, the rats gained weight and increased their RBC count, and with no toxicity problems. The next steps will be to discover whether iron at the nanoscale can be absorbed successfully by the human body, and whether it alters the taste and consistency of food.

Ideal Properties of Material for Drug Delivery

Properties of an ideal macromolecular drug delivery or biomedical vector are as follows:

- Structural control over size and shape of drug or imaging-agent cargo space
- Biocompatible, nontoxic polymer/pendant functionality
- Precise, nanoscale-container and/or scaffolding properties with high drug or imaging-agent capacity features
- Well-defined scaffolding and/or surface modifiable functionality for cell-specific targeting moieties
- Lack of immunogenicity
- Appropriate cellular adhesion, endocytosis, and intracellular trafficking to allow therapeutic delivery or imaging in the cytoplasm or nucleus
- Acceptable bioelimination or biodegradation
- Controlled or triggerable drug release
- Molecular-level isolation and protection of the drug against inactivation during transit to target cells
- Minimal nonspecific cellular and blood-protein binding properties
- Ease of consistent, reproducible, clinical grade synthesis

Nanomaterials and Nanobiotechnologies Used for Drug Delivery

Various nanomaterials and nanobiotechnologies used for drug delivery are listed in Table 4.1.

Table 4.1 Nanomaterials and nanobiotechnologies used for drug delivery

Structure	Size (nm)	Role in drug delivery
Bacteriophage NK97 (a virus that attacks bacteria)		Teams at Scripps Research Institute (San Diego, CA) and Stanford University (Palo Alto, CA) are investigating bacteriophage NK97, which is harmless to humans. Emptied of its own genetic material, HK97, which is covered by 72 interlocking protein rings, can act as a nanocontainer to carry drugs and chemicals to targeted locations
Canine parvovirus (CPV)-like particles	26	CPV binds to transferrin receptors, which are overexpressed by a variety of tumor cells and are being widely investigated for tumor-targeted drug delivery.
Carbon magnetic nanoparticles	40–50	For drug delivery and targeted cell destruction
Dendrimers	1–20	Holding therapeutic substances such as DNA in their cavities
Ceramics nanoparticles	~35	Accumulate exclusively in the tumor tissue and allow the drug to act as sensitizer for PDT without being released
HTCC nanoparticles	110–180	Encapsulation efficiency is up to 90%. In vitro release studies show a burst effect followed by a slow and continuous release
Liposomes	25–50	A new generation of liposomes that incorporate fullerenes to deliver drugs that are not water-soluble and that tend to have large molecules
Micelle/Nanopill	25–200	Made from two polymer molecules—one water-repellant and the other hydrophobic—that self-assemble into a sphere called a micelle that can deliver drugs to specific structures within the cell
Low-density lipoproteins	20–25	Drugs solubilized in the lipid core or attached to the surface
Nanocochleates		Nanocochleates facilitate delivery of biologicals such as DNA and genes
Nanocrystals	<1,000	NanoCrystal technology (Elan) has the potential to rescue a significant number of poorly soluble chemical compounds by increasing solubility
Nanoemulsions	20–25	Drugs in oil and/or liquid phases to improve absorption

Table 4.1 (continued)

Structure	Size (nm)	Role in drug delivery
Nanolipispheres	25–50	Carrier incorporation of lipophilic and hydrophilic drugs
Nanoparticle composites	~40	Attached to guiding molecules such as MAbs for targeted drug delivery
Nanoparticles	25–200	Act as continuous matrices containing dispersed or dissolved drug
Nanopore membrane		An implanted titanium device using silicone nanopore membrane can release encapsulated protein and peptide drugs
Nanospheres	50–500	Hollow ceramic nanospheres created by ultrasound
Nanostructured organogels	50	Gels created by mixing olive oil and liquid solvents and adding a simple enzyme to chemically activate a sugar and used to encapsulate drugs
Nanotubes	20–60	Resemble tiny drinking straws and are alternatives that might offer advantages over spherical nanoparticles for some applications
Nanovalve	500	Externally controlled release of drug into a cell
Nanovesicles	25–3,000	Single or multilamellar bilayer spheres containing the drugs in lipids
Polymer nanocapsules	50–200	Enclosing drugs
PEG-coated PLA nanoparticles		PEG coating improves the stability of PLA nanoparticles in the gastrointestinal fluids and helps the transport of encapsulated protein across the intestinal and nasal mucous membranes.
Superparamagnetic iron oxide nanoparticles	10–100	As drug carriers for intravenous injection to evade RES of the body as well as penetrate the very small capillaries within the body tissues and therefore offer the most effective distribution in certain tissues

HTCC, *N*-(2-hydroxyl) propyl-3-trimethyl ammonium chitosan chloride; PEG, poly(ethylene glycol); PLA, poly(lactic acid); RES, reticuloendothelial system.
Source: Jain PharmaBiotech.

Viruses as Nanomaterials for Drug Delivery

Specific targeting of tumor cells is an important goal for the design of nanotherapeutics for the treatment of cancer. Recently, viruses have been explored as nanocontainers for specific targeting applications; however, these systems typically require modification of the virus surface using chemical or genetic means to achieve tumor-specific delivery. However, there is a subset of viruses with natural affinity for receptors on tumor cells that could be exploited for nanotechnology applications,

e.g., the CPV for targeted drug delivery in cancer, which is described later in this chapter under drug delivery for cancer.

Nanoparticle-Based Drug Delivery

Trend toward miniaturization of carrier particles had started prior to the introduction of nanotechnology in drug delivery. As a part of introduction, microparticles and nanoparticles will be compared for their role as carriers of therapeutic substances.

The suitability of nanoparticles for use in drug delivery depends on a variety of characteristics, including size and porosity. Acusphere Inc (Watertown, MA, USA) is creating porous particles that are smaller than RBCs. Nanoparticles can be used to deliver drugs to patients through various routes of delivery. Nanoparticles are important for delivering drugs intravenously so that they can pass safely through the body's smallest blood vessels, for increasing the surface area of a drug so that it will dissolve more rapidly, and for delivering drugs via inhalation. Porosity is important for entrapping gases in nanoparticles, for controlling the release rate of the drug and for targeting drugs to specific regions.

It is difficult to create sustained-release formulations for many hydrophobic drugs because they release too slowly from the nanoparticles used to deliver the drug, diminishing the efficacy of the delivery system. Modifying water uptake into the nanoparticles can speed the release, while retaining the desired sustained-release profile of these drugs. Water uptake into nanoparticles can be modified by adjusting the porosity of the nanoparticles during manufacturing and by choosing from a wide variety of materials to include in the shell.

Gold Nanoparticles as Drug Carriers

Research is being conducted at the Center for Nanoscience and Nanotechnology of Melbourne University in Australia to develop the intelligent delivery systems by lining the walls of microscopic polymer "delivery-vehicle" particles with gold nanoparticles (Radt et al 2004). By simply shining a laser on loaded delivery vehicles (i.e., particles filled with various contents, such as an enzyme or a drug), the walls could be opened and the contents released. This technique has been used successfully for the release of an encapsulated enzyme on demand with a single nanosecond laser pulse. In contrast to the common approach for drug release by changes in the local environment at the site where drug delivery is needed, gold nanoparticle technology enables externally controlled drug release. In addition to drugs, these gold-coated vehicles could be used for the controlled delivery of a wide range of other substances including genes, pesticides, cosmetics, and food stuffs. There is no risk that the laser energy will be significantly absorbed by biological

structures such as bodily organs because the absorption of the gold-coated delivery vehicles in the NIR light region is intentionally engineered in the wavelength regime for which light has a maximum penetration depth in tissue.

Calcium Phosphate Nanoparticles

BioSante Pharmaceuticals Inc (Lincolnshire, IL, USA) has developed BioOral, an oral delivery system for insulin, based on its proprietary calcium phosphate (CAP) nanoparticles. CAP particles containing insulin were synthesized in the presence of PEG and modified by aggregating the particles with caseins to obtain the CAP-PEG-insulin-casein (CAPIC) oral insulin delivery system. Single doses of CAPIC formulation were tested in nonobese diabetic mice under fasting or fed conditions to evaluate the glycemic activity (Morcol et al 2004). Based on the results obtained, the authors propose the following:

1. The biological activity of insulin is preserved in CAPIC formulation.
2. Insulin in CAPIC formulations, but not the free insulin, displays a prolonged hypoglycemic effect after oral administration to diabetic mice.
3. CAPIC formulation protects insulin from degradation while passing through the acidic environment of the gastrointestinal tract until it is released in the less acidic environment of the intestines where it can be absorbed in its biologically active form.
4. CAPIC formulation represents a new and unique oral delivery system for insulin and other macromolecules.

Cyclodextrin Nanoparticles for Drug Delivery

Cyclodextrins are a family of cyclic oligosaccharides with a hydrophilic outer surface and a lipophilic central cavity. Cyclodextrin molecules are relatively large with a number of hydrogen donors and acceptors, and thus, in general they do not permeate lipophilic membranes. Cyclodextrins have mainly been used as complexing agents to increase aqueous solubility of poorly soluble drugs and to increase their bioavailability and stability.

Amphiphilic cyclodextrin nanoparticles, resulting from the esterification of primary hydroxyl groups by hydrocarbon chains varying from C6 to C14, are capable of forming spontaneously nanoparticles, which can be loaded with drugs. The drug can be released in a controlled manner following targeting delivery by the oral or parenteral route. For injectable preparations, sterile filtration is not feasible since nanoparticle sizes are larger than the filter pore size and the yield after sterilization is very low. However, blank as well as drug-loaded cyclodextrin nanospheres and nanocapsules are capable of being sterilized by gamma irradiation with no effect on particle size, drug loading, and drug-release properties (Memisoglu-Bilensoy and Hincal 2006).

Dendrimers for Drug Delivery

The unique properties of dendrimers such as high degree of branching and well-defined molecular weight make them ideal scaffolds for drug discovery (Gillies and Frechet 2005). Advantages of dendrimers over linear polymers are as follows:

- The controlled multivalency of dendrimers can be used to attach several drug molecules to the periphery of the dendrimer in a well-defined manner.
- Because of their well-defined molecular weight, they provide reproducible pharmacokinetic behavior compared with linear polymers containing fractions within a sample that vary greatly in molecular weight.
- The globular structure of dendrimers, as contrasted with the coil structure of most linear polymers, can modify their biological properties, enabling discovery of new effects related to macromolecular architecture.

Dendrimers are particularly useful for the delivery of anticancer drugs such as cisplatin and doxorubicin as well as agents for boron neutron capture therapy (BNCT). Their newest application is in PDT for cancer.

DNA-Assembled Dendrimers for Drug Delivery

Researchers at the University of Michigan have developed a faster, more efficient way to produce a wide variety of nanoparticle drug delivery systems, using DNA molecules to bind the dendrimers together (Choi et al 2005b). Nanometer-scaled dendrimers can be assembled in many configurations by using attached lengths of ssDNA molecules, which naturally bind to other DNA strands in a highly specific fashion. This approach enables targeting of a wide variety of molecules—drugs, contrast agents—to almost any cell. Nanoparticle complexes can be specifically targeted to cancer cells and are small enough to enter a diseased cell, either killing it from within or sending out a signal to identify it. However, construction of the particles is difficult and time-consuming.

Two different functional dendrimers were constructed—one designed for imaging and the other for targeting cancer cells. Each of the dendrimers also carried a noncoding synthesized ssDNA. The dangling lengths of DNA, typically 34–66 bases long, found complementary sequences on other dendrimers and knitted together, forming barbell-shaped two-dendrimer complexes with folate on one end and fluorescence on the other end. Folate receptors are overexpressed on the surface of cancer cells, so these dendrimer clusters would tend to flock to the diseased cells. The other end of the complex carries a fluorescent protein so that the researchers can track their movement. A series of experiments using cell sorters, 3D microscopes, and other tools verified that these dendrimers hit their targets, were admitted into the cells, and gave off their signaling light. The self-assembled dendrimer clusters were shown to be well formed and functional. The researchers plan to create a library of single-functional dendrimers that can be synthesized in parallel, rather than sequentially, and then linked together in many different combinations with the

DNA strands. A nanoparticle cluster is foreseen in which a single dendrimer carries three ssDNAs, each with a sequence specific to the DNA attached to other kinds of dendrimers. Once placed into solution with other components, the molecule would self-assemble into a four-dendrimer complex carrying one drug, one target, and one fluorescent protein.

Fullerene Conjugate for Intracellular Delivery of Peptides

Cell walls, or membranes, form a protective covering around the cell's inner machinery and its DNA blueprints. Drugs are far more effective if they are delivered through the membrane directly into the cell, but this is difficult. A fullerene–peptide conjugate formed via the incorporation of a fullerene-substituted phenylalanine derivative, "Bucky amino acid" (Baa), to a cationic peptide, acts as a passport for intracellular delivery, enabling transport of peptides that, in the absence of the fullerene amino acid, cannot enter the cell (Yang et al 2007). Delivery of the fullerene species to either the cytoplasm or nucleus of the cell has been demonstrated. The hydrophobic nature of the fullerene assisting peptide transport is suggested by the effect of gamma-cyclodextrin in lowering the efficacy of transport. These data suggest that the incorporation of a fullerene-based amino acid provides a route for the intracellular delivery of peptides and as a consequence the creation of a new class of cell-penetrating peptides. The peptides were found effective at penetrating the defenses of both liver cancer cells and neuroblastoma cells.

Polymer Nanoparticles

Biodegradable polymer nanoparticles are (i) poly(ethylene glycol) (PEG)-coated poly(lactic acid) (PLA) nanoparticles, chitosan (CS)-coated poly(lactic acid–glycolic acid (PLGA) nanoparticles and CS nanoparticles. These nanoparticles can carry and deliver proteins to them in an active form, and transport them across the nasal and intestinal mucosae. Additionally, PEG coating improves the stability of PLA nanoparticles in the gastrointestinal fluids and helps the transport of the encapsulated protein, tetanus toxoid, across the intestinal and nasal mucous membranes. Furthermore, intranasal administration of these nanoparticles provided high and long-lasting immune responses.

Chitosan Nanoparticles

N-(2-Hydroxyl) propyl-3-trimethyl ammonium chitosan chloride (HTCC) is water-soluble derivative of CS, synthesized by the reaction between glycidyl -trimethyl-ammonium chloride and CS. HTCC nanoparticles have been formed based on ionic gelation process of HTCC and sodium tripolyphosphate (TPP). Bovine serum albumin (BSA), as a model protein drug, was incorporated into the HTCC nanoparticles. HTCC nanoparticles were 110–180 nm in size, and their

encapsulation efficiency was up to 90%. In vitro release studies showed a burst effect and a slow and continuous release followed. Encapsulation efficiency was obviously increased with increase of initial BSA concentration (Xu et al 2003).

Coating of PLGA nanoparticles with the mucoadhesive CS improves the stability of the particles in the presence of lysozyme and enhanced the nasal transport of the encapsulated tetanus toxoid. Nanoparticles made solely of CS are stable upon incubation with lysozyme. Moreover, these particles are very efficient in improving the nasal absorption of insulin as well as the local and systemic immune responses to tetanus toxoid, following intranasal administration.

A group of researchers at the Interdisciplinary Nanoscience Center (iNANO) at the University of Aarhus in Denmark have discovered that by encapsulating drugs in CS cubes, the cells of the body are tricked into absorbing drugs that could not normally be transported across the cell membrane. After delivering its load directly into the cells affected, CS is broken down in the body and disappears without a trace. In animal experiments, they demonstrated that CS capsules can transport siRNA into the cell to switch off faulty genes selectively and cure the diseases caused by these genes. Human clinical trials using this technology are anticipated by the year 2010.

Polymeric Micelles

Micelles that are biocompatible nanoparticles varying in size from 50 to 200 nm in which poorly soluble drugs can be encapsulated represent a possible solution to the delivery problems associated with such compounds and could be exploited to target the drugs to particular sites in the body, potentially alleviating toxicity problems. pH-sensitive drug delivery systems can be engineered to release their contents or change their physicochemical properties in response to variations in the acidity of the surroundings. One example of this is the preparation and characterization of novel polymeric micelles (PMs) composed of amphiphilic pH-responsive poly(N-isopropylacrylamide) (PNIPAM) or poly(alkyl(meth)acrylate) derivatives (Dufresne et al 2004). On one hand, acidification of the PNIPAM copolymers induces a coil-to-globule transition that can be exploited to destabilize the intracellular vesicle membranes. PNIPAM-based PMs, loaded with either doxorubicin or aluminium chloride phthalocyanine, are cytotoxic in murine tumor models. On the other hand, poly(alkyl(meth)acrylate) copolymers can be designed to interact with either hydrophobic drugs or polyions and release their cargo upon an increase in pH. Micelle-forming polymeric drugs such as NK911 (doxorubicin-incorporating micelle) and NK105 (taxol-incorporating micelle) are in clinical trials sponsored by Nippon Kayaku Co. and conducted at the National Cancer Center Hospital, Tokyo, Japan (Matsumura 2006).

Ceramic Nanoparticles

Ceramic (inorganic) particles with entrapped biomolecules have potential pharmaceutical applications including drug delivery. Ceramic nanoparticles have several advantages:

- Manufacture processes are relatively similar to the well-known sol-gel process, require ambient temperature condition, and can be easily prepared with the desired size, shape, and porosity.
- Their small size (<50 nm) can help them to evade being trapped by the RES of the body.
- There is no swelling or change in porosity with change in pH.
- These particles effectively protect doped molecules (enzymes, drugs, etc.) against denaturation induced by external pH and temperature.
- Such particles, including silica, aluminum, and titanium, are known for their compatibility with biological systems.
- Their surfaces can be easily modified for conjugation to monoclonal antibodies or ligands to target them to desired sites in vivo.

Nanocrystals

Nanocrystalline Silver

Silver has been valued for centuries for its medicinal properties. From ancient Greece to the American settlers, silver was used as a preservative for drinking water and other liquid storage. Decades ago, doctors would apply a thin layer of silver to large wounds to prevent infection and promote healing. Nucryst's silver nanocrystalline technology decreases the particle size, thus changing the physical and chemical properties. As the proportion of atoms on the surface increases, the result is a more powerful compound than from conventional silver treatments. In vitro tests have demonstrated that active silver clusters of ions begin providing antimicrobial activity immediately and kill many organisms in 30 min, faster than other forms of silver.

Silcryst™ nanocrystals (Nucryst Pharmaceuticals, Wakefield, MA, USA) release sustained, uniform doses of silver. Silver nanocrystalline technology is capable of delivering a sustained release of active silver to the dressings over a longer period of time than any other silver treatment. Other treatments, such as Silver Sulfadiazine and Silver Nitrate, are characterized by the rapid depletion of active silver, forcing the regular scraping of creams or applications of solutions to open wounds multiple times per day. This process is labor intensive and extremely traumatic for patients. Silver nanocrystalline technology dressings cover the wound providing sustained release of silver to the dressing, acting as a barrier to infection for up to 7 days.

Acticoat™ (Smith & Nephew, London, UK) dressings for burns and chronic wounds use Nucryst's proprietary Silcryst™ silver nanocrystalline technology. In vitro studies of Acticoat have demonstrated the following:

- Extensive antimicrobial spectrum of 150 different pathogens
- Rapid kill rates
- Effective against drug-resistant forms of bacteria, such as (methicillin-resistant *Staphylococcus aureus* MRSA) and (vancomycin-resistant *Enterococci* VRE), sometimes referred to as superbugs

- Fast-acting release of ionic silver to the dressing over a sustained period of time (effective for up to 7 days)

The company also is conducting preclinical studies on the use of nanocrystalline silver inhaled into the lungs for the treatment of serious lung infection or lung inflammation. In the future, the company plans to conduct research on the nanocrystalline structures of other metals, including gold, which is well known as a treatment for arthritis, and platinum, which is a wellknown treatment for cancer, to determine whether the behavior and performance of these metals also can be enhanced.

NanoCrystal Technology

NanoCrystal® (Elan) particles are small particles of drug substance, typically $<1{,}000$ nm in diameter, which are produced by milling the drug substance using a proprietary milling technique. The NanoCrystal® particles of the drug are stabilized against agglomeration by surface adsorption of selected GRAS (Generally Regarded As Safe) stabilizers. The end result is a suspension of the drug substance that behaves like a solution—a NanoCrystal® colloidal dispersion, which can be processed into dosage forms for all routes of administration. NanoCrystal® technology is being used by Johnson & Johnson Pharmaceutical Research & Development (Raritan, NJ, USA) in a phase III clinical trial of a long-acting injectable formulation of its paliperidone palmitate in patients with schizophrenia.

NanoCrystal® technology represents a valuable, enabling technology to evaluate new chemical entities that exhibit poor water solubility and is also a valuable tool for optimizing the performance of established drugs. NanoCrystal technology has the potential to rescue a significant number of poorly soluble chemical compounds. The drug in nanoform can be incorporated into common dosage forms, including tablets, capsules, inhalation devices, and sterile forms for injection, with the potential for substantial improvements to clinical performance. There are currently two pharmaceutical products that have been commercialized incorporating NanoCrystal technology, with several additional product launches anticipated over the near future. Advantages of this technology are as follows:

- More rapid absorption of active drug substance
- Higher dose loading with smaller dose volume
- Aqueous based with no organic solvents needed
- Capability for sterile filtering
- Longer dose retention in blood and tumors for some compounds

Nanoparticles Bound Together in Spherical Shapes

Altair Nanotechnologies Inc (Reno, NV, USA) has developed unique micron-size structures (TiNano Spheres™) made by its patented “growth-in-film”

nanotechnology. They consist of hundreds of nanoparticles bound together in spherical and near-spherical shapes and are capable of carrying active pharmaceutical ingredients (APIs), biocides, fungicides, or pesticides on either the interior or the exterior surface. The nanoparticles have a very high surface area and when coated with an API delivers a very large amount of drug to biosystem interface. This larger interface could improve solubility and/or reaction rates.

Altair's nanotechnology is used to create competent porous microstuctures consisting of high-surface-area nano primary particles to enable new applications for hard-to-dissolve drugs. A sustained release of drugs is possible by applying the drug to the inside of the TiNano Spheres™. Dual-action properties are possible by applying one drug to the inside and another to the outside of the TiNano Sphere. Altair has successfully deposited at least one of these drugs on the surface of TiNano Spheres™. Some of the many possible applications of TiNano Spheres™ are as follows:

- Drug delivery by topical applications
- Sustained release of antibiotics and fungicides
- Sustained release of drugs for cholesterol lowering
- Pain and itch preparations with sustained-release action
- Sunscreen and after-sun care

Encapsulating Water-Insoluble Drugs in Nanoparticles

Many of the most potent anticancer agents are poorly soluble in water, presenting a challenge for medicinal chemists who must develop methods of delivering these drugs in the watery environment of the human body. Nanoparticles appear to be perfectly suited to this task, and indeed, numerous research groups are developing nanoparticles specifically for delivering water-insoluble drugs to tumors.

As a means of helping researchers develop nanoparticle-based formulations of water-insoluble drugs, investigators at the University of Texas (Austin, TX) have conducted a systematic study to quantify some of the key parameters involved in forming stable nanoparticles containing maximum levels of water-insoluble drug molecules (Matteucci et al 2006). They studied how water-insoluble drugs become incorporated into nanoparticles. Using a variety of chemical, temperature, and mixing conditions, the investigators create nanoparticles in which drug molecules account for as much as 86% of the final weight of the drug–nanoparticle combination. Over the course of these experiments, the researchers found for example that slowing the formation of the nanoparticles themselves had a large, positive effect on final drug loading levels. Mixing energy also had a large effect, as did the addition of polymer stabilizers, which led to the production of smaller nanoparticles. A fundamental understanding of particle size control in antisolvent precipitation is beneficial for designing mixing systems and surfactant stabilizers for forming nanoparticles of poorly water-soluble drugs with the potential for high dissolution rates.

A team at the École Polytechnique Fédérale (Lausanne, Switzerland) has developed inverse emulsion photopolymerization—a method that uses light to create a well-defined polymeric nanoparticle with internal spaces that can provide a friendly environment to water-insoluble drugs and channels through which the entrapped drugs can escape into malignant cells (Missirlis et al 2006). The investigators created these nanoparticles from two different polymers that crosslink to each other when exposed to light from an argon laser for 1 h. They then added the nanoparticles to a solution of doxorubicin and evaporated the solvent used to dissolve the anticancer drug. Nearly half of the drug in solution became encapsulated within the nanoparticles. The researchers note that the resulting nanoparticles contain a protein-repelling surface coating that should result in favorable pharmacokinetic behavior. Experiments to test the drug-release characteristics of these nanoparticles showed that maximum release occurred at approximately 8 h and then remained close to that level for a week. The data imply that release occurs through a diffusion mechanism, that is, drug travels through channels in the nanoparticle to the nanoparticle surface, as opposed to a disintegration mechanism in which the nanoparticle falls apart and releases drug. This novel colloidal system can be used as a controlled delivery system for small hydrophobic drugs for cancer.

Trojan Nanoparticles

Trojan particles combine the drug release and delivery potential of nanoparticle systems with the ease of flow, processing, and aerosolization potential of large porous particle systems by spray-drying solutions of polymeric and nonpolymeric nanoparticles into extremely thin-walled macroscale structures (Tsapis et al 2002). These hybrid particles exhibit much better flow and aerosolization properties than the nanoparticles; yet, unlike the large porous particles, which dissolve in physiological conditions to produce molecular constituents, the hybrid particles dissolve to produce nanoparticles, with the drug release and delivery advantages associated with nanoparticle delivery systems. Formation of the large porous nanoparticle aggregates occurs via a spray-drying process that ensures the drying time of the sprayed droplet is sufficiently shorter than the characteristic time for redistribution of nanoparticles by diffusion within the drying droplet. Additional control over the physical characteristics is achieved by adding other components to the spray-dried solutions, including sugars, lipids, polymers, and proteins. The ability to produce large porous nanoparticles appears to be largely independent of molecular component type as well as the size or chemical nature of the nanoparticles.

These particles range in size from 25 nm to several hundred nanometers and can be used to deliver drugs to specific sites within the body. They are robust drug delivery systems that can be used to encapsulate drugs of varying chemistry and molecular weights.

Self-Assembling Nanoparticles for Intracellular Drug Delivery

Researchers at the University of Ulsan College of Medicine (Seoul, Korea) have developed self-assembling nanoparticles that can sense the low pH of endosomes and disintegrate, which not only releases their drug payload but enables it to exit the endosomes. CS serves as the starting material for these self-assembling nanoparticles. The investigators modify the polymer by attaching a chemical derivative of the amino acid histidine to each of the sugar units in the CS backbone. At neutral pH, histidine is hydrophobic, or poorly soluble in water. The presence of multiple histidines on the water-soluble, or hydrophilic, CS backbone creates a molecule that naturally self-assembles into a structure that surrounds the hydrophobic histidines with a protective shell of hydrophilic CS. When added to cells grown in culture, the nanoparticles fuse with the cell membrane, forming endosomes inside the cell. At the low pH found inside an endosome, histidine takes on a positive charge and also becomes hydrophilic. As a result, the physical forces that held together the self-assembling nanoparticle no longer exist and the nanoparticle falls apart. Any drug molecules entrapped within the nanoparticle is then released into the endosomes.

Particle Replication in Nonwetting Templates

Most current techniques for particle formation are incompatible with organic materials because they involved baking, etching, or processing robust metals using solvents that destroy fragile organic matter such as genes or drugs. Chemists at the University of North Carolina (Chapel Hill, NC) have developed a breakthrough method of creating the tiniest manufactured particles for delivering drugs and genetic material into the human body (Rolland et al 2005). The tiny bits are so small and they can be designed and constructed to measure <200 nm in diameter. The new method avoids harsh treatment but also allows formation of uniform particles in any shape that designers choose: spheres, rods, cones, trapezoidal solids, etc. The relatively simple process, called Particle Replication in Nonwetting Templates (PRINT), also avoids creating films or "scum layers" that would clump particles together rather than allowing them to be harvested independent of one another. PRINT affords the simple, straightforward encapsulation of a variety of important bioactive agents, including proteins, DNA, and small-molecule therapeutics, which indicates that PRINT can be used to fabricate next-generation particulate drug delivery agents. Besides drug delivery, this technology will have a profound impact on human healthcare in areas such as chemotherapy, gene therapy, and disease detection. Particles injected into the body can be designed to be biodegradable and incorporate as "cargo" any biological material that designers want to introduce into patients' bloodstreams for more efficient uptake by cells for diagnostic testing or therapy. Studies with various organic compounds have been successful and studies on mice have started.

Flash NanoPrecipitation

Flash NanoPrecipitation produces stable nanoparticles at high concentrations using amphiphilic diblock copolymers to direct self-assembly (Prudhomme et al 2006). In NanoPrecipitation, two streams of liquid are directed toward one another in a confined area. The first stream consists of an organic solvent that contains the medicines and imaging agents, as well as long-chain molecules called polymers. The second stream of liquid contains pure water. When the streams collide, the hydrophobic medicines, metal imaging agents, and polymers precipitate out of solution in an attempt to avoid the water molecules. The technique has been applied to the anticancer agent paclitaxel. The polymers immediately self-assemble onto the drug and imaging agent cluster to form a coating with the hydrophobic portion attached to the nanoparticle core and the hydrophilic portion stretching out into the water. By carefully adjusting the concentrations of the substances, as well as the mixing speed, the researchers are able to control the sizes of the nanoparticles. Uniform particles with tunable sizes from 50 to 500 nm can be prepared. The key to the process is the control of time scales for micromixing, polymer self-assembly, and particle nucleation and growth. The diffusion-limited assembly enables particles of complex composition to be formed. The stretched hydrophilic polymer layer keeps the particles from clumping together and prevents recognition by the immune system so that the particles can circulate through the bloodstream. The hydrophobic interior of the particles ensures that they are not immediately degraded by watery environments, though water molecules will, over time, break the particles apart, dispersing the medicine. Ideally, the particles would persist for 6–16 h after they are administered intravenously, which would allow enough time for the potent packages to slip into the solid tumor cells whenever they encounter them throughout the body.

Applications include controlled delivery of multiple drugs from nanoparticles as well as aerosol drug delivery. It enables the simultaneous encapsulation and controlled release of both hydrophobic and hydrophilic actives. The incorporation of gold nanoparticles and organic compounds into single nanoparticles enables simultaneous delivery and medical imaging. Finally, the ability to dry the nanoparticles by lyophilization or spray-drying and to reconstitute them without aggregation greatly enhances the applicability of the technology.

These nanoparticles can deliver medicine deep into the lungs or infiltrate cancer cells while leaving normal ones alone. Only 100–300 nm wide, the particles can be loaded with medicines or imaging agents, like gold and magnetite, that will enhance the detection capabilities of CT scans and MRIs. The nanoparticles are too large to pass through the membrane of normal cells but will pass through larger defects in the capillaries in rapidly growing solid tumors.

Particles in this size range also could improve the delivery of inhaled drugs because they are large enough to remain in the lungs, but too small to trigger the body's lung-clearing defense systems. This trait could maximize the effectiveness of inhaled, needle-free vaccination systems. It has potential applications in the development of nanoparticle-based aerosol vaccines for tuberculosis and diphtheria. Because of their potential for use on a large scale at a relatively low cost, these systems are particularly attractive for use in the developing world.

Nanoparticle Combinations for Drug Delivery

Therapeutic Protein Delivery from Nanoparticle–Protein Complexes

Scientists at the US Department of Energy's Brookhaven National Laboratory have attached gold nanoparticles to proteins to form sheets of protein–gold arrays (Hu et al 2007). The nanoparticle–protein complexes can be used to identify functional parts of proteins and to construct new protein complexes, which can be used as precision vehicles for targeted drug delivery.

Therapeutic protein delivery from nanoparticle aggregates is being developed by Uhru Inc (Texas, USA) as Nanoparticle Aggregate Technology (acquired from Access Pharmaceuticals, Dallas, TX, USA). It is based on several independent variables including nanoparticle size and chemical composition of the particle. The nanoparticle aggregate technology allows remarkable versatility in protein loading and subsequent release. Optimization of a formulation can be achieved in a relatively short time for a given protein drug. Importantly, this technology can substantially reduce the "burst release" of the protein, which occurs with other delivery systems. In preclinical animal studies the ability to control the release of the protein for periods of 3 months and greater has been established. Additional preclinical animal studies have shown that the materials used to produce the aggregates, which are included in several FDA-approved products, are biocompatible and therefore suitable for use in a drug delivery system. It represents one of the simplest delivery systems with great versatility for incorporating and delivering proteins. The ability to load the protein in a cost-effective manufacturing process without using solvents or polymerization and to tightly control the drug-release profile are potentially very significant advantages over other protein delivery technologies. Protein delivery is important as therapeutic proteins are being increasingly used to treat a wide variety of disease including cancer, infections, rheumatoid arthritis, and autoimmune diseases.

Prolonging Circulation of Nanoparticles by Attachment to RBCs

Polymeric nanoparticles are used as carriers for systemic and targeted drug delivery. They protect drugs from degradation until they reach their target and provide sustained release of drugs. However, applications of nanoparticles are limited by their short in vivo circulation lifetimes. They are quickly removed from the blood, sometimes in minutes, rendering them ineffective in delivering drugs. It is now possible to dramatically improve the in vivo circulation lifetime of polymeric nanoparticles by attaching them to the surface of RBCs, without affecting their circulation (Chambers and Mitragotri 2007). The particles remain in circulation as long as they remain attached to RBCs, theoretically up to the circulation lifetime of an RBC, which is 120 days. Particles eventually detach from RBCs due to shear forces and cell–cell interactions and are subsequently cleared in the liver and spleen.

The researchers have learned that particles adhered to RBCs can escape phagocytosis because RBCs have a knack for evading macrophages. Nanoparticles are not the first to be piggybacking on RBCs; the strategy has already been adopted by

certain bacteria, such as hemobartonella, that adhere to RBCs and can remain in circulation for several weeks. Using RBCs to extend the circulation time of the particles avoids the need to modify the surface chemistry of the entire particle, which offers the potential to attach chemicals to the exposed surface for targeting applications. The exposed surface of the particles could be used to immobilize enzymes and improve their in vivo circulation lifetime. The enzyme would have direct access to plasma in the systemic circulation. RBC-mediated prolonged circulation may also be applied to gene delivery applications in which extended circulation times are difficult to achieve. Synthetic gene delivery vectors suffer from rapid clearance by the RES, restricting transfection to the liver and lung. RBC attachment of gene vectors may provide a long-circulating depot, thereby increasing their residence time in blood. This technique could be applied for the delivery of drugs and circulating bioreactors in a wide variety of conditions such as cancer and heart disease.

Nanoparticles Plus Bacteria for Drug Delivery into Cells

Nanoparticles and bacteria have been independently used to deliver genes and proteins into mammalian cells for monitoring or altering gene expression and protein production. Harmless strains of bacteria could be used as vehicles, harnessing bacteria's natural ability to penetrate cells and their nuclei. Researchers at Purdue University's Birck Nanotechnology Center have demonstrated the simultaneous use of nanoparticles and bacteria to deliver nucleic acid-based model drug molecules into cells in mice (Akin et al 2007). In this approach, the gene or cargo is loaded onto the nanoparticles, ranging in size from 40 to 200 nm, which are attached to the bacteria with linker molecules. The bacteria successfully deliver the molecules, and the genes are released from the nanoparticles and expressed in cells. When the cargo-carrying bacteria attach to the recipient cell, they are engulfed by its outer membrane, forming "vesicles," or tiny spheres that are drawn into the cell's interior. Once inside the cell, the bacteria dissolve the vesicle membrane and release the cargo as shown in Fig. 4.1.

This technique may be used to deliver different types of cargo into a variety of cells and live animals for gene therapy without the need for complicated genetic manipulations. This delivery system is also more efficient than techniques using viruses as they usually incorporate only one copy of a gene cargo to virus particle. In this approach, bacteria can carry hundreds of nanoparticles, each of which can in turn carry hundreds of drug molecules, depending on the size of the nanoparticles. Released cargo can be designed to be transported to different locations in the cells to carry out disease detection and treatment simultaneously. The method might be used to take images of diseased tissues by inserting a cargo of fluorescent molecules into tumors that are ordinarily too small to be detected. It could enable insertion of relatively large structures, such as biosensors into the interiors of cells for the early detection of cancer and other diseases and to monitor the progress of disease as well

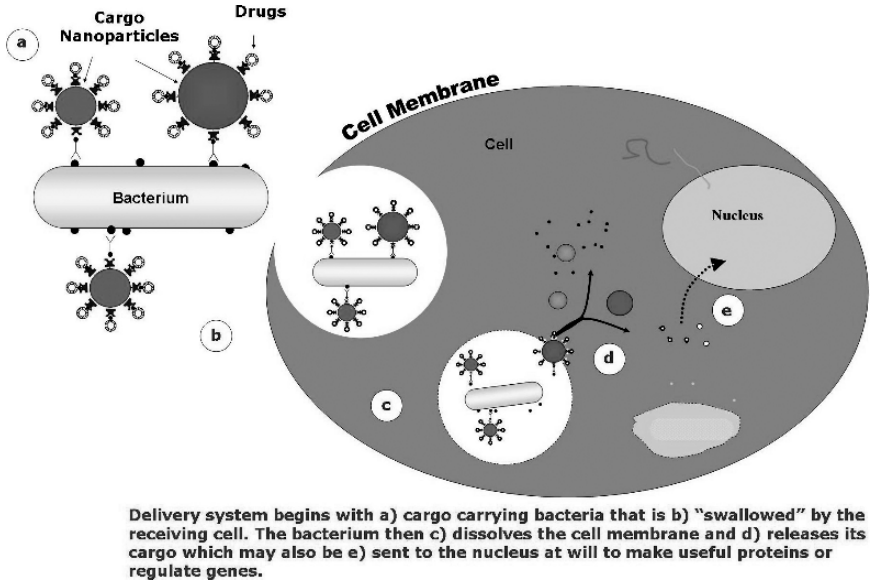

Fig. 4.1 Bacteria plus nanoparticles for drug delivery into cells
Source: Akin et al 2007.

as response to drug therapy. The CNTs could be delivered into diseased cells and then exposed to light, causing them to heat up and selectively kill only the diseased cells.

Liposomes

Liposome properties vary substantially with lipid composition, size, surface charge, and the method of preparation. They are therefore divided into three classes based on their size and number of bilayers.

1. Small unilamellar vesicles are surrounded by a single lipid layer and measure 25–50 nm in diameter.
2. Large unilamellar vesicles are a heterogeneous group of vesicles similar to and are surrounded by a single lipid layer.
3. Multilamellar vesicles consist of several lipid layers separated from each other by a layer of aqueous solution.

Lipid bilayers of liposomes are similar in structure to those found in living cell membranes and can carry lipophilic substances such as drugs within these layers in the same way as cell membranes. The pharmaceutical properties of the liposomes depend on the composition of the lipid bilayer and its permeability and fluidity. Cholesterol, an important constituent of many cell membranes, is frequently included in liposome formulations because it reduces the permeability and increases

the stability of the phospholipid bilayers. Until recently, the use of liposomes as therapeutic vectors was hampered by their toxicity and the lack of knowledge about their biochemical behavior. The simplest use of liposomes is as vehicles for drugs and antibodies targeted for the targeted delivery of anticancer agents. The use of liposomes may be limited because of problems related to stability, the inability to deliver to the right site, and the inability to release the drug when it gets to the right site. However, liposome surfaces can be readily modified by attaching PEG units to the bilayer (producing what is known as stealth liposomes) to enhance their circulation time in the bloodstream. Furthermore, liposomes can be conjugated to antibodies or ligands to enhance target-specific drug therapy.

Liposomes Incorporating Fullerenes

C60 has created "buckysomes," a new generation of liposomes that incorporate fullerenes to deliver drugs that are not water-soluble, that tend to have large molecules, and that are very hard to get into the body. Buckysomes appear to have much more flexibility in incorporating a wider range of drugs, as well as large-molecule drugs, and delivering and releasing them more effectively. The company is studying these to deliver cancer and anesthesia therapeutics.

Polymerized Liposomal Nanoparticle

Polymerized liposomal nanoparticle (PLN) of NanoMed Technologies LLC (Columbus, OH, USA) is a nonviral nanoparticle technology incorporating a customizable drug delivery system for chemotherapeutic applications. The nanoparticles created using the self-assembling ability of a unique class of diacetylenic lipids that can be polymerized into stable, bimolecular membrane structures are capable of delivering a drug payload. The PLN technology lends itself especially well to the display of multiple functionalities. These particles are composed of individual lipid monomers, part of which are functionalized for the purpose of targeting, and may include additional moieties to control other physical properties such as surface charge, polarity, and fluidity. Different functionalized lipids can be rapidly mixed and matched in an infinite number of combinations and relative concentrations to create tailor-made particles with desirable targeting and circulation properties. The nanoparticles are nonimmunogenic, display no acute toxicity, and can be highly concentrated. Intracellular degradation and excretion rates of the particles can be modulated by controlling the degree of polymerization.

Stabilization of Phospholipid Liposomes Using Nanoparticles

An innovative strategy of mixing lipids and nanoparticles to produce new drug and agricultural materials and delivery vehicles has been developed by researchers at the University of Illinois (Urbana-Champaign, IL). The simple strategy of mixing

phospholipid liposomes with charged nanoparticles and using sonication to mix them at low volume fraction produces particle-stabilized liposomes that repel one another and do not fuse (Zhang and Granick 2006). Subsequently, the volume fraction can be raised as high as 50%, reversibly, still without fusion. The nanoparticles adhere to the capsules and prevent further growth, freezing them at the desired size. The lipid concentration can then be increased without limits. As proof of concept, fluorescent dyes were encapsulated within lipid capsules. No leakage occurred, and the lipids proved stable against further fusion. Although these particle-stabilized liposomes were stable against fusion, 75% of the outer liposome surface remained unoccupied.

This opens the door to using particle-stabilized liposomes in various applications. The biocompatible containers could carry cargo such as enzymes, DNA, proteins, and drug molecules throughout living organisms. They could also serve as surrogate factories where enzyme-catalyzed reactions are performed. By attaching biomolecules to the capsule's surface, novel colloidal-size sensors could be produced. An additional use for stabilized lipid capsules is the study of behavior of a drug contained in this nano environment.

Applications of Lipid Nanoparticles

The nanoparticle technology has broad therapeutic and diagnostic applications. The multivalent presentation of ligands or antibodies on nanoparticles makes this new class of drug ideally suited to treat diseases, which involve proliferation of blood vessels such as cancer, atherosclerosis, apoptosis, inflammation, rheumatoid arthritis, macular degeneration, unstable plaque, stroke, heart disease, and psoriasis.

When nanoparticles are used in the treatment of cancer, their powerful targeting ability and potential for large cytotoxic payload dramatically enhance the efficacy of conventional pharmaceuticals as well as novel therapeutic approaches, such as gene therapy, radioimmunotherapy, and PDT. Integrin-targeted nanoparticles can be used for site-specific delivery of a therapeutic payload by using an anticancer gene (Hood et al 2002). These targeted nanoparticles can deliver radionuclides and chemotherapeutics to tumors. Further applications are discussed under drug delivery for cancer.

Lipid Nanocapsules

Due to their small size, lipid nanocapsules (LNCs) might be promising as an injectable as well as for an oral drug delivery system. LNCs provides sufficient drug solubility to avoid embolization during intravenous injection and facilitates drug absorption after oral administration. Biocompatible ibuprofen LNC was developed in a size range of $\sim$50 nm with a new preparation method (Lamprecht et al 2004). Drug incorporation into LNC was successful to a high degree in all formulations tested. The pain relief after intravenous administration was prolonged by at least 2 h when administering LNC formulation. A drug delivery system for intravenous

administration of ibuprofen has been developed, which exhibits sustained release properties by either oral or intravenous route and could be useful in the treatment of postoperative pain.

Lipid Emulsions with Nanoparticles

Scientists at Nippon Shinyaku Co. (Kyoto, Japan) have developed an artificial lipoprotein-like particle, lipid nanosphere (LNS), incorporating dexamethasone palmitate (Seki et al 2004). LNS is 25–50 nm in diameter and is composed of soybean oil and egg lecithin. Because of the lower uptake of LNS particles by the liver, LNS showed good recovery from the liver and prolonged the plasma half-life of dexamethasone palmitate after intravenous injection. In addition, higher efficiency in the targeting of dexamethasone palmitate into inflammation sites and higher anti-inflammatory efficacy were observed in LNS. Thus, LNSs easily and selectively passed through the leaky capillary wall by passive diffusion depending on the plasma concentration. Nanometer-sized lipid emulsion particles, LNS, seem to be a promising carrier system for passive drug targeting of lipophilic drugs.

LNS has been studied as a low-dose therapeutic system with for amphotericin B (AmB), a potent antifungal drug. LNS, a small-particle lipid emulsion, is taken up by the liver to a lesser extent than was a conventional lipid emulsion. As a result, LNS yields higher plasma concentrations of a radiochemical tracer than does the conventional lipid emulsion. In one study, LNS incorporating AmB (LNS-AmB) was prepared and was found to be a homogeneous emulsion with mean particle diameters ranging from 25 to 50 nm (Fukui et al 2003). LNS-AmB yielded higher plasma concentrations of AmB than did Fungizone, a conventional intravenous dosage form of AmB, after intravenous administration to mice, rats, dogs, and monkeys. This difference between LNS-AmB and Fungizone was also observed for constant intravenous infusion. In contrast to Fungizone, LNS-AmB showed a linear relationship between dose and AUC. These pharmacokinetic characteristics of LNS-AmB make it a suitable candidate for an effective low-dose therapeutic system for AmB.

Nanolipispheres (Eurand, Milan, Italy) are colloidal systems of drugs in a solid lipid matrix. These systems possess a submicron mean diameter and a uniform size distribution. The patented microemulsion–solidification process for manufacture uses low-melting, waxy, or composite materials to produce a suspension of solid nanoparticles. This suspension is then dried to obtain physically stable Nanolipispheres in a powder form. Nanolipispheres is an enabling technology that provides for

- carrier incorporation of lipophilic and hydrophilic drugs;
- oral delivery of macromolecules that can be absorbed as a whole or as fragments through the gastrointestinal tract;
- therapeutic efficacy of some drugs by preferential and consistent absorption and metabolism through the lymphatic system; and
- modified drug release.

Liposome–Nanoparticle Hybrids

Small iron nanoparticles, QDs, liposomes, silica, and polystyrene nanoparticles have been incorporated into liposomes for a variety of applications. Different techniques to achieve encapsulation of solid or semisolid nanoparticles within liposomes have been described. These offer improvements in nanoparticle aqueous solubilization and offer a viable platform (the liposome surface) for further bioconjugation. Moreover, these hybrids have increased survival time in blood circulation following systemic administration and accumulate at sites of leaky vasculature such as in tumors or inflammatory lesions, providing opportunities for a combination of diagnostic imaging and therapeutics (Al-Jamal and Kostarelos 2007).

Nanospheres

Using high-intensity ultrasound, researchers at the University of Illinois have created hollow ceramic nanospheres (50–500 nm) and the first hollow nanocrystals, which could be used in drug delivery (Dhas and Suslick 2005). A hollow nanocrystal of molybdenum oxide is prepared using high-intensity ultrasound to form a layer of amorphous material around a silica nanosphere. The nanosphere is then dissolved away with hydrofluoric acid, and upon heating, the shell crystallizes into a single hollow nanocrystal. TEM studies on the hollow ceramic materials indicate the formation of dispersed free spheres with a hollow core.

Nanosphere Protein Cages

Protein cages (SpeciGen Inc, Palo Alto, CA, USA) are being developed for targeted drug delivery with controlled release. Similar to viruses, the protein cages seek to exploit three interfaces: the exterior, the interior, and the interface between subunits that comprise the containers. The size of the viruses, which range from 20 to 50 nm offers a library of platforms for a variety of applications. This device can attach and selectively release the chemotherapeutic doxorubicin (Flenniken et al 2005). It has potential applications in gene therapy also.

Nanovesicle Technology for Delivery of Peptides

Biodegradable nanovesicles, or nanocapsules, have been synthesized and demonstrated to encapsulate and deliver insulin, as a model peptide, through the intestinal mucosa, delivering the agent systemically to test animals and successfully reducing glucose levels. The proprietary Nanocaplet spheroid (Benteley Pharmaceuticals, Exeter, NH, USA) has a diameter of 100 nm. The small size enables uptake via

biochemical pathways unavailable to larger diameter particles. Nanocaplets have been successfully observed and measured utilizing AFM and electron beam microscopic techniques. Advantages of this approach are as follows:

- Nanovesicles are hollow, with sizes that are controllable in the nanoscale range, even below 100 nm.
- The building blocks are composed of biodegradable segments.
- The payloads are protected and leakage is insignificant as is the case with liposomes.
- The technology can be used for a wide range of macromolecules, including nucleotides and proteins.

The nanocapsules have also been successfully tagged by simple chemistry, indicating that ligands targeted to specific areas can be affixed to the outside of the nanocapsules and delivered parenterally. Theoretically, this may enable the blood–brain (BBB) to be crossed and carry large molecules such as antisense RNA or other therapeutics to specific cells of the brain. Nanocaplets may be prepared sterile and/or freeze-dried and appear to be cost-effective to manufacture.

Nanotubes

Micro- and nanotubes or structures that resemble tiny drinking straws are alternatives that might offer advantages over spherical nanoparticles for some applications. When a PEG–silane is attached to the silica nanotubes, adsorption of IgG is strongly suppressed relative to nanotubes that do not contain the attached PEG (Martin and Kohli 2003). This has potential usefulness for the delivery of biopharmaceuticals. A payload can be incorporated into the nanotubes by either covalent bonding or other chemical interactions between the payload and the inside walls of the nanotubes. For some applications, it might be useful to fill the nanotubes with the payload and then to apply caps to the nanotubes to keep the payload encapsulated. The uncapping and release of payload can be triggered by a biochemical signal.

Researchers from the University of Louisville and Rensselaer Polytechnic Institute have come up with a useful twist on CNTs (Mani et al 2003). Their nanopipettes grow thicker at one end to form microscopic cones that have central channels. CNTs are ready-made, strong, electrically useful microscopic tubes that form naturally in soot from sheets of carbon atoms. Nanopipettes could eventually deliver tiny amounts of fluids under the skin, sense chemicals at very specific locations, form electrodes for retinal stimulation, and be tips for atomic force, scanning tunneling, and near-field scanning optical microscopes.

The researchers found that when they immersed platinum wires in methane /hydrogen plasma, sheets of carbon atoms wound around a central nanotube to make nanotubes whose outer walls tapered from as many as 700 nm to only a few nanometers. The nanopipettes were as long as 6,000 nm. The researchers are now working

on controlling the length of the pipettes and on growing them on flat substrates with an eye toward growing long, dense arrays of pipettes that could be used for drug delivery.

Tubular structure of nanoparticles is highly attractive due to their structural attributes, such as the distinctive inner and outer surfaces, over conventional spherical nanoparticles. Inner voids can be used for capturing, concentrating, and releasing species ranging in size from large proteins to small molecules. Distinctive outer surfaces can be differentially functionalized with environment-friendly and/or probe molecules to a specific target. By combining the attractive tubular structure with magnetic property, the magnetic nanotube can be an ideal candidate for the multifunctional nanomaterial toward biomedical applications, such as targeting drug delivery with MRI capability. Magnetic silica–iron oxide composite nanotubes have been successfully synthesized and shown to be useful for magnetic field-assisted chemical and biochemical separations, immunobinding, and drug delivery (Son et al 2005).

Lipid–protein Nanotubes for Drug Delivery

Bionanotubes, with open or closed ends, can be developed for drug or gene delivery applications (Raviv et al 2005). The nanotubes could be designed to encapsulate and then open up to deliver a drug or gene in a particular location in the body. By manipulating the electrical charges of lipid bilayer membranes and microtubules from cells, scientists can create open or closed bionanotubes, or nanoscale capsules. The self-assembly of cationic liposome–microtubule (MT) complexes was studied, using synchrotron x-ray scattering and electron microscopy. Vesicles were found to either adsorb onto MTs, forming a "beads on a rod" structure, or undergo a wetting transition and coating the MT. Tubulin oligomers then coat the external lipid layer, forming a tunable lipid–protein nanotube. The beads on a rod structure is a kinetically trapped state. The energy barrier between the states depends on the membrane bending rigidity and charge density. By controlling the cationic lipid/tubulin stoichiometry, it is possible to switch between two states of nanotubes with either open ends or closed ends with lipid caps, a process that forms the basis for controlled chemical and drug encapsulation and release. The inner space of the nanotube in these experiments measures $\sim$16 nm in diameter. The whole capsule is $\sim$40 nm in diameter. Taxol is one type of drug that could be delivered with these nanotubes.

Single Wall Carbon Nanotubes for Drug Delivery

Various proteins adsorb spontaneously on the sidewalls of SWCNTs, enabling protein–nanotube conjugates. Proteins can be readily transported inside various mammalian cells via the endocytosis pathway with nanotubes acting as the transporter. CNTs thus represent a new class of molecular transporters potentially useful

for future in vitro and in vivo protein delivery applications (Kam and Dai 2005). CNTs, used as liquid-filled nanoparticles, act as absorption enhancers and improve the bioavailability of erythropoietin to 11.5% following administration into the small intestinal in experimental animals (Venkatesan et al 2005).

CNTs can pierce cell membranes like tiny needles without damaging the cell. If proteins or nucleic acids are attached to the nanotubes, they can also go right through the cell membrane. Nanotubes can also carry small pharmaceutical molecules such as antibiotics or cancer drugs directly into cells and have been successfully used to inject antifungal agents into cells (Wu et al 2005). It is also possible to attach two agents to nanotubes enabling combination therapies or to trace the uptake of a drug by adding a marker.

Halloysite Nanotubes for Drug Delivery

Halloysite is a natural clay material typically used in ceramics. Some clay reserves contain halloysite in the form of naturally occurring nanotubes that are approximately 10–100 nm in internal diameter and vary in length from a few hundred nanometers to several micrometers. Biophan Technologies Inc (West Henrietta, NY, USA) has patented technologies for combining halloysite nanotubes with existing pharmaceuticals for expanded drug delivery options. Halloysite nanotubes can be loaded with drugs for sustained release, extending the effective life of drugs as they migrate out of the tubes over time. Once loaded, these tubes can also be encapsulated to further influence the rate of elution. This enables alteration of the drug-release profile and extends the effectiveness of drugs without increasing potency. Compared with CNTs, halloysite nanotubes are far less expensive and have an extraordinarily large surface area. This feature promises significant advantages for drug delivery applications, since surface area contact allows for greater control of drug loading and elution profiles.

Loaded nanotubes can also be combined with other technologies for noninvasive activation. Biophan's intellectual property includes coating the nanotubes with nanomagnetic material that can subsequently be heated selectively and noninvasively using specific electromagnetic energy. Heating can thus provide elution on demand. The benefits of using naturally occurring halloysite material for specific drug delivery applications are longer delivery times, more control of the drug-release profile, and improved safety profiles. This technology can be applied to several product platforms, including transdermal drug delivery and drug-loaded wound care products. Transdermal delivery with halloysite nanotubes can enable a more controlled elution profile with several potential benefits:

- Eliminates the high initial delivery rate and improves the safety profile, particularly with drugs such as stimulants or hormones.
- More uniform delivery can result in better maintenance of the effective clinical dose.

- Less drug loading is required per patch. Since much of the drug is discarded when the patch is removed, this can lead to reduced costs.

Wound care products range from simple bandages to long-term treatments to promote healing and reduce the chances of infection and scarring. Biophan's halloysite drug delivery system is designed to offer superior clinical benefits over current wound care systems, especially in the area of burn care. Drugs loaded into halloysite tubes and embedded into the base layer of a bandage can be released over an extended time period. This increases the duration of drug effectiveness and reduces the frequency with which a bandage needs to be changed. This novel delivery form can provide new dosage formulations with several advantages:

- Linear release ensures maintenance of clinically effective doses
- Compliance and ease of use—longer elution times mean fewer bandage changes
- Uniformity of drug delivery—elution from halloysite

Nanocochleates

Cochleate, a lipid-based delivery system, is formed as a result of interaction between cations, e.g., Ca^{2+} and negatively charged phospholipids such as phosphatidylserine. Cochleates are stable precipitates with a unique structure consisting of a large, continuous, solid, lipid bilayer sheet rolled up in spiral, with no internal aqueous space. They are nontoxic and noninflammatory and have been used as vehicles for oral and parenteral delivery of protein and peptide antigens (Delmarre et al 2004).

Smart Pharmaceuticals™ (BioDelivery Sciences International, Raleigh, NC, USA) are based on Bioral™ Technology, which uses nanocochleates and allows biopharmaceutical manufacturers to offer biologically active compounds with unparalleled convenience, shelf-life, and reduced side effects. This drug delivery technology has been applied to generic, off-patent injectable drugs to make them patent-protected oral drugs. The company has developed food processing technology to encochleate sensitive and easily degraded nutrients (β-carotene, antioxidants, etc.) for addition to processed food and beverages. Nanoencochleation's all–natural process encochleates and preserves essential nutrients like antioxidants into a protected "shell" for in high-temperature/ pressure canning and bottling applications.

Bioral® technology was used to encapsulate and deliver an siRNA therapeutic in a mouse model of influenza. The siRNA targets critical gene segments shared by avian influenza (H5N1). A single intranasal dose of encochleated siRNA administered 4 h after influenza exposure reduced virus titers in the lung by 200 times. It was 25 times more effective in reducing the virus than intravenous delivery as siRNA is destroyed rapidly when introduced into the blood directly.

Nanobiotechnology-Based Transdermal Drug Delivery

Transdermal drug delivery is described in a special report on this topic (Jain 2007c). Nanoparticles and nanoemulsions have better skin penetration than larger particles. Nanotherapeutics (Alachua, FL) is already using nanoparticle technology to prepare cosmetic skin creams for prevention of wrinkles.

Controlled release delivery systems such as solid lipid nanoparticle (SLN) and microemulsion have been developed. Scientists in China have described the preparation and some characterization of specialized delivery systems for triptolide, an anti-inflammatory and immunosuppressive agent (Mei et al 2003). SLN dispersions and microemulsions were shown to be efficient promoters for the penetration of triptolide into the skin.

Indomethacin-loaded poly *n*-butylcyanoacrylate nanocapsules can improve the transdermal delivery of indomethacin compared with a conventional gel formulation using Pluronic F-127 (Miyazaki et al 2003). This might be due to their ultrafine particle size and their hydrophilic and hydrophobic surface characteristics.

There is experimental evidence for the potential of nanoparticles as delivery vectors for antigens and DNA for the purpose of transdermal vaccination protocols. Fluorescent particles ranging in size and charge were applied to the surface of full-thickness pig skin in a diffusion chamber and the receptor fluid was assayed to determine permeation (Kohli and Alpar 2004). Fluorescence microscopy was used to visualize the skin after experiments. The results showed that only 50 and 500 nm particles that were negatively charged were able to permeate the skin, indicating that negative particles with sufficient charge may be ideal carriers for this purpose.

Delivering genes and drugs within cells with devices approaching the nanoscale allows for new levels of precision and minimal damage to cells. The "nanopatches" being researched at the University of Queensland, Australia, target immunologically sensitive cells and could be used in the treatment of malaria and allergies. This technology has the potential to be applicable to a range of diseases. Initially, the focus will be on diseases benefiting from immunotherapeutics, some of which do not currently have adequate treatment. Examples include DNA vaccination against malaria and HIV. It will also enable pain-free and needle-free immunotherapy of asthma.

Delivery of Nanostructured Drugs from Transdermal Patches

Nanotechnology Victoria Ltd (Clayton, Victoria, Australia) is applying nanotechnology to the painless transdermal delivery of vaccines, peptide hormones, and other drugs. The patches are structured on the skin side with microprotrusions, which hold the drugs to be delivered. The protrusion face of the patch is applied to the skin where they cross the outer surface layer of the skin only reaching as far as

the interstitial space avoiding nerves and blood vessels. In this interstitial space, the nanostructured drugs are released from the surface of the protrusions, and as the biocompatible polymer biodegrades the drugs are released continuously from the body of the protrusions. The nanostructured drugs are taken up by the cells of the immune system (for vaccination applications) or they flow through the interstitial fluid to other compartments in the body.

NanoCyte Transdermal Drug Delivery System

NanoCyte drug delivery system (NanoCyte Inc, Zemach, Jordan Valley, Israel) is based on a sophisticated injection system developed by the sea anemone during million years of evolution. The NanoCyte natural substance is extracted from aquatic invertebrates. Each microcapsule contains a coiled microscopic nanotube, which unfolds on activation—a process whereby high pressure of 200 atm is developed within the microcapsule. The long thin nanotube evaginates out of the microcapsule and penetrates the skin at an acceleration of 40,000 *g* to deliver the drug efficiently in a fraction of a second into the epidermis skin layer. NanoCyte can be formulated as a suspension, lotion, creme, or a stick. NanoCyte can also be activated after attaching to an adhesive patch. Advantages of this system are follows:

- Immediate intradermal delivery
- Deviceless active delivery
- Painless administration
- Avoidance of large dosages and side effects
- Treatment of large skin areas
- Multipoint penetration using nanoinjectors
- Ease of use

Ethosomes for Transdermal Drug Delivery

Ethosomes—soft, malleable vesicles with size ranging from 30 nm to a few microns—form the basis of Ethosome Delivery System (Novel Therapeutic Technologies). Ethosomal systems were found to be significantly superior at delivering drugs through the skin in terms of both quantity and depth when compared with liposomes and many commercial transdermal and dermal delivery systems. Visualization by dynamic light scattering showed that ethosomes could be unilamellar or multilamellar through to the core. These novel delivery systems contain soft phospholipid vesicles in the presence of high concentrations of ethanol. Ethosomal systems are sophisticated conceptually but characterized by simplicity in their preparation, safety, and efficiency—a rare combination that can expand their applications.

Because of their unique structure, ethosomes are able to encapsulate and deliver through the skin highly lipophilic molecules such as cannabinoids, testosterone,

and minoxidil, as well as cationic drugs such as propranolol and trihexyphenidil. Results obtained in a double-blind two-armed randomized clinical study showed that treatment with the ethosomal acyclovir formulation significantly improved all the evaluated parameters (Godin and Touitou 2003).

Ethosomes penetrate cellular membrane, releasing the entrapped molecule within cells. Studies focused on skin permeation behavior of fluorescently labeled bacitracin from ethosomal systems through human cadaver and rat skin demonstrated that the antibiotic peptide was delivered into deep skin layers through intercorneocyte lipid domain of stratum corneum (Godin and Touitou 2004). Ethosomal delivery systems could be considered for the treatment of a number of dermal infections, requiring intracellular delivery of antibiotics, whereby the drug must bypass two barriers: the stratum corneum and the cell membrane. Ethosomal formulation of testosterone could enhance testosterone systemic absorption and also be used for designing new products that could solve the weaknesses of the current testosterone replacement therapies (Ainbinder and Touitou 2005).

Advantages of ethosomes over other transdermal delivery systems are as follows:

- Enhanced permeation
- Platform for the delivery of large and diverse group of drugs including peptides and very lipophilic molecules
- Safe and approved components
- Passive, noninvasive delivery system
- Available for immediate commercialization
- High patient compliance
- High cost-to-benefit ratio

Nanoparticle-Based Drug Delivery to the Inner Ear

Drug delivery to the inner ear is important for the treatment of inner ear disorders such as those involving hearing. Another disorder, tinnitus, is a problem in management and several innovative approaches are under investigation. NeuroSystec Inc (Valencia, CA) has devised a therapy that inhibits spontaneous firing of the spiral ganglion, which is thought to be the source of the erroneous perception of sound arriving at the auditory cortex. The company has licensed an osmotic pump from Durect Corporation (Cupertino, CA, USA) and plans to target the *N*-methyl-D-aspartate (NMDA) receptor with nanoliter delivery of an NMDA antagonist.

An obstacle to effective treatment of inner ear diseases is the atraumatic delivery of therapeutics into inner ear perilymph. It is feasible to use superparamagnetic nanoparticles as drug delivery vehicles. Biostable magnetically responsive nanodevices (NanoBioMagnetics Inc, Edmond, OK, USA) have been investigated as components of implantable hearing devices.

Pulmonary Drug Delivery by Nanoparticles

There has been growing interest in the potential for the systematic delivery of drugs and therapeutic agents (e.g., peptides and proteins) via pulmonary (inhalation) means. Research and development of pulmonary drug delivery by surface acoustic wave (SAW) technology is being conducted at the Micro/Nanophysics Research Laboratory of Monash University (Victoria, Australia). The SAW technique enables well-controlled generation of fine particles and is ideal for this pulmonary drug delivery, particularly for a number of drugs that require frequent dosing. The major focus of this project will be on the development of a device producing insulin nanoparticles delivered across the pulmonary alveoli. There is already evidence for increased efficacy of inhaled insulin compared with that of injected insulin, due to faster uptake and clearance. An economically viable microdevice for portable pulmonary drug delivery for human use based on SAW technique has significant commercial potential. Another advantage of using noninvasive techniques as alternatives to frequent injections would be the profound impact on a child's willingness to comply with diabetic treatment.

Nanotechnology Victoria has started funding of this 3-year project, which commenced in January 2007 and will conclude in December 2010. It is expected that an initial prototype of this unit will be available within the first year of the project, with subsequent improvement, validation, and commercialization to be completed within the following 2 years.

Nasal Drug Delivery Using Nanoparticles

The nasal cavity is an ideal site for delivery of both locally and systemically acting drugs. Topical administration includes agents for the treatment of nasal congestion, rhinitis, sinusitis, and related allergic and other chronic conditions. Various medications include corticosteroids, antihistaminics, anticholinergics, and vasoconstrictors. The focus in recent years has been on the use of nasal route for systemic drug delivery. Intranasal route is considered for drugs that are ineffective orally, are used chronically, and require small doses, and is used where rapid entry into the circulation is desired. The rate of diffusion of the compounds through the nasal mucous membranes, like other biological membranes, is influenced by the physicochemical properties of the compound. Impressive improvements in bioavailability have been achieved with a range of compounds.

CS, a naturally occurring polysaccharide derived from chitin, is used as an absorption enhancer for transnasal drug delivery. CS is bioadhesive and binds to the mucosal membrane, prolonging retention time of the formulation on the nasal mucosa. It may also facilitate absorption through promoting paracellular transport. The CS nasal technology can be exploited as solution, dry powders, or nanoparticle formulations to further optimize the delivery system for individual compounds. For compounds requiring rapid onset of action, the nasal CS technology can provide a

fast peak concentration compared with oral or subcutaneous administration. Density and the size of PEG coating of poly(lactic acid)-poly(ethylene glycol) (PLA-PEG) nano- and microparticles has an important effect on their transport across the nasal mucosa. PLA-PEG particles with a high PEG coating density and a small size are more significantly transported than noncoated PLA nanoparticles or PLA-PEG nanoparticles with a lower coating density (Vila et al 2004).

Consort Medical plc (Buckinghamshire, UK) has licensed technology from the MIT for the nasal delivery of nanoparticles of encapsulated drugs. This formulation technology offers systemic delivery using the nasal mucous membrane, with the potential benefits of sustained release once the nanoparticles are in the bloodstream.

Nanoparticles made of low molecular weight CS are promising carriers for nasal vaccine delivery. Compacted DNA nanoparticles, encoding CF transmembrane regulator gene, can be safely administered by perfusion to the nose of CF subjects (Konstan et al 2004). A double-blind, dose escalation gene therapy trial with this technique showed evidence of vector gene transfer and partial correction of nasal potential difference that is typical of subjects with classic CF.

Mucosal Drug Delivery with Nanoparticles

The layers of mucus that protect sensitive tissue throughout can also prevent the entry of drugs into the body. The role of nanoparticles as drug delivery vehicles has been explored to overcome this hurdle. Cervicovaginal mucus was used for these investigations because its viscoelastic properties and mucin concentration are similar to those in many other human mucous secretions. Large nanoparticles, 500 and 200 nm in diameter, if coated with PEG, diffused through mucus with an effective diffusion coefficient (D_{eff}) only 4- and 6-fold lower than that for the same particles in water (Lai et al 2007). In contrast, for smaller but otherwise identical 100-nm coated particles, D_{eff} was 200-fold lower in mucus than in water. For uncoated particles 100–500 nm in diameter, D_{eff} was 2,400- to 40,000-fold lower in mucus than in water. Much larger fractions of the 100-nm particles were immobilized or otherwise hindered by mucus than the large 200- to 500-nm particles. Thus, in contrast to the prevailing belief, these results demonstrate that large nanoparticles, if properly coated, can rapidly penetrate physiological human mucus, and they offer the prospect that large nanoparticles can be used for mucosal drug delivery.

Future Prospects of Nanotechnology-Based Drug Delivery

A desirable situation in drug delivery is to have smart drug delivery systems that can integrate with the human body. This is an area where nanotechnology will play an extremely important role. Even time-release tablets, which have a relatively simple coating that dissolves in specific locations, involve the use of nanoparticles. Pharmaceutical companies are already involved in using nanotechnology to create

intelligent drug-release devices. For example, control of the interface between the drug/particle and the human body can be programmed so that when the drug reaches its target, it can then become active. The use of nanotechnology for drug-release devices requires autonomous device operation. For example, in contrast to converting a biochemical signal into a mechanical signal and being able to control and communicate with the device, autonomous device operation would require biochemical recognition to generate forces to stimulate various valves and channels in the drug delivery systems, so that it does not require any external control.

It is now appears that we are on the verge of bioengineering molecular motors for specialized applications on nanoscale. These systems might be the key to yet unsolved biomedical applications that include nonviral gene therapy and interneuron drug delivery (Cohen et al 2005). Examples of some potential nanotechnology-based drug delivery systems are discussed below:

Nanomolecular Valves for Controlled Drug Release

A macroscopic valve is a device with a movable control element that regulates the flow of gases or liquids by blocking and opening passageways. Construction of such a device on the nanoscale level requires (i) suitably proportioned movable control elements, (ii) a method for operating them on demand, and (iii) appropriately sized passageways. These three conditions can be fulfilled by attaching organic, mechanically interlocked, linear motor molecules that can be operated under chemical, electrical, or optical stimuli to stable inorganic porous frameworks (i.e., by self-assembling organic machinery on top of an inorganic chassis). A reversibly operating nanovalve has been demonstrated that can be turned on and off by redox chemistry (Nguyen et al 2005). It traps and releases molecules from a maze of nanoscopic passageways in silica by controlling the operation of redox-activated bistable rotaxane molecules tethered to the openings of nanopores leading out of a nanoscale reservoir. Future applications could include nanofluidic systems and the controlled release of drugs from implants with nanoscopic properties.

Nanomotors for Drug Delivery

Basics of nanomotors—nanometer-scale machines, which are powered by chemical reactions—have been described in Chapter 3. Researchers from the University of Georgia (Atlanta, GA) have demonstrated a new way to create catalytic nanomotors using dynamic shadowing growth (He et al 2007). The technique involves a simple modification of existing methods that allows for greater flexibility in designing desired nanomotor structures. These could be used as tools to open constricted or clogged blood vessels too small for conventional stents, or they could deliver drugs by drilling through the cell wall of an organism. The researchers looked at the hundreds of moving parts in an automobile for designing each part of a nanomotor so

as to achieve a controlled, flexible range of motion for the parts to work together. After successfully using the new technique to design nanorods to rotate, scientists broke the symmetry of the rods to form L-shaped rods that could then be aggregated to form larger particles. Then they transformed the rod into a spiral shape so that its rotation would mimic the turning of a drill. The team used the new technique to deposit a platinum or silver catalyst on different portions of the L-shaped rods, and then designed different experiments to test their ability to control the motion. In a solution of hydrogen peroxide, they captured images of the nanorods turning precisely in the directions proscribed by the catalyst depositions.

Chapter 5
Role of Nanotechnology in Biological Therapies

Introduction

Biological therapies mean the application of molecular biology in therapeutics. Broadly speaking, it includes vaccines, cell therapy, gene therapy, antisense therapy, and RNA interference (RNAi). Some of these involve the use of nucleic acids and proteins, whereas others involve genetic manipulation. Biological therapies, particularly their delivery, can be refined by use of nanobiotechnology.

Vaccination

DNA vaccines, also referred to as genetic vaccines, are generating significant preclinical and clinical interest. It has been proven that the expression of an antigen or antigens from plasmid DNA (pDNA) may elicit both humoral and cellular immune responses. Therefore, DNA vaccines may have potential as new vaccines for important diseases such as HIV, hepatitis C, tuberculosis, and malaria. However, DNA vaccine delivery is not satisfactory. Relatively high doses of pDNA are needed to elicit a response. The clinical results using "naked" pDNA have been disappointing in the breadth and depth of the immune response. Clinical trials with the gene gun have been promising, but it is unclear whether this technology will be commercially viable. Therefore, there is a need for new vaccine delivery systems that can be administered at low doses to elicit strong humoral and cellular immune responses. Use of nanobiotechnology has been explored for this purpose.

Nanobiotechnology for Vaccine Delivery

Nanoparticles for DNA Vaccines

Microparticles and nanoparticles are promising as delivery systems for DNA vaccines (Cui and Mumper 2003). Nanoemulsions or nanoparticle aerosol vaccines are also under development.

K.K. Jain, *The Handbook of Nanomedicine*,

A team of biomedical engineers and cell biologists at Yale Institute for Nanoscience and Quantum Engineering (Hartford, CT) received a $1 million award in October 2006 from the National Science Foundation to develop "smart" nanoparticles for the delivery of vaccines. The 2-year, Nanoscale Interdisciplinary Research Team funding will be applied to develop a new class of nanomaterials with properties that mimic biological vectors like bacteria and viruses. Although previous research has shown that safe, biocompatible materials can be engineered into nanoparticles that contain drugs or vaccines, the Yale will develop new materials for vectors that interact specifically and predictably with cells. Nanosystems will be designed to evade the normal barriers and stimulate antigen-presenting cells of the immune system. Working together, biomedical and materials scientists at Yale are looking to these "smart" targetable nanoparticles as devices that could seek out and destroy individual cancer cells while bypassing healthy ones.

Bacterial Spores for Delivery of Vaccines

Bacterial spores as described in Chapter 2 can be used for vaccine delivery. The spore coat can act as a vehicle for heterologous antigen presentation and protective immunization. Use of bacterial spores will solve the problem of stability and transport in the developing countries as they do not require refrigeration and the administration is needle free.

Proteosomes™ as Vaccine Delivery Vehicles

Components of the immune system recognize particles more efficiently than soluble proteins. Proteosomes™ (GlaxoSmithKline, Brentford, Middlesex, UK) serve as vaccine delivery vehicles by virtue of their nanoparticulate nature, forming vesicles and vesicle clusters comparable to the size of small viruses. Proteosome™ vaccine vesicles and vesicle clusters may range in size from 20 to 800 nm, depending on the type and amount of antigen formulated with the Proteosomes™. The hydrophobic nature of the Proteosome™ porin proteins also contributes to vaccine delivery capabilities by facilitating interaction of the vaccine particles with, and uptake of the vaccine by, cells that initiate immune responses. The fact that Proteosomes™ are effective nasal vaccines is considered to be particularly related to this enhanced recognition and uptake afforded by Proteosomes' particulate and hydrophobic nature. This technology is being applied to develop vaccines for influenza, allergy, plague, respiratory syncitial virus, and AD.

Nanospheres for Controlled Release of Viral Antigens

SuperFluids (Aphios Corporation, Woburn, MA, USA), which are supercritical, critical, or near-critical fluids with or without polar cosolvents are being developed for nanoencapsulating potent viral antigens in biodegradable polymer nanospheres for controlled release. The use of SuperFluids reduces exposure of viral antigens such as HIV and influenza to potentially denaturing organic solvents such as methylene

chloride and ethyl acetate and improves the stability of protein antigens in the body at ambient temperature for long periods, thereby enhancing the capability of nanoencapsulated vaccine antigens to induce the production of protective and neutralizing antibodies. This controlled release vaccine delivery technology has the capability to deliver different types and combinations of HIV or influenza vaccine candidates including whole inactivated virus particles, DNA plasmids, and/or subunit protein antigens. SuperFluids polymer nanoencapsulation technology will reduce cost by eliminating unnecessary processing steps while improving the manufacturing environment. Unlike currently available technologies, this technology is portable, inexpensive, and amenable to large-scale processing.

Cell Therapy

Cell therapy is the prevention or treatment of human disease by the administration of cells that have been selected, multiplied, and pharmacologically treated or altered outside the body (ex vivo). The scope of cell therapy can be broadened to include methods, pharmacological as well as nonpharmacological, to modify the function of intrinsic cells of the body for therapeutic purposes. The aim of cell therapy is to replace, repair, or enhance the function of damaged tissues or organs. The cells used can originate from the patient or from a donor or from another species. Other sources include cell lines and cell from patients' tumors to make cancer vaccines. Cells can be encapsulated in selectively permeable membranes that block the entry of immune mediators but allow the outward diffusion of active molecules produced by the cells. Genetic engineering of cells is part of ex vivo gene therapy. The cells may be introduced by various routes into the body and selectively implanted at the site of action. More recently, cell therapies have expanded to replace some conventional procedures. Bone marrow transplants are being replaced by peripheral blood stem cell transplants. Most of the interest in cell therapy centers on stem cells. Cell therapy is described in detail in a special report on this topic (Jain 2007g).

Cell transplantation to treat diseases has made considerable advances. There is a need for methods to protect the transplanted allogeneic or xenogeneic cells from rejection by the host immune system, techniques to enhance cellular integration of the transplant within the host tissue, strategies for in vivo detection and monitoring of the cellular implants, and new techniques to deliver genes to cells without eliciting a host immune response. Further investigations in this area by visualizing and controlling cellular interactions at a submicron level are desirable.

Nanobiotechnology and Cell Transplantation

Nanobiotechnology is well suited to optimize the generally encouraging results already achieved in cell transplantation (Halberstadt et al 2006). The small size of nanometer material constructs provides an increasing number of options to label,

transfect, visualize, and monitor cells and tissues used in transplantation. Nanoparticles are by their very nature well suited to interact with cells. Antibodies (10 nm) and viruses (100 nm) can easily interact with cells and transport across cell membranes. Nanosized constructs that are 20 nm in size can even cross the endothelial barrier.

Nanobiotechnology in Stem Cell-Based Therapies

The role of nanobiotechnology in tracking stem cells introduced into the human body is described in Chapter 3. Nanobiotechnology can be applied to delivery of gene therapy using genetically modified stem cells.

In their natural environment in the body, stem cells transform into other cell types based on chemical triggers they receive from their surroundings. The nature and the location of these triggers are not known for most stem cells. The current ability to introduce specific chemicals at select locations on a cell is also very limited as one must bathe the entire surface of stem cells in various chemicals to search for a response. Scientists at Stanford University are developing a nano laboratory to experiment with individual adult stem cells. Each laboratory essentially consists of a capsule on a silicon chip, around which up to 1,000 nanoreservoirs hold roughly a millionth of a billionth of a milliliter of liquid, comparable to the size of secretions cells use to communicate. They are in essence building an artificial cell-interface unit to a stem cell to establish chemical communication in much the same way real cells do. Nanotechnology is essential for this approach. Larger systems cannot provide the number of different reservoirs and chemicals within a space small enough to select different areas on a cell.

Scientists at Ohio State University are also developing nanofibrous scaffolds for stem cells, which mimic the nanometer-scale fibers normally found in that matrix (Kang et al 2005). They are creating biodegradable scaffolds to nurture stem cells derived from adipose tissue. Preadipocytes grown on 3D matrices acquire morphology and biological features of mature adipocytes. This new culture model should have significant utility for in vitro studies of adipocyte cell biology and development.

One study has assessed bone formation from mesenchymal stem cells (MSCs) on a novel nanofibrous scaffold in a rat model (Shin 2004). A highly porous, degradable poly(caprolactone) scaffold with an ECM-like topography was produced by electrostatic fiber spinning. MSCs derived from the bone marrow of neonatal rats were cultured, expanded, and seeded on the scaffolds. The cell–polymer constructs were cultured with osteogenic supplements and they maintained the size and shape of the original scaffolds when explanted. Morphologically, the constructs were rigid and had a bone-like appearance. Cells and ECM formation were observed throughout the constructs. In addition, mineralization and type I collagen were also detected. This study establishes the ability to develop bone grafts on electrospun nanofibrous scaffolds in a well-vascularized site using MSCs.

Gene Therapy

Gene therapy can be broadly defined as the transfer of defined genetic material to specific target cells of a patient for the ultimate purpose of preventing or altering a particular disease state. Vectors are usually viral, but several nonviral techniques are being used as well. Genes and DNA are now being introduced without the use of vectors, and various techniques are being used to modify the function of genes in vivo without gene transfer. Nanoparticles and other nanostructures can be used for gene delivery.

Nanoparticle-Mediated Gene Therapy

The success of the gene therapy for clinical applications, in part, would depend on the efficiency of the expression vector as determined by the level as well as the duration of gene expression. Although various cationic polymers and lipid-based systems are being investigated, most of these systems exhibit higher-level but transient gene expression. Most often, the emphasis is on the level of gene expression rather than on the duration of gene expression. In certain disease conditions, a relatively low level of gene expression (therapeutic level) but for a sustained duration may be more effective than higher-level but transient gene expression. Therefore, a gene expression system that can modulate the level as well as the duration of gene expression in the target tissue is desirable. Polymer-based sustained release formulations such as nanoparticles have the potential of developing into such a system.

Nanoparticles escape rapidly (within 10 min) from the endolysosomal compartment to the cytoplasmic compartment following their intracellular uptake via an endocytic process (Panyam et al 2002). The escape of nanoparticles is attributed to the reversal of their surface charge from anionic to cationic in the acidic pH of the endolysosomal compartment, causing nanoparticles to interact with the endolysosomal membrane and then escape into the cytoplasmic compartment. The rapid escape of nanoparticles from the endolysosomal compartment could protect nanoparticles as well as the encapsulated DNA from the degradative environment of the endolysosomes. Nanoparticles localized in the cytoplasmic compartment would release the encapsulated DNA slowly, thus resulting in sustained gene expression. Sustained gene expression could be advantageous, especially if the half-life of the expressed protein is very low and/or a chronic gene delivery is required for therapeutic efficacy. Examples of application of nanoparticles for gene therapy are listed in Table 5.1 and described in the following text.

Nanoparticles as Nonviral Vectors for CNS Gene Therapy

Use of organically modified silica (ORMOSIL) nanoparticles ($\approx$30 nm) has been reported as a nonviral vector for efficient in vivo gene delivery without toxic effects and with efficacy equaling or exceeding that obtained in studies using a

Table 5.1 Examples of application of nanoparticles for gene therapy

Nanoparticle	Application
Poly(D,L-lactide-co-glycolide) nanoparticles loaded with wild-type p53 DNA	Inhibition of cell proliferation in cancer due to sustained gene expression following intracellular release of p53
Intravenous liposomal DOTAP:Chol-FUS1 complex of tumor suppressor gene FUS1	Suppresses tumor growth and has led to tumor regression in mouse models of metastatic lung cancer
Fluorescently labeled organically modified silica nanoparticles as a nonviral vector	For gene delivery and optical monitoring of intracellular trafficking and gene transfection
Cationized gelatin nanoparticles	Nonviral and nontoxic vectors for gene therapy
Calcium phosphate nanoparticles	Nonviral vectors for targeted gene therapy of liver
Nanotube spearing is based on the penetration of nickel-embedded nanotubes into cell membranes by magnetic field driving	This technique may provide a powerful tool for highly efficient gene transfer into a variety of cells, especially the hard-to-transfect cells (Cai et al 2005)
Polyamidoamine dendrimers: nanoparticles that can hold DNA in their cavities	Nonimmunogenic vector for in vivo gene transfer
Nanoparticles: EGF–PEG–biotin–streptavidin–PEI–DNA complexes	Exhibit high transfection efficiency with no particle aggregation
Compacted DNA nanoparticles (20–25 nm): Each DNA molecule is wrapped in a coat of positively charged peptides	Nanoparticles pass through a nuclear pore with thousands-fold enhancement of gene expression compared to naked DNA. Used for transnasal gene therapy in cystic fibrosis
Cochleate delivery system is formed as a result of interaction between cations and negatively charged phospholipids	In vivo lipid-based delivery of DNA plasmids and antisense DNA
Nanorod binds plasmid DNA as well as a proteins in spatially defined regions	A versatile gene delivery system that increases the plasmid's cellular internalization and cytoplasmic release
Combination of a gene, nanoparticle, and surfactant	Facilitation of gene transfer in the brain across the blood–brain barrier
Integrin-targeted nanoparticles	Site-specific delivery of anticancer genes
Nanocomposites: nanoparticles of titanium dioxide combined with oligonucleotide DNA that can be activated by light or radiation	Antisense genes can be delivered to a particular intracellular site and in combination with radiotherapy with the purpose of killing the cell in cancer patients
Nonionic polymeric micelles of poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide)	Stable gene transfer to the gastrointestinal tract can be achieved in mice by oral delivery

EGF, epidermal growth factor; PEG, polyethylene glycol; PEI, polyethylenimine.
Source: Jain PharmaBiotech.

viral vector (Bharali et al 2005). Highly monodispersed, stable aqueous suspension of nanoparticles, surface-functionalized with amino groups for binding of DNA, were prepared and characterized. Intraventricular and intracerebral stereotaxic injections of nanoparticles, complexed with DNA encoding for enhanced green fluorescent protein (EGFP), were made into the mouse brain. Use of an optical fiber in vivo imaging technique, CellviZio, developed by Mauna Kea Technologies (Paris, France), enabled observation of the brain cells expressing genes without having to sacrifice the animal. The ORMOSIL-mediated transfections were also used to manipulate the biology of the neural stem/progenitor cells in vivo. Transfection of a plasmid expressing the nucleus-targeting fibroblast growth factor receptor type 1 resulted in significant inhibition of the in vivo incorporation of bromodeoxyuridine into the DNA of the cells in the subventricular zone and the adjacent rostral migratory stream. Targeted dopamine neurons, which degenerate in PD, took up and expressed a fluorescent marker gene, demonstrating the ability of nanoparticle technology to deliver effectively genes to specific types of cells in the brain. The gene–nanoparticle complexes were shown to activate adult brain stem/progenitor cells in vivo, which could be effective replacements for those destroyed by neurodegenerative diseases. Thus ORMOSIL nanoparticles have a potential for effective therapeutic manipulation of the neural stem/progenitor cells as well as in vivo-targeted brain therapy. The structure and composition of ORMOSIL allows for the development of an extensive library of tailored nanoparticles to target gene therapies for different tissues and cell types.

A European research team led by researchers from the Scuola Superiore Sant'Anna, CRIM lab (Italy) is developing nonviral vectors based on CNTs for a safe and efficient gene transfer. The CNT-based vectors should be able to perform an "enhanced" gene transfer in target cells. The project results will be first validated in vivo on two specific neurological disorders cerebral ischemia and Rett syndrome.

The Department of Neurosurgery of University of Kentucky is conducting a research project titled "Nanoparticle Gene Therapy for Parkinson's Disease," which is testing the feasibility of delivering condensed DNA nanoparticles that encode for a neurotrophic factor to the brain as a means to halt or prevent the neurodegenerative process in an animal model of PD. The technology has been developed by Copernicus Therapeutics Inc (Cleveland, OH, USA) and this will be one of the first applications of this technology to CNS disorders. This gene therapy strategy holds potential to help repair faulty genes. The aim of using this technology is to transduce brain cells so that they express proteins beneficial for the cell's survival.

Lipid Nanoparticles for Targeted Delivery of Nucleic Acids

Targeted liposomal nanoparticle vectors are being tested at Yale University (New Haven, CT) as nonviral vectors to deliver Icon gene in animal models of human metastatic prostate cancer. The advantage of nanoparticle vectors is that they do not reproduce, are not immunogenic, and are easier to produce than viral vectors. The nanoparticles have a tag on their outside that binds to the tumor blood vessels. Icon gene, delivered to tumor cells, activates an immune response that destroys the tumor.

Protiva's SNALPs (stable nucleic acid-lipid particles) are specialized lipid nanoparticles that fully encapsulate and systemically deliver a variety of nucleic acid molecules such as siRNA, aptamers, and DNA. Because the SNALP particles are small (~100 nm), have a uniform size, low surface charge, are stable, and do not aggregate, they remain intact in circulation for many hours. These features of SNALPs allow the particles to accumulate at target sites. This technology utilizes a mechanism referred to as the "enhanced permeability and retention effect," which occurs because these nucleic acid-containing particles have a long circulation time in the blood, resulting in accumulation at sites of vascular leak such as those found at sites of tumor cell growth, infection, inflammation, and in the normal liver. The newly formed vessels in growing tumors are characterized by disorganized endothelial layers, with holes or gaps called fenestrae where the tumor cells are in direct contact with the blood. These fenestrated vascular beds are the sites where SNALPs leave the blood vessel and accumulate in the diseased tissue. These factors result in unparalleled potency, achieving increases of more than 100-fold over other delivery systems. Once SNALPs have accumulated the target site, the cells take up the particle by endocytosis in which the cell's membrane surrounds the SNALP particles, wrapping it in an envelope of membrane lipids that are similar to those of SNALPs. This envelope or endosome pinches off from the cell's membrane and migrates to the inside of the cell. The internalized SNALP undergoes an interaction with the endosomal membrane. The lipids of the SNALP bilayer interact with the endosomal membrane and the two fuse with one another. In the process the nucleic acid payload is liberated and released inside the cell's cytoplasm. The released nucleic acid molecules disperse throughout the cell. In the case of siRNA, they engage the RNA-induced silencing complex (RISC) in the cytoplasm, mediating RNAi. In the case of DNA, the plasmid must enter the nucleus of the cell, where it is expressed.

Carbonate Apatite Nanoparticles for Gene Delivery

Biocompatible, inorganic nanoparticles of carbonate apatite have the unique features essentially required for smart delivery, as well as for the expression of genetic material in mammalian cells (Chowdhury 2007). The newly developed carbonate apatite, as with hydroxyapatite, adsorbs DNA, but, unlike the latter, it can prevent the growth of its crystals to a significant extent, enabling the synthesis of nanosized crystals to effectively carry the associated DNA across the cell membrane. It also possesses a high dissolution rate in endosomal acidic pH, leading to the rapid release of the bound DNA for a subsequent high level of protein expression. Carbonate apatite is a natural component of the body and is usually found in the hard tissues, such as bone and teeth. Moreover, because of their nanosize dimensions and sensitivity to low pH, particles of carbonate apatite are quickly degraded when taken up by cells in their acidic vesicles, without any indication of toxicity. Apatite nanoparticles are promising candidates for nonviral gene delivery and are superior to polymer- or lipid-based systems that are generally non-biodegradable and inefficient.

Nanoparticles Linked to Viral Vectors for Photothermal Therapy

Hyperthermia can be produced by NIR laser irradiation of gold nanoparticles present in tumors and thus induce tumor cell killing via a bystander effect. However, selective delivery and physical targeting of gold nanoparticles to tumor cells are necessary to improve therapeutic selectivity. Covalent coupling of gold nanoparticles to retargeted adenoviral vectors enables selective delivery of the nanoparticles to tumor cells, thus facilitating hyperthermia and gene therapy as a combinatorial therapeutic approach. For this, sulfo-*N*-hydroxysuccinimide-labeled gold nanoparticles were linked to adenoviral vectors encoding a luciferase reporter gene driven by the cytomegalovirus promoter (Everts et al 2006). The covalent coupling retains virus infectivity and ability to retarget tumor-associated antigens. These results show the feasibility of using adenoviral vectors as carriers for gold nanoparticles.

Nanoparticles for p53 Gene Therapy of Cancer

One of the important considerations in p53 gene delivery for tumor growth inhibition would be the sustained expression of the p53 protein in the target cells. A single-dose regimen results in only a weak and transient inhibition of cell proliferation. Multiple doses are required to obtain inhibition of cell proliferation comparable to that with viral vectors. Several mechanisms have been attributed to wt-p53 gene-mediated cancer therapy such as apoptosis of cancer cells, cell cycle arrest, and/or the antiangiogenic effect of the protein. Gene delivery with nanoparticles would require direct intratumoral injection in the case of a solid tumor or delivery via a catheter to an accessible diseased tissue. However, tumor targeting via intravascular administration would be possible if nanoparticle surface is modified to avoid extravasation by the reticuloendothelial system.

In one study, poly(D,L-lactide-co-glycolide) nanoparticles loaded with wt-p53 DNA demonstrated a sustained antiproliferative effect whose magnitude increased with incubation time in a breast cancer cell line (Prabha and Labhasetwar 2004). Inhibition of cell proliferation was found to be due to sustained gene expression following slow intracellular release of the encapsulated DNA from nanoparticles. The results of the study suggest that wt-p53 DNA-loaded nanoparticles could be potentially useful in the therapy of breast and other cancers that are ascribed to mutation in the p53 gene.

Immunolipoplex for Delivery of p53 Gene

A sterically stabilized immunolipoplex, containing an anti-Trf single-chain antibody fragment-PEG molecule, has been developed to specifically and efficiently deliver a therapeutic gene to tumor cells (Yu et al 2004). Lipoplex nanoparticles resembling virus particles can penetrate deeply into the tumor and move efficiently into cells. The immunolipoplex is spiked on the outside with antibody molecules that will seek out, bind to, and then enter cancer cells including metastases wherever they hide in the body. These molecules bind to the receptor for transferrin that is present

in large numbers on cancer cells. Once inside, the immunolipoplex will deliver its payload, the p53 gene, whose protein helps to signal cells to self-destruct when they have the kind of genetic damage characterized by cancer and by cancer therapies. Immunolipoplex has shown promising results in animal tumor models, and a phase I clinical trial in patients with advanced solid cancers has been approved. The trial is supported by the NIH and SynerGene Therapeutics (Qormi, MALTA). Among the solid tumors approved for testing in the clinical trial are head and neck, prostate, pancreatic, breast, bladder, colon, cervical, brain, melanoma, liver, and lung cancers.

Immunolipoplex-based gene transfer represents an advance over the viral vectors that have been used to deliver gene therapy, because these liposomes do not produce the immunologic response seen when disabled viruses are used to carry the payload. Immunolipoplex also substantially improves the anticancer effects of both chemotherapy and radiation therapy. These agents work synergistically with traditional therapies because the restoration of p53 protein helps push cancer cells that are now damaged to self-destruct. This approach will make it difficult for the cancer cells to become resistant to therapy and will be less likely to recur after therapy is complete.

Intravenous Nanoparticle Formulation for Delivery of FUS1 Gene

INGN 401 (Introgen, Austin, TX, USA) is an intravenous nanoparticle formulation (liposomal DOTAP:Chol-FUS1 complex) of the tumor suppressor gene FUS1, which suppresses tumor growth and has led to tumor regression in mouse models of metastatic lung cancer (Ito et al 2004). Mice treated with INGN 401 survived almost 70% longer than untreated mice. Phase I clinical trials in patients with lung cancer have started and initial survival results are encouraging. Combining the systemic delivery capabilities of the nanoparticle delivery with the cancer-specific activity of tumor suppressor genes, such as FUS1, may provide a new approach to meet the challenge of treating metastases as it has the ability to attack cancer at multiple sites throughout the body without causing systemic toxicity. However, systemic administration of DNA-nanoparticles induced multiple signaling molecules both in vitro and in vivo that are associated with inflammation. The use of small-molecule inhibitors against the signaling molecules resulted in their suppression and thereby reduced inflammation without affecting transgene expression. These results provide a rationale to use small-molecule inhibitors to suppress nanoparticle-mediated inflammation when administered systemically (Gopalan et al 2004).

Coexpression of FUS1 and p53 by *N*-[1-(2,3-dioleoyloxyl)propyl]-*N,N,N*-trimethylammoniummethyl sulfate:cholesterol nanoparticle-mediated gene transfer significantly and synergistically inhibits the development and growth of tumors in a mouse model of human lung cancer (Deng et al 2007). These results provide new insights into the molecular mechanism of FUS1-mediated tumor suppression activity and imply that a molecular therapy combining two or more functionally synergistic tumor suppressors may constitute a novel and effective strategy for cancer treatment.

Silica Nanoparticles for Gene Delivery

Core–shell silica particles with a hydrodynamic diameter of 28 nm have been synthesized. The role of freeze-drying for the conservation of zwitterionic nanoparticles and the usefulness of different lyoprotective agents (LPAs) for the minimization of particle aggregation were studied. The activity of the nanoparticles was measured as DNA-binding capacity and transfection efficiency in Cos-1 cells before and after lyophilization. It was found that massive aggregation occurred in the absence of LPA. Of the various LPAs screened in the investigations, trehalose and glycerol were found to be well suited for conservation of cationically modified silica nanoparticles with simultaneous preservation of their DNA-binding and transfection activity in Cos-1 cells (Sameti et al 2003).

A multidisciplinary approach has been used to produce fluorescently labeled organically modified silica nanoparticles as a nonviral vector for gene delivery as well as biophotonics methods to optically monitor intracellular trafficking and gene transfection (Roy et al 2005). Highly monodispersed, stable aqueous suspensions of organically modified silica nanoparticles, encapsulating fluorescent dyes and surface-functionalized by cationic-amino groups, are produced by micellar nanochemistry. Gel-electrophoresis studies reveal that the particles efficiently complex with DNA and protect it from enzymatic digestion of DNase 1. The electrostatic binding of DNA onto the surface of the nanoparticles, due to positively charged amino groups, is also shown by intercalating an appropriate dye into the DNA and observing the FRET between the dye (energy donor) intercalated in DNA on the surface of nanoparticles and a second dye (energy acceptor) inside the nanoparticles. Imaging by fluorescence confocal microscopy shows that cells efficiently take up the nanoparticles in vitro in the cytoplasm, and the nanoparticles deliver DNA to the nucleus. This work shows that the nanomedicine approach, with nanoparticles acting as a drug delivery platform combining multiple optical and other types of probes, provides a promising direction for targeted therapy with enhanced efficacy as well as for real-time monitoring of drug action.

Gelatin Nanoparticles for Gene Delivery

Construction of gelatin nanoparticles as biodegradable and low cell toxic alternative carrier to existing DNA delivery system has been described (Zwiorek et al 2005). In order to bind DNA by electrostatic interactions onto the surface of the gelatin nanoparticles, the quaternary amine cholamine was covalently coupled to the particles. The modified nanoparticles were loaded with different amounts of plasmid in varying buffers and compared to polyethyleneimine–DNA complexes (PEI polyplexes) as gold standard. In contrast to PEI polyplexes, cationized gelatin nanoparticles almost did not show any significant cytotoxic effects. Cationized gelatin nanoparticles have the potential of being a new effective carrier for nonviral gene delivery. The major benefit of gelatin nanoparticles is not only the very low cell toxicity, but also their simple production combined with low costs and multiple modification opportunities offered by the matrix molecule.

The potential of engineered gelatin-based nanoparticles, nanovectors, has been investigated to deliver therapeutic genes to human breast cancer tumors implanted in mice (Kommareddy and Amiji 2007). DNA encoding for the soluble form of the extracellular domain of VEGF-R1 or sFlt-1 was encapsulated in the control and PEG-modified gelatin-based nanoparticles. Following intravenous administration in female Nu/Nu mice bearing orthotopic MDA-MB-435 breast adenocarcinoma xenografts, 15% of the dose found its way into the tumor. In vivo expression of sFlt-1 pDNA was therapeutically active as shown by suppression of tumor growth and microvessel density measurements. The results of this study show that PEG-modified gelatin-based nanovectors can serve as a safe and effective systemically administered gene delivery vehicle for solid tumor. Clinical trials of this method are expected in the near future.

Calcium Phosphate Nanoparticles as Nonviral Vectors

Calcium phosphate nanoparticles present a unique class of nonviral vectors, which can serve as efficient and alternative DNA carriers for targeted delivery of genes. Design and synthesis of very small, highly monodispersed DNA-doped calcium phosphate nanoparticles ~80 nm in diameter has been reported (Roy et al 2003b). The DNA encapsulated inside the nanoparticle is protected from the external DNase environment and could be used safely to transfer the encapsulated DNA under in vitro and in vivo conditions. Moreover, the surface of these nanoparticles could be suitably modified by adsorbing a highly adhesive polymer like polyacrylic acid followed by conjugating the carboxylic groups of the polymer with a ligand such as *p*-amino-1-thio-beta-galactopyranoside using 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride as a coupling agent. The authors demonstrated that these surface-modified calcium phosphate nanoparticles could be used in vivo to target genes specifically to the liver.

Dendrimers for Gene Transfer

Dendrimers are nanoparticles ranging in size from 1 to 20 nm and are capable of holding therapeutic substances such as DNA in their cavities. They are made up of precise 3D branches called dendrons, with structure that mimics the bifurcation of tree branches. The dendrimers are so close in shape and size to a histone cluster that DNA wraps around them as it does around the natural protein complex. Dendrimers show great promise as DNA and drug delivery systems (Cloninger 2002). The following factors, which drive the interest in use of dendrimers as gene, transfer vectors:

1. They can be produced as precise macromolecular structures.
2. They are composed of nanoscopic building blocks or modules.
3. They are nonimmunogenic.

Activated polyamidoamine (PAMAM)-dendrimers provide a new technology for gene transfer that offers significant advantages over classical methods. QIAGEN

reagents based on this technology provide high gene transfer efficiencies, minimal cytotoxicity, and can be used with a broad range of cell types. This technology could also be useful for in vivo gene transfer in gene therapy applications (Dennig and Duncan 2002).

DNA–PEG Complexes as Nanoparticles

An efficient receptor-mediated nonviral gene delivery formulation based on mono-pegylated recombinant human epidermal growth factor (EGF) was developed using a streptavidin–biotin system (Lee et al 2002). Biotin-derived and mono-pegylated EGF was prepared by conjugating a biotin–PEG–NHS derivative to EGF and purified through a chromatographic method. Luciferase pDNA and PEI were complexed to form positively charged nanoparticles on which negatively charged streptavidin was first coated and then biotin–PEG–EGF conjugate was immobilized via streptavidin–biotin interaction. The EGF–PEG–biotin–streptavidin–PEI–DNA complexes were characterized in terms of their effective diameter and surface zeta potential value under various formulation conditions. The formulated complexes exhibited high transfection efficiency with no particle aggregation. This was attributed to enhanced cellular uptake of the resultant complexes via receptor-mediated endocytosis. Furthermore, in the presence of serum proteins, a slight decrease in transfection efficiency was observed due to the presence of PEG chains on the surface.

Compacted DNA Nanoparticles

Compacted DNA nanoparticles (Copernicus' PLASmin^{TM} Complexes) can be safely administered to the nose of CF subjects as shown in a phase I/II clinical trial. This was a joint collaboration of Copernicus, University Hospitals of Cleveland, Case Western Reserve University School of Medicine, The Children's Hospital of Denver, and Cystic Fibrosis Foundation Therapeutics Inc Copernicus formulation of compacted DNA was stable upon storage at 4° C for well over 1 year. This property is important for successful commercial development of the product. A single DNA molecule is wrapped in a coat of positively charged peptides. The resulting particle has a diameter of only 20–25 nm, small enough to pass through a nuclear pore. The compacted DNA nanoparticles robustly transfect postmitotic human cells, achieving up to a 6900-fold enhancement of gene expression when compared with naked DNA.

Cochleate-Mediated DNA Delivery

Cochleate, a lipid-based delivery system, is formed as a result of interaction between cations, e.g., Ca^{2+} and negatively charged phospholipids such as phosphatidylserine. Cochleates are stable precipitates with a unique structure consisting of a large, continuous, solid, lipid bilayer sheet rolled up in spiral, with no internal aqueous space. They are nontoxic and noninflammatory and have been used as vehicles for oral and parenteral delivery of protein and peptide antigens. Cochleate-mediated in

vivo delivery of DNA plasmids and antisense DNA is under investigation. Protein and DNA cochleates are highly effective vaccines when given via mucosal or parenteral routes, including oral, intranasal, intramuscular, or subcutaneous (Delmarre et al 2004).

Nanorod Gene Therapy

Gene therapy success has been limited in both viral and synthetic methods. In light of current gene therapy challenges, synthetic transfection systems provide several advantages over viral methods including ease of production, as well as reduced risk of cytotoxicity and immune responses. The drawbacks of synthetic vectors greatly stem from difficulty of controlling the vectors' properties at the nanoscale. The nanorod greatly overcomes those drawbacks by binding pDNA as well as a potential variety of proteins in spatially defined regions. The proteins the nanorod binds can increase the plasmid's cellular internalization, cytoplasmic release, and/or nuclear internalization. The nanorods can be further guided via their magnetic properties. The potential of this versatile gene delivery system with precise composition and size has been demonstrated in cell transfection studies.

Nanomachines for Gene Delivery

Nanomachines such as F1-ATPase rotor (http://www.res.titech.ac.jp/∼seibutu/) and molecular shuttles as described in the web sites (http://depts.washington.edu/chemcrs/bulkdisk/chem560C_spr04/handout_05-13-04_Hess.pdf, http://www.nanomat.mat.ethz.ch/) can be used for nonviral method of gene delivery. A molecular motor can be used as a biomolecular adaptor for retrograde transport (BART), which is a method of attaching synthetic cargo to the molecular motor for intracellular transport. The advantages are that the motor is used in a native environment and the expedited transport of DNA to nucleus could improve gene transfer. It is hypothesized that a BART peptide based on either Tctex-1 or LC8 binding peptide sequences can be used to link a conjugated molecule or particle to dynein and this would result in the transport of the conjugate molecule toward the interior of the cell in a manner similar to that of a virus vector (Cohen et al 2005). The disadvantage is that this approach requires engineered molecular motors for use in cells or covalent modification of DNA.

Application of Pulsed Magnetic Field and Superparamagnetic Nanoparticles

A simple approach has been reported that enhances gene delivery using permanent and pulsating magnetic fields (Kamau et al 2006). DNA plasmids and novel DNA fragments (PCR products) containing sequence encoding for green fluorescent

protein were coupled to PEI-coated SPIONs. The complexes were added to cells that were subsequently exposed to permanent and pulsating magnetic fields. The presence of these magnetic fields significantly increased the transfection efficiency 40 times more than in cells not exposed to the magnetic field. The transfection efficiency was highest when the nanoparticles were sedimented on the permanent magnet before the application of the pulsating field, both for small (50 nm) and large (200–250 nm) nanoparticles. The highly efficient gene transfer already within 5 min shows that this technique is a powerful tool for future in vivo studies, where rapid gene delivery is required before systemic clearance or filtration of the gene vectors occurs.

Nanocomposites for Gene Therapy

The behavior of 45-A nanoparticles of titanium dioxide (TiO_2) semiconductor combined with oligonucleotide DNA into nanocomposites has been studied in vivo and in vitro. TiO_2 possesses several attributes that could make it a molecule of choice in biological systems. First, it is relatively inert biologically and is well tolerated in vivo. Secondly, it acts as a semiconductor and, particularly, when presented as a nanoparticle, can act as a miniaturized electrochemical cell. When linked to an organic modifier and exposed to incident energy greater than the band-gap, semiconduction can occur through both the TiO_2 and the attached modifier. Attachment of such a nanocomposite to a nucleic acid might lead to a multifunctional molecule possessing the specificity inherent to Watson–Crick base pairing. These nanocomposites not only retain the intrinsic photocatalytic capacity of TiO_2 and the bioactivity of the oligonucleotide DNA (covalently attached to the TiO2 nanoparticle) but also possess the chemically and biologically unique new property of a light-inducible nucleic acid endonuclease, which could become a new tool for gene therapy (Paunesku et al 2003). Antisense genes attached to the TiO_2 nanoparticle can be delivered to a particular intracellular site and, when illuminated, the antisense genes or the drugs will "fall off in the right location." And in the long run, because ionizing radiation also seems to photoactivate the nanodevice, "the delivery of gene therapy or drugs to an intracellular target with the purpose of killing the cell could be done in combination with radiotherapy" in cancer patients.

Although this is a unique concept, the use of TiO_2 nanocomposites has a long way to go before practical use in gene therapy. Researchers are first trying to get these devices into mitochondria in the cell and also testing the ability of the nanodevice to act as an alternative to RNAi by cleaving specific RNAs and interfering with RNA synthesis in cancer cells in vitro. Demonstrating the unique properties of the nanodevice in an in vivo model would be very important.

Nonionic Polymeric Micelles for Oral Gene Delivery

Gene delivery through the gastrointestinal tract not only has many potential applications, but is also less invasive and more easily performed. Nanotechnology and

nanomaterials are being developed as advanced approaches to overcome some of the problems associated with oral viral gene. The feasibility of using nonionic PMs of poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide) (PEO-PPO-PEO) as a carrier for oral DNA delivery in vivo has been investigated in experimental animals (Chang et al 2004). After oral administration, the highest expression of transferred gene lacZ was seen in the villi, crypts, and goblet cells of the duodenum and in the crypt cells of the stomach. Reporter gene activity was seen in the duodenum, stomach, liver, brain, and testis. lacZ mRNA was detected in these five organs and in the blood by RT–PCR. These results show that efficient, stable gene transfer can be achieved in mice by oral delivery of PEO-PPO-PEO PMs.

Nanocarriers for Simultaneous Delivery of Anticancer Drugs and DNA

Scientists at the Institute of Bioengineering and Nanotechnology (IBN, Singapore) have developed nanoparticles that can carry both small-molecule anticancer drugs and nucleic acids simultaneously for improved cancer therapy. The uniqueness of the technology from IBN lies in the design of a special biodegradable carrier (cationic core-shell nanoparticle), which can enclose drug molecules and enable therapeutic nucleic acids to bind onto it. It can efficiently introduce DNA into a cell to be incorporated into its genetic makeup, i.e., induce high gene expression level, especially in both human and mouse breast cancer cell lines, and mouse breast cancer model. The codelivery of small-molecule drugs with nucleic acids can improve gene transfection efficiency, reduce side effects of these drugs, and achieve the synergistic effect of drug and gene therapy for a more effective treatment of cancer. Results have shown that the co-delivery of an anticancer drug (paclitaxel) with a highly potent anticancer "messenger molecule" (IL-12-encoded plasmid) using the carrier suppressed cancer growth more efficiently than the delivery of either paclitaxel or the plasmid in mice bearing 4T1 breast cancer. Experiments have also been conducted to codeliver paclitaxel and siRNA targeting a protein that prevents cell death (Bcl-2) to MDA-MB-231 human breast cancer cell line. The cancer cells became more susceptible to the effects of the drug, due to the additional effect of the siRNA targeting Bcl-23. This special carrier can also be potentially used to codeliver therapeutic nucleic acids to prevent cancer cells from developing resistance to multiple drugs. This, coupled with the simultaneous delivery of specific anticancer drugs, could enhance the therapeutic effects of such drugs.

Antisense Therapy

Antisense molecules are synthetic segments of DNA or RNA, designed to mirror specific mRNA sequences and block protein production. One way to target the genetic material is to block the mRNA by using "antisense DNA," which prevents

the message from ever becoming a protein. The use of antisense drugs to block abnormal disease-related proteins is referred to as antisense therapeutics. Synthetic short segments of DNA or RNA are referred to as ODNs. Whereas typical drugs target the proteins, it is possible through antisense gene therapy to target the genetic material itself before it is ever made into copies of harmful proteins. Antisense drugs have the promise to be more effective than conventional drugs, but one of the problems with antisense therapy is delivery. The efficacy of antisense ODNs is limited by the poor stability of the natural oligomers and the low efficacy of their cellular uptake. Nanotechnology has been used to improve this situation.

Antisense Nanoparticles

Scientists at Northwestern University have described the use of gold nanoparticle–oligonucleotide complexes, antisense nanoparticles, as intracellular gene regulation agents for the control of protein expression in cells (Rosi et al 2006). Once inside cells, the DNA-modified nanoparticles act as mRNA "sponges" that bind to their targets and prevent them from being converted into proteins. By chemically tailoring the density of DNA bound to the surface of gold nanoparticles, they have demonstrated a tunable gene knockdown. In the future, this exciting new class of antisense material could be used for the treatment of cancer and other diseases that have a genetic basis. Advantages of attaching multiple strands of antisense DNA to the surface of a gold nanoparticle over conventional antisense ODNs are as follows:

- The DNA becomes more stable and can bind to the target mRNA more effectively than DNA that is not attached to a nanoparticle surface as in commercial agents such as Lipofectamine and Cytofectin.
- They are less susceptible to degradation by nuclease activity.
- They exhibit >99% cellular uptake.
- They can introduce ODNs at a higher effective concentration than conventional transfection agents and are nontoxic to the cells under the conditions studied.

Dendrimers for Antisense Drug Delivery

Polypropylenimine dendrimers have been used for delivering a 31 nt triplex-forming oligonucleotide (ODN) in breast, prostate, and ovarian cancer cell lines, using 32P-labeled ODN (Santhakumaran et al 2004). Dendrimers enhanced the uptake of ODN by 14-fold compared with control ODN uptake. Dendrimers exerted their effect in a concentration- and molecular weight-dependent manner, with generation 4 (G-4) dendrimer having maximum efficacy. The dendrimers had no significant effect on cell viability at concentrations at which maximum ODN uptake occurred. Gel electrophoretic analysis showed that ODN remained intact in cells even after 48 h of treatment. The hydrodynamic radii of nanoparticles formed from ODN in the presence of the dendrimers were in the range of 130–280 nm, as determined by dynamic

laser light scattering. Taken together, these results indicate that polypropylenimine dendrimers might be useful vehicles for delivering therapeutic ODNs in cancer cells.

Polymethacrylate Nanoparticles for Antisense Delivery System

Polymethacrylate nanoparticles appeared to be a promising vehicle for delivery of antisense ODNs. In one technique, nanoparticles were prepared by evaporating ethanol solution containing Eudragit RL100 or RS100, and then mixed with ODNs (Wang et al 2003). The morphology and size were investigated by a TEM and Mastersizer particle characterization systems, and the cytotoxicity was evaluated by Trypan Blue staining and hemolysis test. The flow cytometer was used to determine the uptake of fluorescence-labeled ODNs. The morphology of nanoparticles showed that they were spherical in shape with an average diameter of 127 nm. Optimal antisense loading co-occurred when nanoparticle:ODN was 6.6. The uptake of ODN was significantly increased when loaded by nanoparticles, which also depended on the nanoparticles concentration. Slight cytotoxicity was observed when high doses of nanoparticles were used.

RNAInterference

siRNAs can be used to silence gene expression in a sequence-specific manner in a process that is known as RNAi. RNAi is a powerful tool to reduce the mRNA level of specific genes. The finding that siRNAs of 21 nt as well as stable, endogenously expressed, large dsRNA can specifically induce gene silencing in mammalian cells offered the possibility of expanding this technique to mammalian systems. Detailed description of RNAi is given in a special report on the topic (Jain 2007f). Delivery is an important problem in effective siRNA use.

Nanoparticle siRNA Delivery

Potent sequence selective gene inhibition by siRNA "targeted" therapeutics promises the ultimate level of specificity, but siRNA therapeutics is hindered by poor intracellular uptake, limited blood stability, and nonspecific immune stimulation. To address these problems, ligand-targeted, sterically stabilized nanoparticles have been adapted for siRNA (Schiffelers et al 2004). Self-assembling nanoparticles with siRNA were constructed with polyethyleneimine (PEI) that is PEGylated with an Arg-Gly-Asp peptide ligand attached at the distal end of the PEG, as a means to target tumor neovasculature expressing integrins and used to deliver siRNA inhibiting VEGF receptor-2 expression and thereby tumor angiogenesis. Cell delivery and activity of PEGylated PEI was found to be siRNA sequence specific and depend on the presence of peptide ligand and could be competed by free peptide.

Intravenous administration into tumor-bearing mice gave selective tumor uptake, siRNA sequence-specific inhibition of protein expression within the tumor, and inhibition of both tumor angiogenesis and growth rate. The results suggest achievement of two levels of targeting: tumor tissue-selective delivery via the nanoparticle ligand and gene pathway selectivity via the siRNA oligonucleotide. This opens the door for better targeted therapeutics with both tissue and gene selectivity, also to improve targeted therapies with less than ideal therapeutic targets.

Overexpression of RhoA in cancer indicates a poor prognosis, because of increased tumor cell proliferation and invasion and tumor angiogenesis. Anti-RhoA siRNA inhibits aggressive breast cancer more effectively than conventional blockers of Rho-mediated signaling pathways. A study reports the efficacy and lack of toxicity of intravenously administered encapsulated anti-RhoA siRNA in chitosan-coated polyisohexylcyanoacrylate (PIHCA) nanoparticles in xenografted aggressive breast cancers (Pille et al 2006). The siRNA treatment inhibited the growth of tumors by 90% and necrotic areas were observed in tumors, resulting from angiogenesis inhibition. In addition, this therapy was found to be devoid of toxic effects. Because of its efficacy and the absence of toxicity, it is suggested that this strategy of anti-RhoA siRNA holds significant promise for the treatment of aggressive cancers.

Intravenous Targeted Nanoparticles Containing siRNA

The effects of administering escalating, intravenous (IV) doses of targeted nanoparticles containing an siRNA targeting the M2 subunit of ribonucleotide reductase to nonhuman primates have been studied by using a synthetic delivery system that uses a linear, cyclodextrin-containing polycation, Tf protein targeting ligand, and siRNA (Heidel et al 2007a). The nanoparticles are well tolerated except that elevated levels of blood urea nitrogen and creatinine were observed that are indicative of kidney toxicity. Mild elevations in alanine amino transferase and aspartate transaminase at this dose level indicate that the liver is also affected to some extent. Analysis of complement factors does not reveal any changes that are clearly attributable to dosing with the nanoparticle formulation. Overall, no clinical signs of toxicity clearly attributable to treatment were observed. Multiple administrations spanning a period of 17–18 days enable assessment of antibody formation against the human Tf component of the formulation. Low titers of anti-Tf antibodies indicate that this response is not associated with any manifestations of a hypersensitivity reaction upon readministration of the targeted nanoparticle. Taken together, the data show that multiple, systemic doses of targeted nanoparticles containing nonchemically modified siRNA can safely be administered to nonhuman primates.

Further studies have identified an anti-RRM2 siRNA duplex (Calando Pharmaceuticals' CALAA-01, Pasadena, CA, USA) that exhibits significant antiproliferative activity in cancer cells of varying human type and species (mouse, rat, monkey); these findings suggest that this duplex is a promising candidate for therapeutic development (Heidel et al 2007b).

Delivery of siRNA by Nanosize Liposomes

siRNA incorporated into the neutral nanosize liposome 1,2-dioleoyl-sn-glycero-3-phosphatidylcholine (DOPC) has been used for efficient in vivo siRNA delivery. Getting the siRNA to the targeted protein tumor cells, focal adhesion kinase (FAK), is difficult as it is located inside the cell, rather than on the cell surface where most proteins targeted by cancer drugs are found. FAK, which is difficult to target with a drug, can be attacked with the liposomal siRNA approach, which penetrates deeply into the tumor (Halder et al 2006). Mice infected with three human ovarian cancer cell lines derived from women with advanced cancer were treated with liposomes that either contained the FAK siRNA, a control siRNA, or were empty. Some mice received siRNA liposomes plus the chemotherapy docetaxel. Mice receiving the FAK-silencing liposome had reductions in mean tumor weight ranging from 44 to 72% compared with mice in the control groups. Combining the FAK-silencing liposome with docetaxel boosted tumor weight reduction to the 94–98% range. In addition to its anticancer effect, the therapeutic liposome also had an antiangiogenic effect when combined with chemotherapy. By inducing apoptosis among blood vessel cells, the treatment steeply reduced the number of small blood vessels feeding the tumor, cut the percentage of proliferating tumor cells, and increased cell suicide among cancer cells. Two advantages of this approach are as follows:

1. The FAK-targeting liposome ranges between 65 and 125 nm in diameter. Blood vessels that serve tumors are more porous than normal blood vessels, with pores of 100–780 nm wide. The liposomes do not enter the normal blood vessel, whose pores are $\leq$2 nm in diameter.
2. The liposome DOPC has no electrical charge. Its neutrality provides an advantage over positively or negatively charged liposomes when it comes to binding with and penetrating cells.

These studies show the feasibility of siRNA as a clinically applicable therapeutic modality. The next step for the FAK siRNA-DOPC liposome is toxicity testing. In addition to ovarian cancer, FAK is overexpressed in colon, breast, thyroid, and head and neck cancers.

Quantum Dots to Monitor RNAi Delivery

A critical issue in using RNAi for identifying genotype/phenotype correlations is the uniformity of gene silencing within a cell population. Variations in transfection efficiency, delivery-induced cytotoxicity, and "off target" effects at high siRNA concentrations can confound the interpretation of functional studies. To address this problem, a novel method of monitoring siRNA delivery has been developed that combines unmodified siRNA with semiconductor QDs as multicolor biological probes (Chen et al 2005b). siRNA was cotransfected with QDs using standard transfection techniques, thereby leveraging the photostable fluorescent nanoparticles to

track delivery of nucleic acid, sort cells by the degree of transfection, and purify homogenously silenced subpopulations. Compared with alternative RNAi tracking methods (codelivery of reporter plasmids and end-labeling the siRNA), QDs exhibit superior photostability and tunable optical properties for an extensive selection of nonoverlapping colors. This simple, modular system can be used for multiplexed gene knockdown studies, as demonstrated in a two-color proof-of-principle study with two biological targets. When the method was applied to investigate the functional role of T-cadherin (T-cad) in cell–cell communication, a subpopulation of highly silenced cells obtained by QD labeling was required to observe significant downstream effects of gene knockdown.

QDs are compatible with a variety of transfection techniques (other reagents, electroporation, and microinjection) and therefore amenable to nucleic acid monitoring in cells that are susceptible to liposome-triggered cytotoxicity. Primary cells may be particularly suited to benefit from this method, as nonviral delivery of siRNA has to date been technically difficult.

Chapter 6
Nanodevices for Medicine and Surgery

Nanodevices for Clinical Nanodiagnostics

The role of nanotechnology in molecular diagnostics was discussed in Chapter 3. This will have a tremendous impact on the practice of medicine. Apart from laboratory tests, several devices based on nanobiotechnology will be used for clinical diagnostics. Biosensor systems based on nanotechnology could detect emerging disease in the body at a stage that may be curable. This is extremely important in the management of infections and cancer. Some of the body functions and responses to treatment will be monitored without cumbersome laboratory equipments. Some examples are a nanoradiotransmitter small enough to put into a cell and nanoacoustical devices to measure and record the noise a heart makes.

Nanoendoscopy

NanoRobotics Laboratory at the Carnegie–Mellon University (CMU, Pittsburgh, PA) is developing endoscopic microcapsules that can be ingested and precisely positioned. A control system will allow the capsule to attach to the walls of the digestive tract and move within its lumen. Several different methods are being researched for the attachment of microcapsules including both dry and wet adhesion as well as mechanical methods such as a set of tripod legs with adhesive on feet. A simple model with surface characteristics similar to those of the digestive tract will be constructed to test these methods.

Precisely positioned microcapsules would enable physicians to view any part of the inside lining of the digestive tract in detail resulting in a more efficient, accurate, and less invasive diagnosis. In addition, these capsules could be modified to include treatment mechanisms as well, such as the release of a drug or chemical near an abnormal area.

Given Imaging Ltd. (Yoqneam, Israel) has pioneered imaging capsules. Its PillCam^{TM} capsule endoscope, a tool to visualize small intestine abnormalities, was approved in 2001. Other companies are now producing ingestible capsules for this purpose. The patient ingests the capsule, which contains a tiny camera and

K.K. Jain, *The Handbook of Nanomedicine*,

intestinal peristalsis propels the capsule for ~8 h. During this time, the camera snaps the pictures and images that are transmitted to a data recorder worn by the patient. The physicians can review the images later on to make the diagnosis, but some abnormalities may be missed as this method has only a 50 % success rate in detection of diseases. Controlling the positioning and movement on a nanoscale will greatly improve the accuracy of this method. Video capsule endoscopy is a major innovation that provides high-resolution imaging of the entire small intestine in its entirety (Raju and Nath 2005). In the 4 years since its introduction, capsule endoscopy has demonstrated its viability as a first-line investigation in patients with obscure gastrointestinal bleeding after a negative esophagogastroduodenoscopy and colonoscopy, and it has a positive impact on the outcome. Video capsule endoscopy is also useful in the evaluation of inflammatory and neoplastic disorders of the small bowel.

The so-called gutbot in development at CMU is based on nanotechnology including nanosensors and sticking devices. If this device is successful, its use may be extended to the large intestine. Although colon is currently examined by colonoscopy, physicians might be interested in introducing a pill-sized camera through the anus to visualize the suspicious area. Similar nanorobots are under development for other parts of the body.

Application of Nanotechnology in Radiology

X-ray radiation is widely used in medical diagnosis. The basic design of the x-ray tube has not changed significantly in the last century. Now medical diagnostic x-ray radiation can be generated using CNT-based field emission cathode. The device can readily produce both continuous and pulsed x-rays with programmable waveform and repetition rate. The x-ray intensity is sufficient to image a human extremity. The CNT-based cold-cathode x-ray technology can potentially lead to portable and miniature x-ray sources for industrial and medical applications.

Xintek Inc has invented a new x-ray device based on CNTs that emit a scanning x-ray beam composed of multiple smaller beams while also remaining stationary (Zhang et al 2005b). As a result, the device can create images of objects from numerous angles and without mechanical motion, which is a distinct advantage for any machine since it increases imaging speed, can reduce the size of the device, and requires less maintenance. This technology can lead to smaller and faster x-ray imaging systems for tomographic medical imaging such as CT scanners. Other advantages will be that scanners will be cheaper, use less electricity, and produce higher-resolution images.

A device has been constructed that can readily produce both continuous and pulsed x-ray with a programmable waveform and repetition rate (Yue et al 2002). A total emission current of 28 mA was obtained from a 0.2 cm^2 area CNT cathode. The x-ray intensity is sufficient to image human extremity at 14 kVp and 180 mAs. Pulsed x-ray with a repetition rate >100 kHz was readily achieved by programming the gate voltage. The CNT-based cold-cathode x-ray technology can potentially lead

to portable and miniature x-ray sources for industrial and medical applications. It can create high-resolution CT images at less power than traditional scanners. This has potential for developing scanners for medical imaging and homeland security that are smaller, faster, and less expensive to operate.

High-Resolution Ultrasound Imaging Using Nanoparticles

Early diagnoses would involve using nanotechnology to improve the quality of images produced by one of the most common diagnostic tools used in physicians' offices—the ultrasound machine. In a study at the Ohio State University, mice were injected intravenously with silica nanospheres (100 nm) suspensions dispersed in agarose and imaged by a high-resolution ultrasound imaging system (Liu et al 2006). B-mode images of the livers were acquired at different time points after particle injection. An automated computer program was used to quantify the gray-scale changes. Ultrasonic reflections were observed from nanoparticle suspensions in agarose gels. The image brightness, i.e., mean gray-scale level, increased with particle size and concentration. The mean gray scale of mouse livers also increased after particle administration. These results indicated that it is feasible to use solid nanoparticles as contrast-enhancing agents for ultrasonic imaging. The long-term goal is to use this technology to improve the ability to identify cancers and other diseases at a very early stage. The ultimate aim is to identify disease at its cellular level, at its very earliest stage.

Nanobiotechnology and Drug Delivery Devices

There is a need to improve devices introduced into the human body. Some drug delivery devices are implanted in the body for the release of therapeutic substances. The lining of these devices can be improved by nanotechnology. For example, implants can be coated by nanolayers of polymers.

Coating of Implants by Ultrafine Layers of Polymers

Pulsed Laser Assisted Surface Modification (PLASM^{TM}), in development by Nanotherapeutics Inc, is a fast, dry process for applying thin coatings to biomedical devices. Several techniques such as gamma irradiation, plasma treatment, radio-frequency magnetron sputtering, dip-coating, and chemical modification techniques have been researched over the past several years to produce biocompatible coatings onto medical devices such as stents, catheters, vascular grafts, contact lenses, ocular implants, oral implants, hip implants, pacemakers/defibrillators, and bone fixation devices. Unfortunately, these processes are limited by poor control of adhesion and composition, rigorous processing conditions, and long reaction times.

PLASM^{TM} provides a new method for changing the surface properties of implants by depositing ultrafine layers of polymers through a nonaqueous, nonsolvent technique near atmospheric pressure. It is a vapor deposition process that uses a pulsed laser under controlled conditions. Among the many advantages of PLASM^{TM} are control of both the thickness and uniformity of the polymer coating on any surface, as well as including drugs in a biodegradable or biocompatible polymer for release over several days.

Nanotherapeutics Inc is also developing Pulsed Laser Assisted Surface Functionalization (PLASF^{TM}), which is similar to PLASM^{TM}. It is a fast method to create even more complex surfaces, i.e., a protein or catalytic enzyme attached to a polymer coating. PLASF^{TM} has also been used to change the normal structure of a polymer coating, thus imparting novel surface properties, like increasing the wettability or the adhesion properties of an implanted device. Both processes offer tailored surface properties to large or small devices in a matter of minutes.

Nanoencapsulation

The Nanocoat^{TM} process (Nanotherapeutics Inc) is a patented, solvent-free encapsulation system for coating micron- and submicron- size powders. Adapted from commercial fluidization systems, a core nanoparticle/microparticle is excapsulated with a thin layer of a coating material, such as a surfactant or a biodegradable polymer. The coating may be applied to slow the rate of release of an active component, improve the dispersion/flow properties, or increase the absorption into the systemic circulation. The Nanocoat^{TM} process has been shown to provide a method of producing complete, or continuous, coated drug particles of high encapsulation efficiency while requiring minimal processing. The process also has several advantages over current techniques:

- It is a fast process with run-times on the order of minutes.
- A variety of coating materials can be used, making it possible to produce films from materials with proven biocompatibility.
- It is a dry, solvent-free technique that can be conducted under a sterile cGMP conditions.
- Particle agglomeration/adhesion can be minimized by applying coatings that affect the bonding nature and electrostatic charge on the surface.

Formation of microcapsules by depositing coatings onto the particle surface will make it possible to control drug release kinetics by (i) diffusion of the drug though a polymeric coating and (ii) degradation of a biodegradable polymer coating on the drug particles, releasing the core drug material.

GeneSegues' nanocapsules are designed using a flexible formulation process and have the following characteristics:

- The drug is condensed to a small molecule <50 nm in diameter.
- The drug is fully encapsulated in a stable, controlled-release capsule.

- Ability to carry large or small molecules.
- Capsule coating can be made of ligands for receptor-mediated targeted delivery to different organs, tissues, and cells.
- Choices of route of administration include topical application, tablets, intravenous, or via devices.

Researchers from the University of Hamburg and Max Planck Institute in Germany, and the Russian National Research Center for Antibiotics, have made a microcapsule that is able to communicate its progress in delivering drugs to biological tissue. The microcapsule communicates via light waves. The key was finding nontoxic materials that emit infrared light at wavelengths that can easily be measured because they are not absorbed by water and biological tissue. The researchers' prototype microcapsules include some that emit a single wavelength, or color, and others that emit several in the 750- to 1,200-nm range (visible light ranges from 400 nm blue to 700 nm red). These could eventually be used to monitor combinations of drugs.

They made the microcapsules by causing oppositely charged layers of material to form a shell around carbonate particles 3.7 μm in diameter, which is a little smaller than RBC. The researchers added negatively charged light-emitting nanocrystals, which, attracted by positively charged portions of the microcapsule, embedded in the shell. They dissolved the carbonate particles to leave hollow microcapsules. The microcapsules could be ready for use by the year 2008.

Polymer Nanocontainers

DNA-containing polymeric nanocontainers or nanotraps can preserve the full activity of an encapsulated enzyme against hostile outside environments and the release can be controlled according to demand. A phage transfection strategy has been used to demonstrate that DNA translocation is possible across a completely synthetic block copolymer membrane (Graff et al 2002). For this purpose the bacterial channel forming protein LamB was reconstituted in ABA-triblock copolymer vesicles. The outer membrane protein LamB is a specific transporter for maltodextrins but also serves as a receptor for lambda phage to trigger the ejection of lambda phage DNA. The functionality of the LamB protein is fully preserved despite the artificial surrounding. This leads to a type of polymeric vehicle for DNA that could be useful for gene therapy. Nanocontainers with asymmetric membranes can enable direct protein insertion and chemical modification (Stoenescu et al 2004).

Microcontainer Delivery Systems for Cell Therapy

Like gene therapy vectors, cells may deliver therapeutics, but there is also a need for drug delivery systems for cell and gene therapies. Johns Hopkins researchers have devised a self-assembling cube-shaped perforated container, no larger than a dust speck, that could serve as a delivery system for medications and cell therapy

(Gimi et al 2005). The relatively inexpensive microcontainers can be mass-produced through a process that mixes electronic chip-making techniques with basic chemistry. Because of their metallic nature, the cubic container's location in the body could easily be tracked by MRI. In the tests, the hollow cubes housed and then dispensed microbeads and live cells commonly used in medical treatment. This group has developed a new process for fabricating 3D micropatterned containers for cell encapsulation and drug delivery. The long-term goal is to be able to implant a collection of these therapeutic containers directly at the site of an injury or an illness. The microcontainers could someday incorporate electronic components that would allow the cubes to act as biosensors within the body or to release medication on demand in response to a remote-controlled radio-frequency signal.

The tiny cubes are coated with a very thin layer of gold, so that they are unlikely to pose toxicity problems within the body. The microcontainers have not yet been implanted in humans or animals, but the researchers have conducted laboratory tests to demonstrate how they might work in medical applications. Micropipettes are used to insert into the cubes a suspension containing microbeads that are commonly used in cell therapy. These beads could be released from the cubes through agitation. The researchers also inserted human cells, similar to the type used in medical therapy, into the cubes. A positive stain test showed that these cells remained alive in the microcontainers and could easily be released. MRI technology was used to locate and track the metallic cubes as they moved through a sealed microscopic S-shaped fluid channel. This demonstrated that physicians will be able to use noninvasive technology to see where the therapeutic containers go within the body. Some of the cubes (those made mostly of nickel) are magnetic, and it should be possible to guide them directly to the site of an illness or injury. The researchers are now refining the microdevices so that they have nanoporous surfaces. Nanoporous devices can be used for cell encapsulation in hormonal therapy. Biosensors mounted on these devices can be used for noninvasive signal detection.

Nanopore Membrane in Implantable Titanium Drug Delivery Device

An implanted titanium drug delivery device (NanoGATE) uses silicon nanopore membrane to control the release by diffusion of an encapsulated drug at a nearly constant rate. It can achieve nearly zero-order drug kinetics over long periods of time. It is suitable for the delivery of protein and peptide drugs and avoids the poor pharmacokinetics associated with injections, providing an optimized method of delivery for these compounds. The drug can be formulated as a dry powder or a concentrated suspension and maintains its stability. The drug is protected from the immunological reaction of the body by the nanopore membrane, which releases the drug but excludes the entry of unwanted cells.

Measuring the Permeability of Membranes

In order to design molecular transport systems effectively, one needs to know how big the pores in the vehicle's membranes are and how easily the contents can pass through them. This has proved quite difficult. A new method for determining the permeability of thin films has been developed. A molecular beacon immobilized inside a porous silica particle that is subsequently encapsulated within a thin film can be used to determine the size of DNA that can permeate through the film (Johnston and Caruso 2005). Using this technique, it has been determined that over 3 h, molecules >4.7 nm do not permeate 15-nm-thick polyelectrolyte multilayers and after 75 h molecules >6 nm were excluded. This technique has applications for determining the permeability of films used for controlled drug and gene delivery. A molecular beacon made from single DNA strands has been used to measure how easily DNA or genes can pass through the wall of drug delivery particles.

The beacons used are single DNA strands that have a light-emitting molecule (a fluorophore) at one end and a quencher at the other. The DNA strand self-assembles so that the two end segments pair up, forming a loop in the centre—much like the shape of a round-bottomed flask. This is the closed molecular beacon. When the beacon is closed, the fluorophore on one end of the DNA strand is close to the "quencher" on the other end, which stops the fluorophore from giving off light. To determine the permeability of the capsule, the molecular beacons are placed inside the delivery vehicle. If DNA passes through the capsule wall, the beacon opens and the fluorophore emits light. So when DNA passes through the capsule, the beacon is switched "on." If no DNA passes through the capsule, the beacon remains switched off. This technique can be used in the design of intelligent drug delivery systems that can transport medicine to target locations and release the contents in a controlled way.

Nanovalves for Drug Delivery

UCLA chemists have created the first nanovalve that can be opened and closed at will to trap and release molecules (Nguyen et al 2005). A nano valve potentially could be used as a drug delivery system. This nano valve consists of moving parts—switchable rotaxane molecules that resemble linear motors designed by California NanoSystems Institute—attached to a tiny piece of glass (porous silica), which measures about 500 nm. It is big enough to let molecules in and out, but small enough so that the switchable rotaxane molecules can block the hole. The valve is uniquely designed so one end attaches to the opening of the hole that will be blocked and unblocked, and the other end has the switchable rotaxanes whose movable component blocks the hole in the down position and leaves it open in the up position. The researchers used chemical energy involving a single electron as the power supply to open and shut the valve, and a luminescent molecule that allows them to tell from emitted light whether a molecule is trapped or has been released. The nanovalve is much smaller than living cells. A nano valve combined with biomolecules could be inserted into a cell and activated by light to release a drug inside a cell.

Switchable rotaxanes are molecules composed of a dumbbell component with two stations between which a ring component can be made to move back and forth in a linear fashion. Switchable rotaxanes have been used in molecular electronics and are now being adapted for use in the construction of artificial molecular machinery. Further research will test the size hole that can be blocked to see whether larger molecules such as enzymes can be transported inside the container.

Nanochips for Drug Delivery

MicroCHIPS Inc (Bedford, MA) is working on a silver dollar-size device to implant under a patient's skin or in the abdomen that would provide tiny, precise doses of hormones, pain medication, or other pharmaceuticals. The chips, made of silicon or polymer, feature hundreds of tiny micromachined wells that can be loaded with a mixture of medicines. A microcontroller could release small amounts of different chemicals on a customizable schedule. Or biosensors could trigger releases by detecting blood sugar levels or other biochemical conditions. If approved in 4–5 years, such a device could provide diabetics with doses of insulin so that they could forgo daily injections for as much as a year. Or it could help liberate AIDS patients from following complicated daily regimens of multiple medications. To more closely imitate how the body releases hormones, the device could dispense compounds such as estrogen in periodic bursts.

Products currently in development include external and implantable microchips for the delivery of proteins, hormones, pain medications, and other pharmaceutical compounds. Potential advantages of these microchips include small size, low power consumption, absence of moving parts, and the ability to store and release multiple drugs or chemicals from a single device.

Tools for Nanosurgery

Historically, surgery was macrosurgery and most of general surgery still involves gross manipulation of organs and tissues by human hands and handheld instruments. Some branches of surgery such as ophthalmology and otorhinolaryngology started to miniaturize early and start using microsurgery. In the last quarter of the twentieth century, miniaturization started to develop most branches of surgery. The basic feature was minimization of trauma to the body tissues during surgery. Trends were small incisions, laparoscopic surgery by fiber-optic visualization through tubular devices, vascular surgery by catheters, and microsurgery under operating microscopes to refine the procedures and reduce trauma. Many of the devices such as robotics and implants will be a part of this miniaturization process.

Nanotechnology for Hemostasis During Surgery

There are few effective methods to stop bleeding during surgery without causing tissue damage. More than 57 million Americans undergo nonelective surgery each

year, and as much as 50% of surgical time is spent working to control bleeding. Current tools used to stop bleeding include clamps, pressure, cauterization, vasoconstriction, and sponges. MIT and Hong Kong University researchers have shown that some simple liquids composed of peptides, when applied to open wounds in rodents, self-assemble into a nanoscale protective barrier gel that seals the wound and stop bleeding in <15 s. Once the injury heals, the nontoxic gel is broken down into molecules that cells can use as building blocks for tissue repair. The exact mechanism of the solutions' action is still unknown, but one explanation is that the peptides interact with the ECM surrounding the cells. The hemostatic action was demonstrated in open wounds in several different types of tissue: brain, liver, skin, spinal cord, and intestine.

Catheters with Biosensors for Minimally Invasive Surgery

Surgery is continuously moving toward more minimally invasive methods. The main driver of this technical evolution is patient recovery: the lesser the trauma inflicted on the patient, the shorter is the recovery period. Minimally invasive surgery, often performed by use of catheters navigating the vascular system, implies that the operator has little to no tactile or physical information about the environment near or at the surgical site. This information can be provided by biosensors implanted in the catheters. Verimetra Inc is developing such devices. Nanotechnology will play an important role in the construction of miniaturized biosensing devices. These nanosensors improve outcomes, lower risk, and help control costs by providing the surgeon with real-time data about the following:

- Instrument force and performance
- Tissue density, temperature, or chemistry
- Better or faster methods of preparing tissue or cutting tissue
- Extracting tissue and fluids

Examples of procedures and applications where such an approach would be useful are as follows:

- Cardiovascular surgery
- Stent insertion
- Percutaneous transluminal coronary angioplasty
- Coronary artery bypass graft (CABG)
- Atrial fibrillation
- Cardiac surgery in utero
- Cerebrovascular surgery
- Surgery of intracranial aneurysms
- Embolization of intracranial vascular malformations

Nanoscale Laser Surgery

Scalpel and needle may remain adequate instruments for most surgery work, and biological compounds may still be needed to produce cells to certain actions. Introduction of lasers in surgery more than a quarter of century ago has refined surgery and experimental biological procedures to enable manipulations beyond the capacity of the human handheld instruments. Laser microsurgery was used for both ablation and repair of tissues (Jain 1983). Mechanical devices such as microneedles are too large for the cellular scale, while biological and chemical tools can only act on the cell as a whole rather than on any one specific mitochondrion or other structure.

Further developments are leading to manipulation of cellular structures at the micrometer and nanometer scale. This is opening up the field of nanoscale laser surgery.

Femtosecond (one millionth of a billionth of a second) laser pulses can selectively cut a single strand in a single cell in the worm and selectively knock out the sense of smell. One can target a specific organelle inside a single cell (a mitochondrion, e.g., or a strand on the cytoskeleton) and zap it out of existence without disrupting the rest of the cell. The lasers can neatly zap specific structures without harming the cell or hitting other mitochondria only a few hundred nanometers away. It is possible to carve channels slightly $<1\ \mu$m wide, well within a cell's diameter of 10–20 μm. By firing a pulse for only 10–15 s in beams only 1 μm wide, the amount of photons crammed into each burst becomes incredibly intense: 100 quadrillion watts per square meter, 14 orders of magnitude greater than outdoor sunlight. That searing intensity creates an electric field strong enough to disrupt electrons on the target and create a micro-explosion. But because the pulse is so brief, the actual energy delivered into the cell is only a few nanojoules. To achieve the same intensity with nanosecond or millisecond pulses would require so much more energy that the cell would be destroyed.

Femtosecond laser opens the door to researching how cytoskeletons give a cell its shape, or how organelles function independently from each other rather than a whole system. The technology might be scaled up to do surgery without scarring or perhaps to deliver drugs through the skin. NIR femtosecond laser pulses have been applied in a combination of microscopy and nanosurgery on fluorescently labeled structures within living cells (Sacconi et al 2005). Femtolasers are already in use in corneal surgery.

Nanorobotics for Surgery

Robotics is already developing for applications in life sciences and medicine. Robots can be programmed to perform routine surgical procedures. Nanobiotechnology introduces another dimension in robotics, leading to the development of nanorobots also referred to as nanobots. Instead of performing procedures from outside the body, nanobots will be miniaturized for introduction into the body through

the vascular system or at the end of catheters into various vessels and other cavities in the human body. A surgical nanobot, programmed by a human surgeon, could act as an autonomous on-site surgeon inside the human body. Various functions such as searching for pathology, diagnosis, and removal or correction of the lesion by nanomanipulation can be performed and coordinated by an onboard computer. Such concepts, once science fiction, are now considered to be within the realm of possibility. Nanorobots will have the capability to perform precise and refined intracellular surgery that is beyond the capability of manipulations by the human hand.

NanoRobotics Laboratory at the CMU is developing a device for facilitating minimally invasive beating-heart intrapericardial interventions (Riviere et al 2004). This is based on the concept of an endoscopic robotic device that adheres to the epicardium by suction and navigates by crawling like an inchworm to any position on the surface under the control of a surgeon. This approach obviates cardiac stabilization, lung deflation, differential lung ventilation, and reinsertion of laparoscopic tools for accessing different treatment sites, thus offering the possibility of reduced trauma to the patient. The device has a working channel through which various tools can be introduced for treatment. The current prototype demonstrated successful prehension, turning, and locomotion on beating hearts in a limited number of trials in a porcine model.

Chapter 7
Nano-Oncology

Introduction

Application of nanotechnology in cancer can be termed nanooncology. This includes both diagnostics and therapeutics. Various applications in diagnosis and drug delivery for cancer are described in preceding chapters. Two nanotechnology-based products are already approved for the treatment of cancer: Doxil (a liposome preparation of doxorubicin [DOX]) and Abraxane (paclitaxel in nanoparticle formulation). Approximately 150 drugs in development for cancer are based on nanotechnology. Some of the nanotechnologies and their applications in developing cancer therapies are described in this chapter. The most important factor in the fight against cancer, besides prevention, is early detection. Cancer is easier to treat and less likely to develop drug resistance when treatment is started very early. Cancer cells in very early stages are less likely to have mutations that make them resistant to treatment.

Nanotechnology for Detection of Cancer

Nanobiotechnology offers a novel set of tools for detection of cancer. A workshop held at the National Institutes of Standards and Technology of United States in 2001 made the following recommendations (Srinivas et al 2002):

1. Nanotechnology can complement existing technologies and significantly contribute to cancer detection, prevention, diagnosis, and treatment.
2. Nanotechnology would be extremely useful in the area of biomarker research and provide additional sensitivity in assays with relatively small sample volumes.
3. Specific nanotechnology applications that would impact on biomarker research are (i) nanostructures such as biopores, (ii) nanoprobes such as scanning tunnel microscopy, (iii) nanosources such as laser-induced fluorescence, and (iv) nanomaterials such as QDs.

K.K. Jain, *The Handbook of Nanomedicine*,

QDs for Cancer Diagnosis

QDs, coated with a polyacrylate cap and covalently linked to antibodies or streptavidin, have been used for immunofluorescent labeling of breast cancer marker Her2 (Wu et al 2003). Labeling was highly specific and was brighter and more stable than that of other fluorescent markers. Recent advances have led to QD bioconjugates that are highly luminescent and stable. These bioconjugates raise new possibilities for studying genes, proteins, and drug targets in single cells, tissue specimens, and even in living animals and enable visualization of cancer cells in living animals (Gao and Nie 2003). Her2 and a method for detecting protein analytes has been developed that relies on magnetic microparticle probes with antibodies that specifically bind a target of interest (PSA in case of prostate cancer).

QDs can be combined with fluorescence microscopy to follow cells at high resolution in living animals. These offer considerable advantages over organic fluorophores for this purpose. QDs and emission spectrum scanning multiphoton microscopy have been used to develop a means to study extravasation in vivo (Voura et al 2004). Tumor cells labeled with QDs were intravenously injected into mice and followed as they extravasated into lung tissue. The behavior of QD-labeled tumor cells in vivo was indistinguishable from that of unlabeled cells. QDs and spectral imaging allowed the simultaneous identification of five different populations of cells using multiphoton laser excitation.

Dendrimers for Sensing Cancer Cell Apoptosis

Investigators at the University of Michigan have developed a nanoscale sensor to detect apoptosis or programmed cell death. This sensor is built on the same biocompatible polymeric dendrimer platform they have used to image and treat tumors. Most approaches to detect apoptosis rely on the human protein annexin V, which binds to a hidden cell membrane component revealed in the initial stages of apoptosis. The team took a different approach, looking for the appearance of a protein called caspase-3, an enzyme activated early in the apoptosis process. This enzyme cleaves the bond between two specific amino acids, and researchers have capitalized on this specificity to design fluorescence-based assays for caspase-3: for example, FRET, which generates a bright fluorescent signal only when a specific chemical bond breaks. In this case, fluorescence appears only when caspase-3 breaks a valine-aspartic acid bond in a specially designed substrate for this enzyme. To create their tumor-specific apoptosis detector, the investigators attached folic acid and the caspase-3 substrate to a so-called PAMAM dendrimer. Folic acid acts as a tumor-targeting agent, binding to a folic acid that many types of tumor cells produce in abundance. When the researchers added this dendrimer to apoptotic tumor cells bearing this folic acid receptor, the cells took up the dendrimer and fluoresced brightly. In contrast, apoptotic cells lacking the folic acid receptor did not fluoresce. The investigators note that they have recently developed an optical

fiber device capable of detecting FRET emissions in tumors. They have used this device to quantify apoptosis in live mice with tumors bearing the folic acid receptor.

Gold Nanoparticles for Cancer Diagnosis

Gold nanoparticles conjugated to monoclonal anti-epidermal growth factor receptor (anti-EGFR) antibodies after incubation in cell cultures with malignant as well as nonmalignant epithelial cell lines. However, the anti-EGFR MAb-conjugated nanoparticles specifically and homogeneously bind to the surface of the cancer-type cells with 600% greater affinity than to the noncancerous cells. This specific and homogeneous binding is found to give a relatively sharper SPR absorption band with a red-shifted maximum compared with that observed when added to the noncancerous cells (El-Sayed et al 2005). The particles that worked the best were 35 nm in size. These results suggest that SPR scattering imaging or SPR absorption spectroscopy generated from antibody-conjugated gold nanoparticles can be useful in molecular biosensor techniques for the diagnosis and investigation of oral epithelial living cancer cells in vivo and in vitro. Advantages of this technique are as follows:

- It is not toxic to human cells. A similar technique using QDs uses semiconductor crystals to mark cancer cells, but the semiconductor material is potentially toxic to the cells and humans.
- It does not require expensive high-powered microscopes or lasers to view the results. All it needs is a simple, inexpensive microscope and white light.
- The results are instantaneous. If a cancerous tissue is sprayed with gold nanoparticles containing the antibody, the results can be seen immediately. The scattering is so strong that a single particle can be detected.

Gold nanoparticles can be heated rapidly whenever exposed to infrared light of the right wavelength. Heating of the gold results in a varying pressure near the particle. This pressure change in turn is expressed in the generation of ultrasound—a phenomenon called plasmon resonance. The shape of the particles determines the wavelength at which this happens. In this way, light from a laser results in sound. By attaching MAbs to gold nanoparticles or nanorods, which can recognize a specific cancer cell, the heating phenomenon can be used in cancer detection. This acoustic signal gives valuable information about the presence of cancer cells. Scientists at the University of Twente (UT) in the Netherlands expect better results with this approach than is currently possible with imaging techniques. The temperature rise can be up to 100°C. Photothermal therapy would use the heated gold to destroy the tumor. Another option would be to include gold particles in capsules filled with cancer medication: the capsule attaches to the cancer cell, it is heated, and the medicine is released locally. Both diagnostic and therapeutic applications will be investigated by the UT scientists together with colleagues from the Erasmus Medical Center in Rotterdam and two companies: Esoate Europe and Luminostix.

Nanotubes for Detection of Cancer Proteins

Scientists at the University of Delaware, the Thomas Jefferson University (Philadelphia, PA), and Christiana Care Hospital (Wilmington, DE) are developing single-wall carbon nanoatomic tubes for monitoring cancer-specific proteins. These are hundreds of times smaller than nanocantilevers, highly sensitive to single-protein-binding events, and can be massively multiplexed with millions of tubes per chip for proteomic profiling. The tubes have extraordinary strength, unique electronic properties, and the ability to tag cancer-specific proteins to their surface. These tubes can be fabricated by decomposition of carbon-based gas in a furnace, using iron nanoparticles as catalyst material. With diameter of 1 nm and length of 1 μm, these tubes are smaller than a ss DNA. In other words, this tube is an atomic arrangement of one layer of carbon atoms, which are on the surface. Protein-binding events occurring on the surface of these nanoatomic tubes produce a measurable change in the mechanical and electrical properties.

By coating the surfaces of tiny CNTs with MAbs, biochemists and engineers at Jefferson Medical College (Philadelphia, PA) and the University of Delaware have teamed up to detect cancer cells circulating in the blood. The group took advantage of a surge in electrical current in nanotube–Ab networks when cancer cells bind to the Abs. They placed microscopic CNTs between electrodes and then covered them with MAbs that home in on target protein "antigens" on the surface of cancer cells. The Abs were specific for insulin-like growth factor-1 receptor (IGF-1), which is commonly found at high levels on cancer cells. They then measured the changes in electrical current through the Ab–nanotube combinations when two different types of breast cancer cells were applied to the devices. The increase in current through the Ab–nanotube devices was proportional to the number of receptors on the cancer cell surfaces. One type, human BT474 breast cancer cells, which do not respond to estrogen, had moderate IGF-1R levels, while the other type, MCF7, which needs estrogen to grow, had high IGF-1R levels. The BT474 cancer cells, which had less IGF-1R on their surfaces, caused a 3-fold jump in current. The MCF7 cells showed an 8-fold increase.

This method could be used for detection of recurring circulating tumor cells or micrometastases remaining from the originally treated tumor. The technique could be cost-effective and could diagnose whether cells are cancerous or not in seconds versus hours or days required for conventional histology examination. It will enable large-scale production methods to make thousands of sensors and have microarrays of these to detect the fingerprints of specific kinds of cancer cells. Ultimately, the researchers would like to design an assay that can detect cancer cells circulating in the human bloodstream on a handheld device no bigger than a cell phone. Limitation of the technique is that it may not detect more than one antigen at a time on a single cell.

Nanoparticles for the Optical Imaging of Tumors

A fluorescent peptide–magnetic nanoparticle conjugate has been designed to image E-selectin expression in mouse xenograft models of Lewis lung carcinoma (LLC)

by fluorescence reflectance imaging (Funovics et al 2005). It was synthesized by attaching the E-selectin-binding peptide (ESBP) to a CLIO(Cy5.5) nanoparticle to yield ESBP-CLIO(Cy5.5). Internalization by activated human umbilical vein endothelial cells (HUVECs) was rapid and specifically mediated by E-selectin, indicated by the lack of uptake of nanoparticles bearing similar numbers of a scrambled peptide. E-selectin expression in both endothelial cells and cancer cells in human prostate cancer specimens was demonstrated by immunohistochemistry. Thus ESBP-CLIO(Cy5.5) is a useful probe for imaging E-selectin associated with the LLC tumor, which is expressed not only on endothelial cells but also on LLC cells and human prostate cancer specimens.

Nanolaser Spectroscopy for Detection of Cancer in Single Cells

Nanolaser scanning confocal spectroscopy can be used to identify a previously unknown property of certain cancer cells that distinguishes them with single-cell resolution from closely related normal cells (Gourlay et al 2005). This property is the correlation of light scattering and spatial organization of mitochondria; normally, it is well scattered, but in cancer cells the mitochondria are disorganized and scatter light poorly. These optical methods are promising powerful tools for detecting cancer at an early stage.

Nanotechnology-Based Single-Molecule Assays for Cancer

Information about the biological processes in living cells is required for the detection and diagnosis of cancer for the following reasons:

- To recognize the important changes, which occur when cells undergo malignant transformation.
- There are situations when primary cells from a surgical procedure cannot be propagated due to the type of cell or the low number of cells available.
- Detection of cancer at an early stage is a critical step for improving cancer treatment.

Early detection will require sensitive methods for isolating and interrogating individual cells with high spatial and temporal resolution without disrupting their cellular biochemistry. Probes designed to penetrate a cell and report on the conditions within that cell must be sufficiently small, exceedingly bright, and stable for a long time in the intracellular environment without disrupting the cell's normal biochemical functioning. A series of silver nanoparticles have been prepared that meet many of the criteria listed above (Xu and Patel 2005). Although <100 nm in diameter, these particles are bright enough to be seen by eye using optical microscopy. Unlike fluorophores, fluorescent proteins, or QDs, silver nanoparticles do not photodecompose during extended illumination. Therefore, they can be used as a probe to continuously monitor dynamic events in living cells during studies that last for weeks or even months. Because the color of the scattered light from

nanoparticles depends upon their size, they have been used to measure the change in single-membrane pores in real time using dark-field optical microscopy. Intracellular and extracellular nanoparticles can also be differentiated by the intensity of light scattering. Next challenge is to develop methods for modifying the surface of the nanoparticles to make them more biocompatible, so that biological processes can be observed without disturbing or destroying the cell's intrinsic biochemical machinery. Ultimately, these probes may be combined to produce highly sensitive assays with high spatial and temporal resolution. This advance will enable researchers to study the interactions of multiple genes in the same cell simultaneously by using different-colored reporter molecules. In addition to transcription and translation, similar live-cell single-molecule assays will offer the prospect of studying more complex cellular processes, such as cell signaling. Continuous advances and evolution along these research fronts are necessary to unravel biochemical processes in vivo, and to develop tools that can be used to detect and diagnose cancer using only a single cell from the patient.

Implanted Magnetic Sensing for Cancer

An implant for magnetic sensing for cancer, now in development at the MIT, contains nanoparticles that can be designed to test for different substances, including metabolites such as glucose and oxygen that are associated with tumor growth. It can also be used to test the effects of anticancer drugs in individual patients; the implant could reveal how much of a drug has reached the tumor. The nanoparticles are encased in a silicone delivery device, enabling their retention in patients' bodies for an extended period of time. The device can be implanted directly into a tumor, allowing a more direct look at what is happening in the tumor over a period of time. The technique makes use of detection nanoparticles composed of iron oxide and coated with dextran. Antibodies specific to the target molecules are attached to the surface of the particles. When the target molecules are present, they bind to the particles and cause them to clump together. That clumping can be detected by MRI. The nanoparticles are trapped inside the silicone device, which is sealed off by a porous membrane. The membrane allows molecules <30 nm to get in, but the detection particles are too large to get out. In addition to monitoring the presence of chemotherapy drugs, the device could also be used to check whether a tumor is growing or shrinking, or whether it has spread to other locations, by sensing the amount and location of tumor biomarkers. Preclinical testing is planned for this device human chorionic gonadotropin that can be considered a biomarker for cancer because it is produced by tumors but not normally found in healthy individuals except in pregnant women.

Nanowire Biochips for Early Detection of Cancer Biomarkers

Cancer cells themselves may be difficult to detect at an early stage, but they leave a fingerprint, i.e., a pattern of change in biomarker proteins that circulate in the blood.

There may be 20–25 biomarkers, which may require as many as 500 measurements, all of which should be made from a drop of blood obtained by pinprick. Thus nanoscale diagnostics will play an important role in this effort.

Nanowire biosensors are in development for very early diagnosis of cancer, when there are just a few thousand cells. Nanowires can electronically detect a few protein molecules along with other biochemical markers that are early signs of cancer. Nanowires in a set are coated with different compounds, each of which binds to a particular biomarker and changes the conductivity of the nanowire that can be measured. Thousands of such nanowires are combined on a single chip that enables identification of the type of cancer. Currently, such a biochip can detect between 20 and 30 biomarkers and is being used for the early diagnosis of brain cancer.

Nanotechnology-Based Imaging for Management of Cancer

The role of nanotechnology in diagnostic imaging of cancer, particularly MRI, has already been described in Chapter 3. Nanotechnology-based cancer imaging will lead to sensitive and accurate detection of early-stage cancer. Nanoparticle-enabled imaging can help accurate delivery of cancer therapy.

Nanoparticle-MRI for Tracking Dendritic Cells in Cancer Therapy

Scientists at Johns Hopkins developed several techniques that allow an effective cellular internalization of clinical SPIO formulations without affecting cell proliferation, differentiation, and function, with "magnetoelectroporation" being the most recent labeling paradigm. Animal studies have shown that the MR distribution pattern is reliable when cells have limited cell division, as validated by conventional histological techniques. Magnetically labeled stem cells are not yet in clinical use because of safety concerns about the in vivo behavior of stem cells. Regarding other therapeutic cells, a clinical study using SPIO-labeled dendritic cells has now been completed in Europe (de Vries et al 2005). Autologous dendritic cells were labeled with a clinical SPIO formulation or ^{111}In-oxine and were coinjected intranodally in melanoma patients under ultrasound guidance. This phase I trial has shown the feasibility and safety of imaging intranodal cell trafficking in patients. In contrast to scintigraphic imaging, MRI allowed assessment of the accuracy of dendritic cell delivery and of inter- and intranodal cell migration patterns MRI cell tracking using iron oxides appears clinically safe and well suited to monitor cellular therapy in humans. It is believed that MRI cell tracking will become an important technique that someday may become a routine in standard radiological practice once stem cell therapy enters clinical practice.

Nanoparticle-CT scan

Use of nanomaterials for one of the most common imaging techniques, CT, has remained unexplored. Current CT contrast agents are based on small iodinated

molecules. They are effective in absorbing x-rays, but nonspecific distribution and rapid pharmacokinetics have rather limited their microvascular and targeting performance. While most of the nanoparticles are designed to be used in conjunction with MRI, bismuth sulfide (Bi_2S_3) nanoparticles naturally accumulate in lymph nodes containing metastases and show up as bright white spots in CT images (Rabin et al 2006). A polymer-coated Bi_2S_3 nanoparticle preparation has been proposed as an injectable CT imaging agent. This preparation demonstrates excellent stability at high concentrations, high x-ray absorption (5-fold better than iodine), very long circulation times (>2 h) in vivo and an efficacy/safety profile comparable to or better than iodinated imaging agents. The utility of these polymer-coated Bi_2S_3 nanoparticles for enhanced in vivo imaging of the vasculature, the liver, and lymph nodes has been demonstrated in mice. These nanoparticles and their bioconjugates are expected to become an important adjunct to in vivo imaging of molecular targets and pathological conditions. Tumor-targeting agents are now being added to the surfaces of these polymer-coated Bi_2S_3 nanoparticles.

QDs Aid Lymph Node Mapping in Cancer

Using QDs that emit NIR light, researchers have developed an improved method for performing sentinel lymph node biopsy, which depends on illuminating lymph nodes during cancer surgery (Kim et al 2004). The infrared QDs were developed and synthesized at the MIT Department of Chemistry in collaboration with Quantum Dot Corporation, which is now taken over by Invitrogen Corporation. The first challenge was that the particles had to be rendered soluble, which was achieved by using a polydentate phosphene coating. The QDs were engineered to emit NIR light, a part of the spectrum that is transmitted through biological tissue with minimal scattering. The study describes how the QDs were injected into live pigs and followed visually to the lymph system just beneath the skin of the animals. The new imaging technique allowed the surgeons to clearly see the target lymph nodes without cutting the animals' skin. Sentinel lymph node (SLN) mapping, the surgical technique employed in the study, is a common procedure used to identify the presence of cancer in a single, "sentinel" lymph node, thus avoiding the removal of a patient's entire lymph system. SLN mapping relies on a combination of radioactivity and organic dyes, but the technique is inexact during surgery, often leading to the removal of much more of the lymph system than necessary, causing unwanted trauma. This study is a significant improvement over the dye/radioactivity method currently used to perform SLN mapping.

Throughout the procedure, the QDs were clearly visible using the imaging system, allowing the surgeon to see not only the lymph nodes, but also the underlying anatomy. The imaging system and QDs allowed the pathologist to focus on specific parts of the SLN that would most likely contain malignant cells, if cancer were present. The imaging system and QDs minimized inaccuracies and permitted real-time confirmation of the total removal of the target lymph nodes, drastically reducing the potential for repeated procedures.

Different varieties of PEG-coated QDs have been injected directly into tumors in mouse models of human cancer and their course has been tracked through the skin using NIR fluorescence microscopy to image and map SLNs (Ballou et al 2007). In tumors that drained almost immediately to the SLNs, the QDs were confined to the lymphatic system, mapping out the connected string of lymph nodes. This provided easy tagging of the SLNs for pathology and there was little difference in results among the different QD types used. Examination of the SLNs identified by QD localization showed that at least some contained metastatic tumor foci. The animals used in this study have been followed for over 2 years, with no evidence of toxicity, even though QDs can still be observed within the animals.

SLN mapping has already revolutionized cancer surgery. NIR QDs have the potential to improve this important technique even further. Because the QDs in the study are composed of heavy metals, which can be toxic, they have not yet been approved for use in humans. The next step is to develop QDs that can be used safely in human trials.

Nanoparticles Designed for Dual-Mode Imaging of Cancer

Scientists at Yonsei University, Korea, have combined the best characteristics of QDs and magnetic iron oxide nanoparticles to create a single nanoparticle probe that can yield clinically useful images of both tumors and the molecules involved in cancer (Choi et al 2006). They start by synthesizing 30-nm-diameter silica nanoparticles impregnated with rhodamine, a bright fluorescent dye, and 9-nm-diameter water-soluble iron oxide nanoparticles. They then mix these two nanoparticles with a chemical linker, yielding the dual-mode nanoparticle. On average, 10 magnetic iron oxide particles link to a single-dye-containing silica nanoparticle, and the resulting construct is $\sim$45 nm in diameter. The combination nanoparticle performed better in both MRI and fluorescent imaging tests than did the individual components. In MRI experiments, the combination nanoparticle generated an MRI signal that was over 3-fold more intense than did the same number of iron oxide nanoparticles. Similarly, the fluorescent signal from the dual-mode nanoparticle was almost twice as bright as that produced by dye molecules linked directly to iron oxide nanoparticles. Next, the researchers labeled the dual-mode nanoparticles with an antibody that binds to molecules known as polysialic acids, which are found on the surface of certain nerve cell and lung tumors. These targeted nanoparticles were quickly taken up by cultured tumor cells and were readily visible using fluorescence microscopy.

Role of Nanoparticle-Based Imaging in Oncology Clinical Trials

Currently, CT scans are used as surrogate end points in cancer clinical trials. The size of the tumor gives only limited information about the effectiveness of therapy. New imaging agents could speed the clinical trials process in two ways: (i) better

imaging data could help oncologists better select which therapies to use on a particular patient and (ii) increasingly sensitive and specific imaging agents will be able to provide real-time information about whether a therapy is working. Currently, oncologists and their patients must wait months to determine whether a given therapy is working. Shorter clinical trials would mean that effective new drugs would reach patients quicker and ineffective drugs would be dropped from clinical trials sooner, allowing drug discoverers to better focus their efforts on more promising therapies.

Nanotechnology for Cancer Therapy

Nanoparticles for Targeting Tumors

Nanoparticles can deliver chemotherapy drugs directly to tumor cells and then give off a signal after the cells are destroyed. According to the work done in 2004 at Center for Biological Nanotechnology of the University of Michigan, drugs delivered this way are 100 times more potent than standard therapies. Tests in mice have been encouraging, and human trials are expected within a couple of years.

Scientists at Nanoprobes Inc (Yaphank, NY, USA) and University of Connecticut Health Center have shown that gold nanoparticles can help x-rays kill cancerous cells more effectively in mice (Hainfeld et al 2004). The team hopes to refine the technique so that it will eventually work on humans. First cancer cells were injected into the mice, followed by a salt solution containing gold nanoparticles. Two minutes later, the mice were irradiated with high-energy (250 kV) x-rays. The team found that the combination of nanoparticles followed by x-ray treatment reduced the size of the tumors, or completely eradicated them, whereas tumors that had received only x-ray therapy continued to grow. The gold nanoparticles had no therapeutic effect on their own. Another finding was that the 1-year survival rate for the combined treatment was 86%, compared with 20% for x-ray therapy alone, and zero for nanoparticles without x-rays. The technique works because gold, which strongly absorbs x-rays, selectively accumulates in tumors. This increases the amount of energy that is deposited in the tumor compared with nearby normal tissue. The team now plans to improve targeting of the nanoparticles to tumors and to work toward applications for humans. Since the gold also shows up on CT and planar x-rays, it can be useful for early imaging and detection of tumors. An x-ray manufacturer is considering a modification that would optimize gold nanoparticle radiotherapy for patients.

Efficient conversion of strongly absorbed light by plasmonic gold nanoparticles to heat energy and their easy bioconjugation suggest their use as selective photothermal agents in molecular cancer cell targeting (El-Sayed et al 2006). Two oral squamous carcinoma cell lines and one benign epithelial cell line were incubated with anti-EGFR antibody-conjugated gold nanoparticles and then exposed to continuous visible argon ion laser at 514 nm. Malignant cells required less than half the laser energy to be killed than the benign cells after incubation with anti-EGFR

antibody-conjugated gold nanoparticles. No photothermal destruction was observed for all types of cells in the absence of nanoparticles at four times energy required to kill the malignant cells with anti-EGFR/Au conjugates bonded. Au nanoparticles thus offer a novel class of selective photothermal agents using a CW laser at low powers. The ability of gold nanoparticles to detect cancer was demonstrated previously. Now it will be possible to design an "all in one" active agent that can be used to noninvasively find the cancer and then destroy it. This selective technique has a potential in molecularly targeted photothermal therapy in vivo.

Nanoshell-Based Cancer Therapy

Nanoshells for Thermal Ablation in Cancer

Metal nanoshells belong to a class of nanoparticles with tunable optical resonances that have been used for thermal ablative therapy for cancer. Nanoshells can be tuned to strongly absorb light in the NIR, where optical transmission through tissue is optimal. Nanoshells placed at depth in tissues can be used to deliver a therapeutic dose of heat by using moderately low exposures of extracorporeally applied NIR. In vivo studies under magnetic resonance guidance have revealed that exposure to low doses of NIR light in solid tumors treated with metal nanoshells reached average maximum temperatures capable of inducing irreversible tumor destruction within 4–6 min (Hirsch et al 2003). Gold nanoshells are ~120 nm in diameter and a cancer cell is 170 times bigger. Therefore, nanoshells can penetrate the tumor capillaries and lodge in the tumor. Application of NIR light, which passes through the skin harmlessly, heats the nanoshells and kills the tumor cells. Since no drug is used, the cancer cells are unlikely to develop drug resistance.

The ability to control both wavelength-dependent scattering and absorption of nanoshells offers the opportunity to design nanoshells that provide both diagnostic and therapeutic capabilities in a single nanoparticle. A nanoshell-based all-optical platform technology can integrate cancer imaging and therapy applications. Immunotargeted nanoshells, engineered both to scatter light in the NIR range enabling optical molecular cancer imaging and to absorb light, enable selective destruction of targeted carcinoma cells through photothermal therapy (Loo et al 2005). In a proof-of-principle experiment, dual-imaging/therapy immunotargeted nanoshells were used to detect and destroy breast carcinoma cells that overexpress HER2, a clinically relevant cancer biomarker. This approach has some significant advantages over alternatives that are under development. For example, optical imaging is much faster and less expensive than other medical imaging techniques. Gold nanoparticles are also more biocompatible than other types of optically active nanoparticles, such as QDs.

Nanospectra Biosciences Inc (Houston, TX, USA) is already developing nanoshells for the targeted destruction of various cancers using Nanoshells (AuroShell^{TM}). AuroLase^{TM} Cancer Therapy combines the unique physical and optical properties of AuroShell^{TM} microparticles with an NIR laser source to thermally destroy cancer

cells without significant damage to surrounding tissue. AuroShell™ microparticles are injected intravenously and they specifically collect in the tumor through the associated leaky vasculature (the enhanced permeability and retention effect, or EPR). After the particles accumulate in a tumor, the area is illuminated with an NIR laser at wavelengths chosen to allow the maximum penetration of light through tissue. Unlike solid metals and other materials, AuroShell™ microparticles are designed to specifically absorb this wavelength, converting the laser light into heat. This results in the rapid destruction of the tumor along its irregular boundaries. The basics of this approach have been tested experimentally.

The blood vessels inside tumors develop poorly, allowing small particles like nanoshells to leak out and accumulate inside tumors. An animal trial involved 25 mice with tumors ranging in size from 3 to 5.5 mm. The mice were divided into three groups. The first group was given no treatment. The second received saline injections, followed by 3 min exposure to NIR laser light. The final group received nanoshell injections and laser treatments. In the test, researchers injected nanoshells into the mice, waited 6 h to give the nanoshells time to accumulate in the tumors and then applied a 5-mm-wide laser on the skin above each tumor. Surface temperature measurements taken on the skin above the tumors during the laser treatments showed a marked increase that averaged about 46°F (7.7°C) for the nanoshells group. There was no measurable temperature increase at the site of laser treatments in the saline group. Likewise, sections of laser-treated skin located apart from the tumor sites in the nanoshells group also showed no increase in temperature, indicating that the nanoshells had accumulated as expected within the tumors. All signs of tumors disappeared in the nanoshells group within 10 days. These mice remained cancer-free after treatment. Tumors in the other two test groups continued to grow rapidly. All mice in these groups were euthanized when the tumors reached 10 mm in size. The mean survival time of the mice receiving no treatment was 10.1 days; the mean survival time for the group receiving saline injections and laser treatments was 12.5 days. The advantages of Nanoshell-based tumor cell ablation include the following:

- Targeting to specific cells and tissues to avoid damage to surrounding tissue
- Less adverse effects than targeted chemotherapeutic agents or PDT
- Repeatability because of lack of "tissue memory" as in radiation therapy and biocompatibility
- Ability to treat such as glioblastoma multiforme, metastases, and inoperable tumors.

Nanoshells Combined with Targeting Proteins

Nanoshells may be combined with targeting proteins and used to ablate target cells. This procedure can result in the destruction of solid tumors or possibly metastases not otherwise observable by the oncologist. In addition, Nanoshells can be utilized to reduce angiogenesis present in cancer. Experiments in animals in vitro and in tissue demonstrate that specific cells (e.g., cancer cells) can be targeted and destroyed

by an amount of infrared light that is otherwise not harmful to surrounding tissue. This procedure may be performed using an external (outside the body) infrared laser. Prior research has indicated the ability to deliver the appropriate levels of infrared light at depths of up to 15 cm, depending upon the tissue. Photothermal tumor ablation in mice has been achieved by using NIR-absorbing nanoparticles (O'Neal et al 2004). The advantages of Nanoshell-based tumor cell ablation are as follows:

- Targeting to specific cells and tissues to avoid damage to surrounding tissue
- Superior side-effect profile than targeted chemotherapeutic agents or PDT
- Repeatability because of
  - no "tissue memory" as in radiation therapy
  - biocompatibility
  - ability to treat metastases and inoperable tumors

Nanoshells enabled a seamless integration of cancer detection and therapy. The US Department of Defense awarded Prof. Naomi Halas of Rice University $3 million innovator's grant in 2004 to develop breast cancer therapies using nanoshells.

Nanobody-Based Cancer Therapy

A nanobody with subnanomolar affinity for the human tumor-associated carcinoembryonic antigen (CEA) has been identified (Cortez-Retamozo et al 2004). This nanobody was conjugated to *Enterobacter cloacae* beta-lactamase, and its site-selective anticancer prodrug activation capacity was evaluated. The conjugate was readily purified in high yields without aggregation or loss of functionality of the constituents. In vitro experiments showed that the nanobody–enzyme conjugate effectively activated the release of phenylenediamine mustard from the cephalosporin nitrogen mustard prodrug 7-(4-carboxybutanamido) cephalosporin mustard at the surface of CEA-expressing LS174T cancer cells. In vivo studies demonstrated that the conjugate had an excellent biodistribution profile and induced regressions and cures of established tumor xenografts. The easy generation and manufacturing yield of nanobody-based conjugates together with their potent antitumor activity make nanobodies promising vehicles for new-generation cancer therapeutics.

Nanobomb for Cancer

The University of Delaware scientists have developed a unique nanobomb that can literally blow up tumors. The research started with the use of CNTs as drug delivery vehicles. While experimenting with the molecules and considering optical and thermal properties, they found that they could trigger microscopic explosions of nanotubes in a wide variety of conditions. Explosions in air of loosely packed nanotubes are known to occur in an oxygen environment. However, the localized thermal energy imbalance sets off explosions that are intrinsic in nature. The nanobombs are

tiny bombs on nanoscale, which are selective, localized, and minimally invasive. Like cluster bombs, they start exploding one after another once they are exposed to light and the resulting heat. The nanobomb holds great promise as a therapeutic agent for killing cancer cells, particularly breast cancer cells, because its shockwave kills the cancerous cells as well as the biological pathways that carry instructions to generate additional cancerous cells and the small blood vessels that nourish the tumor. Its effect can be spread over a wide area to create structural damage to the surrounding cancer cells. In another approach, Nanoclusters (gold nanobombs) can be activated in cancer cells only by confining NIR laser pulse energy within the critical mass of the nanoparticles in the nanocluster (Zharov et al 2005). Once the nanobombs are exploded, they kill cancer cells and macrophages can effectively clear the cell debris and the exploded nanotube along with it.

Nanobiotechnology-Based Drug Delivery in Cancer

Drug delivery in cancer is important for optimizing the effect of drugs and reducing toxic side effects. Several nanobiotechnologies, mostly based on nanoparticles, have been used to facilitate drug delivery in cancer (Jain 2005d). A classification of the nanotechnologies for drug delivery in cancer is summarized in Table 7.1.

Nanoparticle Formulations for Drug Delivery in Cancer

Anticancer Drug Particles Incorporated in Liposomes

Several injectable and biodegradable systems have been synthesized based on incorporation of antiestrogens (AEs) in nanoparticles and liposomes. Both nanospheres and nanocapsules (polymers with an oily core in which AEs were solubilized) incorporated high amounts of 4-hydroxy-tamoxifen (4-HT) or RU 58668 (Maillard et al 2005). Liposomes containing various ratios of lipids enhanced the apoptotic activity of RU 58668 in several multiple myeloma cell lines tested by flow cytometry. These cell lines expressed both estrogen receptor alpha and beta subtypes. RU-loaded liposomes administered intravenously in an animal model induced the arrest of tumor growth. Thus, the drug delivery of AEs enhances their ability to arrest the growth of tumors which express estrogen receptors and are of particular interest for estrogen-dependent breast cancer treatment. In addition, it represents a new potent therapeutic approach for multiple myeloma.

SuperFluids™ technology (Aphios Corporation) is based on advanced liquid–liquid technology using supercritical or near-critical fluids with or without cosolvents. The patented technology can be utilized to form stable biocompatible aqueous formulations of poorly soluble anticancer drugs such as paclitaxel and camptothecin (CPT). The process has been used for the nanoencapsulation of paclitaxel in a formulation called Taxosomes™, which has been tested in nude mice with breast cancer xenografts. Taxosomes™ will lead to (i) enhanced therapeutic

Table 7.1 Classification of nanobiotechnology approaches to drug delivery in cancer

Nanoparticles
Nanoparticle formulations of anticancer drugs, e.g., paclitaxel
Exosomes for cancer drug delivery
Nanoencapsulation and enclosure of anticancer drugs
Enclosing drugs in lipid nanocapsules
Encapsulating drugs in hydrogel nanoparticles
Micelles for drug delivery in cancer
Targeted delivery of anticancer therapy
Targeted drug delivery with nanoparticles
Pegylated nanoliposomal formulation
Folate-linked nanoparticles
Carbon magnetic nanoparticles for targeted drug delivery in cancer
Targeted drug delivery with nanoparticle–aptamer bioconjugates
Nanodroplets for site-specific cancer treatment
Lipid-based nanocarriers
Targeted antiangiogenic therapy using nanoparticles
Nanoparticles for delivery of drugs to brain tumors
Combination of nanoparticles with radiotherapy
Combination with boron neutron capture therapy
Nanoengineered silicon for brachytherapy
Combination with physical modalities of cancer therapy
Combination with laser ablation of tumors
Combination with photodynamic therapy
Combination with thermal ablation
Combination with ultrasound
Nanoparticle-mediated gene therapy
p53 gene therapy of cancer
Immunolipoplex for delivery of p53 gene
Intravenous delivery of FUS_1 gene
Strategies combining diagnostics and therapeutics
Nanoshells as adjuncts to thermal tumor ablation
Perfluorocarbon nanoparticles
Nanocomposite devices

efficacy (ii) elimination of premedication to counteract castor oil (iii) reduction of drug toxicity side effects (iv) prolonged circulation time and therapeutic effect and (v) improved quality of life.

The process has also been used for the nanoencapsulation of CPT, a potent and exciting anticancer agent, in a stable aqueous liposomal formulation called Camposomes^{TM}. Water-soluble derivatives of CPT, a unique topoisomerase 1 inhibitor, have recently been approved by the FDA for use in colorectal cancer. Camposomes^{TM} have been shown to be very effective against lymphomas in nude mice.

The development of less toxic, liposomal formulations of cisplatin has been hampered by the low water solubility and low lipophilicity of cisplatin, resulting in very

low encapsulation efficiencies. A novel method enables the efficient encapsulation of cisplatin in a lipid formulation; it is based on repeated freezing and thawing of a concentrated solution of cisplatin in the presence of negatively charged phospholipids (Burger et al 2002). The method is unique in that it generates nanocapsules, which are small aggregates of cisplatin covered by a single lipid bilayer. The nanocapsules have an unprecedented drug-to-lipid ratio and an in vitro cytotoxicity up to 1,000-fold higher than the free drug. Analysis of the mechanism of nanocapsule formation suggests that the method may be generalized to other drugs showing low water solubility and lipophilicity.

In Protein Stabilized Liposome (PSL^{TM}) nanotechnology of Azaya Therapeutics, the liposome product is prepared in a single step that encapsulates the active drug Docetaxel (ATI-1123) in the lipid layer of the liposome while forming active nanoparticles in situ (100–130 nm). This process is geared toward the formulation of hydrophobic molecules that would otherwise have limited success as developmental drugs using traditional formulation methodologies. Azaya intends to use its PSL nanotechnology to improve the performance and reduce the nonspecific cytotoxicity of leading marketed chemotherapeutics such as Taxotere (Docetaxel) and CAMPTOSAR®, as well as several experimental drugs that have been withdrawn from development because of their nonspecific cytotoxicity and formulation difficulties.

Encapsulating Drugs in Hydrogel Nanoparticles

NOF Corporation (Tokyo, Japan) has developed Hydrogel-Nanoparticles based on hydrophobic polysaccharides (PUREBRIGHT®) for drug delivery in which proteins and/or antibodies can be encapsulated. Cholesterol pullulan shows unique characteristics. In the water four cholesterol molecules gather to form hydrophobic core, then usually 11 units of these core make polymers self-aggregated with localizing pullulan outside. Resulting cholesterol nanoparticles can stabilize proteins and/or antibodies by forming hybrid complex. These particles also stimulate immune system as they are easily trapped by dendritic cells in the blood, so that vaccine therapy for cancer may be facilitated by encapsulating cancer-specific MAbs such as Her2.

Alnis Biosciences Inc, has encapsulated magnetic material within nanoparticulate hydrogels (NanoGels) to create a particle ~25 nm to which 5–100 ligands can be attached—MagNaGel particles. The particles have iron oxide core ~10 nm in diameter with polymer coating and can be loaded with up to 1,000 anticancer drug molecules. MagNaGel particles can be tracked by MRI as they accumulate in tumors. Then, by alternating magnetic fields, the SPIONs can be heated to facilitate the penetration of anticancer agent into the tumor. SPIONs can also be separated, purified, and enable MagNaGel binding assays. Fluorescent MagNaGel is endocytosed in 30 min to 24 h. In cancer cell culture experiments, anti-HER2 binding requires 30 min incubation time. MagNaGel particles demonstrate high chemotherapeutic loading, tumor-associated biomolecular binding, good magnetic susceptibility, and attractive toxicity and circulation profiles in mouse models (Sunderland et al 2006).

In vivo studies show that MagNaGel displays prolonged circulation time and 33% of the drug is still retained in the body 22 h after administration. High payload is delivered to the tumor relative to the organs that are prone to toxic effects, e.g., the concentration in the tumor is 10 times that in the kidney. MagNaGel can be injected into the skin by intradermal microneedles for imaging of lymph nodes by MRI. MagNaGel is being investigated in various cancer models including those of ovarian cancer and brain cancer involving choroid plexus. MagNaGel, a drug–device hybrid, is part of multimodal therapy of cancer involving diagnostic MRI, chemotherapy, as well as hyperthermia.

Curcumin, an element found in the cooking spice turmeric, has long been known to have potent anticancer properties as demonstrated in several human cancer cell line and animal carcinogenesis models. Nevertheless, widespread clinical application of this relatively efficacious agent in cancer and other diseases has been limited because of poor aqueous solubility, and consequently, minimal systemic bioavailability. This problem has been overcome by encapsulating free curcumin with a polymeric nanoparticle, creating nanocurcumin (Bisht et al 2007). Furthermore, nanocurcumin's mechanisms of action on pancreatic cancer cells mirror those of free curcumin, including induction of cellular apoptosis, blockade of nuclear factor kappa B (NF-κB) activation, and downregulation of steady-state levels of multiple proinflammatory cytokines (IL-6, IL-8, and TNF-α). No evidence of toxicity was found in tests with empty versions of the polymeric nanoparticle. Their findings show no evidence of weight loss, organ changes, or behavioral changes in live mice after administering a relatively large dosage of the empty nanoparticles. Nanocurcumin provides an opportunity to expand the clinical repertoire of this efficacious agent by enabling ready aqueous dispersion. Future studies utilizing nanocurcumin are warranted in preclinical in vivo models of cancer and other diseases that might benefit from the effects of curcumin.

Researchers from the University of Michigan have developed a versatile chemical technique for creating ultrafine nanosized hydrogels, essentially a network of polymer chains that absorb as much as 99% of their weight in water (Gao et al 2007). They used polyacrylamide to create 2-nm-diameter nanoparticles that have no charge on their surfaces. This lack of charge prevents blood proteins from sticking to the surface of the nanoparticles. Combined with the fact that these nanoparticles are too small to be recognized by the immune system, the result is a nanoscale drug delivery vehicle with the ability to remain in circulation long enough to reach and permeate tumors before being excreted through the kidneys. These nanoscale hydrogels were first tested as a drug delivery vehicle for a water-insoluble photosensitizer called *m*-tetra(hydroxyphenyl) chlorin (mTHPC), which is approved in the European Union for use in treating head and neck cancer. mTHPC produces cell-killing reactive oxygen when irradiated with red light, but not without serious side effects resulting from the method now used to deliver this drug to tumors. When added to the chemical mixture used to create the nanoparticles, mTHPC becomes trapped within the polymer framework. Characterization experiments showed that this photosensitizer does not escape from the nanoparticles but is still capable of producing the same amount of reactive oxygen as if it were free in solution. When added

to human brain cancer cells growing in culture and irradiated with red light, this formulation kills the cells rapidly. Empty nanoparticles had no effect on the cells. Neither did drug-loaded nanoparticles added to the cells that were kept in the dark.

Exosomes

Exosomes are small (50–100 nm), spherical vesicles produced and released by most cells to facilitate intercellular communication. These vesicles are of endosomal origin and are secreted in the extracellular milieu following fusion of late endosomal multivesicular bodies with the plasma membrane. They have a defined protein composition, which confers specific biological activities contingent on the nature of the producing cell. Although exosomes express tumor antigens, leading to their proposed utility as tumor vaccines, they also can suppress T-cell-signaling molecules and induce apoptosis (Taylor and Gercel-Taylor 2005). The first phase I clinical trial using autologous exosomes pulsed with MAGE 3 peptides for the immunization of stage III/IV melanoma patients has shown the feasibility of large-scale exosome production and the safety of exosome administration (Escudier et al 2005).

Exosomes produced by dendritic cells are called dexosomes and contain essential components to activate both adaptive and innate immune responses. Anosys is developing dexosome vaccines that use patient-specific dexosomes loaded with tumor antigen-derived peptides to treat cancer. Exosome research continues to reveal unique properties that broaden their fields of application. Anosys' Exosome Display Technology provides the ability to manipulate exosome composition and tailor exosomes with new desirable properties opening up opportunities in the field of recombinant vaccine and MAb preparation. This is achieved by generating genes coding for chimeric proteins linking an exosome addressing sequence to antigens or biologically active proteins. The resulting proteins are targeted to exosomal compartment and released in the extracellular milieu bound to exosomes.

Folate-Linked Nanoparticles

PEG-coated biodegradable nanoparticles can be coupled to folic acid to target the folate-binding protein; this molecule is the soluble form of the folate receptor that is overexpressed on the surface of many tumor cells. The specific interaction between the conjugate folate-nanoparticles and the folate-binding protein has been evaluated by SPR and confirmed a specific binding of the folate-nanoparticles to the folate-binding protein (Stella et al 2000). Thus, folate-linked nanoparticles represent a potential new drug carrier for tumor cell-selective targeting.

Iron Oxide Nanoparticles

A novel water-dispersible oleic acid (OA)-Pluronic-coated iron oxide magnetic nanoparticle formulation can be loaded easily with high doses of water-insoluble anticancer agents (Jain et al 2005). Drug partitions into the OA shell surrounding iron oxide nanoparticles and the Pluronic that anchors at the OA–water interface confer

aqueous dispersity to the formulation. Neither the formulation components nor the drug loading affects the magnetic properties of the core iron oxide nanoparticles. Sustained release of the incorporated drug is observed over 2 weeks under in vitro conditions. The nanoparticles have further demonstrated sustained intracellular drug retention relative to drug in solution and a dose-dependent antiproliferative effect in breast and prostate cancer cell lines. This nanoparticle formulation can be used as a universal drug carrier system for systemic administration of water-insoluble drugs while simultaneously enabling magnetic targeting and/or imaging.

Lipid-Based Nanocarriers

LiPlasome Pharma's proprietary prodrug and drug delivery technology is based on smart lipid-based nanocarriers (LiPlasomes) that can be applied for targeted transport of anticancer drugs (Andresen et al 2005). The targeted drug delivery principle consists of long-circulating nanoparticles such as liposomes or micelles that accumulate in porous cancer tissue with a high PLA2 activity. The carrier nanoparticles are composed of special prodrug lipids whose degradation products, after exposure to PLA2, are converted to active drugs such as anticancer lysolipids and/or fatty acid drug derivatives The PLA2 hydrolysis products will furthermore act as locally generated permeability enhancers that promote the absorption of the released drugs across the cancer cell membranes into putative intracellular target sites. This innovative prodrug and drug delivery concept allows for intravenous transport of high concentrations of anticancer drugs directly to the tumor target. It enables, without any prior knowledge of the position and size of the tumor, the release of anticancer drugs specifically at the tumor target site.

Micelles for Drug Delivery in Cancer

Block-copolymer micelles are spherical supramolecular assemblies of amphiphilic copolymers that have a core–shell architecture. The core is a loading space that can accommodate hydrophobic drugs, and the shell is a hydrophilic brush-like corona that makes the micelle water soluble, thereby allowing delivery of the poorly soluble contents (Fig. 7.1).

However, a key issue with the contained cytotoxic drugs is an understanding of how the micelle and the micelle-incorporated agent are distributed. By using fluorescently labeled polymer and organelle-specific dyes in combination with confocal microscopy, it was shown that the micelles localized in several cytoplasmic organelles, including the mitochondria, but not the nucleus (Savic et al 2003). Further experiments confirmed that the micelles increased the amount of a model agent delivered to the cells, indicating that these micelles might be worth investigating for their potential to deliver drugs to particular subcellular targets. Antibodies can be attached to the polymers that make up the micelles. Administering immunomicelles loaded with the sparingly soluble anticancer drug taxol to mice with lung carcinoma resulted in increased accumulation of taxol in the tumor compared with free taxol or taxol in nontargeted micelles, and enhanced

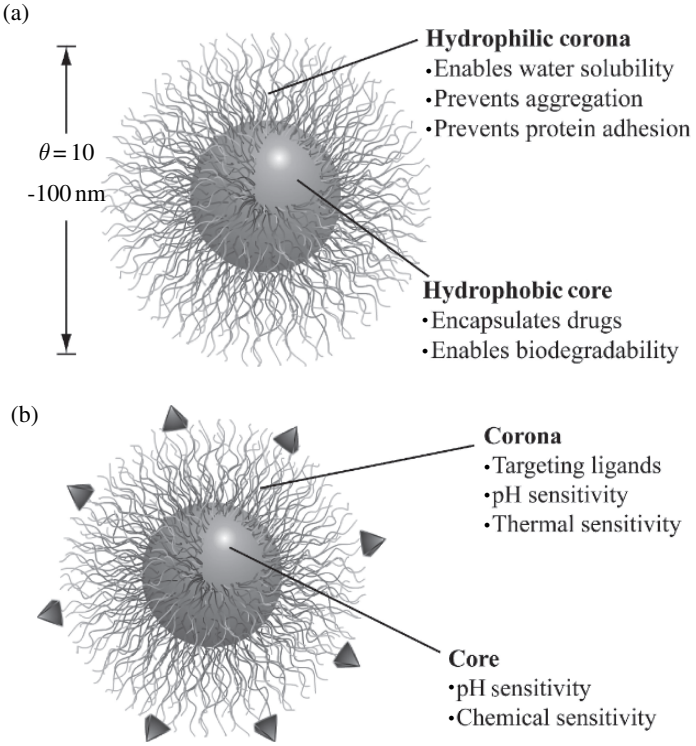

Fig. 7.1 Use of micelles for drug delivery
Source: Sutton et al 2007.

inhibition of tumor growth, illustrating the therapeutic potential of such micelle-based approaches (Torchilin et al 2003). PMs incorporating cisplatin (CDDP) were prepared by NanoCarrier Ltd. (Chiba, Japan) through the polymer–metal complex formation between CDDP and PEG-poly(glutamic acid) block copolymers, and their utility as a tumor-targeted drug delivery system was investigated by scientists at the National Cancer Research Institute (Tokyo, Japan). CDDP-incorporated micelles had a size of 28 nm and exhibited a sustained drug release accompanied with the decay of the carrier itself in physiological saline. These micelles showed remarkably prolonged blood circulation and effectively accumulated in solid tumors (LLC cells) in a passive targeting manner (Nishiyama et al 2003). These data suggest that CDDP/m could be a promising formulation for the targeted therapy of solid tumors.

DACH-platin-PEG-poly(glutamic acid) (DACH Platin Medicelle^{TM}) from NanoCarrier (Chiba, Japan), based on Medicelle^{TM} technology, has demonstrated enhanced permeability and retention of the compound in the tumor, leading to improved efficacy and toxicity profiles in animal experiments. The mechanism of action of Medicelle^{TM} delivery system is based on the formation of micelles, including hydrophilic–hydrophobic block copolymers, with a hydrophobic inner core and hydrophilic outer shell. This allows the chemical entrapment of various drugs into the

micelles. The drugs are then released slowly into the organism. This product will be developed for clinical application in collaboration with Debiopharm (Lausanne, Switzerland).

Scientists at the University of Wisconsin (Madison, WI) have coaxed water-insoluble anticancer drugs inside PMs, which can circulate in the bloodstream for long periods. Enveloped by the micelle's hydrated outer shell, the drug then becomes much more water-soluble than it would be normally. Besides being safer and easier to administer, PMs maintain anticancer drugs like rapamycin in blood plasma for longer periods than do standard formulations.

CPT is a topoisomerase I inhibitor that is effective against cancer, but clinical application of CPT is limited by insolubility, instability, and toxicity problems. Biocompatible, targeted sterically stabilized micelles (SSMs) have been used as nanocarriers for CPT (CPT-SSM). CPT solubilization in SSMs is reproducible and is attributed to the avoidance of drug aggregate formation. Furthermore, SSMs composed of polyethylene glycol (PEGylated) phospholipids are attractive nanocarriers for CPT delivery because they are sufficiently small ($\sim$14 nm) to extravasate through the leaky microvasculature of tumor and inflamed tissues for passive targeting of solid cancers in vivo, resulting in high drug concentration in tumors and reduced drug toxicity to the normal tissues (Koo et al 2006).

Shape may be important in designing better nanotechnology-based drug delivery vehicles. A study in rodents has compared highly stable, polymer micelle assemblies known as filomicelles with spheres of similar chemistry and shown that filomicelles persisted in the circulation up to 1 week after intravenous injection (Geng et al 2007). This is about 10 times longer than their spherical counterparts and is more persistent than any known synthetic nanoparticle. Under fluid flow conditions, spheres and short filomicelles are taken up by cells more readily than longer filaments because the latter are extended by the flow. Preliminary results further demonstrate that filomicelles can effectively deliver the anticancer drug paclitaxel and shrink human-derived tumors in mice. Although these findings show that long-circulating vehicles need not be nanospheres, they also lend insight into possible shape effects of natural filamentous viruses.

Stealth micelle formulations have stabilizing PEG coronas to minimize opsonization of the micelles and maximize blood circulation times. Currently, clinical data have been reported on three stealth micelle systems: SP1049C, NK911, and Genexol-PM (Sutton et al 2007). SP1049C is formulated as DOX-encapsulated pluronic micelles, NK911 is DOX-encapsulated micelles from a copolymer of PEG and DOX-conjugated poly(aspartic acid), and Genexol-PM is a paclitaxel-encapsulated PEG-PLA micelle formulation.

Polymer micelles are becoming a powerful nanotherapeutic platform that affords several advantages for targeted drug delivery in cancer, including increased drug solubility, prolonged circulation half-life, selective accumulation at tumor sites, and a decrease in toxicity. However, the technology still lacks tumor specificity and controlled release of the entrapped agents. Therefore, the focus has gradually shifted from passive targeting micelles to active targeting and responsive systems that carry additional mechanisms for site-specific release. Ligand-targeted, pH-sensitive

formulations are examples of how versatility of micelles can lead to a fusion of chemical customization with biological insight to achieve targeted drug delivery.

Nanomaterials for Delivery of Poorly Soluble Anticancer Drugs

Nanomaterials have been successfully manipulated to create a new drug delivery system that can solve the problem of poor water solubility of most promising currently available anticancer drugs and thereby increase their effectiveness. The poorly soluble anticancer drugs require the addition of solvents in order for them to be easily absorbed into cancer cells. Unfortunately, these solvents not only dilute the potency of the drugs but create toxicity as well. Researchers from UCLA's California NanoSystems Institute have devised a novel approach using silica-based nanoparticles to deliver the anticancer drug CPT and other water-insoluble drugs into human cancer cells (Lu et al 2007). The method incorporates a hydrophobic anticancer drug CPT into the pores of fluorescent mesoporous silica nanoparticles and delivers the particles into a variety of human cancer cells to induce cell death. The results suggest that the mesoporous silica nanoparticles might be used as a vehicle to overcome the insolubility problem of many anticancer drugs.

Nanoparticle Formulations of Paclitaxel

Paclitaxel is active and widely used to treat multiple types of solid tumors. The commercially available paclitaxel formulation uses cremophor/ethanol (C/E) as solubilizers. Other formulations including nanoparticles have been introduced. A study evaluated the effects of nanoparticle formulation of paclitaxel on its tissue distribution in experimental animals (Yeh et al 2005). The nanoparticle and C/E formulations showed significant differences in paclitaxel disposition; the nanoparticles yielded 40% smaller area under the blood concentration–time curve and faster blood clearance of total paclitaxel concentrations (sum of free, protein-bound, and nanoparticle-entrapped drug). Tissue specificity of the two formulations was different. The nanoparticles showed longer retention and higher accumulation in organs and tissues, especially in the liver, small intestine, and kidney. The most striking difference was an 8-fold greater drug accumulation and sustained retention in the kidney. These data indicate that nanoparticulate formulation of paclitaxel affects its clearance as well as distribution in tissues with preferential accumulation in the liver, spleen, small intestine, and kidney. Solid tumors have unique features, such as leaky tumor blood vessels and defective lymphatic drainage, that promote the delivery and retention of macromolecules or particles, a phenomenon recognized as the enhanced permeability and retention effect. Tissue specificity of the gelatin nanoparticles warrants further investigations before using nanoparticle formulations of anticancer drugs for tumors in various organs.

AI-850 (Acusphere Inc) is a rapidly dissolving porous particle formulation of paclitaxel, created by using the company's Hydrophobic Drug Delivery Systems. The patented spray-drying technology embeds small drug particles inside hydrophobic water-soluble matrices so that the whole composition is a mixture of microparticles

and nanoparticles. AI-850 was compared to Taxol following intravenous administration in a rat pharmacokinetic study, a rat tissue distribution study, and a human xenograft mammary tumor model in nude mice (Straub et al 2005). The volume of distribution and clearance for paclitaxel following intravenous bolus administration of AI-850 were 7-fold and 4-fold greater, respectively, than following intravenous bolus administration of Taxol. There were no significant differences between AI-850 and Taxol in tissue concentrations and area under the curve for the tissues examined. Nude mice implanted with mammary tumors showed improved tolerance of AI-850, enabling higher administrable dose of paclitaxel, which resulted in improved efficacy as compared with Taxol administered at its maximum tolerated dose.

Nanoparticles Containing Albumin and Antisense Oligonucleotides

Nanoparticles consisting of human serum albumin (HSA) and containing different antisense ODNs (ASOs) have been used for drug delivery to tumors (Wartlick et al 2004). The preparation process was optimized regarding the amount of solving agent, stabilization conditions, as well as nanoparticle purification. The glutaraldehyde crosslinking procedure of the particle matrix was identified as a crucial parameter for biodegradability and drug release of the nanoparticles. The drug loading efficiency increased with longer chain length and employment of a phosphorothioate backbone. The resulting nanoparticles were tested in cell cultures for cytotoxicity and cellular uptake. In different tumor cell lines no cytotoxic effect was observed up to nanoparticle concentrations of 5,000 μg/ml. All cell lines showed a significant cellular uptake of HSA nanoparticles. The entrapment of a fluorescent labeled oligonucleotide within the particle matrix was used for the detection of the intracellular drug release of the carrier systems. Confocal laser scanning microscopy revealed that nanoparticles crosslinked with low amounts of glutaraldehyde, rapidly degraded intracellularly, leading to a significant accumulation of the ASO in cytosolic compartments of the tumor cells.

Nonaggregating Nanoparticles

Medisperse parenteral formulations (Medisperse LLC) consist of spherical, amorphous nanoparticles, which do not aggregate, so circulation throughout the vasculature is safe. The need for using a cosolvent to solubilize a compound is eliminated, thereby reducing the overall toxicity of the formulation. Reducing overall toxicity potentially enables higher dose administration, which could improve efficacy. Eliminating the use of a cosolvent also eliminates any risk that the compound will precipitate in situ upon contact with blood, which again improves the safety profile for this drug. Some cosolvents require special administration sets to eliminate the risk of leaching plasticizer during infusion. Medisperse formulations do not require special infusion sets. For most therapeutic applications, uptake by the RES should be avoided. Certain procedures, such as liver imaging, benefit by RE uptake. The Medisperse technology is designed to minimize or maximize RE uptake depending on the pharmacological objective.

Pegylated Nanoliposomal Formulation

Collaborative research between the NCI and Northeastern University has shown that PEG-coated nanoparticles remain in the tumors and bloodstream longer when compared with gelatin nanoparticles. This discovery could lead to more effective nanoparticles with tumor-targeting properties.

Ceramide, an antimitogenic and proapoptotic sphingolipid, accumulates in cancer tissues and helps to kill cancer cells when patients undergo chemotherapy and radiation. Although the mechanism remains unknown, ceramide is inherently attracted to tumor cells. In vitro tumor cell culture models have shown the potential therapeutic utility of raising the intracellular concentration of ceramide. However, therapeutic use of systemically delivered ceramide is limited by its inherent insolubility in the blood as it is a lipid as well as by its toxicity when injected directly into the bloodstream. Packaging ceramide in nanoliposome capsules allows them to travel through the bloodstream without causing toxicity and release the ceramide in the tumor. Systemic intravenous delivery of C6-ceramide (C6) in a pegylated liposomal formulation significantly limited the growth of solid tumors in a syngeneic BALB/c mouse tumor model of breast adenocarcinoma (Stover et al 2005). A pharmacokinetic analysis of systemic liposomal-C6 delivery showed that the pegylated liposomal formulation follows first-order kinetics in the blood and achieves a steady-state concentration in tumor tissue. Intravenous liposomal-C6 administration was also shown to diminish solid tumor growth in a human xenograft model of breast cancer. In this study in mice, the ceramide bundles targeted and destroyed only breast cancer cells, sparing the surrounding healthy tissue. Together, these results indicate that bioactive ceramide analogues can be incorporated into pegylated liposomal vehicles for improved solubility, drug delivery, and antineoplastic efficacy. The next step is to explore how additional chemotherapeutic agents could be incorporated into the liposomes for a more lasting effect.

Perfluorocarbon Nanoparticles

The contrast agents in development by Kereos Inc comprise tiny perfluorocarbon nanoparticles suspended in an emulsion. Agents such as Technetium-99m, may be attached to the nanoparticles to provide the contrast that allows for imaging. In addition, nanoparticles are labeled with a specific ligand that causes the agent to target newly developing blood vessels. When injected into the body, the resulting agent will find and illuminate these vessels. Anticancer drugs and therapeutic radionuclides may also be incorporated into the nanoparticles to deliver therapy directly and selectively.

Protosphere Nanoparticle Technology

Protosphere^{TM} nanoparticle technology (Abraxis Bioscience Inc) was used to integrate biocompatible proteins with drugs to create the nanoparticle form of the drug having a size of about 100–200 nm (~1/100th the size of a single RBC).

The product Abraxane (ABI-007) is a patented albumin-stabilized nanoparticle formulation of paclitaxel (nab-paclitaxel) designed to overcome insolubility problems encountered with paclitaxel. The solvent C/E, used previously in formulations of paclitaxel, causes severe hypersensitivity reactions. To reduce the risk of allergic reactions when receiving Taxol, patients must undergo premedication using steroids and antihistamines and be given the drug using slow infusions. The active component (paclitaxel) can be delivered into the body at a 50% higher dose over 30 min. This contrasts with Taxol infusions, which can take up to 3 h. Because Abraxane is solvent-free, solvent-related toxicities are eliminated, premedication is not required, and administration can occur more rapidly. Abraxane also has a different toxicity profile than solvent-based paclitaxel, including a lower rate of severe neutropenia. In a randomized phase III trial, the response rate of Abraxane was almost twice that of the solvent-containing drug Taxol. Because Abraxane does not contain solvents, higher doses of paclitaxel could be given which may account in part for its increased anticancer activity. In addition, albumin is a protein that normally transports nutrients to cells and has been shown to accumulate in rapidly growing tumors. Therefore, Abraxane's increased effectiveness may also be due to preferential delivery of albumin-bound paclitaxel to cancer cells. In addition to the standard infusion formulation of Abraxane, oral and pulmonary delivery formulations are also being investigated.

A pivotal randomized controlled phase III clinical trial compared the safety and efficacy of 260 mg/m^2 of Abraxane with 175 mg/m^2 of Taxol administered every 3 weeks in patients with metastatic breast cancer (Gradishar et al 2005). Abraxane was infused over 30 min without steroid pretreatment and at a higher dose than Taxol, which requires steroid therapy and infusion over 3 h. Abraxane was found to be superior to Taxol on lesion response rate as well as on tumor progression rate. On January 7, 2005, the FDA approved Abraxane for the treatment of metastatic breast cancer. Abraxane also is being evaluated in non–small cell lung, ovarian, melanoma, and cervical cancers.

Multifunctional Nanoparticles for Treating Brain Tumors

Scientists at the University of Michigan (Ann Arbor, MI) have combined two promising approaches for diagnosing and treating cancer, a multidisciplinary research team that has created a targeted multifunctional polymer nanoparticle that successfully images and kills brain tumors in laboratory animals (Reddy et al 2006). The team developed of a 40-nm diameter polyacrylamide nanoparticle loaded with Photofrin, a photosensitizing agent, and iron oxide. When irradiated with laser light, Photofrin, which is used to treat several types of cancer, including esophageal, bladder, and skin cancers, triggers the production of reactive oxygen species that destroy a wide variety of molecules within a cell. The iron oxide nanoparticles function as a MRI contrast agent. As the targeting agent, the researchers used a 31-amino acid–long peptide developed by members of the NCI-funded Center of Nanotechnology for Cancer at the University of California. This peptide targets an unknown receptor found on the surface of new blood vessels growing around tumors and also triggers

cell uptake of nanoparticles attached to it. Researchers tested the nanoparticles in cell cultures and animal models. The studies showed that the nanoparticles traveled to the tumor, resulting in less Photofrin exposure throughout the body and enhanced exposure within the tumor. This allowed a larger window for activating the drug with light, which was accomplished by threading a fiber-optic laser into the brain. In humans, this approach could reduce or eliminate a common side effect of PDT, in which healthy skin becomes sensitive to light.

Nanoparticles for Targeted Delivery of Drugs into the Cancer Cells

Nanosystems are emerging that may be very useful for tumor-targeted drug delivery: novel nanoparticles are preprogrammed to alter their structure and properties during the drug delivery process to make them most effective for the different extra- and intracellular delivery steps (Wagner 2007). This is achieved by the incorporation of molecular sensors that are able to respond to physical or biological stimuli, including changes in pH, redox potential, or enzymes. Tumor-targeting principles include systemic passive targeting and active receptor targeting. Physical forces (e.g., electric or magnetic fields, ultrasound, hyperthermia, or light) may contribute to focusing and triggered activation of nanosystems. Biological drugs delivered with programmed nanosystems also include plasmid DNA, siRNA, and other therapeutic nucleic acids.

A drug delivery system developed by scientists at the University of Wyoming (Laramie, WY) may destroy tumors more effectively by using synthesized smart nanoparticles that target and kill cancer cells while sparing healthy cells. The system is intended to improve the efficiency of cancer treatment. These particles are injected intravenously into the blood circulation. Each of the particles can recognize the cancer cell, anchor itself to it, and diffuse inside the cell. Once inside, the particle disintegrates, causing a nearly instantaneous release of the drug precisely where it is needed. Nanoparticles are chemically programmed to have an affinity for the cell wall of tumors. To be effective, the particles must evade the body's immune system, penetrate into the cancer cells, and discharge the drugs before being recognized by the cancer cells. Advantages of this system are as follows:

- This system can fool cancer cells, which are very good at detecting and rejecting drugs.
- It provides very rapid drug delivery at sufficiently high concentration that can overwhelm the cancer cell's resistance mechanisms.
- It should reduce side effects because it targets only the cancer cells.

This method has been tested in vitro and in vivo in mouse cancer models. In one preliminary study, the experimental treatment reduced the number of tumors in mice from 60 to 10, while traditional therapies reduced the number of tumors from 60 to 30. According to the research proposals submitted to the NIH in 2005, the focus is on ovarian cancer applications, but the approach is applicable to other forms of cancer.

Another study, conducted by scientists at Harvard Medical School (Boston, MA) and the MIT, involved engineering nanoparticles embedded with the cancer drug Taxotere. The nanoparticles are made of a hydrogen and carbon polymer with bits of drug bound up in its fabric and attached to a substance that targets cancer cells. The polymer gradually dissolves, exposing the nuggets of drug little by little. The particles were injected directly into human tumors created from prostate cancer cell lines and implanted into the flanks of mice. The mice were observed for 100 days. In the group of mice that had their tumors injected with the targeted nanoparticles containing the drug, the tumor completely disappeared. There is a plan to test the approach in prostate cancer patients within 2 years. One major problem in perfecting the blood injections is that the nanoparticles end up in the liver and spleen—an unwanted side effect because once they dissolve in those organs, they release toxic levels of chemotherapy to healthy tissue.

Antiangiogenic Therapy Using Nanoparticles

Integrin-targeted nanoparticles can be used for site-specific delivery of a therapeutic payload. Selective targeting of upregulated $\alpha_v\beta_3$ and Flk-1 on the neovasculature of tumors is a novel antiangiogenesis strategy for treating a wide variety of solid tumors. A study provides proof of principle that targeted radiotherapy works using different targeting agents on a nanoparticle (NP), to target both the integrin $\alpha_v\beta_3$ and the vascular EGFR (Li et al 2004). These encouraging results demonstrate the potential therapeutic efficacy of the IA-NP-90Y and anti-Flk-1 MAb-NP-90Y complexes as novel therapeutic agents for the treatment of a variety of tumor types.

The synthetic peptide bearing Arg-Gly-Asp (RGD) sequence is considered to specifically bind to avb3 integrin expressed on endothelial cells in the angiogenic blood vessels, which provides a potential to inhibit the tumor growth. Hydrophobically modified glycol chitosan (HGC) capable of forming nanosized self-aggregates was used as a carrier for the RGD peptide, which was labeled with fluoresein isothiocyanate (FITC-GRGDS) and loaded into self-aggregates by solvent evaporation methods (Park et al 2004). The self-aggregates loaded with FITC-GRGDS might be useful for monitoring or destroying the angiogenic vessels surrounding the tumor tissue.

Bacterial Nanoparticles for Encapsulation and Drug Delivery

Indiscriminate drug distribution and severe toxicity of systemic administration of chemotherapeutic agents can be overcome through encapsulation and cancer cell-specific targeting of chemotherapeutics in bacterially derived 400-nm minicells—EnGeneIC (Sydney, Australia) Delivery Vehicle. Scientists at EnGeneIC (Sydney, Australia) discovered that minicells can be packaged with therapeutically significant concentrations of chemotherapeutics of differing charge, hydrophobicity, and solubility (Macdiarmid et al 2007). Targeting of minicells via bispecific antibodies to receptors on cancer cell membranes results in endocytosis, intracellular degradation,

and drug release. Doses of drugs delivered via minicells are ~1,000 times less than the dose of the free drug required for equivalent or better tumor shrinkage. It produces significant tumor growth inhibition and regression in mouse xenografts and lymphoma in dogs despite administration of minute amounts of drug and antibody, a factor critical for limiting systemic toxicity that should allow the use of complex regimens of combination chemotherapy. Human trials may be carried out if approval is gained from Australian, US, European, and Japanese regulatory authorities.

Canine Parvovirus as a Nanocontainer for Targeted Drug Delivery

The CPV utilizes transferrin receptors (TfRs) for binding and cell entry into canine as well as human cells. TfRs are overexpressed by a variety of tumor cells and are widely being investigated for tumor-targeted drug delivery. To explore the natural tropism of CPV to TfRs for targeting tumor cells, CPV virus-like particles (VLPs) produced by expression of the CPV-VP2 capsid protein in a baculovirus expression system were examined for attachment of small molecules and delivery to tumor cells (Singh et al 2006a). Structural modeling suggested that six lysines per VP2 subunit are presumably addressable for bioconjugation on the CPV capsid exterior. Between 45 and 100 of the possible 360 lysines/particle could be routinely derivatized with dye molecules depending on the conjugation conditions. Dye conjugation also demonstrated that the CPV-VLPs could withstand conditions for chemical modification on lysines. Attachment of fluorescent dyes neither impaired binding to the TfRs nor affected internalization of the 26-nm-sized VLPs into several human tumor cell lines. CPV-VLPs therefore exhibit highly favorable characteristics for development as a novel nanomaterial for tumor targeting.

Carbon Magnetic Nanoparticles for Targeted Drug Delivery in Cancer

Using dense-medium plasma technology, carbon magnetic nanoparticles (CMNPs) have been synthesized at room temperature and atmospheric pressure. Results of x-ray photoelectron spectroscopy, Fourier transform infrared spectroscopy, and scanning electron microscopy show that these nanoparticles are composed of spherical particles, 40–50 nm in diameter, with iron/iron oxide particles dispersed in a carbon-based host structure (Ma et al 2004). Thermal gravimetry/differential thermal gravimetry analysis shows these nanoparticles are stable to temperatures as high as 600°C. The synthesized CMNP were treated by argon-plasma, aminated with ethylene diamine and subsequently activated by generating aldehyde groups on them. Free DOX molecules are then immobilized onto the surfaces of activated CMNP to form CMNP–DOX conjugates. The in vitro antiproliferative activity of immobilized DOX in the conjugates has been demonstrated in tumor cell cytotoxicity assays. It is suggested that this CMNP–DOX system can be used for targeted drug delivery systems in cancer.

Carbon Nanotubes for Targeted Drug Delivery to Cancer Cells

The University of Surrey in the United Kingdom has been awarded an EU grant under Marie Curie scheme as a part of an international project: "Multifunctional Carbon Nanotubes for Biomedical Applications (CARBIO)." CNTs have already found applications in engineering, but so far biological application has been hampered by their poor interaction with biological systems. The Surrey team has overcome this problem by wrapping DNA and RNA around CNTs making them biocompatible. The aim of the project is to attach additional molecules to the RNA-wrapped carbon nanotubes to target them toward cancer cells. In combination with laser treatment, the CNTs may then be used to destroy the cancer cells. Although considerable further work is required before any new drugs based on this technology are developed, it is hoped that it will eventually lead to more effective treatments for cancer.

Cyclosert System for Targeted Delivery of Anticancer Therapeutics

Cyclosert™ (Insert Therapeutics, Pasadena, CA, USA) is the first nanoparticle drug transport platform to be designed de novo and synthesized specifically to overcome limitations in existing technologies used for the systemic transport of therapeutics to targeted sites within the body. Based on small cyclic repeating molecules of glucose called cyclodextrins, Cyclosert promotes the ability of cytotoxic drugs to inhibit the growth of human cancer cells while reducing toxicity and remaining nonimmunogenic at therapeutic doses. In particular, the system is designed to reduce the toxicity of the drugs until they actually reach the targeted tumor cells where the active drug is released in a controlled fashion. Animal studies have shown that the Cyclosert system can safely deliver tubulysin A, a potent, but highly toxic, antitumor agent. In vitro studies have shown the tubulysin–Cyclosert conjugate to be effective against multiple human cancer cell lines. The conjugate is stable and 100 times more water soluble than the free drug. Calando is developing CALAA01, an siRNA, for anticancer use using Cyclosert as a delivery system.

IT-101 (Insert Therapeutics) is a de novo-designed experimental therapeutic comprised of linear, cyclodextrin (CD)-containing polymer conjugates of CPT that assemble into 40-nm-diameter nanoparticles via polymer–polymer interactions that involve inclusion complex formation between the CPT and the CD. Particle size, near-neutral surface charge, and CPT release rate were specifically designed into IT-101. Cyclosert platform forms nanoscale constructs with hydrodynamic diameters between 30 and 60 nm. This makes Cyclosert-based drugs ideal for effective delivery to solid tumors. Preclinical animal studies show extended circulation times, tumor accumulation, slow release of the CPT, and anticancer efficacy that directly correlate to the properties of the nanoparticle. Release of CPT can disassemble the nanoparticle into individual polymer chains $\sim$10 nm in size that are capable of renal clearance. IT-101 has been evaluated in patients with relapsed or refractory cancer following two cycles of therapy by intravenous infusion. Interim analysis shows that IT-101 is well tolerated and pancytopenia is the dose-limiting toxicity

(Yen et al 2007). Pharmacokinetics data were favorable and consistent with results from preclinical animal studies. In the patients studied, IT-101 showed longer half-life, lower clearance, and lower volume of distribution than seen in patients treated with other CPT-based drugs. The preliminary results of this phase I study warrant continued enrollment that is ongoing. Phase II studies are expected to follow.

Dendrimers for Anticancer Drug Delivery

Earlier studies of dendrimers in drug delivery systems focused on their use for encapsulating drug molecules. However, it was difficult to control the release of the drug. One solution to this problem involves the use of dendrimers with pH-sensitive hydrophobic acetal groups on the dendrimer periphery. The loss of acetal group at mildly acidic pH triggers the disruption of micelles and release of the drug. Another approach is to attach the drug to the periphery of the dendrimer so that the release of the drug can be controlled by incorporating a degradable linkage between the drug and the dendrimer. Dendrimers have been used to facilitate BNCT as well as PDT of cancer.

New developments in polymer and dendrimer chemistry have provided a new class of molecules called "dendronized polymers," i.e., linear polymer that bear dendrons at each repeat unit. Their behavior differs from that of linear polymers and provides drug delivery advantages because of their longer circulation time and numerous possibilities of peripheral attachments of drugs (Gillies and Frechet 2005).

At the Michigan Nanotechnology Institute for Medicine and the Biological Sciences (Ann Arbor, MI), modified PAMAM dendritic polymers <5 nm in diameter have been used as drug carriers (Kukowska-Latallo et al 2005). They were conjugated to folic acid as a targeting agent and then coupled to methotrexate and injected intravenously into animals bearing tumor that overexpress the folate receptor. Folate molecules bind to receptors on tumor cell membranes and facilitate the transport of methotrexate to inside of the tumor cell.

DOX has been conjugated to a biodegradable dendrimer with optimized blood circulation time through size and molecular architecture, drug loading through multiple attachment sites, solubility through PEGylation, and drug release through the use of pH-sensitive hydrazone linkages (Lee et al 2006). In culture, dendrimer–DOX was >10 times less toxic than free DOX toward colon carcinoma cells. Upon intravenous administration to tumor-bearing mice, tumor uptake of dendrimer–DOX was 9-fold higher than intravenous free DOX. In efficacy studies it caused complete tumor regression and 100% survival of the mice over the 60-day experiment. No cures were achieved in tumor-implanted mice treated with free DOX, drug-free dendrimer, or dendrimer-DOX in which the DOX was attached by means of a stable carbamate bond. The antitumor effect of dendrimer-DOX was similar to that of an equimolar dose of liposomal DOX (Doxil). The remarkable antitumor activity of dendrimer-DOX results from the ability of the dendrimer to favorably modulate the pharmacokinetics of attached DOX.

A synthetic vector system based on polypropylenimine dendrimers has the desired properties of a systemic delivery vehicle and mediates efficient transgene

expression in tumors after intravenous administration (Dufes et al 2005). Specifically, the systemic injection of dendrimer nanoparticles containing a TNF-α expression plasmid regulated by telomerase gene promoters (hTR and hTERT) leads to transgene expression, regression of remote xenograft murine tumors, and long-term survival of up to 100% of the animals. The combination of pharmacologically active synthetic transfection agent and transcriptionally targeted antitumor gene creates an efficacious gene medicine for the systemic treatment of experimental solid tumors. The promising results of these experiments could make it possible to treat inaccessible tumors in humans using gene therapy in the future. This new treatment can selectively target cancer cells, without causing damage to surrounding healthy cells.

Fullerenes for Enhancing Tumor Targeting by Antibodies

Although it was previously possible to attach drug molecules directly to antibodies, scientists have not been able to attach more than a handful of drug molecules to an antibody without significantly changing its targeting ability. That happens, in large part, because the chemical bonds that are used to attach the drugs—strong, covalent bonds—tend to block the targeting centers on the antibody's surface. If an antibody is modified with too many covalent bonds, the chemical changes will destroy its ability to recognize the cancer it was intended to attack.

In order to overcome this limitation, a new class of anticancer compounds have been created that contain both tumor-targeting antibodies and nanoparticles called fullerenes (C60), which can be loaded with several molecules of anticancer drugs like Taxol® (Ashcroft et al 2006). It is possible to load as many as 40 buckyballs into a single skin-cancer antibody called ZME-018, which can be used to deliver drugs directly into melanoma tumors. Certain binding sites on the antibody are hydrophobic (water repelling) and attract the hydrophobic fullerenes in large numbers so multiple drugs can be loaded into a single antibody in a spontaneous manner. No covalent bonds are required, so the increased payload does not significantly change the targeting ability of the antibody. The real advantage of fullerene immunotherapy over other targeted therapeutic agents is likely to be the fullerene's potential to carry multiple drug payloads, such as Taxol plus other chemotherapeutic drugs. Cancer cells can become drug resistant, and one can cut down on the possibility of their escaping treatment by attacking them with more than one kind of drug at a time. The first fullerene immunoconjugates have been prepared and characterized as an initial step toward the development of fullerene immunotherapy.

Gold Nanoparticles for Targeted Drug Delivery in Cancer

Gold and silica composite nanoparticles have been investigated as nanobullets for cancer. Gold atoms bind to silicon atoms with dangling bonds and serve as seeds for the growth of Au islands. The large electron affinity of gold causes a significant change in the electronic structure of silica, resulting in a substantial reduction in the highest occupied and the lowest unoccupied molecular orbital and the optical gap, thus allowing it to absorb NIR radiation. This suggests that a small cluster can

have a similar effect in the treatment of cancer as the large-size nanoshell, but with a different mechanism (Sun et al 2004).

The unique chemical properties of colloidal gold (cAu) make it a promising targeted delivery approach for drugs or genes to specific cells. The physicochemical properties of cAu permit more than one protein molecule to bind to a single particle of cAu. CytImmune Sciences Inc has shown that TNF can be bound to gold nanocrystals and delivered safely and effectively to tumor-burdened mice and dogs (Paciotti et al 2004). CytImmune scientists have characterized and modified the cAu particles to optimize binding of TNF to the nanocrystals and also the targeting of the particles to the tumor. The therapeutic compounds that CytImmune is developing are new formulations of the TNF-α—a naturally occurring cytokine causes the death of tumors but is also very toxic to healthy organs. By coupling TNF-α to cAu the company believes that this will be a future safe and effective anticancer therapy. Specifically, two drugs are in development: Aurimune-T and AuriTax. Aurimune-T is manufactured by covalently linking molecules of TNF-α and thiol-derivatized polyethylene glycol (PEG-THIOL) onto the surface of 25 nm cAu. Intravenously administered Aurimune-T rapidly accumulates in solid tumors implanted in mice and shows little to no accumulation in the RES or in other healthy organs. Coincident with the sequestration of gold is a 10-fold accumulation of TNF-α in the tumor when compared with animals treated with native TNF-α. By getting more TNF-α to the tumor Aurimune-T improves the safety and efficacy of TNF-α treatment since maximal tumor responses were achieved at lower doses of the drug. The second nanoparticle drug, AuriTax, consists of TNF-α, a chemotherapeutic (paclitaxel), and PEG-THIOL, which are bound to the same cAu nanoparticle. Like Aurimune-T, AuriTax delivers 10-fold more TNF-α and paclitaxel to the solid tumor when compared with each drug alone. These data support the continued development of the cAu platform and show the potential uses of TNF-α as a tumor targeting ligand and a cancer therapy.

Lipoprotein Nanoparticles Targeted to Cancer-Associated Receptors

A lipoprotein-based nanoplatform generated by conjugating tumor-homing molecules to the protein components of naturally occurring lipoproteins reroutes them from their normal lipoprotein receptors to other selected cancer-associated receptors (Zheng et al 2005). Multiple copies of these targeting moieties may be attached to the same nanoparticle, or a variety of different targeting moieties can be attached. Such a diverse set of tumor-homing molecules could be used to create a variety of conjugated lipoproteins as multifunctional, biocompatible nanoplatforms with a broad application to both cancer imaging and treatment. This technology is being developed by Marillion Pharmaceuticals (Malvern, PA, USA). The same principle can be applied to imaging and treatment of other diseases and for monitoring specific tissues. To validate this concept, the authors prepared a low-density lipoprotein (LDL)-based folate receptor (FR)-targeted agent by conjugating folic acid to the Lys residues of the apoB-100 protein. To demonstrate the ability of the lipoprotein-based nanoplatform to deliver surface-loaded and core-loaded payloads,

the particles were labeled either with the optical reporter 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine that was intercalated in the phospholipid monolayer or with the lipophilic PDT agent, tetra-*t*-butyl-silicon phthalocyanine bisoleate that was reconstituted into the lipid core. Cellular localization of the labeled LDL was monitored by confocal microscopy and flow cytometry in FR-overexpressing KB cells, in FR-nonexpressing CHO and HT-1080 cells, and in LDL receptor-overexpressing HepG2 cells. These studies demonstrate that the folic acid conjugation to the Lys side-chain amino groups blocks binding to the normal LDL receptor and reroutes the resulting conjugate to cancer cells through their FRs.

Nanocarriers to Improve Cancer-Targeting Therapy

TGF-β plays a pivotal role in the regulation of progression of cancer through effects on tumor microenvironment as well as on cancer cells. TGF-β inhibitors have recently been shown to prevent the growth and metastasis of certain cancers. However, there may be adverse effects caused by TGF-β signaling inhibition, including the induction of cancers by the repression of TGF-β-mediated growth inhibition. A short-acting, small-molecule TGF-β type I receptor (TβR-I) inhibitor has been used at a low dose in treating several experimental intractable solid tumors, including pancreatic adenocarcinoma and diffuse-type gastric cancer, characterized by hypovascularity and thick fibrosis in tumor microenvironments (Kano et al 2007). Low-dose TβR-I inhibitor altered neither TGF-ββ signaling in cancer cells nor the amount of fibrotic components. However, it decreased pericyte coverage of the endothelium without reducing endothelial area specifically in tumor neovasculature and promoted accumulation of macromolecules, including anticancer nanocarriers, in the tumors. Compared with the absence of TβR-I inhibitor, anticancer nanocarriers exhibited potent growth inhibitory effects on these cancers in the presence of TβR-I inhibitor. The use of TβR-I inhibitor combined with nanocarriers may thus be of significant clinical and practical importance in treating intractable solid cancers.

Nanocell for Targeted Drug Delivery to Tumor

Simultaneous delivery of chemotherapeutic and antiangiogenic drugs is clearly beneficial, but because chemotherapy is bloodborne, shutting down the tumor's blood supply with antiangiogenic drugs may decrease the delivery of drugs designed to kill the tumor cells. A more effective strategy would be to use a delivery vehicle that became concentrated in tumors before the vasculature shuts down, and allows the staged release of the two drugs (Sengupta et al 2005). The delivery of the antiangiogenic factor could lead to a collapse of the vascular network and imprison the vehicle, which would still be carrying its second payload of chemotherapeutic drug, inside the tumor. The subsequent release of the latter drug within the tumor would kill the cancer cells.

To prove this hypothesis, the researchers created composite vehicle particles of 80–120 nm—a nanocell consisting of a solid biodegradable polymer core surrounded by a lipid membrane. The nanocell was constructed as a balloon within

a balloon that resembles an actual cell. The outer membrane of the nanocell is loaded with the antiangiogenic drug combretastatin and the inner membrane with the chemotherapy drug DOX.

A "stealth" surface chemistry on the nanocell enables it to avoid attracting the attention of the immune system. While the nanocells are small enough to pass through tumor blood vessels, they are too large to pass through the pores of healthy, normal vessels. Once inside the tumor, the nanocell's outer membrane disintegrates, releasing the antiangiogenic drug and causing the collapse of the blood vessels feeding the tumor. The collapsed blood vessels trap the nanocell inside the tumor. The nanocell then slowly releases the chemotherapy drug.

Effects of the drugs were examined on two types of tumor in mice. Either drug alone was shown to slow tumor growth, but when the drugs were delivered simultaneously, there was an additive effect. The staged release of the two drugs using the new delivery vehicle improved the outcome still further and the survival time increased from $\sim$30 days when the drugs were delivered simultaneously to $>$60 days when they were released sequentially. The delivery vehicles tended to accumulate in the tumors, rather than in other body tissues, and the drugs they transported killed both endothelial and cancer cells.

Although the effect of the sequential delivery of these two drugs on tumor growth is dramatic, these results cannot be quickly translated into therapy for humans. There is a concern that antiangiogenic drugs may promote the spread of tumors to other tissues. Also, in contrast to combretastatin, many antiangiogenic drugs require prolonged tissue exposure to shut down the vasculature, and so may not be amenable to this particular approach with a short exposure time. Nevertheless, this method needs to be pursued further. Appropriate design of drugs will allow targeting of cancer cells or other specific cell types and the delivery device could readily be modified for this. It may also be necessary to target multiple aspects of angiogenesis, either by using several drugs or by using a drug that interferes with several pathways to prevent tumors from switching on alternative angiogenesis pathways. Ultimately, combining the development of advanced drug delivery systems with the identification of early markers of cancer may allow early and highly effective intervention with prospects of cure for cancer.

Nanodroplets for Site-Specific Cancer Treatment

ImaRx Therapeutics (Tucson, AZ) is developing nanodroplets for site-specific cancer treatment under a contract, jointly awarded by NASA and the National Cancer Institute in 2003 with funding for 3 years. The nanodroplets will be loaded with cancer drugs and targeted to specifically seek out cancer cells and release the drug payload. The therapy will also use ultrasound to further aid in specifically targeting cancer cells. ImaRx will be collaborating with scientists at the University of Arizona and the University of California at Davis.

One purpose of this research is to develop a nanoparticle delivery system for CPT-based drugs because their poor solubility and labile lactone ring pose challenges for drug delivery. After initial investigations SN-38 was selected as the

candidate camptotheca alkaloid for further development. Nanoparticles comprising SN-38, phospholipids and PEG were developed and studied in vitro and in vivo (Williams et al 2003). The SN-38 formulations were stable in HSA and high lactone concentrations were observed even after 3 h. In vivo studies in nude mice showed prolonged half-life of the active (lactone form) drug in whole blood and increased efficacy compared with Camptosar in a mouse xenograft tumor model.

Polymer Nanoparticles for Targeted Drug Delivery in Prostate Cancer

A prostate-specific, locally delivered gene therapy has been developed for the targeted killing of prostate cells using C32/DT-A, a degradable polymer of a nanoparticulate system, to deliver a diphtheria toxin suicide gene (DT-A) driven by a prostate-specific promoter to cells (Peng et al 2007). These nanoparticles were directly injected to the normal prostate and to prostate tumors in mice. Nearly 50% of normal prostates showed a significant reduction in size, attributable to cellular apoptosis, whereas injection with naked DT-A-encoding DNA had little effect. A single injection of C32/DT-A nanoparticles triggered apoptosis in 80% of tumor cells present in the tissue. It is expected that multiple nanoparticle injections would trigger a greater percentage of prostate tumor cells to undergo apoptosis. These results suggest that local delivery of polymer/DT-A nanoparticles may have application in the treatment of benign prostatic hypertrophy and prostate cancer.

Polymersomes for Targeted Cancer Drug Delivery

Polymersomes, hollow shell nanoparticles, have unique properties that allow them to deliver two distinct drugs—paclitaxel and DOX directly to tumors implanted in mice (Ahmed et al 2006). Loading, delivery, and cytosolic uptake of drug mixtures from degradable polymersomes are shown to exploit both the thick membrane of these block-copolymer vesicles and their aqueous lumen as well as pH-triggered release within endolysosomes. Drug-delivering polymersomes break down in the acidic environment of the cancer cells, resulting in targeted release of these drugs within tumor cells. While cell membranes and liposomes (vesicles often used for drug-delivery) are created from a double layer of fatty molecules called phospholipids, a polymersome is composed of two layers of synthetic polymers. The individual polymers are degradable and considerably larger than individual phospholipids but have many of the same chemical features. The large polymers making up the shell allow paclitaxel, which is water-insoluble, to embed within the shell. DOX, which is water-soluble, stays within the interior of the polymersome until it degrades. The polymersome and drug combination is self-assembling the structure spontaneously forms when all of the components are suitably mixed together. Recent studies have shown that cocktails of paclitaxel and DOX lead to better tumor regression than either drug alone, but previously there was no carrier system that could carry both drugs as efficiently to a tumor. Polymersomes get around those limitations.

Another approach is by assembling diverse bioactive agents, such as DNA, proteins, and drug molecules, into core–shell multifunctional polymeric nanoparticles (PNPs) that can be internalized in human breast cancer cells (Bertin et al 2006). Using ring-opening metathesis polymerization, block copolymers containing small-molecule drug segments (>50% w/w) and tosylated hexaethylene glycol segments were prepared and assembled into PNPs that allowed for the surface conjugation of DNA sequences and/or tumor-targeting antibodies. The resulting antibody-functionalized particles were readily uptaken by breast cancer cells that overexpressed the corresponding antigens.

Quantum Dots and Rods for Targeted Drug Delivery in Cancer

A single-particle QD conjugated with a tumor-targeting MAb (anti-HER2) has been tracked in tumors of live mice (Tada et al 2007). The researchers used a dorsal skinfold chamber and a high-speed confocal microscope with a high-sensitivity camera to track the antibody-labeled QDs and made 30-frame-per-second movies of these nanoparticles as they traveled through the bloodstream. The HER2 MAb binds to a protein found on the surface of certain breast and other tumors. This was injected, conjugated to the QDs, into mice with HER2-overexpressing breast cancer to analyze the molecular processes of its mechanistic delivery to the tumor. The investigators identified six distinct "stop-and-go" steps in the process involved in the antibody-labeled QDs traveling from the injection site to the cell where they bind HER2: within a blood vessel in the circulation, during extravasation, in the extracellular region, binding HER2 on the cell membrane, moving into the perinuclear region and within the perinuclear region. The image analysis of the delivery processes of single particles in vivo thus provides valuable information on antibody-conjugated therapeutic nanoparticles, which will be useful in increasing therapeutic efficacy.

Water-soluble CdSe/CdS/ZnS QRs have been developed as targeted probes for imaging cancer cell lines using two-photon fluorescence imaging. The researchers first developed a new method of creating QRs that would remain well dispersed in water and then refined the technique to allow the attachment of targeting molecules (in this case, transferrin, which binds to a receptor that is overexpressed in many types of cancer cells) to the QR surface. QRs, similar to the spherical QDs, fluoresce and can be made to fluoresce in a range of colors. However, since QRs have larger dimensions than QDs, they are easier to excite with incoming light than QDs. This research showed that the QRs were only taken up by targeted transferrin-positive cells and accumulated within these cells, being easily visible using low-intensity NIR light, which helps to protect cell integrity. If future research can further our understanding of QDs and QRs following these studies, it is hoped that we could then improve the ability of nanoparticles to deliver drugs specifically to tumors, thus resulting in improved cancer diagnostics and therapeutics.

Targeted Drug Delivery with Nanoparticle–Aptamer Bioconjugates

Nucleic acid ligands (aptamers) are potentially well suited for the therapeutic targeting of drug-encapsulated controlled release polymer particles in a cell- or tissue-specific manner. Scientists at the MIT have synthesized poly(lactic acid)-block-polyethylene glycol (PLA-PEG) copolymer with a terminal carboxylic acid functional group (PLA-PEG-COOH), and encapsulated rhodamine-labeled dextran (as a model drug) within PLA-PEG-COOH nanoparticles (Farokhzad et al 2004). These nanoparticles have the following desirable characteristics:

- Negative surface charge, which may minimize nonspecific interaction with the negatively charged nucleic acid aptamers
- Carboxylic acid groups on the particle surface for potential modification and covalent conjugation to amine-modified aptamers
- Presence of PEG on particle surface, which enhances circulating half-life while contributing to decreased uptake in nontargeted cells

Nanoparticle–aptamer bioconjugates were generated with RNA aptamers that bind to the prostate-specific membrane antigen (PSMA), a well-known prostate cancer tumor marker that is overexpressed on prostate acinar epithelial cells. These bioconjugates could efficiently target and are taken up by the prostate epithelial cells, which express the PSMA protein. The uptake of these particles was not enhanced in cells that do not express the PSMA protein. This represents the first report of targeted drug delivery with nanoparticle–aptamer bioconjugates.

Numerous investigators have used aptamers as replacements for antibodies in various therapeutic and diagnostic applications, and now a team at McMaster University has found a third use for these versatile molecules—as the heart of a DNA–protein nanoengine that can be programmed to release therapeutically useful molecules in response to a programmed molecular signal (Nutiu and Li 2005). To construct their nanoengine, the researchers first create an aptamer that binds to a molecule that would signal "release cargo here". The researchers call this signaling molecule the "input." The researchers then prepare a complementary piece of DNA that binds to the aptamer according to the Watson–Crick rules. A drug molecule, or even a therapeutic gene, can be linked to this piece of DNA, and the combination is called the "output." When the output piece of DNA is then mixed with the aptamer, the two bind to one another until the aptamer comes in contact with the input signal. The aptamer folds around the input signal, causing it to release its cargo, the output DNA–drug molecule combination. As an example, the researchers used this construct to carry and release an enzyme.

Nanoparticle-Based Anticancer Drug Delivery to Overcome MDR

Although MDR is known to develop through a variety of molecular mechanisms within the tumor cell, many tend to converge toward the alteration of apoptotic signaling. The enzyme glucosylceramide synthase (GCS), responsible for bioactivation

of the proapoptotic mediator ceramide to a nonfunctional moiety glucosylceramide, is overexpressed in many MDR tumor types and has been implicated in cell survival in the presence of chemotherapy.

A study has investigated the therapeutic strategy of coadministering ceramide with paclitaxel in an attempt to restore apoptotic signaling and overcome MDR in the human ovarian cancer cell line using modified poly(epsilon-caprolactone) (PEO-PCL) nanoparticles to encapsulate and deliver the therapeutic agents for enhanced efficacy (van Vlerken et al 2007). Results show that indeed the complete population of MDR cancer cells can be eradicated by this approach. Moreover, with nanoparticle drug delivery, the MDR cells can be resensitized to a dose of paclitaxel near the IC50 of non-MDR (drug-sensitive) cells, indicating a 100-fold increase in chemosensitization via this approach. Molecular analysis of activity verified the hypothesis that the efficacy of this therapeutic approach is due to a restoration in apoptotic signaling, although the beneficial properties of PEO-PCL nanoparticle delivery enhanced the therapeutic success even further, showing the promising potential for the clinical use of this therapeutic strategy to overcome MDR.

Devices for Nanotechnology-Based Cancer Therapy

Convection-Enhanced Delivery with Nanoliposomal CPT-11

Combination of convection-enhanced delivery (CED) with a novel, highly stable nanoparticle/liposome containing CPT-11 (nanoliposomal CPT-11) is a potential dual-drug delivery strategy for brain tumor treatment. After CED in rat brains, tissue retention of nanoliposomal CPT-11 was shown to be greatly prolonged, with $>20\%$ injected dose remaining at 12 days (Noble et al 2006). In contrast, CED of free CPT-11 resulted in rapid drug clearance. At equivalent CED doses, nanoliposomal CPT-11 increased area under the time–concentration curve by 25-fold and tissue $t1/2$ by 22-fold over free CPT-11; CED in intracranial U87 glioma xenografts showed even longer tumor retention. Plasma levels were undetectable after CED of nanoliposomal CPT-11. Importantly, prolonged exposure to nanoliposomal CPT-11 resulted in no measurable CNS toxicity at any dose tested, whereas CED of free CPT-11 induced severe CNS toxicity. In the intracranial U87 glioma xenograft model, a single CED infusion of nanoliposomal CPT-11 resulted in significantly improved median survival compared with CED of control liposomes. The study concluded that CED of nanoliposomal CPT-11 greatly prolonged tissue residence while also substantially reducing toxicity, resulting in a highly effective treatment strategy in preclinical brain tumor models.

Nanocomposite Devices

The Center for Biologic Nanotechnology at the University of Michigan is synthesizing and using novel nanocomposite devices (NCDs) as smart agents for the diagnosis and therapy of cancer. The goal is to develop NCDs to deliver radioisotopes selectively to tumors by exploiting differences between normal and tumor

vasculature. These NCDs (within the range of 5–100 nm) will have a specific surface to permit both size-related and/or surface recognition targeting of the encapsulated radioisotopes. They will have the ability to carry different isotopes either individually or in combinations to their targets. So far bismuth- and gold-containing nanocomposite particles with neutral and positive surfaces have been synthesized. Activation of gold metal atoms in the composite nanoparticle into Au-198 was achieved by direct irradiation in a neutron beam. Currently, the targeting capabilities of a series of PAMAM (polyamidoamine) dendrimer-derived nanoparticles with various size/charge and other antigenic characteristics are being examined. Delivery, biodistribution, and cytotoxicity of the radioactive nanoparticle was observed in the melanoma-bearing C57BL6/J mice. The dendrimers localized to major organs and tumor tissue, and stable levels were maintained for up to 7 days without in vivo toxicity. Biodistribution experiments with gold/PAMAM nanocomposites are in progress.

Chemical–biological hybrid nanocomposites can be introduced into cells and subsequently used to initiate intracellular processes or biochemical reactions. Such nanocomposites would advance medical biotechnology, just as they are improving microarray technology and imaging in biology and medicine, and introducing new possibilities in chemistry and material sciences.

Nanoengineered Silicon for Brachytherapy

BrachySil™ (^{32}P BioSilicon) is a nanoparticle in which the isotope 32-phosphorus is immobilized. It demonstrates a very high degree of isotope retention following injection into the liver, thus reducing the risk of soluble radioactive material affecting healthy hepatic tissue, or entering the circulation and causing systemic toxicity. Unlike titanium seeds, which remain forever in the body, phosphorus seeds degrade over time and enable repetition of treatment if necessary. Other treatments for primary liver cancer include a variety of embolization and radio-frequency ablation techniques. BrachySil offers a more versatile and safer product for the treatment of such tumors. The procedure is undertaken without surgery under local anesthetic and patients can be discharged the following day. A phase IIa trial in primary liver cancer has shown that it is safe and effective in tumor regression with increased efficacy. Following the completion of analysis of the final phase IIa trial results, a dose profiling study was conducted in 2005 followed by multicenter pivotal registration trials to obtain data to support registration of BrachySil as an approved treatment for primary liver cancer. Its use will be expanded for a wider range of solid tumor indications. BioSilicon™ is being evaluated for brachytherapy of prostate cancer at Singapore General Hospital, Singapore.

Nanoparticles Combined with Physical Agents for Tumor Ablation

Carbon Nanotubes for Laser-Induced Cancer Destruction

Biological systems are known to be highly transparent to 700–1,100-nm NIR light. It is shown here that the strong optical absorbance of SWNTs in this special spectral

window, an intrinsic property of carbon nanotubes, can be used for optical stimulation of nanotubes inside living cells to afford multifunctional nanotube biological transporters. For ODNs transported inside living cells by nanotubes, the oligos can translocate into cell nucleus upon endosomal rupture triggered by NIR laser pulses. Continuous NIR radiation can cause cell death because of excessive local heating of carbon nanotubes in vitro. Selective cancer cell destruction can be achieved by functionalization of CNTs with a folate moiety, selective internalization of CNTs inside cells labeled with folate receptor tumor markers, and NIR-triggered cell death, without harming receptor-free normal cells. Thus, the transporting capabilities of CNTs combined with suitable functionalization chemistry and their intrinsic optical properties can lead to new classes of novel nanomaterials for drug delivery and cancer therapy (Kam et al 2005). One example for application is lymphoma, as lymphoma cells have well-defined surface receptors that recognize unique antibodies. When attached to a CNT, the antibody would play the role of a Trojan horse. This approach is being tested in laboratory mice with lymphoma. The researchers want to determine whether shining NIR on the animal's skin will destroy lymphatic tumors, while leaving normal cells intact. CNTs also can be delivered to diseased cells by direct injection. The idea is to use the nanotube to deliver therapeutic molecules of DNA, RNA, or protein directly into the cell nucleus to fight various infections and diseases.

Nanoparticles and Thermal Ablation

An experimental procedure for the treatment of breast cancer called "magnetic thermal ablation" has been examined under in vivo animal conditions. MNPs are promising tools for the minimal invasive elimination of small tumors in the breast using magnetically induced heating. The approach complies with the increasing demand for breast-conserving therapies and has the advantage of offering a selective and refined tuning of the degree of energy deposition allowing an adequate temperature control at the target (Hilger et al 2005).

Anti-HER2 antibody can induce antitumor responses and can be used in delivering drugs to HER2-overexpressing cancer. Anti-HER2 immunoliposomes containing magnetite nanoparticles, which act as tumor-targeting vehicles, have been used to combine anti-HER2 antibody therapy with hyperthermia (Ito et al 2004a). When introduced into SKBr3 breast cancer cells in vitro, 60% of magnetite nanoparticles incorporated into SKBr3. The cells were then heated at 42.5°C under an alternating magnetic field, resulting in strong cytotoxic effects. These results suggest that this novel therapeutic tool is applicable to treatment of HER2-overexpressing cancer.

An innovation of thermal ablation developed by Triton BioSystems (Chelmsford, MA, USA) is a noninvasive method of killing cancer using localized lethal heat with negligible damage to healthy tissues. This approach bonds iron nanoparticles and MAbs into bioprobes. The company's product is referred to as the Targeted Nano-Therapeutics^{TM} (TNT) system. The TNT system consists of two components:

1. The injectable component referred to as T-probes. Each T-probe is made of two parts: (i) an MAb that acts only as a guidance system directing the T-probe (40 nm long) to cancer cells and (ii) a nanoscale particle made of a special material composite that constitutes the lethal payload when activated.
2. The magnetic field device that serves to activate the T-probes in the treatment area.

Trillions of T-probes are dispensed into the body in serum form by infusion into the bloodstream. Once the T-probes are attached to cancer cells, a focused magnetic field selectively activates their magnetic particles. The magnetic field energy is converted to lethal heat by the particles causing a rapid temperature increase to $> 170^{\circ}C$ at the surface of the cancer cells, killing them and their blood supply with negligible damage to surrounding healthy tissues. To evaluate the potential of TNT for in vivo tumor, targeting, efficacy, and predictive radionuclide-based heat dosimetry were studied using ^{111}In-ChL6 bioprobes (ChL6 is chimeric L6) in a human breast cancer xenograft model (Denardo et al 2007). Mice in the study received a series of alternating magnetic field (AMF) bursts in a single 20-min treatment. Dosing was calculated using an equation that included tumor concentration of bioprobes, heating rate of particles at different amplitudes, and the spacing of AMF bursts. MAb-guided bioprobes (iron oxide nanoparticles) effectively targeted the tumors without causing particle-related toxicity. Tumor total heat dose, calculated using empirically observed ^{111}In-bioprobe tumor concentration and in vitro nanoparticle heat induction by AMF, correlated with tumor growth delay. The biggest problems of thermotherapy of cancer have been how to apply it to the tumor alone, how to predict the amount needed, and how to determine its effectiveness. By combining nanotechnology, focused AMF therapy, and quantitative molecular imaging techniques, a safe technique has been developed that could be considered for clinical use as a treatment for breast and other cancers.

Nanoparticles Combined with Ultrasound Radiation of Tumors

Nanoparticles have been introduced in tumors followed by ultrasound-induced cavitation for safe and efficient drug and gene delivery. In a study on athymic nude mice bearing human colon KM20 tumors, polystyrene nanoparticles (100 and 280 nm in diameter) were injected intravenously in combination with ultrasound to enhance delivery of chemotherapeutic agent 5-fluorouracil (Larina et al 2005). This combination significantly decreased tumor volume and resulted in complete tumor regression at optimal irradiation conditions.

Nanoparticles as Adjuncts to Photodynamic Therapy of Cancer

PDT uses light-activated drugs called photosensitizers to treat a range of diseases characterized by rapidly growing tissue, including the formation of abnormal blood vessels, such as cancer and age-related macular degeneration. The more traditional name for this therapy is photoradiation therapy. Treatment with PDT consists of a

two-step process that starts with the administration of the drug, or photosensitizer, by intravenous injection. Once the drug enters the bloodstream, it attaches itself to LDLs already circulating. As cells undergoing rapid growth require an above-average supply of lipoproteins, the drug reaches these types of cells more quickly and in higher concentrations. Once the necessary level of concentration is attained, the second step is to activate the drug with a specific dose of light of a particular wavelength. This causes the conversion of normal oxygen found in tissue to a highly energized form called singlet oxygen, which in turn disrupts normal cellular functions. Neither the drug nor the light exerts any effect until combined.

Numerous studies have used liposomes, oils, and PMs as encapsulation methods, with some success. However, all of these techniques suffer from one unpleasant side effect: after controlled release and photosensitization, the drug is free to circulate the body, accumulating in the eyes and skin. This leads to phototoxic side effects, rendering the patient highly sensitive to light. A further disadvantage is that liposomes can be engulfed and destroyed by cells of the RES. Such problems have limited the emerging field of PDT, but the combination of this technique with nanotechnology is promising.

The possibility of improving dendrimers through appropriate functionalization of their periphery makes them promising carriers of PDT. The use of 5-aminolevulinic acid (ALA) is one approach to PDT based on dendrimers. ALA is a natural precursor of the photosensitizer protoporphyrin IX (PIX) and its administration increases the cellular concentrations of PIX. Cellular uptake of the dendrimer occurs through endocytic routes predominantly via a macropinocytosis pathway. A dendrimer conjugate, which incorporated 18 aminolaevulinic acid residues attached via ester linkages to a multipodent aromatic core, has been investigated (Battah et al 2007). The ability of the dendrimer to deliver and release 5-ALA intracellularly for metabolism to the photosensitizer, PIX, was studied in the transformed PAM 212 murine keratinocyte and A431 human epidermoid carcinoma cell lines. The macromolecular dendritic derivatives were shown to be capable of delivering 5-ALA efficiently to cells for sustained porphyrin synthesis.

An approach to deep tissue penetration is based on two-photon excitation with NIR lasers. Multivalent aspects of dendrimer scaffold have been used to conjugate several two-photon absorbing chromophores to the porphyrin core (Dichtel et al 2004). This system has been shown to generate singlet oxygen efficiently on light irradiation at 780 nm wavelength.

Poly(lactic-co-glycolic acid) nanoparticles have been produced, which encapsulate the photosensitizer meso-tetraphenylporpholactol and are stable and non-phototoxic upon systemic administration (McCarthy et al 2005). Upon cellular internalization, the photosensitizer is released from the nanoparticle and becomes highly phototoxic. Irradiation with visible light results in cell-specific killing of several cancer cell lines. In vivo experiments have show complete eradication of cancers in mouse models. The concept of photosensitizers with selective phototoxicity should have widespread applications in cancer therapy.

Dynamic ceramics is a new development that addresses the various problems of previous PDT applications has been described by a team from the State University of

New York's Institute for Lasers, Photonics, and Biophotonics in collaboration with members of Roswell Park Cancer Institute, which is the birthplace of PDT (Roy et al 2003a). For the drug, they chose 2-devinyl-2-(1-hexyloxyethyl) pyropheophorbide (HPPH), which is undergoing phase I/II clinical trials for esophageal cancer. The drug is encapsulated within a ceramic-based nanoparticle, the physical properties of which make it ideal for the job. The spherical particles of ~35 nm diameter are made from silica, or similar materials, and are readily prepared at ambient temperature using a simple procedure. They are stable to fluctuations in temperature and pH and small enough to evade the RES. The shape, size, and porosity can all be tailored, and the exterior of each particle can be functionalized to improve targeting. The success of the technique relies upon the tiny pores in the ceramic particle, which range from 0.5 to 1.0 nm. These are too small to allow the drug to escape its encapsulation but small enough to enable oxygen to diffuse back and forth. Thus, HPPH can exert tumor-killing effects without being released into the bloodstream. So far, the technique has only been demonstrated in vitro. The HPPH-containing nanoparticles were actively taken up by cultured UCI-107 and HeLa tumor cells. Irradiation at 650 nm with a laser caused significant tumor cell death, leaving fewer than 10% of HeLa cells viable. Preliminary in vivo tests have now begun. The ceramic nanoparticles accumulate exclusively in the tumor tissue without the need for active targeting. After phototherapy, the ceramic spheres retain the drug and are unlikely to cause the side effects that were seen with earlier encapsulation methods. In an extension of the technique, a magnetic core is encapsulated within a silica nanoparticle and targeted to tumor tissue. The magnetically impregnated tumor cells could then be killed by exposing the tissue to a DC magnetic field. This is a potentially complementary technique to PDT.

A nanocarrier consisting of PMs of diacylphospholipid-poly(ethylene glycol) (PE-PEG) coloaded with the photosensitizer drug 2-[1-hexyloxyethyl]-2-devinyl pyropheophorbide-a (HPPH) and magnetic Fe_3O_4 nanoparticles has been used for guided drug delivery together with light-activated PDT for cancer (Cinteza et al 2006). The nanocarrier shows excellent stability and activity over several weeks. The loading efficiency of HPPH is practically unaffected upon coloading with the magnetic nanoparticles, and its phototoxicity is retained. The magnetic response of the nanocarriers was demonstrated by their magnetically directed delivery to tumor cells in vitro. The magnetophoretic control on the cellular uptake provides enhanced imaging and phototoxicity. These multifunctional nanocarriers demonstrate the exciting prospect offered by nanochemistry for targeting PDT.

Nanoparticles for Boron Neutron Capture Therapy

BNCT offers a potential method for localized destruction of tumor cells. The technology is based on the nuclear reaction between thermal neutrons and boron-10 (10B) to yield alpha particles and lithium-7 nuclei. The destructive effect of this reaction is limited to a range of about the diameter of a single cell. In order for BNCT to be effective in cancer therapy, there must be selective delivery of an adequate concentration of 10B to tumors. Various types of antibodies as well as EGF

have been utilized to investigate receptor-mediated boron delivery; however, in vivo studies have demonstrated that only a small percentage of the total administered dose actually accumulates in tumors, while high concentrations end up in the liver.

In normal as well as cancer cells, the low molecular weight vitamin, folic acid, is required for a number of enzymatic pathways. Cell membrane receptors mediating endocytic transport of folic acid into cells are expressed in elevated levels in a variety of human tumors. Folic acid conjugates with macromoledules such as toxins, enzymes, antibodies, genes, and liposomes have been shown to be internalized into tumor cells overexpressing folate receptors. These strategies have been employed to enhance the effect of BNCT. The use of dendrimers as boron carriers for antibody conjugation is based on their well-defined structure and multivalency.

The use of dendrimers as boron carriers for antibody conjugation is based on their well-defined structure and multivalency. Boronated PAMAM dendrimers have been designed to target the EGFR, a cell surface receptor that is frequently overexpressed in brain tumor cells.

Boron carbide nanoparticles are proposed as a system for T-cell-guided BNCT (Mortensen et al 2006). Nanoparticles were produced by ball milling in various atmospheres of commercially available boron carbide. The physical and chemical properties of the particles were investigated using TEM, photon correlation spectroscopy, x-ray photoelectron spectroscopy, x-ray diffraction, vibrational spectroscopy, gel electrophoresis, and chemical assays and revealed profound changes in surface chemistry and structural characteristics. In vitro thermal neutron irradiation of B16 melanoma cells incubated with sub-100-nm nanoparticles induced complete cell death. The nanoparticles alone induced no toxicity.

RNA Nanotechnology for Delivery of Cancer Therapeutics

RNA has immense promise as a therapeutic agent against cancer, but the problem has been to have an efficient system to bring multiple therapeutic agents directly into specific cancer cells where they can perform different tasks. The 25-nm RNA nanoparticles enable repeated long-term administration and avoid the problems of short retention time of small molecules and the difficulties in the delivery of particles >100 nm. Scientists at Purdue University have used these nanoparticles, which are assembled from three short pieces of RNA and resemble miniature triangles. The microscopic particles possess both the right size to gain entry into cells and the right structure to carry other therapeutic strands of RNA inside with them, where they are able to halt viral growth or cancer's progress. RNA molecules come in many variant forms, and the one mimicked from the phi29 virus—called pRNA—also can be linked to other types of RNA to form longer, hybrid strands with properties that could be assigned. Incubation of cancer with the pRNA dimer, one subunit of which harbored the receptor-binding moiety and the other harboring the gene-silencing molecule, resulted in their binding and entry into the cells, and subsequent silencing of anti/proapoptotic genes. The chimeric pRNA complex was found to be

processed into functional double-stranded siRNA by Dicer (RNA-specific endonuclease). Animal trials have confirmed the suppression of tumorigenicity of cancer cells by ex vivo delivery (Guo et al 2005).

RNA nanotechnology has been used to engineer both therapeutic siRNA and a receptor-binding RNA aptamer into individual pRNAs of phi29's motor (Khaled et al 2005). The RNA building block harboring siRNA or other therapeutic molecules was fabricated subsequently into a trimer through the interaction of engineered right and left interlocking RNA loops. The incubation of the protein-free nanoscale particles containing the receptor-binding aptamer or other ligands results in the binding and coentry of the trivalent therapeutic particles into cells, subsequently modulating the apoptosis of cancer cells and leukemia model lymphocytes in cell culture and animal trials. The use of such antigenicity-free 20–40 nm particles holds promise for the repeated long-term treatment of chronic diseases.

Nanocarriers for Simultaneous Delivery of Multiple Anticancer Agents

Researchers from the Northeastern University, MIT, and Massachusetts General Hospital (Boston, MA), supported by NCI Platform Partnership award, have already designed nanoscale carrier molecules to deliver several drugs to tumor cells all at once, providing not just a one-two punch, but three, four, or five "knockout" steps. Once the cargo has been delivered, the nanocarrier is destroyed, leaving no trace. The next step is to take this technology to the clinic with the goal of treating breast and ovarian cancers that have not responded to current therapies.

Combination of Diagnostics and Therapeutics for Cancer

Biomimetic Nanoparticles Targeted to Tumors

Nanoparticle-based diagnostics and therapeutics hold great promise because multiple functions can be built into the particles. One such function is an ability to home to specific sites in the body. Biomimetic particles that not only home to tumors but also amplify their own homing have been described (Simberg et al 2007). The system is based on a peptide that recognizes clotted plasma proteins and selectively homes to tumors, where it binds to vessel walls and tumor stroma. Iron oxide nanoparticles and liposomes coated with this tumor-homing peptide accumulate in tumor vessels, where they induce additional local clotting, thereby producing new binding sites for more particles. The system mimics platelets, which also circulate freely but accumulate at a diseased site and amplify their own accumulation at that site. The self-amplifying homing is a novel function for nanoparticles. The clotting-based amplification greatly enhances tumor imaging, and the addition of a drug carrier function to the particles is envisioned.

Dendrimer Nanoparticles for Targeting and Imaging Tumors

Dendrimer nanoparticles have been used to entrap metal nanoparticles, a combination that could serve as a potent imaging and thermal therapy agent for tumors if it were not for associated toxicity issues. To eliminate the toxicity associated with dendrimer–metal nanoparticle combinations, scientists from the University of Michigan have developed methods for modifying the surface of dendrimers laden with gold nanoparticles. This chemical treatment greatly reduces the toxicity of the hybrid nanoparticle, without changing its size. The investigators also found that the chemical modification they developed makes it possible to add targeting molecules to the surface of the dendrimer. Between four and five folic acid molecules were added to the surface of the dendrimer. Folic acid binds to a high-affinity receptor found on many types of tumor cells. Nanoparticles coated with folic acid target tumors and preferentially deliver their drug or imaging payloads to cancer cells. After folic acid conjugated with dendrimers loaded with gold nanoparticles, the resulting construct is stable across a wide range of pH and the nanoparticles do not clump together under physiological conditions. When these nanoparticles are added to tumor cells bearing the high-affinity folic acid receptor, gold-laden nanoparticles accumulate within the cells. Microscopic studies show that the nanoparticles accumulate within intracellular lysosomes. These experiments are being repeated in animal models of human cancer.

Gold Nanorods for Diagnosis plus Photothermal Therapy of Cancer

Photothermal therapy is based on the enhancement of electromagnetic radiation by noble metal nanoparticles due to strong electric fields at the surface. The nanoparticles also absorbed the laser light more easily, so that the coated malignant cells only required half the laser energy to be killed compared with the benign cells. This makes it relatively easy to ensure that only the malignant cells are being destroyed. These unique properties provide the potential of designing novel optically active reagents for simultaneous molecular imaging and photothermal cancer therapy. Gold nanorods with suitable aspect ratios (length divided by width) can absorb and scatter strongly in the NIR region (650–900 nm). Changing the spheres into rods lowers the frequency to which the nanoparticles respond from the visible light spectrum used by the nanospheres to the NIR spectrum. Since these lasers can penetrate deeper under the skin than lasers in the visible spectrum, they can reach tumors that are inaccessible to visible lasers.

In vitro studies have demonstrated that gold nanorods are novel contrast agents for both molecular imaging and photothermal cancer therapy (Huang et al 2006). Nanorods are synthesized and conjugated to anti-EGFR MAbs and incubated in cancer cell cultures. The anti-EGFR antibody-conjugated nanorods bind specifically to the surface of the malignant-type cells with a much higher affinity due to the

overexpressed EGFR on the cytoplasmic membrane of the malignant cells. As a result of the strongly scattered red light from gold nanorods in dark field, observed using a laboratory microscope, the malignant cells are clearly visualized and diagnosed from the nonmalignant cells. It is found that, after exposure to continuous red laser at 800 nm, malignant cells require about half the laser energy to be photothermally destroyed than the nonmalignant cells. Thus, both efficient cancer cell diagnostics and selective photothermal therapy are realized at the same time.

Magnetic Nanoparticles for Imaging as Well as Therapy of Cancer

Scientists at the Michigan Nanotechnology Institute for Medicine and Biological Sciences (Ann Arbor, MI) are developing tumor-targeting dendrimers that contain both imaging agent and therapeutic agent. DNA-linked dendrimer nanocluster platform enables the delivery of drugs, genetic materials, and imaging agents to cancer cells, offering the potential for developing combinatorial therapeutics (Choi and Baker 2005). A dendrimer linked to a fluorescent imaging agent and paclitaxel can identify tumor cells and kill them simultaneously. Teams at other universities in the United States are also developing multifunctional nanoparticles for simultaneous imaging and therapeutic applications.

In the future it may be possible for a patient to be screened for breast cancer using MRI techniques with engineered enhanced ferrites as the MRI contrast agent. Enhanced ferrites are a class of ferrites that are specially engineered to have enhanced magnetic or electrical properties and are created through the use of core–shell morphology. MNPs are coupled to the radio frequency of the MRI, which converts the radio frequency into heat. If a tumor is detected, the physician could increase the power to the MRI coils and localized heating would destroy the tumor without damage to the surrounding healthy cells. The only hindrance to the development of enhanced ferrites for 100-MHz applications is a lack of understanding of the growth mechanisms and synthesis–property relationships of these nanoparticles. By studying the mechanism for the growth of the enhanced ferrites, it will be possible to create shells that help protect the metallic core from oxidation in biologically capable media.

pHLIP Nanotechnology for Detection and Targeted Therapy of Cancer

The pH-selective insertion and folding of a membrane peptide, pHLIP (pH low insertion peptide), can be used to target acidic tissue in vivo, including acidic foci in tumors. pHLIP nanotechnology is considered to be a promising approach for mapping areas of elevated acidity in the body. The peptide has three states: soluble in water, bound to the surface of a membrane, and inserted across the membrane. At physiological pH, the equilibrium is toward water, which explains its low affinity

for cells in healthy tissue; at acidic pH, the equilibrium shifts toward membrane insertion and tissue accumulation. This peptide acts like a nanosyringe to deliver tags or therapy to cells. Tumors can be detected by labeling pHLIP peptide with Cy5.5 and imaging by use of NIR fluorescence with wavelengths in the range of 700–900 nm. In a mouse breast adenocarcinoma model, fluorescently labeled pHLIP detects solid acidic tumors with high accuracy and accumulates in them even at a very early stage of tumor development (Andreev et al 2007). The fluorescence signal is stable and is approximately five times higher in tumors than in healthy counterpart tissue. Tumor targeting is based on the fact that most tumors, even very small ones, are acidic as a result of the way they grow, known as the Warburg effect (awarded the Nobel Prize in 1931). Tumors may be treated by attaching and delivering anticancer agents with pHLIP. The advantage of this approach is that hypoxia and acidosis are uniformly present biomarkers of cancer, whereas gene signatures are variable.

Targeted Therapy with Magnetic Nanomaterials Guided by Antibodies

The example of this method is TNT (Triton BioSystems), which are bioprobes with two components: a magnetic nanomaterial and an antibody that acts as a guidance system to find cancer cells. The biprobes can be injected into a cancer patient with cancer metastases. Once the probes attach themselves to cancer cells, exposure to a magnetic field produces local heat to selectively kill cancer cells without harming the normal cells. This approach will eliminate many of the side effects of currently used chemotherapy and radiotherapy.

Ultrasonic Tumor Imaging and Targeted Chemotherapy by Nanobubbles

Drug delivery in PMs combined with tumor irradiation by ultrasound results in effective drug targeting, but this technique requires prior tumor imaging. A new targeted drug delivery method uses ultrasound to image tumors, while also releasing the drug from nanobubbles into the tumor (Rapoport et al 2007). Mixtures of drug-loaded PMs and perfluoropentane (PFP) nanobubbles stabilized by the same biodegradable block copolymer were prepared. Size distribution of nanoparticles was measured by dynamic light scattering. Cavitation activity (oscillation, growth, and collapse of microbubbles) under ultrasound was assessed based on the changes in micelle/nanobubble volume ratios. The effect of the nanobubbles on the ultrasound-mediated cellular uptake of DOX in MDA-MB-231 breast tumors in vitro and in vivo (in mice bearing xenograft tumors) was determined by flow cytometry. Phase state and nanoparticle sizes were sensitive to the copolymer/perfluorocarbon volume ratio. At physiologic temperatures, nanodroplets converted into nanobubbles. DOX was localized in the nanobubble walls formed by

the block copolymer. Upon intravenous injection into mice, DOX-loaded micelles and nanobubbles extravasated selectively into the tumor interstitium, where the nanobubbles coalesced to produce microbubbles. When exposed to ultrasound, the bubbles generated echoes, which made it possible to image the tumor. The sound energy from the ultrasound popped the bubbles, releasing DOX, which enhanced intracellular uptake by tumor cells in vitro to a statistically significant extent relative to that observed with unsonicated nanobubbles and unsonicated micelles and resulted in tumor regression in the mouse model. In conclusion, multifunctional nanoparticles that are tumor-targeted drug carriers, long-lasting ultrasound contrast agents, and enhancers of ultrasound-mediated drug delivery have been developed and deserve further exploration as cancer therapeutics.

Nanoparticles for Chemoradioprotection

Chemotherapy and radiotherapy are the standard treatments for cancer, but they have severe adverse effects on the body. Radiation can damage epithelial cells and lead to permanent hair loss, among other effects, and certain types of systemic chemotherapy can produce hearing loss and damage to a number of organs, including the heart and kidneys. Only one drug, Amifostine, has been approved to date by the FDA to help protect normal tissue from the side effects of chemotherapy and radiation, and there is a need for new and improved agents. Molecular mechanisms responsible for cellular damage from radiation are being explored in tiny zebrafish embryos, which are transparent and enable scientists to closely observe damage produced by cancer treatments to organs. Using zebrafish embryos, researchers at Jefferson Medical College (Philadelphia, PA) have shown that the nanoparticle, fullerene CD60_DF1 (Tego Biosciences Corporation, Pasadena, CA, USA), can help fend off damage to normal tissue from radiation. It acts like an "oxygen sink," binding to dangerous oxygen radicals produced by radiation. Fullerenes can be considered as a potentially "new class of radioprotective agents." CD60_DF1 given before and even immediately after exposure to x-rays reduces organ damage by one-half to two-thirds, which is equal to the level of protection given by Amifostine. Moreover, the fullerene provides organ-specific protection, e.g., the kidney as well as certain parts of the nervous system. One mechanism by which radiation frequently damages cells and tissues is by producing "reactive oxygen species"—oxygen radicals, peroxides, and hydroxyls. The research team showed that zebrafish embryos exposed to ionizing radiation had $<50\%$ reactive oxygen species when compared with untreated embryos. Tego Biosciences' technology enabling certain molecules to be attached to the fullerenes, which enable targeting to specific organs and tissues. The investigators are using a mouse model to find out whether fullerene protects the entire animal from radiation and examine organ-specific effects, e.g., protecting of the lungs. They also are interested in exploring its ability to prevent some of the long-term side effects of radiation, such as fibrosis in the leg.

Nanosensor Device as an Aid to Cancer Surgery

Scientists at the University of Nebraska–Lincoln have developed a high-resolution touch sensor, one that uses a self-assembling nanoparticle device and acts much like a human finger. The self-assembly process developed by the research team involves no complex lithography, thus proving to be cost-effective and would be relatively easy to reproduce. This device has the ability to sense texture by touch, which is vital for surgeons who need the "touch sensation" in order to operate with precision and accuracy, such as when it comes to detecting and removing cancer cells from the body. One of the most important applications of this newly created sensor is the potential it holds for cancer surgeons, who are faced with the difficult task of knowing where to stop cutting when removing cancer cells in the body. In the development of artificial skin, the nanodevice structure can attain resolution of $\lesssim$20 μm. As this dimension is comparable to single-cell dimension, one can hope to "see" a single cancer cell in a tissue. The next goal is "to make a high-resolution thermal imaging device and develop an ultrasound detector with a much better image resolution to enable detection of malignant tumors at early stages."

A Cancer-Killing Device Based on Nanotechnology

It is within the realm of possibility to use molecular tools to design a miniature device that can be introduced in the body, locate and identify cancer cells, and finally destroy them. The device would have a biosensor to identify cancer cells and a supply of anticancer substance that could be released on encountering cancer cells. A small computer could be incorporated to program and integrate the combination of diagnosis and therapy and provide the possibility to monitor the in vivo activities by an external device. Since there is no universal anticancer agent, the computer program could match the type of cancer to the most appropriate agent. Such a device could be implanted as a prophylactic measure in persons who do not have any obvious manifestations of cancer. It would circulate freely and could detect and treat cancer at the earliest stage. Such a device could be reprogrammed through remote control and enable change of strategy if the lesion encountered is other than cancer.

Chapter 8
Nanoneurology

Introduction

Diseases of the nervous system are an important part of medicine. In spite of all the advances in neurology, particularly in the last decade of the twentieth century (Decade of the Brain), there are serious deficiencies in our understanding of the pathomechanism of several neurological disorders as well as our ability to diagnose and treat these disorders. Nanobiotechnology will have an impact on improving our understanding of the nervous system and developing new treatments—both medical and surgical—for disorders of the nervous system (Jain 2006a).

Nanobiotechnology for Study of the Nervous System

Nanowires for Monitoring Brain Activity

Electrical recording from spinal cord vascular capillary bed has been achieved demonstrating that the intravascular space may be utilized as a means to address brain activity without violating the brain parenchyma. Working with platinum nanowires and using blood vessels as conduits to guide the wires, researchers have successfully detected the activity of individual neurons lying adjacent to the blood vessels (Llinás et al 2005). This can provide an understanding of the brain at the neuron-to-neuron interaction level with a nonintrusive, biocompatible and biodegradable nanoprobes. This technique may one day enable monitoring of individual brain cells and perhaps provide new treatments for neurological diseases. Because the nanowires can deliver electrical impulses as well as receive them, the technique has potential as a treatment for Parkinson's disease (PD). It has already been shown that patients with PD can experience significant improvement from direct stimulation of the affected area of the brain. But the stimulation is currently carried out by inserting wires through the skull and into the brain, a process that can cause scarring of the brain tissue. By stimulating the brain with nanowires threaded through blood vessels, patients can receive benefits of the treatment without the damaging side effects. The challenge is to precisely guide the nanowire probes

K.K. Jain, *The Handbook of Nanomedicine*,

to a predetermined spot through the thousands of branches in the brain's vascular system. One solution is to replace the platinum nanowires with new conducting polymer nanowires. Not only do the polymers conduct electrical impulses, they change shape in response to electrical fields, which would allow the researchers to steer the nanowires through the brain's circulatory system. Polymer nanowires have the added benefit of being 20–30 times smaller than the platinum ones used in the reported laboratory experiments. They are biodegradable and therefore suitable for short-term brain implants.

Nanoparticles and MRI for Macrophage Tracking in the CNS

Activated macrophages, acting in concert with other immune competent cells, are an index of inflammatory/immune reaction in central nervous system (CNS) disorders such as multiple sclerosis, ischemic stroke lesions, and tumors. The MRI detection of brain macrophages defines precise spatial and temporal patterns of macrophage involvement that helps to characterize individual neurological disorders. Macrophage tracking by magnetic MRI with iron oxide nanoparticles has been developed during the last decade for numerous diseases of the CNS. Experimental studies on animal models were confirmed by clinical applications of MRI technology of brain macrophages. This approach is being explored as an in vivo biomarker for the clinical diagnosis of cerebral lesion activity, in experimental models for the prognosis of disease development, and to determine the efficacy of immunomodulatory treatments under clinical evaluation (Petry et al 2007). Comparative brain imaging follow-up studies of blood–brain barrier (BBB) leakage by MRI with gadolinium-chelates, microglia activation by PET with radiotracer ligand PK11195, and MRI detection of macrophage infiltration provide more precise information about the pathophysiological cascade of inflammatory events in cerebral diseases. Such multimodal characterization of the inflammatory events should help in the monitoring of patients, in defining precise time intervals for therapeutic interventions, and in developing and evaluating new therapeutic strategies.

Ultrasmall superparamagnetic iron oxide (USPIO) as a cell-specific contrast agent for MRI. An open-label phase II study has tested the potential of USPIO-enhanced MRI for macrophage imaging in human ischemic lesions (Saleh et al 2004). USPIO-induced signal alterations throughout differed from signatures of conventional gadolinium-enhanced MRI, thus being independent from breakdown of the BBB. Macrophages, as the prevailing inflammatory cell population in stroke, contribute to brain damage. USPIO-enhanced MRI may provide an in vivo surrogate marker of cellular inflammation in stroke and other CNS pathologies. USPIO has favorable properties that result from its intravascular retention and lack of extravasation, allowing optimal contrast between the vessel and the adjacent tissue for several minutes postinjection (Corot et al 2003). SH U 555 C (Bayer Schering Pharma) which is an optimized formulation of carboxydextran-coated ferucarbotran is another USPIO. When injected as a bolus at highest dose of 40 μmol Fe/kg, it has

the capability for depiction at first-pass magnetic resonance angiography and for cardiac perfusion (Reimer et al 2004).

Autologous bone marrow CD34+ cells labeled with magnetic nanoparticles have been delivered into the spinal cord via lumbar puncture in a study on patients with chronic spinal cord injury (SCI). One group received their own labeled CD34+ cells whereas the others received an injection containing only magnetic nanoparticles without stem cells to serve as controls. CD34+ cells were labeled with magnetic nanoparticles coated with a monoclonal antibody specific for the CD34 cell membrane antigen. MRI showed that magnetically labeled CD34+ cells were visible at the lesion site as hypointense signals following transplantation, but these signals were not visible in any patient in the control group (Callera and de Melo 2007). This study shows that autologous bone marrow CD34+ cells labeled with magnetic nanoparticles, when delivered intrathecally migrate into the site of injury in patients with chronic SCI and can be tracked by MRI.

Nanotechnology-Based Drug Delivery to the CNS

Delivery of drugs to the CNS is a challenge and the basics as well as various strategies are discussed in a special report on this topic (Jain 2007d). Molecular motors, operating at nanoscale, can deliver drugs to the CNS by peripheral muscle injection. An advantage is the use of motor in native environment and intraneural drug delivery. The disadvantages are that this approach requires engineered molecular motors for use in cells and neurotoxicity may be a problem. Currently most of the strategies are directed at overcoming the BBB.

Nanoencapsulation for Delivery of Vitamin E for CNS Disorders

Vitamin E is used for the treatment of neurological disorders, particularly those where oxidative stress plays a role. Oxidative stress is an early hallmark of affected neurons in Alzheimer's disease (AD). The antioxidant vitamin E provided limited neuroprotection in AD, which may have derived from its lipophilic nature and resultant inability to quench cytosolic reactive oxygen species (ROS), including those generated from antecedent membrane oxidative damage. Encapsulation into polyethylene glycol (PEG)-based nanospheres, which can enter the cytosol, improved the efficacy of vitamin E against Aβ-induced ROS (Shea et al 2005). These findings suggest that nanosphere-mediated delivery methods may be a useful adjunct for antioxidant therapy in AD.

Nanoparticle Technology for Drug Delivery Across BBB

Blood–brain barrier represents an insurmountable obstacle for a large number of drugs, including antibiotics, antineoplastic agents, and a variety of CNS-active

drugs, especially neuropeptides. One of the possibilities to overcome this barrier is a drug delivery to the brain using nanoparticles. Drugs that have been transported successfully into the brain using this carrier include the hexapeptide dalargin, the dipeptide kytorphin, loperamide, tubocurarine, the NMDA receptor antagonist MRZ 2/576, and doxorubicin.

The mechanism of the nanoparticle-mediated transport of the drugs across the BBB at present is not fully elucidated. The most likely mechanism is endocytosis by the endothelial cells lining the brain blood capillaries. Nanoparticle-mediated drug transport to the brain depends on the overcoating of the particles with polysorbates, especially polysorbate 80 (Aliautdin et al 2003). Overcoating with these materials seems to lead to the adsorption of apolipoprotein E from blood plasma onto the nanoparticle surface. The particles then seem to mimic low density lipoprotein (LDL) particles and could interact with the LDL receptor leading to their uptake by the endothelial cells. After this the drug may be released in these cells and diffuse into the brain interior or the particles may be transcytosed. Other processes such as tight junction modulation or P-glycoprotein (Pgp) inhibition also may occur. Moreover, these mechanisms may run in parallel or may be cooperative thus enabling a drug delivery to the brain.

In vivo studies using the antinociceptive opioid peptide dalargin showed that both empty PBCA nanoparticles and polysorbate-80 did not allow dalargin to enter the brain in quantities sufficient to cause antinociception. Only dalargin preadsorbed to PBCA nanoparticles was able to induce an antinociceptive effect in the animals. At concentrations of PBCA nanoparticles and polysorbate-80 that achieve significant drug delivery to the brain, there is little in vivo or in vitro evidence to suggest that a generalized toxic effect on the BBB is the primary mechanism for drug delivery to the brain (Kreuter et al 2003). The fact that dalargin has to be preadsorbed onto nanoparticles before it is effective in inducing antinociception suggests specific mechanisms of delivery to the CNS rather than a simple disruption of the BBB allowing a diffusional drug entry.

The use of NPs to deliver drugs to the brain across the BBB may provide a significant advantage to current strategies. The primary advantage of NP carrier technology is that NPs mask the BBB-limiting characteristics of the therapeutic drug molecule. Furthermore, this system may slow drug release in the brain, decreasing peripheral toxicity. Various factors that influence the transport include the type of polymer or surfactant, NP size, and the drug molecule (Lockman et al 2002). Currently, reports evaluating NPs for brain delivery have studied anesthetic and chemotherapeutic agents. NP technology appears to have significant promise in delivering therapeutic molecules across the BBB.

Delivery Across BBB Using NanoDel™ Technology

In NanoDel technology (NanoDel Technologies GmbH), the drugs are bound to nanoparticles, which are subsequently coated with a surfactant. The average

nanoparticle diameter is about 200–300 nm. The efficiency of nanoparticles has been proven in several animal studies. Different drugs bound to nanoparticles could be transported across the BBB and cause pharmacological effects in the brain. Efficacy of the nanoparticle approach has been demonstrated in studies with the analgesic drug, dalargin, a peptide as well as with several other drugs. Dalargin normally does not cross the BBB. Therefore, it only shows an analgesic effect when injected directly into the brain. However, when bound to nanoparticles, significant analgesia can be achieved by systemic administration (e.g., by intravenous injection).

NanoMed Technology to Mask BBB-Limiting Characteristics of Drugs

NanoMed Pharmaceuticals is using its Nanotemplate engineering technology to manufacture nanoparticles that mask a drug's BBB-limiting characteristics, enabling targeted delivery via BBB transporters. This would provide a sustained release in the brain tissue, which could reduce dose frequency and adverse effects. NanoMed is developing paclitaxel NP (an approved chemotherapeutic agent) to treat primary and secondary brain tumors. At standard therapeutic doses, paclitaxel is limited in its access to the brain by P-glycoprotein efflux pump. By effectively surmounting the BBB, paclitaxel NP enables a lower and safer drug dose that still maintains efficacy.

Nanotechnology-Based Devices and Implants for CNS

Nanoparticle-mediated drug delivery to the brain, as described in previous sections, will minimize the need for use of invasive delivery devices but there will still be need for implants and direct delivery of drugs to the brain and the cerebral ventricles. Nanomaterials, because of their action in preventing the formation of scar due to astrocyte proliferation, would improve the construction of nonreactive cerebroventricular catheters for administration of drugs into the cerebral ventricles. Nanoengineered probes can deliver drugs at the cellular level using nanofluidic channels.

An implantable chip that can serve as both a prosthetic retina and a drug delivery system has been developed to treat age-related blindness and conditions such as PD. Created by researchers at the Stanford University School of Medicine in California, the chip communicates chemically rather than electrically, using neurotransmitters to stimulate cells (Peterman et al 2004). The team built the computer chip with four tiny openings, which excrete droplets of chemicals using electro-osmosis—the movement of electrically charged particles in a fluid under the influence of an electric field. Because the chip can draw droplets of fluid in as well as out, it could also enable researchers to take samples in real time, giving them a chemical picture of what goes on in living tissues during certain processes. This "artificial synapse chip" is a prototype neural interface. Although designed for retinal drug delivery

for macular dystrophy, the chip could also serve as a drug delivery system for other parts of the CNS. It could distribute small amounts of drugs precisely where they are needed, such as dopamine in the brains of patients with PD.

Nanoparticles and MRI for Tracking Stem Cell Therapy of CNS

Cellular MRI using superparamagnetic iron oxide nanoparticles (SPION) can visualize and track cells in living organisms. MRI studies have been conducted in rat models of CNS injury and stroke to track stem cells that were either grafted intracerebrally, contralaterally to a cortical photochemical lesion, or injected intravenously (Sykova and Jendelova 2007). ESCs and MSCs were labeled with iron oxide nanoparticles (Endorem®) and human $CD34^+$ cells were labeled with magnetic MicroBeads (Miltenyi). During the first posttransplantation week, grafted MSCs or ESCs migrated to the lesion site in the cortex as well as in the spinal cord and were visible in the lesion on MRI as a hypointensive signal, persisting for more than 30 days. In rats with an SCI, an increase in functional recovery was noted after the implantation of MSCs or after an injection of granulocyte colony stimulating factor (G-CSF). Morphometric measurements in the center of the lesions showed an increase in white matter volume in cell-treated animals. Prussian blue staining confirmed a large number of iron-positive cells, and the lesions were considerably smaller than in control animals. To obtain better results with cell labeling, new polycation-bound SPION (PC-SPION) were developed. In comparison with Endorem, PC-SPION demonstrated a more efficient intracellular uptake into MSCs, with no decrease in cell viability. These studies demonstrate that MRI of grafted adult as well as ESCs labeled with iron oxide nanoparticles is a useful method for evaluating cellular migration toward a lesion site.

Application of Nanotechnology for Neuroprotection

Applied nanobiotechnology aimed at the regeneration and neuroprotection of the CNS will significantly benefit from basic nanotechnology research conducted in parallel with advances in cell biology, neurophysiology, and neuropathology (Silva 2005). The ultimate goal is to develop novel technologies that directly or indirectly aid in providing neuroprotection and/or a permissive environment and active signaling cues for guided axon growth. Research at the Silva Research Group for Cellular Neural Engineering of University of California (San Diego, CA) focuses on using nanotechnologies to study neuropathological processes. The aim is to help neuroscientists better understand the physiology of and develop treatments for disorders such as brain injury, SCI, degenerative retinal disorders, and AD. One of the areas of research in this laboratory is neuroprotection. Quantum dot (QD) technology is used to gather information about how the CNS environment becomes inhospitable to neuronal regeneration following injury or degenerative events by studying the

process of reactive gliosis. Glial cells, housekeeping cells for neurons, have their own communication mechanisms that can be triggered to become reactive following injury. QDs are being used to build data capture devices that are easy to use by neuroscientists, and a new protocol has been developed for tracking glial activity. Other research is looking at how QDs might spur growth of neurites by adding bioactive molecules to the QDs, in a way to provide a medium that will encourage this growth in a directed way.

Fullerene-Based Neuroprotective Antioxidants

Water-soluble derivatives of buckminsterfullerene C60 derivatives are a unique class of nanoparticle compounds with potent antioxidant properties. Studies on one class of these compounds, the malonic acid C60 derivatives (carboxyfullerenes), indicated that they are capable of eliminating both superoxide anion and H_2O_2, and were effective inhibitors of lipid peroxidation, as well. Carboxyfullerenes have demonstrated robust neuroprotection against excitotoxic, apoptotic, and metabolic insults in cortical cell cultures. They were also capable of rescuing mesencephalic dopaminergic neurons from both MPP(+) and 6-hydroxydopamine-induced degeneration. Although there is limited in vivo data on these compounds, systemic administration of the C3 carboxyfullerene isomer has been shown to delay motor deterioration and death in a mouse model familial amyotrophic lateral sclerosis. Ongoing studies in other animal models of CNS disease states suggest that these novel antioxidants are potential neuroprotective agents for other neurodegenerative disorders including PD.

Ceria Nanoparticles as Neuroprotective Antioxidants

Ceria nanoparticles from anthanide series have several unique properties that make them highly efficient redox reagents. Several studies have reported the ability of ceria nanoparticles to mitigate oxidative stress at the biological level. Ceria nanoparticles also protect neurons from free radical-mediated damage initiated by ultraviolet (UV) light, H_2O_2, and excitotoxicity, leading to the hypothesis that the mechanism of action is one of free radical scavenging (Rzigalinski et al 2006). When compared with single doses of other free radical scavengers, such as vitamin E, melatonin, and *n*-acetyl cysteine, ceria nanoparticles demonstrated significantly greater neuroprotection after a 5- and 15-min UV insult. A single dose of nanoparticles delivered up to 3 h postinjury also afforded neuroprotection. Ceria nanoparticles were also effective in reducing cell death associated with γ-irradiation. In another study, nanoparticles were shown to directly decrease free radical production (Schubert et al 2006). No toxicity was observed with ceria nanoparticle sizes of 6 and 12 nm and yttria oxide nanoparticles were even more effective than ceria. Ceria nanoparticles larger than 30 nm or nitrates and sulfates of cerium did not have any significant effects.

Several studies also suggest that ceria nanoparticles are potent antiinflammatory agents. Microglia, the immune cells of the brain, are "activated" in response to neuronal damage and show an inflammatory response with release of NO as well as IL-1β. Treatment of injured organotypic cultures with ceria nanoparticles reduced their ability to activate microglia. Further, treatment of activated microglia with ceria nanoparticles reduces production of soluble factors that promoted death in uninjured neurons, including NO and IL-1β. Delivery of nanoparticles to the uninjured neurons also directly affords neuroprotection from the damaging effects of activated microglia. Thus, it appears that nanoparticles may blunt the inflammatory response in immune cells, as well as reduce inflammatory injury to nonimmune cells.

Application of Nanotechnology for Neuroregeneration

One of the major challenges of treating neurological disorders, particularly CNS damage resulting from trauma, is repair and regeneration. At nanoscale, there is little difference between basic building blocks of neuronal structures whether they are created artificially or occur in nature. Improving cell to cell communication with nanoelectronics may enable the creation of a bridge between severed nerves and muscles up to a meter away. This opens up the possibilities of repairing severed spinal cords and rehabilitation of stroke victims. Studies at the Montreal Neurological Institute and McGill Institute for Advanced Materials (Montreal, Canada) have shown that latex nanobeads can attract neurons to extend processes (or neurites) over its surface and some of these processes will later form presynaptic contacts with the beads. The temporary bridges can overcome the body's inhibitory system that prevents regeneration and encourage a nerve axon to grow. The substrate in development is photosensitive and shining a light on it can change its properties so that a cell's growth could be precisely directed.

Nanotube–Neuron Electronic Interface

Thin films of carbon nanotubes deposited on transparent plastic can also serve as a surface on which cells can grow and these nanotube films could potentially serve as an electrical interface between living tissue and prosthetic devices or biomedical instruments. University of Texas Medical Branch's scientists have shown that there is some kind of electrical communication between these two things, by stimulating cells through the transparent conductive layer (Liopo et al 2006). The scientists employed two different types of cells in their experiments, neuroblastoma cells commonly used in test-tube experiments and neurons cultured from experimental rats. Both cell types were placed on 10-layer-thick "mats" of single-walled carbon nanotubes (SWNTs) deposited on transparent plastic. This enabled the researchers to use a microscope to position a tiny electrode next to individual cells and record

their responses to electrical pulses transmitted through the SWNTs. In addition to their electrical stimulation experiments, the scientists also studied how different kinds of SWNTs affected the growth and development of neuroblastoma cells. They compared cells placed on mats made of "functionalized" SWNTs, carbon nanotubes with additional molecules attached to their surfaces that may be used to guide cell growth or customize nanotube electrical properties, to cells cultured on unmodified "native" carbon nanotubes and conventional tissue culture plastic. Native carbon nanotubes supported neuron attachment and growth well better than the two types of functionalized nanotubes tested. Next step in the research is to find a way to functionalize the nanotubes to make neuron attachment and communication better and make these surfaces more biocompatible. If nanotubes turn out to be sensitive enough to record ongoing electrical activity in cells, they could form the basis of a device that can both sense and deliver stimuli to cells for prosthetic control.

Photochemical Activation of Nanoparticle-Film Neuronal Interface

The remarkable optical and electrical properties of nanostructured materials are now considered to be a source for a variety of biomaterials, biosensing, and cell interface applications. Some of the characteristics of nanoparticles can be exploited to custom-build new materials from the bottom up with characteristics such as compatibility with living cells and the ability to turn light into tiny electrical currents that can produce responses in nerves. A study reports construction of a hybrid bionanodevice where absorption of light by thin films of quantum-confined semiconductor nanoparticles of HgTe produced by the layer-by-layer assembly stimulate adherent neural cells via a sequence of photochemical and charge-transfer reactions (Pappas et al 2007). The development opens the door to applying the unique properties of nanoparticles to a wide variety of light-stimulated nerve-signaling devices including the possible development of a nanoparticle-based artificial retina.

Although light signals have previously been transmitted to nerve cells using silicon (whose ability to turn light into electricity is employed in solar cells and in the imaging sensors of video cameras), nanoengineered materials promise far greater efficiency and versatility. It should be possible to tune the electrical characteristics of these nanoparticle films to get properties like color sensitivity and differential stimulation, which are needed for an artificial retina. Creation of an actual implantable artificial retina is, however, a long-range project. But, a variety of less complex applications are enabled by a tiny, versatile light-activated interface with nerve cells, e.g., ways to connect with artificial limbs and new tools for imaging, diagnosis, and therapy. The main advantage of this technology is that remote activation by light is possible without cumbersome wire connections. This type of technology can provide noninvasive connections between the human nervous system and prostheses that are flexible, compact, and reliable. Such tools will provide nanomedicine new capabilities that were not possible with conventional medicine.

Nanoneurosurgery

Neurosurgery is an extension of neurology involving surgery, nanodiagnostics, and application of new technologies for treatment of neurological disorders. Advances in nanobiotechnology have already refined many surgical approaches to diseases of the nervous system and this new field can be called nanoneurosurgery. Examples are applications in brain cancer, neuroregeneration, and CNS implants.

Femtolaser Neurosurgery

Understanding how nerves regenerate is an important step towards developing treatments for human neurological disease, but investigation has so far been limited to complex organisms (mouse and zebrafish) in the absence of precision techniques for severing axons (axotomy). Femtosecond laser surgery has been used for axotomy in the roundworm *Caenorhabditis elegans* and these axons are functionally regenerated after the operation (Yanik et al 2004). Femtolaser acts like a pair of tiny "nanoscissors," which is able to cut nanosized structures like nerve axons.

The pulse has a very short length making the photons in the laser concentrate in one area, delivering a lot of power to a tiny, specific volume without damaging surrounding tissue. Once cut, the axons vaporize and no other tissue is harmed. The researchers cut axons they knew would impair the worms' backward motion. The worms could not move backwards after surgery. But within 24 h, most of the severed axons regenerated and the worms recovered backward movement, confirming that laser's cut did not damage surrounding tissue and allowed the neurons to grow a new axon to reach the muscle. Application of this precise surgical technique should enable nerve regeneration to be studied in vivo.

Nanofibers as an Aid to CNS Regeneration by Neural Progenitor Cells

Researchers at Northwestern University (Chicago, IL) have developed a new nano technology method for growing nerve cells in tissue cultures. Neural progenitor cells were encapsulated in vitro within a three-dimensional network of nanofibers formed by self-assembly of peptide amphiphile molecules (Silva et al 2004). The self-assembly of nanofiber scaffold is initiated by mixing cell suspensions in media with dilute aqueous solutions of the molecules, and cells survive the growth of the nanofibers around them. These nanofibers were designed to present to cells the neurite-promoting laminin epitope. Relative to laminin or soluble peptide, the artificial nanofiber scaffold induced very rapid differentiation of neural progenitor cells into neurons, while discouraging the development of astrocytes.

These new materials, because of their chemical structure, interact with cells of the CNS in ways that may help prevent the formation of scar due to astrocyte proliferation that is often linked to paralysis resulting from traumatic SCI.

The silicon neural electrodes being developed by Spire Corporation are engineered with a nanostructured form of silicon, called porous silicon, which acts as a scaffold that reduces glial scarring from electrode implantation and enhances neural growth at the brain recording sites to create a superior interface with neurons. This would be useful in the procedure of electrode implantation in neurological disorders such as PD and epilepsy.

Nanofiber Brain Implants

Several brain probes and implants are used in neurosurgery. Examples are those for the management of epilepsy, movement disorders, and pain. Many of these implants are still investigational. The ideal inert material for such implants has not yet been discovered. Silicon probes are commonly used for recording of electrical impulses and for brain stimulation. The body generally regards these materials as foreign and the probes gets encapsulated with glial scar tissue, which prevents them from making good contact with the brain tissue.

Scientists at Purdue University (West Lafayette, IN) have conducted an in vitro study to determine cytocompatibility properties of formulations containing carbon nanofibers pertinent to neural implant applications (McKenzie et al 2004). Substrates were prepared from four different types of carbon fibers, two with nanoscale diameters (nanophase, or $\leq$100 nm) and two with conventional diameters (or $>$100 nm). Within these two categories, both a high and a low surface energy fibers were investigated and tested. Astrocytes (glial scar tissue-forming cells) were seeded onto the substrates for adhesion, proliferation, and long-term function studies (such as total intracellular protein and alkaline phosphatase activity). Results provided the first evidence that astrocytes preferentially adhered and proliferated on carbon fibers that had the largest diameter and the lowest surface energy. Formulations containing carbon fibers in the nanometer regime limited astrocyte functions leading to decreased glial scar tissue formation. Positive interactions with neurons, and, at the same time, limited astrocyte functions leading to decreased gliotic scar tissue formation are essential for increased neuronal implant efficacy. Nanotubes, because of the interesting electronic properties and reduction in scar formation, hold great promise for replacing conventional silicon implants.

Nanoparticles as an Aid to Neurosurgery

A research team from Oregon Health & Science University (Portland, OR) has shown that an iron oxide nanoparticle can outline not only brain tumors under

MRI but also other lesions in the brain that may otherwise have gone unnoticed (Neuwelt et al 2004). Ferumoxtran-10 (Combidex®, Advanced Magnetics Inc), a dextran-coated iron oxide nanoparticle, provides enhancement of intracranial tumors by MRI for more than 24 h and can be imaged histologically by iron staining. Each iron oxide nanoparticle is the size of a small virus and is much smaller than a bacterium but much larger than an atom or standard gadolinium contrast molecule. It is an iron oxide crystal surrounded with a carbohydrate or "sugar" coating called dextran, which gives the particle a longer plasma half-life, allowing it to slowly slip through the BBB—a natural defense system that blocks the entry of foreign substances, including therapeutic agents.

In a parallel study by Neuwelt and colleagues, presented in 2004 to the American Society of Neuroradiology, ferumoxtran-10 also was found to provide a "stable imaging marker" during surgery to remove brain tumors, and it remains in the brain long enough for postoperative MR, even after surgical manipulation. The studies' findings have the potential to assist image-guided brain surgery and improve diagnosis of lesions caused by multiple sclerosis, stroke, and other neurological disorders, in addition to residual tumors. This is one of the first biologically specific nanoparticles to be used in clinical trials. Because ferumoxtran-10 can stay in brain lesions for days—it can be administered to patients 24 h before surgery and can image other, noncancerous lesions. It has some advantages over gadolinium, a metal used as an MR contrast agent for 20 years and which must be administered just before surgery. However, it will complement gadolinium, but not replace it. Ferumoxtran-10 gives additional information that cannot be obtained in some patients with gadolinium. Using both the contrast agents, one can get better diagnostic information and that has the potential to improve the patient's outcome. In addition, ferumoxtran-10 can be detected with an iron stain in the tissue removed by biopsy or surgery, allowing physicians to see it in brain tissue samples under a microscope. Unlike any other MRI contrast agent, ferumoxtran-10 enables the comparison of images from an MRI scan with the tissue taken out at surgery. Moreover, it is relatively safe when diluted and administered as an infusion.

Nanoscaffold for CNS Repair

There are several barriers that must be overcome to achieve axonal regeneration after injury in the CNS: (i) scar tissue formation; (ii) gaps in nervous tissue formed during phagocytosis of dying cells after injury; (iii) factors that inhibit axon growth in the mature mammalian CNS; and (iv) failure of many adult neurons to initiate axonal extension.

Using the mammalian visual system as a model, a self-assembling peptide nanofiber scaffold was designed, which creates a permissive environment for axons not only to regenerate through the site of an acute injury but also to knit the brain tissue together. In experiments using a severed optic tract in the hamster, it

was shown that regenerated axons reconnect to target tissues with sufficient density to promote functional return of vision, as evidenced by visually elicited orienting behavior (Ellis-Behnke et al 2006). The peptide nanofiber scaffold not only represents a previously undiscovered nanobiomedical technology for tissue repair and restoration but also raises the possibility of effective treatment of CNS and other tissue or organ trauma. This peptide nanofiber scaffold has several advantages over currently available polymer biomaterials: (i) it forms a network of nanofibers that are similar in scale to the native extracellular matrix and therefore provides an "in vivo" environment for cell growth, migration, and differentiation; (ii) it can be broken down into natural L-amino acids and metabolized by the surrounding tissue; (iii) it is synthetic and free of chemical and biological contaminants that may be present in animal-derived biomaterials such as collagens; and (iv) it appears to be immunologically inert, thus avoiding the problem of neural tissue rejection.

Nanoparticles for Repair of Spinal Cord Injury

Spinal cord injury can lead to serious neurological disability and the most serious form of it is paraplegia or quadriplegia. Currently, over 250,000 persons in the United States and several million worldwide are living with permanent disability due to chronic SCI. There are approximately 12,000 new cases of acute SCI in the United States each year. Over 90% of acute SCI victims now survive their injuries and go on to become part of the chronic SCI population, living paralyzed for an average of more than 40 years after injury.

Local spinal cord lesions are often greatly enlarged by secondary damage, which is accompanied by additional massive cell death that involves neurons, microglia, and macroglia and is virtually complete at 12 h. Immediate care involves stabilization of the patient's general condition by supportive measures. Surgery is carried out in some cases for removal of compressing lesions and stabilization of spinal fractures. A number of neuroprotective strategies are under investigation. Stem cell therapies are also under investigation for neuroregeneration and nanoparticles can be used to track the course of stem cells. There is no therapeutic measure available currently that enhances functional recovery significantly.

Scientists at the Institute for BioNanotechnology in Medicine of Northwestern University (Chicago, IL) have conducted a study in which they injected nanomaterials into the severed spinal cords of mice, enabling them to walk again after several weeks of therapy. The nanomaterials used were designed to self-assemble into nanofibers, which provide the framework for regeneration of nerve fibers. In a nanofiber network, progenitor cells develop into neurons and not astrocytes that form scar tissue and hinder regeneration. The research offers new insights into the near-term research potential of nanotechnology and offers hope for patients with severe neuron damage due to other causes as well.

Nanobiotechnology for Brain Tumor Management

The focus of this section is glioblastoma multiforme (GBM), a primary malignant tumor of the brain. Treatment of GBM is one of the most challenging problems. Surgery remains the basic treatment in which the bulk of the tumor is removed and the peripheral infiltrating part is the target of supplementary treatments. GBM is not easily targeted but advances in nanobiotechnology have improved the prospects of delivery of therapeutics to GBM (Jain 2007h).

Nanoparticles for Delivery of Drugs to Brain Tumors Across BBB

The nanoparticles may be especially helpful for the treatment of the disseminated and very aggressive brain tumors. Intravenously injected doxorubicin-loaded polysorbate 80-coated nanoparticles were able to lead to a 40% cure in rats with intracranially transplanted glioblastomas 101/8 (Kreuter 2001). A further study has evaluated the acute toxicity of doxorubicin (DOX) associated with polysorbate 80-coated nanoparticles in healthy rats and to establish a therapeutic dose range for this formulation in rats with intracranially implanted 101/8 glioblastoma (Gelperina et al 2002). The presence of polysorbate 80 in the formulations was not associated with changes in toxicity compared with free or nanoparticulate drug. The results in tumor-bearing rats were similar to those in healthy rats. These results demonstrate that the cardiotoxicity of the DOX bound to nanoparticles was lower than that of free DOX.

Scientists at the University of Washington (Seattle, WA) are using smart superparamagnetic particle conjugates to locate brain tumors earlier and more accurately than current methods and to target the tumors (Zhang et al 2004). The iron oxide superparamagnetic nanoparticles were prepared using a novel circulating system. A simple dialysis method was developed to immobilize nanoparticles with functional biopolymers and targeting agents, which avoids the use of the normal centrifugation process that may cause particle agglomeration during the coating process. To enhance the specific targeting capability of the nanoparticles, a new chemical scheme was introduced, in which folic acid was chosen as the targeting agent combined with PEG serving to improve biocompatibility of nanoparticles. AFM characterization showed that the nanoparticles produced are well dispersed with a narrow size distribution. The biological part of the study showed that coating nanoparticles with PEG-FA significantly enhanced the intracellular uptake of nanoparticles by target cells. The researchers plan to attach a variety of small molecules, such as tumor receptor target, and even chemotherapy agents, to the nanoparticles.

MRI can detect the incorporation into brain tumor vasculature of systemically administered bone marrow stem cells labeled with SPION as part of ongoing angiogenesis and neovascularization (Anderson et al 2005). This technique can be used to directly identify neovasculature in vivo and to facilitate gene therapy by noninvasively monitoring these cells as gene delivery vectors.

Doxorubicin, an anticancer drug, bound to polysorbate-coated nanoparticles crosses the intact BBB and reaches therapeutic concentrations in the brain. NanoDel

technology has been shown to deliver doxorubicin to brain tumors. Another formulation of doxorubicin with polysorbate-coated nanoparticles has been administered in rat models with implanted brain tumors (Steiniger et al 2004). Rats treated with doxorubicin bound to polysorbate-coated nanoparticles had significantly higher survival times compared with all other groups. Over 20% of the animals in this group showed a long-term remission. Preliminary histology confirmed lower tumor sizes and lower values for proliferation and apoptosis in this group. All groups of animals treated with polysorbate-containing formulations also had a slight inflammatory reaction to the tumor. There was no indication of neurotoxicity. Additionally, binding to nanoparticles may reduce the systemic toxicity of doxorubicin. This study showed that therapy with doxorubicin bound to nanoparticles offers a therapeutic potential for the treatment of human glioblastoma.

Intravenous Gene Delivery with Nanoparticles into Brain Tumors

Brain tumors may be amenable to gene therapy with cytotoxic genes, such as the proapoptotic Apo2 ligand/tumor necrosis factor-related apoptosis-inducing ligand (Apo2L/TRAIL). Gene therapy of gliomas ideally employs intravenously given vectors, thus excluding viral vectors as they cannot cross the BBB. Cationic albumin-conjugated pegylated nanoparticles (CBSA-NP) have been synthesized and shown to accumulate in mouse brain cells upon IV administration. Plasmid pORF-hTRAIL (pDNA) has been incorporated into CBSA-NP, and the resulting CBSA-NP-hTRAIL was evaluated as a nonviral vector for gene therapy of gliomas (Lu et al 2006). Thirty minutes after IV administration of CBSA-NP-hTRAIL to BALB/c mice bearing intracranial C6 gliomas, CBSA-NP-hTRAIL colocalized with glycoproteins in brain and tumor microvasculature and, via absorptive-mediated transcytosis, accumulated in tumor cells. At 24 and 48 h after IV administration of CBSA-NP-hTRAIL, respectively, hTRAIL mRNA and protein were detected in normal brain and tumors. Furthermore, repeated IV injections of CBSA-NP-hTRAIL induced apoptosis in vivo and significantly delayed tumor growth. In conclusion, this study indicates that CBSA-NP-hTRAIL is a promising candidate for noninvasive gene therapy of malignant glioma.

PEBBLEs for Brain Tumor Therapy

PEBBLEs (see Chapter 3) have been designed to carry a variety of agents on their surface, each with a unique function. This multifunctionality is the major potential advantage of using nanoparticles to treat cancer. One target molecule immobilized on the surface could guide the PEBBLE to a tumor. Another agent could be used to help visualize the target using MRI, while a third agent attached to the PEBBLE could deliver a destructive dose of drug or toxin to cancer cells. All three functions can be combined in a single tiny polymer sphere to make a potent weapon against cancer.

The MRI contrast agent, gadolinium, has been incorporated in the PEBBLEs. When injected into the bloodstream, the nanoparticles wend their way through the

bloodstream. But because they can transverse the BBB, and because they have a targeting agent attached, the PEBBLEs accumulate in the brain tumor enabling a clear MRI image within just a few hours. Each PEBBLE carries a photocatalyst. When stimulated by a light source through a micrometer-sized fiber-optic probe inserted into the skull, the photocatalyst converts oxygen into a singlet state, which effectively destroys nearby cells. The PEBBLEs are inert and harmless until the light is switched on. Used in combination with MRI imaging, it enables to kill cancer cells at will, while tracking the effectiveness of the treatment with imaging.

The targeted treatments using nanoparticles offer a number of advantages over traditional chemotherapy. In chemotherapy, the drugs permeate cells throughout the body to damage their DNA and prevent rapid growth, and are only moderately more toxic to cancer cells over normal cells. In contrast, PEBBLEs are highly localized to the cancer target and do very little damage to surrounding healthy tissue. PEBBLEs and other nanoparticle drugs could also avoid another serious problem occurring in traditional chemotherapy—development of multidrug resistance, which occurs when cancer cells mutate and begin to pump the chemotherapy drugs back out before they can destroy the cell. The cancer becomes resistant to the drug. But PEBBLEs act on the outside of the cell, and the toxic payload of oxygen that they deliver acts quickly, without giving the cancer much chance to survive and develop resistance. In rat models of brain cancer, 9L-gliosarcoma, PEBBLE-based treatment can significantly increase survival time from 5 days without treatment to 2 months with MRI image showing elimination of the tumor (Kopelman et al 2005). The investigators hope ultimately to prove the utility and safety of this approach to treating brain cancer in humans.

Fullerenes for Brain Cancer

Scientists at the Virginia Commonwealth University (Richmond, VA) are working on a project using fullerenes (buckyballs) to improve the ability of MRIs to locate brain tumors and deliver a payload of radiation to destroy them. Experiments on rats have shown that fullerenes packed with the MRI contrast metal gadolinium can increase the sensitivity of MRI detection by at least 40-fold. This level of precision is reaching a point at which cancer cells that have spread beyond the margins of the tumor may become visible. Stray cells, left behind after surgery, are thought to be responsible for tumor relapse. Finding and removing these cells could improve a patient's chance of survival. The scientists have created a modified version of the fullerenes with a fluorescent metal atom called terbium, which could guide surgeons to remove tumors with greater precision. Addition of yet another metal, lutetium, would deliver a lethal dose of radiation to the cancer cells, including those missed by the surgeon. The research is a few years away from testing in humans, but the potential is promising.

Chapter 9
Nanocardiology

Introduction

Nanocardiology is the application of nanobiotechnology to cardiovascular diseases. Recent rapid advances in nanotechnology and nanoscience offer a wealth of new opportunities for diagnosis and therapy of cardiovascular, pulmonary, and hematological diseases. To review the challenges and opportunities offered by these nascent fields, the National Heart, Lung, and Blood Institute convened a Working Group on Nanotechnology. Working group participants discussed the various aspects of nanotechnology and its applications to heart, lung, blood disorders, and cardiovascular complications of sleep apnea. An overall recommendation of the working group was to focus on translational applications of nanotechnology to solve clinical problems (Buxton et al 2003). The working group recommended the creation of multidisciplinary research centers capable of developing applications of nanotechnology and nanoscience to research and medicine. Centers would also disseminate technology, materials, and resources and train new investigators. Individual investigators outside these centers should be encouraged to conduct research on the application of nanotechnology to biological and clinical problems.

Nanotechnology-Based Diagnosis and Treatment

Use of Perfluorocarbon Nanoparticles in Cardiovascular Disorders

Perfluorocarbon (PFC) nanoparticles, described in Chapter 3, provide an opportunity for combining molecular imaging and local drug delivery in cardiovascular disorders. Ligands such as MAbs and peptides can be crosslinked to the outer surface of PFCs to enable active targeting to biomarkers expressed within the vasculature. PFC nanoparticles are naturally constrained by size to the circulation, which minimizes unintended binding to extravascular, nontarget tissues expressing similar epitopes. Moreover, their prolonged circulatory half-life of approximately 5 h allows

K.K. Jain, *The Handbook of Nanomedicine*,

saturation of receptors without addition of PEG or lipid surfactant polymerization. The utility of targeted PFC nanoparticles has been demonstrated for a variety of applications in animal models and phantoms, including the diagnosis of ruptured plaque, the quantification and antiangiogenic treatment of atherosclerotic plaque, and the localization and delivery of antirestenotic therapy following angioplasty (Lanza et al 2006).

Cardiac Monitoring in Sleep Apnea

Because sleep apnea is a cause of irregular heartbeat, hypertension, heart attack, and stroke, it is important that patients be diagnosed and treated before these highly deleterious sequelae occur. For patients suspected of experiencing sleep apnea, in vivo sensors could constantly monitor blood concentrations of oxygen and cardiac function to detect problems during sleep. In addition, cardio-specific antibodies tagged with nanoparticles may allow doctors to visualize heart movement while a patient experiences sleep apnea to determine both short- and long-term effects of apnea on cardiac function.

Detection and Treatment of Atherosclerotic Plaques in the Arteries

A key feature of the atherosclerotic process is the angiogenic expansion of the vasa vasorum in the adventitia, which extends into the thickening intimal layer of the atheroma in concert with other neovessels originating from the primary arterial lumen. Magnetic resonance molecular imaging of focal angiogenesis with integrin-targeted paramagnetic contrast agents has been reported with PFC nanoparticles and liposomes. Site-targeted PFC nanoparticles also offer the opportunity for local drug delivery in combination with molecular imaging.

The diagnosis and treatment of unstable plaque is an area in which nanotechnology could have an immediate impact. Fibrin-specific PFC nanoparticles may allow the detection and quantification of unstable plaque in susceptible patients, which may be an important feature of future strategies to prevent heart attacks or stroke. Research is under way using probes targeted to plaque components for noninvasive detection of patients at risk. In an extension of this approach, targeted nanoparticles, multifunctional macromolecules, or nanotechnology-based devices could deliver therapy to a specific site, localized drug release being achieved either passively (by proximity alone) or actively (through supply of energy as ultrasound, near-infrared, or magnetic field). Targeted nanoparticles or devices could also stabilize vulnerable plaque by removing material, e.g., oxidized low-density lipoproteins. Devices able to attach to unstable plaques and warn patients and emergency medical services of plaque rupture would facilitate timely medical intervention.

Nanotechnology-Based Therapeutics for Cardiovascular Diseases

Nanolipoblockers for Atherosclerotic Arterial Plaques

Nanoscale particles can be synthetically designed to potentially intervene in lipoprotein matrix retention and lipoprotein uptake in cells—processes central to atherosclerosis. These micelles can be engineered to present varying levels of anionic chemistry, which is a key mechanism to induce differential retentivity of low-density lipoproteins (LDLs). Scientists of Rutgers University have reported on lipoprotein interactions of nanoscale micelles self-assembled from amphiphilic scorpion-like macromolecules based on a lauryl chloride–mucic acid hydrophobic backbone and poly(ethylene glycol) shell. They have used nanoengineered molecules called nanolipoblockers (NLBs) to attack atherosclerotic plaques due to raised levels of LDLs (Chnari et al 2006). Their approach contrasts with statin drug therapy, which aims to reduce the amount of LDL throughout the body. NLPs compete with oxidized LDLs for a macrophage's attention. The NLBs bind to receptor sites on macrophages, cutting the accumulation of oxidized LDL by as much as 75%.

Nanotechnology-Based Drug Delivery in Cardiovascular Diseases

Liposomal Nanodevices for Targeted Cardiovascular Drug Delivery

High-affinity ligand–receptor interactions have been exploited in the design and engineering of targeting systems that use a liposomal nanodevice for site-specific cardiovascular drug delivery. An example of application is atherothrombosis, a condition in which platelet activation/adhesion/aggregation is closely associated with vascular thrombotic events. Therefore, the majority of antithrombotic therapies have focused on drugs that impede platelet-activation pathways or block ligand-binding platelet integrins. In spite of reasonable clinical efficacy of these therapies, the magic bullet, a single drug and delivery system that selectively targets pathologically thrombotic environment without affecting hemostatic balance, remains elusive. The use of anti-integrin/anticoagulant/anti-inflammatory drugs in conjunction might be necessary to treat the multifactorial nature of pathological thrombogenesis. For this purpose, a nanoscale device that can carry such a combination selectively to a thrombotic site is being developed at the Department of Biomedical Engineering of Case Western Reserve University (Cleveland, OH). The liposomal nanodevice surface is modified by RGD (arginine-glycine-aspartic acid) motifs that specifically targets and binds activated platelets by virtue of the high-affinity interaction between the RGD motif and the integrin GPIIb-IIIa expressed on active platelets, potentially acting as a thrombus-targeted vector. The ability of such liposomes to compete with native ligand fibrinogen in specifically binding activated platelets has been accomplished using both in vitro and in vivo approaches. The results demonstrate feasibility of using liposomes as platelet-targeted devices for

delivery of cardiovascular therapeutics. By utilizing a library of synthetic peptide/peptidomimetic ligands having binding affinity towards specific receptors expressed in cardiovascular biology, it is possible to manipulate the liposome surface-modification and hence dictate targeting specificity and affinity of the liposomal nanodevices.

Antirestenosis Drugs Encapsulated in Biodegradable Nanoparticles

Local delivery of antiproliferative drugs encapsulated in biodegradable nanoparticles has shown promise as an experimental strategy for preventing restenosis development. A novel PDGFR β-specific tyrphostin, AGL-2043 (Calbiochem), was formulated in polylactide-based nanoparticles and administered intraluminally to the wall of balloon-injured rat carotid and stented pig coronary arteries (Banai et al 2005). The antiproliferative effect of nanoencapsulated tyrphostin was found to be considerably higher than that of surface-adsorbed drug. In the pig model, intramural delivery of AGL-2043 resulted in reduced in-stent neointima formation in the coronary arteries as compared to control despite similar degrees of wall injury. The results of this study suggest that locally delivered tyrphostin AGL-2043 formulated in biodegradable nanoparticles may be applicable for antirestenotic therapy independent of stent design or type of injury.

Nanocoated Drug-Eluting Stents

MIV Therapeutics Inc has developed unique coating technologies that utilize hydroxyapatite (HAp) for application on medical devices and drug delivery systems. The lead product in the development is a HAp-coated coronary stent with a nanofilm coating. In November 2006, the results of an independently conducted 4-week porcine study, performed by the Department of Cardiology, Thoraxcenter, Erasmus University Medical Center in the Netherlands, indicated that three variations of MIV's polymer-free drug-eluting coatings were at least as effective as or better than Cypher (Johnson & Johnson). The study concluded that MIV's HAp coating, with or without drugs, demonstrated highly promising performance. A pilot clinical trial was launched on 31 May 2007 and the first HAp-coated was implanted at the Institute Dante Pazzanese of Cardiology in Sao Paulo, Brazil.

ElectroNanoSpray™ formulation technology (Nanocopoeia Inc) produces precise, ultrapure nanoparticles. Particle sizes can be designed from 2 to 200 nm. The device is capable of applying a coating to the particles in a single process step, producing a drug-loaded core. Competitive processes to produce nanoparticles using wet milling and super critical fluid are inherently limited in their ability to produce consistently pure particles within a specified size range and distribution. ElectroNanoSpray™ technology provides a novel approach for applying challenging materials to the surfaces of medical devices. This process can generate both

single- and multiple-phase coatings and apply these with tight control to small, complex surfaces. ElectroNanospray™ process is being developed for applying nanoparticle-based drug-eluting coatings to coronary stents.

Debiotech SA in collaboration with the Laboratory of Powder Technology at Ecole Polytechnique Fédérale de Lausanne (Lausanne, Switzerland) is developing a new type of structured ceramic coatings for drug-eluting stents (DES) and other implants. Ceramics offer unique properties compared to polymers. Polymers dissolve over time and residues provoke inflammation, whereas ceramic is stable and inert when in contact with living tissue. With this coating, one can combine an active release of drug during the first weeks after implantation with the long-term stability of the ceramic. Nanostructured ceramics provide novel properties to biomaterials which are not attainable with other materials. The challenge in this project is to process nanosized ceramic powders to reach unique surface structures, which show a controlled porosity over a size range of 2,000 times between the smallest and the largest pores. Based on results of fundamental research activities in the field of ordered arrangement of nanosized particles at surfaces, the knowledge of processing particles < 10 nm at large scale has been established as a key competence to achieve that goal.

Nanopores to Enhance Compatibility of Drug-Eluting Stents

Scientists at the Forschungszentrum Dresden-Rossendorf in Germany have developed an innovative method to create a large number of nanopores on the surface of stainless steel. Bombarding the surface of a stent from all sides with a high dose of noble gas ions generates a scaffold of nanopores in the material below the surface. The desired porosity can be precisely engineered by tuning the ion energy, the flux, and the temperature during the process. A larger amount of the highly effective drugs can be deposited on the enlarged noble metal surface, due to this nanoporous structure, which enhances the biocompatibility of the implants in the human body. Thus, this treatment results in the release of drugs over a longer period of time. This method is currently being assessed as a platform technology for the next generation of DES by the Boston Scientific Corporation. The objective of this research collaboration is to further develop this technique for commercialization.

Low Molecular Weight Heparin-Loaded Polymeric Nanoparticles

Low molecular weight heparin (LMWH) nanoparticles have been prepared as potential oral heparin carriers. The nanoparticles were formulated using an ultrasound probe by water-in-oil-in-water emulsification and solvent evaporation with polymers (Hoffart et al 2002). The mean diameter of LMWH-loaded nanoparticles ranged from 240 to 490 nm and was dependent on the reduced viscosity of the polymeric organic solution. The highest encapsulation efficiencies were observed

when Eudragit polymers were used in the composition of the polymeric matrix. The in vitro LMWH release in phosphate buffer from all formulations ranged from 10 to 25% and was more important (2- to 3-fold) when esterase was added into the dissolution medium. The in vitro biological activity of released LMWH, determined by the antifactor Xa activity with a chromogenic substrate, was preserved after the encapsulation process, making these NPs good candidates for oral administration.

Injectable Peptide Nanofibers for Myocardial Ischemia

Endothelial cells can protect cardiomyocytes from injury through platelet-derived growth factor (PDGF)-BB signaling. PDGF-BB induces cardiomyocyte Akt phosphorylation in a time- and dose-dependent manner and prevents apoptosis via PI3K/Akt signaling. An experimental study in rats using injectable self-assembling peptide nanofibers, which bound PDGF-BB in vitro, demonstrated sustained delivery of PDGF-BB to the myocardium at the injected sites for 14 days (Hsieh et al 2006). This blinded and randomized rat study showed that injecting nanofibers with PDGF-BB, but not nanofibers or PDGF-BB alone, decreased cardiomyocyte death and preserved systolic function after myocardial infarction. A separate blinded and randomized study showed that PDGF-BB delivered with nanofibers decreased infarct size after ischemia/reperfusion. PDGF-BB with nanofibers induced PDGFR-β and Akt phosphorylation in cardiomyocytes in vivo. These data demonstrate that PDGF-BB signaling and the in vitro finding can be translated into an effective in vivo method of protecting myocardium after infarction. Furthermore, this study shows that injectable nanofibers allow precise and sustained delivery of proteins to the myocardium with potential therapeutic benefits.

Nanotechnology Approach to the Vulnerable Plaque as Cause of Cardiac Arrest

Recent studies have shown that plaque exists in two modes: nonvulnerable and vulnerable. The latter is the probable cause of death in sudden cardiac arrest. Blood passing through an artery exerts a shearing force and can cause vulnerable plaque to rupture, which often leads to occlusion and myocardial infarction. Approximately 60–80% of sudden cardiac deaths can be attributed to the physical rupture of vulnerable plaque.

There is currently no satisfactory solution to the problem of vulnerable plaque but it will be tackled by a “Program of Excellence in Nanotechnology” by the National Heart, Lung, and Blood Institute of the NIH. In concert with the NIH’s strategy to accelerate progress in medical research through innovative technology and interdisciplinary research, cardiac disease was chosen as the focus of the National Heart, Lung, and Blood Institute’s recently established Program of Excellence in Nanotechnology. The program will be a partnership of 25 scientists from The Burnham

Institute (La Jolla, CA), University of California Santa Barbara, and The Scripps Research Institute (San Diego, CA) that will use a $13 million award to design nanotechnologies to detect, monitor, treat, and eliminate "vulnerable" plaques. By focusing on devising nanodevices, machines at the molecular level, the scientists at these institutions will specifically target vulnerable plaque. It is hoped that this work will lead to useful diagnostic and therapeutic strategies for those suffering from this form of cardiac disease. The project team will work on three innovative solutions to combat vulnerable plaque:

1. Building delivery vehicles that can be used to transport drugs and nanodevices to sites of vulnerable plaque
2. Designing a series of self-assembling polymers that can be used as molecular nanostents to physically stabilize vulnerable plaque
3. Creating nanomachines comprised of human proteins linked to synthetic nanodevices for the purpose of sensing and responding to vulnerable plaque

IGF-1 Delivery by Nanofibers to Improve Cell Therapy for Myocardial Infarction

Strategies for cardiac repair include injection of cells, but these approaches have been hampered by poor cell engraftment, survival, and differentiation. To address these shortcomings for the purpose of improving cardiac function after injury, a self-assembling peptide nanofiber was designed for prolonged delivery of insulin-like growth factor 1 (IGF-1), a cardiomyocyte growth and differentiation factor, to the myocardium, using a "biotin sandwich" approach (Davis et al 2006). Biotinylated IGF-1 was complexed with streptavidin and then bound to biotinylated self-assembling peptides. This biotin sandwich strategy enabled binding of IGF-1 but did not prevent self-assembly of the peptides into nanofibers within the myocardium. IGF-1 that was bound to peptide nanofibers activated Akt, decreased activation of caspase-3, and increased expression of cardiac troponin I in cardiomyocytes. In studies on rats, cell therapy with IGF-1 delivery by biotinylated nanofibers improved systolic function after experimental myocardial infarction. This nanobiotechnology approach has the potential to improve the results of cell therapy for myocardial infarction, which is in clinical trials currently.

Tissue Engineering and Regeneration of the Cardiovascular System

Nanotechnology may facilitate repair and replacement of blood vessels, myocardium and myocardial valves. It may also be used to stimulate regenerative processes such as therapeutic angiogenesis for ischemic heart disease. Cellular function is integrally related to morphology, so the ability to control cell shape in tissue engineering is essential to ensure proper cellular function in final products. Precisely constructed

nanoscaffolds and microscaffolds are needed to guide tissue repair and replacement in blood vessels and organs. Nanofiber meshes may enable vascular grafts with superior mechanical properties to avoid patency problems common in synthetic grafts, particularly small-diameter grafts. Cytokines, growth factors, and angiogenic factors can be encapsulated in biodegradable microparticles or nanoparticles and embedded in tissue scaffolds and substrates to enhance tissue regeneration. Scaffolds capable of mimicking cellular matrices should be able to stimulate the growth of new heart tissue and direct revascularization.

Nanostructures promote formation of blood vessels, bolster cardiovascular function after heart attack. Scientists at the Institute of Bionanotechnology in Medicine at Northwestern University (Evanston, IL) have shown that injecting nanoparticles into the hearts of mice that suffered heart attacks helped restore cardiovascular function in these animals. The finding is an important research advance that could one day help rapidly restore cardiovascular function in people who have heart disease. The self-assembling nanoparticles—made from naturally occurring polysaccharides and molecules known as peptide amphiphiles—boost chemical signals to nearby cells that induce formation of new blood vessels and this may be the mechanism through which they restore cardiovascular function. One month following injection, the hearts of the treated mice were capable of contracting and pumping blood almost as well as healthy mice. In contrast, the hearts of untreated mice contracted about 50% less than normal.

Nanobiotechnology in Cardiovascular Surgery

Restenosis After Percutaneous Coronary Angioplasty

Restenosis after percutaneous coronary intervention continues to be a serious problem in clinical cardiology. Recent advances in nanoparticle technology have enabled the delivery of NK911, an antiproliferative drug, selectively to the balloon-injured artery for a longer time (Uwatoku et al 2003). NK911 is a core–shell nanoparticle of PEG-based block copolymer encapsulating doxorubicin. It accumulates in vascular lesions with increased permeability. In a balloon injury model of the rat carotid artery, intravenous administration of NK911 significantly inhibited the neointimal formation. The effect of NK911 was due to inhibition of vascular smooth muscle proliferation but not due to enhancement of apoptosis or inhibition of inflammatory cell recruitment. NK911 was well tolerated without any adverse systemic effects. These results suggest that nanoparticle technology is a promising and safe approach to target vascular lesions with increased permeability for the prevention of restenosis after balloon injury.

Biomedical engineers at Purdue University (Lafayette, IN) have shown that vascular stents used to repair arteries might perform better if their surfaces contained "nanobumps" that mimic tiny features found in living tissues. The stents, which are made of titanium and other metals, enable the arteries to grow new tissue after

vessel-clogging plaque deposits have been removed. A major problem, however, is that the body often perceives the metal devices as foreign invaders, hindering endothelial cells from attaching to the scaffolding and prompting the creation of scar tissue, which can build up inside blood vessels and interfere with blood flow. If a stent does not attach firmly, it can become loose and parts of it will actually break off and go down the bloodstream. There is need for new materials that cause the endothelial cells to attach better to these stents without creating as much dangerous scar tissue. The researchers tested discs of titanium containing surface bumps about as wide as 100 nm. The metals used to make conventional stents have features about 10 times larger or none at all. The nanometer-scale bumps mimic surface features of proteins and natural tissues, prompting cells to stick better. Ideally endothelial cells should quickly attach to stents and form a coating only one cell layer thick. The researchers found that nearly three times as many cells stuck to the discs containing the nanobumps, as compared to ordinary titanium. Further research is planned that will replace the titanium disks with tube-shaped pieces of the nano-featured metal, which will resemble the actual shape of real stents.

Currently available stents have problems with imaging within the stent structure, where potential restenosis can occur. Biophan Technologies Inc has two solutions for stent visibility: a thin-film nanomagnetic particle coating solution and an antiantenna solution. These solutions enable the noninvasive, MRI-based, imaging of these devices which today can only be accomplished through more complicated invasive procedures. These approaches will become an important part of the rapidly growing worldwide market for stents and vascular implants.

NOLabs is developing nitric oxide (NO)-eluting nanofibers. One potential application is incorporation into stents for antithrombogenic action. NO has vasodilating action as well, which may be beneficial in ischemic heart disease.

Nanotechnology-Based Personalized Cardiology

The future of cardiovascular diagnosis is already being impacted by nanosystems that can both diagnose pathology and treat it with targeted delivery systems (Wickline et al 2006). The potential dual use of nanoparticles for both imaging and site-targeted delivery of therapeutic agents to cardiovascular disease offers great promise for individualizing therapeutics. Image-based therapeutics with site-selective agents should enable verification that the drug is reaching the intended target and a molecular effect is occurring. Experimental studies have shown that binding of paclitaxel to smooth muscle cells in culture has no effect in altering the growth characteristics of the cells. If paclitaxel-loaded nanoparticles are applied to the cells, however, specific binding elicits a substantial reduction in smooth muscle cell proliferation, indicating that selective targeting may be a requirement for effective drug delivery in this situation. Similar behavior has been demonstrated for doxorubicin-containing particles. Intravenous delivery of fumagillin (an antiangiogenic agent)-loaded nanoparticles targeted to $\alpha v\beta 3$-integrin epitopes on vasa

vasorum in growing plaques results in marked inhibition of plaque angiogenesis in cholesterol-fed rabbits. The unique mechanism of drug delivery for highly lipophilic agents such as paclitaxel contained within emulsions depends on close apposition between the nanoparticle carrier and the targeted cell membrane and has been described as "contact facilitated drug delivery." In contrast to liposomal drug delivery (generally requiring endocytosis), the mechanism of drug transport in this case involves lipid exchange or lipid mixing between the emulsion vesicle and the targeted cell membrane, which depends on the extent and frequency of contact between two lipidic surfaces. The rate of lipid exchange and drug delivery can be greatly increased by the application of clinically safe levels of ultrasound energy that increase the propensity for fusion or enhanced contact between the nanoparticles and the targeted cell membrane.

The combination of targeted drug delivery and molecular imaging with MRI has the potential to enable serial characterization of the molecular epitope expression based on imaging readouts. Monitoring and confirmation of therapeutic efficacy of the therapeutic agents at the targeted site would permit personalized medical regimens.

Monitoring for Disorders of Blood Coagulation

Patients would benefit greatly from nanotechnological devices that could monitor the body for the onset of thrombotic or hemorrhagic events. Multifunctional devices could detect events, transmit real-time biologic data externally, and deliver anticoagulants or clotting factors to buy critical time.

A gold nanoparticle-based simple assay has been described that enables the visual detection of a protease (Guarise et al 2006). The method takes advantage of the high molar absorptivity of the plasmon band of gold colloids and is based on the color change of their solution when treated with dithiols. Contrary to the native ones, cleaved peptides are unable to induce nanoparticle aggregation; hence, the color of the solution does not change. The assay was used to detect two proteases: thrombin (involved in blood coagulation and thrombosis) and lethal factor (an enzyme component of the toxin produced by *Bacillus anthracis*). The sensitivity of this nanoparticle-based assay is in the low nanomolar range.

Chapter 10
Nano-Orthopedics

Introduction

Reducing Reaction to Orthopedic Implants

In orthopedic implants, biomaterials (usually titanium and/or titanium alloys) often become encapsulated with undesirable soft fibrous but not hard bony tissue. Although possessing intriguing electrical and mechanical properties for neural and orthopedic applications, carbon nanofibers (CNs)/nanotubes have not been widely considered for these applications previously. Researchers at Purdue University (West Lafayette, IN) have developed a CN-reinforced polycarbonate urethane (PU) composite in an attempt to determine the possibility of using CNs as orthopedic prosthetic devices (Webster et al 2004). Mechanical characterization studies determined that such composites have properties suitable for orthopedic applications. These materials enhanced osteoblast (bone-forming cell) functions whereas functions of cells that contribute to fibrous-tissue encapsulation events for bone implants (fibroblasts) decreased on PU composites containing increasing amounts of CNs. In this manner, this study provided the first evidence of the future that CN formulations may have towards interacting with bone cells, which is important for the design of successful orthopedic implants.

Spire Corporation produces superlattice coatings, which are nanostructures composed of alternating layers of dissimilar materials. They exhibit hardness and wear resistance far superior to their constituent materials. This cutting-edge surface technology is expected to significantly increase the service life of spinal implants by controlling the inevitable bearing surface wear that occurs in any articulating system. This technology will have applications across a wide range of orthopedic devices, but disk replacements are targeted in this program due to the heightened risks of revision surgery and the relatively long required service life.

K.K. Jain, *The Handbook of Nanomedicine*,

Enhancing the Activity of Bone Cells on the Surface of Orthopedic Implants

It is very important to increase the activity of bone cells on the surface of materials used in the design of orthopedic implants so that such cells can promote either integration of these materials into surrounding bone or complete replacement with naturally produced bone if biodegradable materials are used. Osteoblasts are bone-producing cells and, for that reason, are the cells of interest in initial studies of new orthopedic implants. If these cells are functioning normally, they lay down bone matrix onto both existing bone and prosthetic materials implanted into the body. It is generally accepted that a successful material should enhance osteoblast function, leading to more bone deposition and, consequently, increased strength of the interface between the material and juxtaposed bone. A study has provided evidence of greater osteoblast function on carbon and alumina formulations that mimic the nanodimensional crystal geometry of hydroxyapatite (HA) found in bone (Price et al 2003).

Nanobone Implants

A nanoscale molecular scaffolding has been designed that resembles the basic structure of bone (Hartgerink et al 2001). It uses the pH-induced self-assembly of a peptide-amphiphile to make a nanostructured fibrous scaffold reminiscent of extracellular matrix. The design of this peptide-amphiphile allows the nanofibers to be reversibly crosslinked to enhance or decrease their structural integrity. After crosslinking, the fibers are able to direct mineralization of HA to form a composite material in which the crystallographic *c* axes of HA are aligned with the long axes of the fibers. This alignment is the same as that observed between collagen fibrils and HA crystals in bone. Nanofibers, approximately 8 nm in size, come in the form of a gel that could be injected into a broken bone to help the fracture-mending crystallization process. The discovery is related to recreating the structure of bone at the nanoscale level and has implications beyond bone repair. It could lead to development of a hardening gel that speeds the healing of fractures. It could help patients avoid conventional surgery or be used to repair bone fractures of soldiers in battlefield.

It shows some features of natural bone both in main composition and hierarchical microstructure, which is nanohydroxyapatite and collagen assembled into mineralized fibril (Liao et al 2004). The three-dimensional porous scaffold materials mimic the microstructure of cancellous bone. Cell culture and animal model tests showed that the composite material is bioactive. The osteoblasts were separated from the neonatal rat calvaria. Osteoblasts adhered, spread, and proliferated throughout the pores of the scaffold material within a week. This was implanted into a bone defect model in the radius of the rabbit and was found to be partially substituted by new bone tissue after 3 months.

The scaffolds or “nanobones” have been successfully implanted in patients in China for repair of bone defects after fractures or tumor removal and also for spinal

fusion. The nanobone material is inserted where the bone needs to heal. The critical material is calcium phosphorus, which is reduced to 30 nm in thickness and 60 nm in width. At this size, the properties of calcium phosphorus change. On a large scale calcium phosphorus does not degrade, but on a nanoscale it does. The nanoscale material degrades after a minimum of 6 months, and the space is filled by natural bone. This technology is better than current methods that use ceramics or metals because those materials remain in the patient's body and can cause infection, pain, and make the repaired bone more vulnerable to fracture.

The technology has been found to be effective in repairing small bones ranging from 1 to 2 cm in length, making the technology useful after removal of bone tumors. Research is currently being performed on larger bones up to 4 cm in length. The nanobone technology, which was approved by China's regulatory agency, is available for commercial use in Chinese hospitals. The cost of the nanobone implant, which is initially high, is expected to be reduced over time to be economically competitive with other technologies available.

Synthetic Nanomaterials as Bone Implants

Argonide Corporation has designed synthetic materials that posses the grain size, shape, and porosity similar to HA, the natural mineral present in bone. The HA in bone has a fibrous shape and is <100 nm in diameter. A study was performed on NanoCeram® fibers, where its cytocompatibility was tested and osteoblast (bone) cell adhesion and proliferation were measured. The study showed that after 1, 3, and 5 days of culture, the number of osteoblasts was significantly greater on nanofiber alumina than nano- or micron-size alumina spheres, metallic titanium, or HA compacts. There were more than two, three, and four times the number of osteoblasts on nanofiberalumina than on titanium after 1, 3, and 5 days, respectively.

Although nanohydroxyapatite has wide range of medical applications, particles mobilization and slow resorption limit its use in certain applications, particularly periodontal and alveolar ridge augmentation. However, the rate of resorption of a composite of HA and chitosan (CS) is quite higher than HA and may have a great impact on human health care systems as bioresorbable bone substitute (Murugan and Ramakrishna 2004). A transparent and slight yellow CS/HA nanocomposite with high performed, potential application as internal fixation of bone fracture was prepared by a novel and simple in situ hybridization (Hu et al 2004). The bending strength and modulus of CS/HA with ratio of 100/5 (wt/wt) were slightly higher than that of pure CS rod.

Carbon Nanotubes as Scaffolds for Bone Growth

Artificial bone scaffolds have been made from a wide variety of materials, such as polymers or peptide fibers. Their drawbacks include low strength and the potential

for rejection in the body. Chemically functionalized single-walled carbon nanotubes (SWNTs) have been used as scaffolds for the growth of artificial bone material (Zhao et al 2005). The strength, flexibility, and light weight of SWNTs enable them to act as scaffolds to hold up regenerating bone. Bone tissue is a natural composite of collagen fibers and crystalline HA, which is a mineral based on calcium phosphate. SWNTs can mimic the role of collagen as a scaffold for inducing the growth of HA crystals. By chemically treating the nanotubes, it is possible to attract calcium ions and to promote the crystallization process while improving the biocompatibility of the nanotubes by increasing their water solubility. SWNTs may lead to improved flexibility and strength of artificial bone, new types of bone grafts, and to inroads in the treatment of osteoporosis and fractures.

Bone cells can grow and proliferate on a scaffold of carbon nanotubes (CNTs). Because CNTs are not biodegradable, they behave like an inert matrix on which cells can proliferate and deposit new living material, which becomes functional, normal bone (Zanello et al 2006). CNTs carrying neutral electric charge sustained the highest cell growth and production of plate-shaped crystals. There was a dramatic change in cell morphology in osteoblasts cultured on multiwalled CNTs, which correlated with changes in plasma membrane functions. CNTs hold promise in the treatment of bone defects in humans associated with the removal of tumors, trauma, and abnormal bone development and in dental implants. More research is needed to determine how the body will interact with CNTs, specifically in its immune response.

Aligning Nanotubes to Improve Artificial Joints

Researchers at Purdue University (Lafayette, IN) have shown that artificial joints might be improved by making the implants out of tiny carbon tubes (diameter 60 nm) and filaments that are all aligned in the same direction, mimicking the alignment of collagen fibers and natural ceramic crystals in real bones. The researchers have already shown in a series of experiments that bone cells in petri dishes attach better to materials that possess smaller surface bumps than are found on conventional materials used to make artificial joints. The smaller features also stimulate the growth of more new bone tissue, which is critical for the proper attachment of artificial joints once they are implanted. Nanotubes and nanofibers are aligned in the same direction and this orientation is similar to the way collagen and natural ceramic crystals, called hydroxyapatite, are aligned in bone. One-third more bone-forming cells (osteoblasts) attach to CNTs that possess surface bumps about as wide as 100 nm than to conventional titanium, which has surface features on the scale of microns. The nanometer-scale bumps mimic surface features of proteins and natural tissues, prompting cells to stick better and promoting the growth of new cells. The findings also suggest that using such nanometer-scale materials might cause less of a rejection response from the body. Rejection eventually weakens the attachment of implants and causes them to become loose and painful, requiring replacement surgery. Aligning the nanotubes to further mimic natural bone might also provide

more strength. There are two methods to align the tiny nanotube structures. In one method, researchers mixed the nanotubes in a polymer and passed an electric current through the mixture. Because nanotubes have the same natural electrical charge, they react to electricity by orienting themselves in the same direction. Once the polymer solidifies, the nanotubes are fixed in the aligned position. In the second method, the nanotubes are poured into grids of tiny channels. Because the channels are so narrow, the tubes can only fit lengthwise, causing them to become aligned. The grids can then be removed, leaving behind the aligned nanotubes. The aligned nanotubes are then added to a suspension of dyed bone cells in a small container. Future research may focus on combining the two methods for aligning nanotubes. Using the grid technique creates a greater number of aligned nanotubes on the surface, which helps to increase bone-cell adhesion and alignment, whereas using electricity could better stimulate the growth of new bone tissue.

Cartilage Disorders of Knee Joint

The meniscus is the knee's shock absorber. It is a cartilage spacer for preventing friction and absorbing approximately one-third of the impact load that the joint cartilage surface experiences. Cartilage injuries of the knees are one of the common injuries in sports, particularly football and hockey. Unlike other body tissues, the meniscus does not repair itself because only a very small part receives blood. The conventional treatment of a torn cartilage is surgical removal of the loose pieces and repair of the tear where possible to save as much as possible of the cartilage. The procedure has become refined with arthroscopy. Although the results are generally good in terms of relief of pain and recovery of function of the joint, there are long-term effects if the cartilage is lost and degenerative changes in the joints may occur.

Several methods have been developed to encourage the regeneration of cartilage defects. Procedures such as debridement, lavage, microfracturing, subchondral bone drilling, and abrasion arthroplasty may perhaps alleviate symptoms, but cannot restore the hyaline articular cartilage. The regenerated tissue formed in response to these procedures consists of fibrocartilage and does not possess the biomechanical or biochemical properties of hyaline articular cartilage. Nanotechnology and cell therapy are being used as refinements of procedures to replace the torn knee cartilage.

Role of Nanotechnology in Engineering of a Replacement for Cartilage

One of the projects at the Australian Institute of Bioengineering and Nanotechnology of the University of Queensland (St Lucia, Australia) is the use of nanotechnology to produce viable structural and functional scaffolds capable of promoting the growth of mesenchymal stem cells (MSCs), and differentiate these cells into meniscal tissue using a specially designed bioreactor. Secondly, the researchers would investigate the way these structures, laden with MSCs, could be incorporated into the body. Alternatively, they will attempt to create an artificial environment

mimicking as closely as possible the meniscus and capable of recruiting cells from within the meniscal cavity to differentiate these cells into the various types that make up a healthy meniscus. A thorough understanding is needed of how MSCs interact with scaffolds and how to optimize conditions promoting the cell growth around these scaffolds, which should not only encourage cell growth, but also degrade at the correct rate so that all that remains is meniscal tissue.

Nanotechnology as an Aid to Arthroscopy

Arthroscopy of joints, particularly the knee joint, is an established procedure for diagnosis and treatment. Nanotechnology has been used to refine this procedure. The first step was the study of cartilage by AFM as a basis for the construction of a scanning force arthroscope.

Cartilage stiffness was measured ex vivo at the micrometer and nanometer scales to explore structure–mechanical property relationships at smaller scales than has been done previously. A method has been developed to measure the dynamic elastic modulus, in compression by indentation-type AFM (Stolz et al 2004). Spherical indenter tips (radius approximately 2.5 μm) and sharp pyramidal tips (radius approximately 20 nm) were employed to probe micrometer-scale and nanometer-scale responses, respectively, on subsurface cartilage from porcine femoral condyles. From results of AFM imaging of cartilage, the micrometer-scale spherical tips resolved no fine structure except some chondrocytes, whereas the nanometer-scale pyramidal tips resolved individual collagen fibers and their 67-nm axial repeat distance. The cartilage compressive stiffness was different at the nanometer scale compared to the overall structural stiffness measured at the micrometer and larger scales because of the fine nanometer-scale structure, and enzyme-induced structural changes can affect this scale-dependent stiffness differently. The collagen fibers were seen to coalesce together as evidence of disease state.

Scanning Force Arthroscope

A prototype of the device constructed at Muller Institute for Structural Biology (Basel, Switzerland) combines both diagnostics and therapeutics in a single tube in contrast to the conventional arthroscopes which have two tubes—one for visualization and the other for manipulation with instruments. There are inflatable balloons to provide an irrigation system. This prototype fulfils the requirements of an ideal arthroscope:

- It is user friendly.
- It provides information not obtainable by conventional methods.
- It is expected to have an affordable price tag.

So far the device has been tested only in models of knee joint. It is expected to be in the market in a decade.

Chapter 11
Nanomicrobiology

Introduction

Microbiology is the study of microorganisms such as bacteria and viruses as well as the diseases caused by them. Application of nanobiotechnology in microbiology can be termed as nanomicrobiology. It includes some of the diagnostic procedures already mentioned in Chapter 3. Role of microorganisms in disease and their management using nanobiotechnology will be discussed in this chapter.

Nanobiotechnology and Virology

Study of Interaction of Nanoparticles with Viruses

Scanning surface confocal microscopy, simultaneous recording of high-resolution topography and cell surface fluorescence in a single scan enables imaging of individual fluorescent particles in the nanometer range on fixed or live cells. This technique has been used to record the interaction of single virus-like particles with the cell surface and demonstrated that single particles sink into the membrane in invaginations reminiscent of pinocytic vesicles (Gorelik et al 2002). This method provides a technique for elucidating the interaction of individual viruses and other nanoparticles, such as gene therapy vectors, with target cells.

Silver nanoparticles undergo a size-dependent interaction with HIV-1 and particles in the range of 1–10 nm attached to the virus (Elechiguerra et al 2005). The regular spatial arrangement of the attached nanoparticles, the center-to-center distance between nanoparticles, and the fact that the exposed sulfur-bearing residues of the glycoprotein knobs would be attractive sites for nanoparticle interaction suggest that silver nanoparticles interact with the HIV-1 virus via preferential binding to the gp120 glycoprotein knobs. Due to this interaction, silver nanoparticles inhibit the virus from binding to host cells, as demonstrated in vitro.

K.K. Jain, *The Handbook of Nanomedicine*,

Study of Pathomechanism of Viral Diseases

Nanobiotechnology-based diagnosis of viral infections described in Chapter 3. Research in nanobiotechnology may be helpful in understanding the pathomechanism of viral diseases and devising strategies for treatment. An example is the neurotropic herpes simplex virus (HSV), which infects mucosal epithelia and enters nerve terminals, from where it travels in axons to dorsal root ganglia neurons and delivers its genome into the nucleus of the cell body. In the nucleus, the genome may give rise to infectious progeny or become latent with little gene expression. The silenced genome can be reactivated upon stress and establish a productive infection in the peripheral nervous system and, later, also in the mucosal periphery. To achieve this, a virus must elude host restrictions at multiple levels, including entry, cytoplasmic transport, replication, innate, and adaptive immune recognition, and egress from the infected cell.

Research on virus nanoparticles has provided cues to the regulation of cytoplasmic transport. Viruses that replicate their genomes in the nucleus make use of the microtubule and the actin cytoskeleton as molecular motors for trafficking toward the nuclear membrane during entry and the periphery during egress after replication. Analyzing the underlying principles of viral cytosolic transport will be helpful in the design of viral vectors to be used in research as well as human gene therapy, and in the identification of new antiviral target molecules (Dohner and Sodeik 2005).

Nanofiltration to Remove Viruses from Plasma Transfusion Products

One of the complications of blood transfusion is transmission of viral infections. Nanofiltration, use of nanotechnology in viral removal filtration systems, is an important safety step in the manufacture of plasma-derived coagulation factor concentrates and other biopharmaceutical products from human blood. Nanofiltration of plasma products has already been carried out since the early 1990s to improve margin of viral safety, as a complement to the viral reduction treatments, such as solvent-detergent and heat treatments, which are applied for the inactivation of HIV, hepatitis B, and hepatitis C viruses. The main reason for the introduction of nanofiltration was the need to improve product safety against nonenveloped viruses and to provide a possible safeguard against new infectious agents potentially entering the human plasma pool. Nanofiltration has gained quick acceptance as it is a relatively simple manufacturing step that consists of filtering protein solution through membranes of a very small pore size (typically 15–40 nm) under conditions that retain viruses by a mechanism largely based on size exclusion. Recent large-scale experience throughout the world has now established that nanofiltration is a robust and reliable viral reduction technique that can be applied to essentially all plasma products. Many of the licensed plasma products are currently nanofiltered.

The technology has major advantages as it is flexible and it may combine efficient and largely predictable removal of more than 4–6 logs of a wide range of viruses, with an absence of denaturing effect on plasma proteins. Compared with other viral reduction means, nanofiltration may be the only method to date permitting efficient removal of enveloped and nonenveloped viruses under conditions where 90–95% of protein activity is recovered. New data indicate that nanofiltration may also remove prions, opening new perspectives in the development and interest of this technique.

Role of Nanobacteria in Human Diseases

Nanobacteria are mineral-forming, sterile-filterable, slow-growing Gram-negative infectious agents (Wilk and Martirosian 2004). They are detected in bovine/human blood and urine. Nanobacteria-like particles have been detected in synovial fluids of arthritis patients and were shown to gradually increase in number and in size in culture (Tsurumoto et al 2006).

According to their 16S rDNA structure, nanobacteria belong to the alpha-2 Proteobacteria, subgroup, which includes the Brucella and Bartonella species. *Nanobacterium sanguineum* (nanobacteria) is the smallest self-replicating organism ever detected—at 50–500 billionths of a meter, 1/1,000th the size of the smallest previously known bacteria. Primordial proteins in nanobacteria, only recently identified in the atmosphere, could play a significant role in clouds, accelerating the formation of cloud droplets and interconnecting nanobacteria (and possibly nanobacteria and other microorganisms), thus enhancing their chances to eventually reach the Earth (Sommer and Wickramasinghe 2005).

Nanobacteria have been implicated in a variety of human diseases associated with pathological calcification. Their most remarkable characteristic is the formation of carbonate apatite crystals of neutral pH and at physiologic phosphate and calcium concentrations. The extracellular mineralization forms a hard protective shelter for these hardy microorganisms and enables them to survive conditions of physical stress that would be lethal to most other bacterial species. The Olavi Kajander group (Finland) suggests that the apatite produced by nanobacteria may play a key role in the formation of all kidney stones, by providing a central calcium phosphate deposit around which other crystalline components can collect. Nanobacteria seem to be causative agents of diseases related to biomineralization processes. Nanobacteria are also associated with calcified geological specimens, human kidney stones, and psammona bodies in ovarian cancer. Much research has focused attention on the potential role these particles may play in the development of urologic pathology, including polycystic kidney disease, renal calculi, and chronic prostatitis. Recent clinical research targeting these agents has proven effective in treating some patients with refractory category III prostatitis (Wood and Shoskes 2006).

Nanobacteria and Kidney Stone Formation

Approximately 12% of men and 5% of women develop kidney stones by the time they reach the age of 70 years but exactly how kidney stones form is not known. Kidney stones can be debilitating and recur in 50% of patients within 5 years. Kidney stone formation is considered to be a multifactorial disease in which the defense mechanisms and risk factors are imbalanced in favor of stone formation. One theory is that if nanoparticles accumulate in the kidney, they can form the focus of subsequent growth into larger stones over months to years. Other factors, such as physical chemistry and protein inhibitors of crystal growth, also play a role.

Mineral forming nanobacteria are active nidi that attach to, invade, and damage the urinary epithelium of collecting ducts and papilla forming the calcium phosphate center(s) found in most kidney stones. Scientists at NASA have used multiple techniques to determine that nanobacteria infection multiplies faster in space flight simulated conditions than on earth (Ciftcioglu et al 2005). Nanobacteria are considered to initiate kidney stone formation as they grow faster in a microgravity environment and may explain why astronauts get kidney stones on space missions. This discovery may prove to be critical for future exploratory missions to the moon and Mars. For further proof to this hypothesis, screening of the nanobacterial antigen and antibody level in flight crew before and after flight would be necessary. This concept also opens the door for new diagnostic and therapeutic techniques addressing nanobacterial infection in kidney stones.

Nanoparticles, isolated from renal stones obtained at the time of surgical resection, have been analyzed and propagated in standard cell culture medium (Kumar et al 2006). Nanoparticles were propagated from the majority of renal stones. Isolates were susceptible to selected metabolic inhibitors and antibiotics and contained conserved bacterial proteins and DNA. These results suggest that renal stone formation is unlikely to be driven solely by physical chemistry; rather, it is critically influenced by specific proteins and cellular responses, and understanding these events will provide clues toward novel therapeutic targets. Using high-spatial and energy resolution near-edge x-ray absorption fine structure at the 25-nm spatial scale, it is possible to define a biochemical signature for cultured calcified bacteria, including proteins, polysaccharides, nucleic acids, and hydroxyapatite (Benzerara et al 2006). These preliminary studies suggest that nanoparticles isolated from human samples share spectroscopic characteristics with calcified proteins.

Nanobacteria in Cardiovascular Disease

Scientists at the Mayo Clinic have examined surgical specimens from patients with cardiovascular pathology to evaluate human vascular tissue for the presence of similar nanometer-scale objects (Miller et al 2004). Analysis of areas with positive immunostaining identified spheres ranging in size from 30 to 100 nm with a spectral pattern of calcium and phosphorus (high-energy dispersive spectroscopy).

Nano-sized particles cultured from calcified but not from noncalcified aneurysms were recognized by a DNA-specific dye, incorporated radiolabeled uridine, and after decalcification, appeared via electron microscopy to contain cell walls. Therefore, nanometer-scale particles similar to those described as nanobacteria isolated from geological specimens and human kidney stones can be visualized in and cultured from human calcified cardiovascular tissue. In a further study nanoparticles were found near plaque-filled arteries in animal models. The study suggests that nanoparticles potentially represent a previously unrecognized factor in the development of arteriosclerosis and calcific arterial disease.

Nanobiotechnology for Detection of Infectious Agents

The rapid and sensitive detection of pathogenic bacteria is extremely important in medical diagnosis and measures against bioterrorism. Limitations of most of the conventional diagnostic methods are lack of ultrasensitivity or delay in getting results. Several nanotechnology-based methods have already been described in this chapter including ferrofluid magnetic nanoparticles, ceramic nanospheres, and nanowire sensors for viruses. A bioconjugated nanoparticle-based bioassay for in situ pathogen quantification can detect a single bacterium within 20 min (Zhao et al 2004). The nanoparticle provides an extremely high fluorescent signal for bioanalysis and can be easily incorporated in a biorecognition molecule such as an antibody. The antibody-conjugated nanoparticles can readily and specifically identify a variety of bacteria such as *Escherichia coli O157:H7* through antibody–antigen interaction and recognition. This method can be applied to multiple bacterial samples with high throughput by using a 384-well microplate format. It has a potential for application in ultrasensitive detection of disease markers and infectious agents.

Detection of single-molecule hybridization has been achieved by a hybridization detection method using multicolor oligonucleotide-functionalized quantum dots (QDs) as nanoprobes (Ho et al 2005). In the presence of various target sequences, combinatorial self-assembly of the nanoprobes via independent hybridization reactions leads to the generation of discernible sequence-specific spectral codings. This method can be used for genetic analysis of anthrax pathogenicity by simultaneous detection of multiple relevant sequences.

Sensing of Phage-Triggered Ion Cascade for Detection of Bacteria

Researchers at Texas A&M University (College Station, TX) have developed a novel nanotechnology to rapidly detect and identify bacteria (Dobozi-King et al 2005). The technique called SEnsing of Phage-Triggered Ion Cascade (SEPTIC) uses a nanowell device with two antenna-like electrodes to detect the electric field fluctuations that result when a bacteriophage infects a specific bacterium and then identifies the bacterium. The technology had a 100% success rate in detecting and

identifying strains of *E. coli* quickly and accurately. The technique works because only a specific phage can infect a specific bacterium. When a bacteriophage infects a bacterium, the phage injects its DNA into the bacterium and "reprograms" it to produce multiple copies of the phage, called virons. During the infection process, about 100 million ions escape from the host cell. This ion leakage causes fluctuations in the electric field around the bacterium, and the nanowell detects these fluctuations. The Texas A&M University System holds a provisional patent on the technology.

Rapid and sensitive identification of bacteria is extremely important in clinical, veterinary, and agricultural practice, as well as in applications to microbiological threat detection and reduction. It will also be useful in the current fight against bioterrorism. Eventually, every medic or soldier may be equipped with a cell phone-like wireless SEPTIC biolab. The researchers' ultimate aim is to have a biochip where hundreds of nanowells and their preamplifiers are integrated. Each nanowell covers a different phage, and if a relevant bacterium is present, the corresponding nanowell will signal and identify the bacterium. This would be a pen-size biolab that would be able to identify hundreds of bacteria in 5 min.

Viral Detection by Surface-Enhanced Raman Scattering

Although surface-enhanced Raman scattering (SERS) is well known, previous attempts to use spectroscopy to diagnose viruses failed because the signal produced is inherently weak. A spectroscopic assay based on SERS using silver nanorods, which significantly amplify the signal, has been developed for rapid detection of trace levels of viruses with a high degree of sensitivity and specificity (Shanmukh et al 2006). The technique measures the change in frequency of a near-infrared laser as it scatters viral DNA or RNA. That change in frequency is as distinct as a fingerprint. This novel SERS assay can detect spectral differences between viruses, viral strains, and viruses with gene deletions in biological media. The method provides rapid diagnostics (60 s or less) for detection and characterization of viruses generating reproducible spectra without viral manipulation. It is also quite cheap and is very reproducible.

Detection of Single Virus Particles

Microfabrication and application of arrays of silicon cantilever beams as microresonator sensors with nanoscale thickness has been applied to detect the mass of individual virus particles (Gupta et al 2004). The dimensions of the fabricated cantilever beams were in the range of 4–5 μm in length, 1–2 μm in width, and 20–30 nm in thickness. The virus particles used in the study were vaccinia virus, which is a member of the Poxviridae family and forms the basis of the smallpox vaccine. The frequency spectra of the cantilever beams, due to thermal and ambient noise, were measured using a laser Doppler vibrometer under ambient conditions. The change in

resonant frequency as a function of the virus particle mass binding on the cantilever beam surface forms the basis of the detection scheme. This device can detect a single vaccinia virus particle with an average mass of 9.5 fg. Such devices can be very useful as components of biosensors for the detection of airborne virus particles. This technology has been refined as described under nanocantilever biosensors.

Rapid, selective, and sensitive detection of viruses is crucial for implementing an effective response to viral infection, such as through medication or quarantine. Established methods for viral analysis include plaque assays, immunological assays, transmission electron microscopy, and PCR-based testing of viral nucleic acids. These methods have not achieved rapid detection at a single virus level and often require a relatively high level of sample manipulation that is inconvenient for infectious materials.

Scientists at the Harvard University (Cambridge, MA) have reported direct, real-time electrical detection of single virus particles with high selectivity by using nanowire field effect transistors (Patolsky et al 2004). Measurements made with nanowire arrays modified with antibodies for influenza A showed discrete conductance changes characteristic of binding and unbinding in the presence of influenza A but not paramyxovirus or adenovirus. Simultaneous electrical and optical measurements using fluorescently labeled influenza A were used to demonstrate conclusively that the conductance changes correspond to binding/unbinding of single viruses at the surface of nanowire devices. pH-dependent studies further show that the detection mechanism is caused by a field effect and that the nanowire devices can be used to determine rapidly isoelectric points and variations in receptor–virus binding kinetics for different conditions. Larger arrays of reproducible nanowire devices might simultaneously screen for the presence of 100 or more different viruses. Finally, studies of nanowire devices modified with antibodies specific for either influenza or adenovirus show that multiple viruses can be selectively detected in parallel. The possibility of large-scale integration of these nanowire devices suggests potential for simultaneous detection of a large number of distinct viral threats at the single virus level.

Fluorescent QD Probes for Detection of Respiratory Viral Infections

Respiratory syncytial virus (RSV) causes about one million deaths annually worldwide. RSV mediates serious lower respiratory tract illness in infants and young children and is a significant pathogen of the elderly and immune compromised. Although it is only life-threatening in one case out of every 100, it infects virtually all children by the time they are 5 year old. Approximately 120,000 children are hospitalized with RSV in the United States each year. Few children in the United States die from RSV but it causes 17,000–18,000 deaths annually among the elderly.

Rapid and sensitive RSV diagnosis is important for infection control and efforts to develop antiviral drugs. Current RSV detection methods are limited by

sensitivity and/or time required for detection, which can take 2–6 days. This can delay effective treatment. Antibody-conjugated nanoparticles rapidly and sensitively detect RSV and estimate relative levels of surface protein expression (Agrawal et al 2005). A major development is use of dual-color QDs or fluorescence energy transfer nanobeads that can be simultaneously excited with a single light source.

A QD system can detect the presence of particles of the RSV in a matter of hours. It is also more sensitive, allowing it to detect the virus earlier in the course of an infection (Bentzen et al 2005). When an RSV virus infects lung cells, it leaves part of its coat containing F and G proteins on the cell's surface. QDs have been linked to antibodies keyed to structures unique to RSV's coat. As a result, when QDs come in contact with either viral particles or infected cells they stick to their surface. In addition, co-localization of these viral proteins was shown using confocal microscopy. The potential benefits for such an early detection system are that it can

1. Increase the proper use of antiviral medicines. Although such medicines have been developed for some respiratory viruses, they are not used often as therapy because they are only effective if given early in the course of infection. By the time current tests identify the virus, it is generally too late for them to work.
2. Reduce the inappropriate use of antibiotics. Currently, physicians often prescribe antibiotics for respiratory illnesses. However, antibiotics combat respiratory illness caused by bacteria and are ineffective on viral infections. An early virus detection method would reduce the frequency with which doctors prescribe antibiotics for viral infections inappropriately, thereby reducing unnecessary antibiotic side-effects and cutting down on the development of antibiotic resistance in bacteria.
3. Allow hospital personnel to isolate RSV patients. RSV is extremely infectious so early detection would allow hospital personnel to keep the RSV patients separate from other patients who are especially susceptible to infection, such as those undergoing bone-marrow transplants.

Currently, there are three diagnostic tests available for identifying respiratory viruses like RSV. The "gold standard" involves incubating an infected sample in a tissue culture for 5 days and then using a fluorescent dye to test for the presence of the virus. The main problem with this technique is that the virus is multiplying in the patient at the same time as it is growing in the culture. This has caused many hospitals to switch to real-time PCR, which is extremely sensitive but still takes 36–48 h because of the need for a technician well trained in molecular biology to conduct the test in a reference laboratory. The third method, the antigen test, takes only 30 min but it is not sensitive enough to detect the presence of the virus at the early stages of an infection. By comparison, the new QD method takes 1–2 h and is even more sensitive than real-time PCR. It can detect the presence of RSV within an hour after the virus is added to a culture. Another advantage of the QD method over detection systems that rely on fluorescent protein is that the protein "bleaches out" in minutes while QDs maintain their brightness for hours.

It is estimated that it will take only 2–3 years to develop and validate the QD test. All the components are available off-the-shelf and any one can put together one of these detection system. The system should also be relatively inexpensive. The most costly ingredient is the QDs: a small bottle that contains enough of the material for about 200 tests costs $300. As a result, this could be one of the earliest medical applications of nanotechnology. The next step will be to develop a QD cocktail capable of simultaneously detecting the presence of at least five major respiratory viruses: influenza A and B, parainfluenza, and metapneumovirus, in addition to RSV. This should be possible because one can use different colors of QDs simultaneously. The colored QDs are attached to different "linker" molecules that bind to different RSV surface structures. QDs are available in a dozen different colors, and antibodies specific to the other four respiratory viruses have been identified and can be used as linker molecules. Such a test would be able to diagnose more than 90% of all the cases of viral respiratory infection. The existence of such a test could encourage the development of improved therapies for respiratory viruses. Without a good diagnostic test for a specific viral infection, pharmaceutical companies are not motivated to develop effective treatments because physicians are unlikely to prescribe them very often.

Nanotechnology-Based Microbicidal Agents

Nanoscale Bactericidal Powders

Certain formulations of nanoscale powders possess antimicrobial properties. These formulations are made of simple, nontoxic metal oxides such as magnesium oxide (MgO) and calcium oxide (CaO, lime) in nanocrystalline form, carrying active forms of halogens, e.g., $MgO{\cdot}Cl_2$ and $MgO{\cdot}Br_2$. When these ultrafine powders contact vegetative cells of *Escherichia coli*, *Bacillus cereus*, or *Bacillus globigii*, over 90% are killed within a few minutes. Likewise, spore forms of the Bacillus species are decontaminated within several hours. Dry contact with aflatoxins and contact with MS2 bacteriophage (surrogate of human enterovirus) in water also causes decontamination in minutes.

A nanopowder of MgO can scour contaminated rooms of anthrax spores (Stoimenov et al 2002). Unlike antibacterial gases and foams, which are messy, corrosive, and ruin electrical equipment, the powder could be sprayed into rooms and swept or vacuumed up. The chemical specks attract oppositely charged spores. The particles then cut open and chemically break down the spores' tough outer shell. The scientists tested the powder by blowing spores into a stainless-steel room, then cleaning them up with a squirt of nanoparticles. Based on this technology, NanoScale Materials Inc plans to market a dry powder dubbed FAST ACT (First Applied Sorbent Treatment Against Chemical Threats) that decomposes toxic chemicals. The powder contains reactive nanoparticles that attract and then break down

at least 24 commonly transported toxic chemicals, including some acids. Unlike foams, the powder need not be wet to be effective and works on liquids and vapors.

Nanotubes for Detection and Destruction of Bacteria

University of Pittsburgh (Pittsburgh, PA) researchers have synthesized a simple molecule from a hydrocarbon and an ammonium compound to produce a unique nanotube structure with antimicrobial capability (Lee et al 2004). The quaternary ammonium compound is known for its ability to disrupt cell membranes and causes cell death whereas the hydrocarbon diacetylene can change colors when appropriately formulated; the resulting molecule would have the desired properties of both a biosensor and a biocide.

The self-assembled nanotubes are perfectly uniform and organize themselves into an expanse of upright clusters that when magnified a million times resemble the fibers of a shag rug leading to the name "nanocarpet." The self-assembling nanotubes all had the same diameter (89 nm) and wall thickness (27 nm). The nanocarpet measures about 1 μm in height, approximately the same height as the free-form nanotubes. This alignment of nanotubes in the absence of a template is unprecedented and represents an important step toward rational design of bioactive nanostructures. In addition, because they form within hours under room-temperature conditions, the significant costs of synthesizing carbon nanotubes can be reduced. Normally a neutral color, when exposed to ultraviolet light the nanotubes changed to a permanent deep blue. The process also chemically altered the nanotubes so that they became polymerized, giving them a more firm structure. Polymerized, these nanotubes could change from blue to other colors, depending on its exposure to different materials. For instance, in tests with acids and detergents, they turned red or yellow.

Because they display sensitivity to different agents by changing color, these nanotubes can be trained to kill bacteria. In the presence of *E. coli*, some strains of which are food-borne pathogens, the nanotubes turned shades of red and pink. Moreover, with the aid of an electron microscope, the researchers observed the tubes piercing the membranes of the bacteria like a needle being inserted into the cell. Both the polymerized (those that can change color) and the unpolymerized nanotube structures were effective antimicrobials, completely killing all the *E. coli* within an hour's time. The findings have implications for developing products that can simultaneously detect and kill biological weapons. The research, funded by the Department of Defense's Army Research Office, has as its goal the development of a paint that in the event of biological or chemical agents being deployed would change color and simultaneously destroy the deadly substances.

A research team at the Scripps Research Institute (La Jolla, CA) has developed antibiotic agents based on self-assembling cyclic peptide nanotubes that attach to, and poke holes through, bacterial cell membranes, thus killing the cell. These

self-assembling peptide nanotubes cleared infections of antibiotic-resistant bacteria in mice, even when injected far from the site of infection (Fernandez-Lopez et al 2001). Another promising example is a vaccine consisting of self-assembling virus-like particles for the prevention of infection of the genital tract by human papilloma virus, which can cause cervical cancer. Such particles are now being developed by MedImmune (Gaithersburg, MD) and GlaxoSmithKline (Uxbridge, United Kingdom).

Carbon Nanotubes for Protection Against Anthrax Attack

There has been significant interest in the binding of anthrax spores by molecular species, but with only limited success. Proteins and more recently peptides were used. However, despite the known presence of carbohydrates on the spore surface, carbohydrate–carbohydrate interactions have hardly been explored likely because of the lack of required specific platform for synthetic carbohydrates. Scientists at Clemson University (Clemson, SC) have reported the successful use of single-walled carbon nanotubes as a truly unique scaffold for displaying multivalent monosaccharide ligands that bind effectively to anthrax spores with divalent cation mediation to cause significant spore aggregation (Wang et al 2006). The work should have far-reaching implications in development of technologies to counteract bioterrorism such as by use of anthrax. For anthrax to be effective, it has to be made into a fine powder that can easily enter the lungs when inhaled. That nanotechnology-based agent clings to the anthrax spores to make their inhalation into the lungs difficult. Similar approach using sugar-coated carbon nanotubes to stop the spread of *E. coli* bacteria has been tested successfully in 2004.

Nanoemulsions as Microbicidal Agents

The antimicrobial nanoemulsions (NanoBio) are emulsions that contain water and soya bean oil with uniformly sized droplets in the 200–400 nm range. These droplets are stabilized by surfactant and are responsible for the microbicidal activity. In concentrated form, the nanoemulsions appear as a white milky substance with a taste and consistency of cream. They can be formulated in a variety of carriers allowing for gels, creams, liquid products, etc. In most applications, the nanoemulsions become largely water-based, and in some cases such as a beverage preservative comprise 0.01% or less of the resultant mixture. Laboratory results indicate a shelf life of at least 2 years and virtually no toxicity. The NanoBio nanoemulsions destroy microbes effectively without toxicity or harmful residual effects (Hamouda et al 2001). The nanoparticles fuse with the membrane of the microbe and the surfactant disrupts the membrane, killing the microbe. The classes of microbes eradicated are virus (e.g., HIV, herpes), bacteria (e.g., *E. coli*, Salmonella), spores (e.g., anthrax), and fungi (e.g., *Candida albicans*, *Byssochlamys fulva*). Clinical trials have shown

efficacy in healing cold sores due to herpes simplex virus 1 and toenail fungus. The nanoemulsions also can be formulated to kill only one or two classes of microbes. Due in large part to the low toxicity profile, the nanoemulsions are a platform technology for any number of topical, oral, vaginal, cutaneous, preservative, decontamination, veterinary, and agricultural antimicrobial applications.

Since it is nontoxic and noncorrosive, nanoemulsion can be used to decontaminate personnel, equipment, terrain, structures, and water. Further, tests by DTRA (Defense Threat Reduction Agency), an agency of the US Department of Defense, have demonstrated that the nanoemulsion is a chemical decontaminating agent. The US Army tested the nanoemulsion and nine other biodecontamination technologies against an anthrax surrogate. The nanoemulsion was one of four technologies that proved effective.

Silver Nanoparticle Coating as Prophylaxis Against Infection

The Institute for New Materials (Saarbrucken, Germany), a research institute specializing in applied nanotechnology applications, has developed a silver nanoparticles surface coating that is deadly to fungi and bacteria. The researchers added the germicidal ability by sprinkling copious amounts of silver nanoparticles through the coating material (every square centimeter contains more than one billion of the invisible particles) and aligning them so that they release a tiny number of silver ions. These ions are the death knell for fungus and bacteria that might have succeeded in gathering on the surface despite its already dirt-repellent qualities. Applications include any surface where germs can gather and possibly endanger people's health. That includes surfaces in hospitals, public buildings, factories, or in the home. The coating could be applied to almost any surface that people touch often such as metal, glass, or plastic and would remove the need for constant cleaning with liquid disinfectants, especially in areas where hygienic conditions are crucial. People who normally cannot use hearing aids that lie inside the ear because of the risk of infection of the auditory canal can safely wear nanocoated appliances.

Bio-Gate (Nürnberg, Germany) produces NanoSilver BG, a nanoporous silver powder with particle size ranging from 50 to 100 nm. It has a homogeneous distribution of nanoparticles in the material and antiinfective properties.

Silver nanoparticles have been incorporated in preparations for wound care to prevent infection. Acticoat bandages (Smith & Nephew) contain nanocrystal silver, which is highly toxic to pathogens in wounds.

AcryMed's silver nanoparticle technology, SilvaGard, involves coating with silver nanoparticles with size range of 2 to 20 nm in a stable solution and antimicrobial treatment levels last for more than a year. With other technologies, nano-based silver coatings must be applied through vapor deposition, which coats only on one side, whereas AcryMed technology is a solution that provides a complete surface treatment rather than a coating.

Nanotechnology-Based Antiviral Agents

Nanocoating for Antiviral Effect

Laboratory testing of the permanent nanocoating developed by researchers at North Carolina State University College of Textiles and Emory University School of Medicine showed the coating kills 99.9% of influenza viruses and 99.99% of vaccinia viruses that cause rash, fever, head and body aches. The development may lead to being able to protect oneself from virtually all viruses and bacteria by simply exposing a surface to light. The technology has been licensed to LaamScience Inc.

In November 2006, Mass Transit Railway (MTR), the corporation that runs Hong Kong subway, announced that Nano Silver–Titanium Dioxide Coating (NSTDC, a nontoxic disinfectant) will be applied to surfaces that passengers commonly touch in order to enhance hygiene levels in MTR stations and trains. The coating is manufactured using nanotechnology, which maximizes coverage and effectiveness of NSTDC. Developed in Japan, NSTDC is certified to be effective in killing a wide range of bacteria, viruses, and mold including the H1N1 influenza virus A. It is used in hospitals, offices, and homes in Japan. NSTDC's main component, titanium dioxide (TiO_2), has been approved for use in foods by the FDA and under the Public Health and Municipal Services Ordinance in Hong Kong.

Fullerenes as Antiviral Agents

A series of bis-fulleropyrrolidines bearing two ammonium groups have been synthesized and their activities against HIV-1 and HIV-2 have been evaluated (Marchesan et al 2005). Two trans isomers were found to have interesting antiviral properties, confirming the importance of the relative positions of the substituent on the C60 cage. None of the compounds showed any inhibitory activity against a variety of DNA and RNA viruses other than HIV.

Cationic, anionic, and amino acid-type fullerene derivatives have shown inhibitory effect against HIV-reverse transcriptase and HCV (Mashino et al 2005). Out of all derivatives of fullerenes, anionic fullerenes, were found to be the most active. All the tried fullerene derivatives were more active than the nonnucleoside analogue of HIV-RT inhibitor. The effect of long alkyl chains on fullerenes was not significant; rather it depressed the inhibition strength. The two important targets for anti-HIV characteristics are the HIV-protease and HIV-reverse transcriptase. The molecular modeling experimental designs exhibit that C60-core could penetrate into hydrophobic binding site of HIV protease. However, the mechanism of this anti-HIV activity is through HIV-protease inhibition, which has not been experimentally demonstrated.

Nanoviricides

NanoViricides Inc is developing nanoviricides, which are nanomedicines that destroy viruses. A nanoviricide is a polymeric single chemical chain with covalently attached ligands that specify the virus target. The antiviral spectrum of the drug is determined by the specificity of the set of ligands attached to the chain, in addition to other functionally important aspects inherent in the chemistries. Nanoviricide is designed to seek a specific virus type, attach to the virus particle, engulf or coat the virus particle, thereby neutralizing the virus's infectivity, destabilize and possibly dismantle the virus particle, and optionally it may also be made capable of attacking the viral genome thereby destroying the virus completely. They are different from any of the other micellar nanotechnologies as there are no metal particles attached and the micelles can penetrate the virus and bind to multiple sites for effective destruction of the virus. Active pharmaceutical ingredients are optional and can be hidden in the core of the nanoviricide missile.

Mechanism of Action of Nanoviricides

For a virus to infect a cell, it needs to bind to more than one site. For example, binding of HIV only to CD4 on T cells is insufficient to cause sustained disease; it needs HIV binding to at least two and possibly three different sites on the T cell and that too, at multiple points. For an antiviral to be effective, it should match the strategy to bind to more than one site on the virus. Ideally it should block all of these to prevent virus from infecting the cell and multiplying. Most of the current antiviral drugs have a single mechanism of action and block a single receptor. Drug combinations from different categories are required to increase the number of receptors blocked. Still this is not fully effective.

In contrast to other approaches, a NanoViricide^{TM} micelle can recognize and bind to more than one type of binding site on the virus. The NanoViricide^{TM} system enables design of a drug that binds to more than one type of site—currently as many as three different sites, on the virus—for a highly effective attack. NanoViricides Inc terms this as "multi-specific targeting."

A NanoViricide^{TM} drug goes much further than just blocking all of the binding sites of the virus. The base material of a NanoViricide^{TM} is a specially designed polymeric micelle material. It has the ability to disassemble an HIV particle by itself. Thus, after coating the virus particle, the NanoViricide^{TM} loosens the virus particle, and weakens it. Some virus particles will even fall apart (uncoat). This provides a further therapeutic benefit. NanoViricides plans to enhance the viral disassembly capabilities of the NanoViricide^{TM} by attaching specially designed "molecular chisels" to the NanoViricide^{TM}. Once the NanoViricide^{TM} micelles coat the virus particle, the attached "molecular chisels" will go to work. They literally insert themselves into the virus coat at specific vulnerable points and pry apart the coat proteins so that the virus particle falls apart readily. The mechanism of action of NanoViricide is depicted schematically in Fig. 11.1. This description is a simplification. There is no fully adequate explanation of the observed efficacy because the

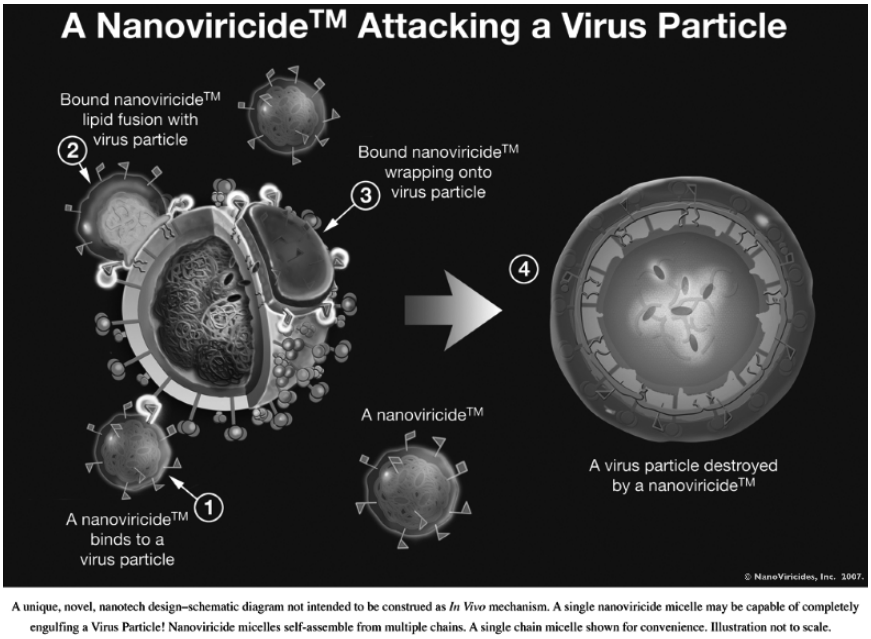

Fig. 11.1 Schematic representation of NanoViricide attacking a virus particle. (**A**) NanoViricide micelles attach to the virus at multiple points with nanovelcro effect and start engulfing the virus. (**B**) Flexible micelle coats and engulfs the virus particle, dismantles and neutralizes it, and fuses with viral lipid coat. Reproduced by permission of NanoViricides Inc

mechanisms of action of nanomaterials as drugs and particularly, NanoViricides in vivo, are multiple and somewhat complex. Targets for this approach include influenzas, HIV, HCV, rabies, and other viruses.

Advantages of NanoViricides

NanoViricides have been compared to current approaches to viral diseases, which are seldom curative and some of the advantages include the following:

- Specific targeting of the virus with no metabolic adverse effects on the host.
- The biological efficacy of NanoViricides drugs may be several orders of magnitude better than that of usual chemical drugs. This in itself may limit the potential for mutant generation.
- There are also other key aspects of the design of NanoViricides that are expected to lead to minimizing mutant generation.
- Nanoviricides are safe because of their unique design and the fact that they are designed to be biodegradable within the body.
- The new technology enables rapid drug development against an emerging virus, which would be important for global biosecurity against natural as well as man-made (bioterrorism) situations. It is possible to develop a research drug against a

novel life-threatening viral disease within 3–6 weeks after the infection is found, i.e., as soon as an antibody from any animal source is available.

- It is possible to make a single NanoViricide drug that responds to a large number of viral threats by using targeting ligands against the desired set of viruses in the construction of the drug. It is possible to "tune" the specificity and range (spectrum) of a NanoViricide drug within a virus type, subtype, or strain, by appropriate choices of the targeting ligand(s).
- The safety of NanoViricide drugs is proven now as they specifically attack the virus and not the host.
- A variety of formulations, release profiles, and routes of administration are possible.
- Low cost of drug development, manufacture, distribution.

NanoViricide drug candidates are currently in preclinical studies. Clinical trials are planned. Initially injectable products are considered to be most effective but alternative routes of administrations such as nasal sprays and bronchial aerosols can also be developed. Various NanoViricide products will be described further along with relevant viral diseases.

Advantages of Nanoviricides over vaccines are as follows:

- Nanoviricides work where vaccines fail and are effective even when the immune system is impaired such as in AIDS.
- Nanoviricides work where effective vaccines are unavailable.
- Sufficient short-term protection for an individual outbreak cluster.
- Treatment can be started after infection.
- No need to vaccinate whole world population for control of a viral epidemic.

Advantages of Nanoviricides over immunoglobulin therapies are as follows:

- Fully chemical, room-temperature stable NanoViricides can be made against many diseases.
- Nanoviricides based on antibody fragment conjugates do not require humanized antibodies. Antibodies from virtually any source can be used for developing NanoViricides, thus significantly reducing time and cost of development.

Immunoglobulin therapies require the patient's immune system (complement system) to function well, which is often not the case in advanced disease states. NanoViricides function completely independently of the human immune system while accomplishing the same goal of reduction in viremia.

Nanotechnology-Based Vaccines

Although a number of adjuvants are currently approved for use in veterinary species, only alum has been widely used in humans. While it induces strong antibody responses, cell-mediated responses are often low and inflammatory reactions at the site of injection are common. Immunological properties of a novel nanobead

adjuvant have been investigated in a large-animal sheep model (Scheerlinck et al 2006). In contrast to alum, antigen covalently coupled to nanobeads induced substantial cell-mediated responses along with moderate humoral responses. No adverse reactions were seen at the site of immunization in the sheep. Thus, nanobead adjuvants in veterinary species may be useful for the induction of immunity to viral pathogens, where a cell-mediated response is required. These findings also highlight the potential usefulness of nanobead vaccines for intracellular pathogens in humans. Nanobeads measure 40 nm. Most adjuvants only stimulate antibodies against a particular disease. The nanobead technology gave the immune system a further boost, also producing T cells which are needed to eliminate viruses or cancer. The size of 40 nm is critical as it is similar in size to many viruses, where the nanobeads are taken up abundantly by the immune system and tricked into producing high levels of many types of T-cells.

Nanofiltration of Blood in Viral Diseases

Nanofiltration is the filtration of minute particles using a filter with nanopores. Scientists at the Queensland University of Technology (QUT) in Australia have developed specially designed ceramic membranes used as nanomesh for nanofiltration, which are so advanced they have the potential to remove viruses from water, air, and blood. Shortcomings of current membranes were that they often formed pin-holes and cracks during the fabrication process, resulting in wasted membranes.

QUT scientists introduced radical changes to the membrane texture because it is crucial for the separation efficiency of the material. Mesh structure, which is the most efficient form of filtration, has been successfully constructed on a nanoscale with ceramic fibers. This modification has increased the rates of flow that pass through the membranes by at least 10 times compared with current ceramic membranes, while maintaining the efficiency of capturing over 96% of the unwanted particles.

This technology could be used to filter airborne viruses such as the severe acute respiratory syndrome (SARS) and the avian flu virus. It may be possible to filter HIV from human blood to treat patients with AIDS.

Nanoparticles to Combat Biological Warfare Agents

Nanosilicate crystals are generated by a nontoxic electrochemical reaction when Bio-DECON™ (Sierra Pacific Research Company) is mixed with water. Although a chemical process creates the silicate crystals, it is a mechanical process that destroys the cellular wall of the spore, virus, or bacteria. Certain types of Gram-negative bacteria do not allow a chemical charge to penetrate the cellular wall. Bio-DECON mechanically penetrates the cellular wall of both Gram-negative as well as Gram-positive bacteria.

Bio-DECON was tested several years ago at the Battelle Memorial Institute as an antibacterial agent against *Bacillus anthracis* vegetative cells and spores. Results revealed that a laboratory-diluted 4% solution Bio-DECON (normal application is a 25% solution) had an immediate deleterious impact on the anthrax colony forming units, disabling 96% of the weapons grade form of *Ames anthrax* within 2 min of exposure. Continued exposure for 60 min increased the "kill ratio" to more than 99%. During a longer period of time, the effects of Bio-DECON continue to kill microorganisms for days, if not weeks.

Nanomaterials could play a role as an anthrax antibiotic. Chemists at Rice University have found that antibodies that latch on to dormant anthrax spores and drugs that destroy anthrax could be linked to the spherical carbon molecule fullerene to make an antibiotic. The drug would kick in when an inhaled spore germinates, killing the anthrax before it releases deadly amounts of toxin. This could be effective if one has just been exposed or the exposure is expected within 24 h following administration of the drug. The research group is in preliminary stages of investigating antibiotics such as vancomycin for anthrax infections.

Preventing the interaction of toxins with their cellular receptors CMG2 and ATR/TEM8 is an important goal for anthrax therapy. Scientists at the Scripps Research Institute (La Jolla, CA) have described novel nanotechnology approaches for the multivalent display of engineered receptor decoys, and their efficacy against anthrax lethal toxin in vitro and in vivo.

Chapter 12
Nano-Ophthalmology

Introduction

Nanotechnology has many applications in disorders of eye. These include drug delivery, study of pathomechanism of eye diseases, regeneration of the optic nerve, and counteracting neovascularization involved in some degenerative disorders.

Nanocarriers for Ocular Drug Delivery

Approximately 90% of all ophthalmic drug formulations are applied as eye drops. While eye drops are convenient, about 95% of the drug contained in the drops is lost through tear drainage, a mechanism for protecting the eye against exposure to noxious substances. Moreover, the very tight epithelium of the cornea compromises the permeation of drug molecules.

Nanocarriers, such as nanoparticles, liposomes, and dendrimers, are used to enhance ocular drug delivery (Vandervoort and Ludwig 2007). Easily administered as eye drops, these systems provide a prolonged residence time at the ocular surface after instillation, thus avoiding the clearance mechanisms of the eye. In combination with a controlled drug delivery, it should be possible to develop ocular formulations that provide therapeutic concentrations for a long period of time at the site of action, thereby reducing the dose administered as well as the instillation frequency. In intraocular drug delivery, the same systems can be used to protect and release the drug in a controlled way, reducing the number of injections required. Another potential advantage is the targeting of the drug to the site of action, leading to a decrease in the dose required and a decrease in side-effects.

Nanoparticle-Based Topical Drug Application to the Eye

Nanoparticle technology has been used for ophthalmic formulations for a decade but research is still in progress to improve the delivery and safety of drugs used for treating disorders of the eye. Topical application of nonsteroidal antiinflammatory

K.K. Jain, *The Handbook of Nanomedicine*,

drugs on the eye is a common treatment used to treat the inflammatory reaction manifested by narrowing of the pupil (miosis) induced by surgical injury such as cataract extraction. With the aim of improving the availability of sodium ibuprofen (IBU) at the intraocular level, IBU-loaded polymeric nanoparticle suspensions were made from Eudragit RS100, an inert polymer resin (Pignatello et al 2002). Nanosuspension particles had a mean size of ~100 nm and a positive charge making them suitable for ophthalmic applications. In vitro dissolution tests indicated a controlled release profile of IBU from nanoparticles. In vivo efficacy was assessed on the rabbit eye after induction of an ocular trauma. An inhibition of the miotic (narrowing of pupil) response to the surgical trauma was achieved, comparable to a control aqueous eye-drop formulation, even though a lower concentration of free drug in the conjunctival sac was reached from the nanoparticle system. Drug levels in the aqueous humor were also higher after application of the nanosuspensions; moreover, IBU-loaded nanosuspensions did not show toxicity on ocular tissues.

Chitosan Nanoparticles for Topical Drug Application to the Eye

Use of chitosan (CS) nanoparticles for ocular drug delivery has been investigated by studying their interaction with the ocular mucosa in vivo and also their toxicity in conjunctival cell cultures (de Campos et al 2004; de Salamanca et al 2006). The in vivo interaction of fluorescent CS (CS-fl) nanoparticles with the rabbit cornea and conjunctiva was analyzed by spectrofluorimetry and confocal microscopy. CS-fl nanoparticles were found to be stable upon incubation with lysozyme and did not affect the viscosity of mucin dispersion. In vivo studies showed that the amounts of CS-fl in cornea and conjunctiva were significantly higher for CS-fl nanoparticles than for a control CS-fl solution, these amounts being fairly constant for up to 24 h. Confocal studies suggest that nanoparticles penetrate into the corneal and conjunctival epithelia. Cell survival at 24 h after incubation with CS nanoparticles was high and the viability of the recovered cells was nearly 100%. These findings indicate that CS nanoparticles are promising vehicles for ocular drug delivery.

Chitosan has been modified by covalent coupling to cholesterol (Yuan et al 2006). These molecules self-aggregate into nanoparticles with a size of approximately 200 nm. Cyclosporin was incorporated with a drug loading of 6.2%. In vitro tests demonstrated that the drug was gradually released over a period of 48 h. Use of SPECT and scintillation counter demonstrated that 71% of the drug was still present at the ocular surface after 112 min.

Polylactide Nanoparticles for Topical Drug Application to the Eye

Poly(lactic/glycolic) acid nanoparticles incorporating flurbiprofen have been prepared by the solvent displacement technique using poloxamer 188 as a stabilizer to improve the availability of the drug for the prevention of the inflammation caused by ocular surgery (Vega et al 2006). Formulations, with particle size of 230 nm, did not show toxicity on ocular tissues. In vivo studies in rabbits demonstrated that the formulations did not induce toxicity or irritation. Nanoparticle formulations were

compared with commercial eye drops (Ocuflur^{TM}) after induction of inflammation by instillation of sodium arachidonate. The commercial eye drop showed a suppression of inflammation, with minimal inflammation reached after 90 min. A comparable nanoparticle formulation demonstrated a higher suppression, which increased throughout the 150-min observation time of the study.

Ophthalmic Drug Delivery Through Nanoparticles in Contact Lenses

Scientists at the Institute of Bioengineering and Nanotechnology in Singapore have invented a simple method of making polymeric lens materials that can be loaded with eye medication for ophthalmic drug delivery applications. The method of drug delivery via the lens using nanoparticles is better than the conventional drug delivery by incorporating the drug into the lens during the manufacture process. The solution to constitute the lens contains a mixture of molecules, which create nanochannels when they set. The channels act as conduits for the drug to be released when the lens comes into contact with eye fluid. The channels also render the lens nanoporous, i.e., tears and gases can cross into and out of lens, making it more compatible with the human eye. By adjusting the channel size, medications can be delivered over hours or days. Other methods that soak the lens in a drug-laden solution provide only uncontrolled release of the medication that lasts a few hours. This method of drug delivery would reduce drug waste and adverse effects of systemic absorption resulting in increased patient compliance.

Scientists at the University of Florida have devised a particle-laden soft contact lens as a new vehicle for ophthalmic drug delivery. Ophthalmic drugs are formulated as nanoparticles and dispersed within the lens material. Drug diffuses into the eye from the lens. These lenses will reduce drug wastage and side-effects and provide controlled drug release for over 5–10 days.

Nanoparticles for Intraocular Drug Delivery

Nanoparticles have also been investigated for intraocular drug delivery to provide controlled drug release, protect the drug against enzymatic degradation, and to direct the drug to the site of action. The kinetics of polylactide (PLA) nanoparticle localization within the intraocular tissues and their potential to release encapsulated material have been studied in experimental animals (Bourges et al 2003). Intravitreous injection of PLA nanoparticles appears to result in transretinal movement, with a preferential localization in the retinal pigmented epithelial (RPE) cells. Encapsulated rhodamine dye diffuses from the nanoparticles and stains the neuroretina and the RPE cells. The findings support the idea that specific targeting of these tissues is feasible. Furthermore, the presence of the nanoparticles within the RPE cells 4 months after a single injection shows that a steady and continuous delivery of drugs can be achieved.

Subconjunctivally administered 200-nm and larger PLA nanoparticles can be almost completely retained at the site of injection in male Sprague–Dawley rats for at least 2 months (Amrite and Kompella 2005). The 20-nm particles disappeared more rapidly, with 8% of the administered dose remaining after 7 days. The

neuroprotective effects of PLGA nanospheres to encapsulate pigment epithelium-derived factor (PEDF) has been evaluated in induced retinal ischemic injury (Li et al 2006). Intravitreal injection of the naked peptides demonstrated a 44% reduction of cell death of the retinal ganglion cells (RGCs) after 48 h. Injection of the encapsulated peptide showed a very similar protective effect that lasted for at least 7 days. The authors attributed the extended effect to the slow release of PEDF from the PLGA particles and to the protection of the peptide against degradation and rapid clearance.

Besides size, grafting polymers with PEG is another method of controlling particle distribution. A hydrophobic polymer, cyanoacrylate-co-hexadecyl cyanoacrylate, was coupled to hydrophilic PEG chains in order to produce tamoxifen-loaded nanoparticles (De Kozak et al 2004). Intraocular injection in rats resulted in a significant inhibition of experimentally induced autoimmune uveoretinitis, whereas injection of the free drug did not alter the disease.

DNA Nanoparticles for Nonviral Gene Transfer to the Eye

The eye is an excellent candidate for gene therapy as it is immune privileged and much of the disease-causing genetics are well understood. Compacted DNA nanoparticles (Copernicus) have been investigated as a system for nonviral gene transfer to ocular tissues. The compacted DNA nanoparticles have already been shown to be safe and effective in a human clinical trial, have no theoretical limitation on plasmid size, do not provoke immune responses, and can be highly concentrated. An experimental study has shown that DNA nanoparticles can be targeted to different tissues within the eye by varying the site of injection (Farjo et al 2006). Almost all cell types of the eye can be transfected by nanoparticles and produce robust levels of gene expression that are dose dependent. Subretinal delivery of these nanoparticles transfects nearly all of the photoreceptor population and produces expression levels almost equal to that of rodopsin, the highest expressed gene in the retina. As no deleterious effects on retinal function have been observed, this treatment strategy appears to be clinically viable and provides a highly efficient nonviral technology to safely deliver and express nucleic acids in the retina and other ocular tissues. These findings have implications for the development of DNA-based therapeutics for various eye disorders, including retinitis pigmentosa, diabetic retinopathy, and macular degeneration.

Nanotechnology-Based Therapeutics for Eye Diseases

Use of Dendrimers in Ophthalmology

Scientists at the Imperial College (London, United Kingdom) are examining the use of anionic dendrimers in ophthalmology. One of the main motives of using these

compounds is to overcome the limitation of agents targeted to a single molecule or receptor. Dendrimers enable polyvalent medicines, larger molecules where several ligands can bind to several receptors in order to get the desired biological response. The use of dendrimers in drug delivery to the eye is also being explored. Dendrimers can be used to prevent scar formation following eye surgery. Another use would be to disrupt inflammation and angiogenesis in the posterior chamber of the eye.

Nanotechnology for Prevention of Neovascularization

Some of the strategies for treatment of eye disorders involve prevention of neovascularization. Examples of how nanotechnology can refine these procedures are as follows.

Photodynamic therapy (PDT) has been used for exudative age-related macular degeneration (AMD). This therapy can be refined by using a supramolecular nanomedical device, i.e., a novel dendritic photosensitizer (DP) encapsulated by a polymeric micelle formulation (Ideta et al 2005). The characteristic dendritic structure of the DP prevents aggregation of its core sensitizer, thereby inducing a highly effective photochemical reaction. With its highly selective accumulation on choroidal neovascularization (CNV) lesions, this treatment results in a remarkably efficacious CNV occlusion with minimal unfavorable phototoxicity.

Dendrimer glucosamine 6-sulfate has been shown to block FGF-2-mediated endothelial cell proliferation and neoangiogenesis in human Matrigel and placental angiogenesis assays (Shaunak et al 2004). When dendrimer glucosamine and dendrimer glucosamine 6-sulfate were used together in a validated and clinically relevant rabbit model of scar tissue formation after glaucoma filtration surgery, they increased the long-term success of the surgery from 30 to 80%. Synthetically engineered dendrimers can be tailored to have defined immuno-modulatory and antiangiogenic properties, and they can be used synergistically to prevent scar tissue formation.

A long-term study was performed into the use of a lipophilic amino-acid dendrimer to deliver an antivascular endothelial growth factor (VEGF) oligonucleotide (ODN-1) into the eyes of rats and inhibit laser-induced CNV (Marano et al 2005). In addition, the uptake, distribution, and retinal tolerance of the dendrimer plus oligonucleotide conjugates were examined. Analysis of fluorescein angiograms of laser photocoagulated eyes revealed that dendrimer plus ODN-1 significantly inhibited the development of CNV for 4–6 months by up to 95% in the initial stages. Eyes similarly injected with ODN-1 alone showed no significant difference. Intravitreally injected ODN-1 was absorbed by a wide area of the retina and penetrated all of the retinal cell layers to the retinal pigment epithelium. Ophthalmological examinations indicated that the dendrimers plus ODN-1 conjugates were well tolerated in vivo, which was later confirmed using immunohistochemistry, which showed no observable increase in antigens associated with

inflammation. The use of such dendrimers may provide a viable mechanism for the delivery of therapeutic oligonucleotides for the treatment of angiogenic eye diseases.

Nanobiotechnology for Regeneration of the Optic Nerve

Self-assembling peptide nanofiber scaffolds (SAPNS) are being used at the Massachusetts Institute of Technology (Cambridge, MA) to nano-neuro knit -damaged tissue deep within the mammalian brain. After transecting the optic nerve in hamsters, they injected SAPN into the area, which allowed the axons in the damaged area to regenerate and knit to the surrounding neural tissue with restoration of vision. Although the technique is still far away from human use, the prospects of application are promising.

DNA Nanoparticles for Gene Therapy of Retinal Degenerative Disorders

A research team at University of Oklahoma Health Sciences Center presented findings at the American Society of Gene Therapy meeting in June 2007 to demonstrate that DNA nanoparticles (Copernicus) corrected vision defects in a mouse model of retinitis pigmentosa by delivery of normal copies of genes into photoreceptor cells. DNA nanoparticles may also offer the potential to provide effective treatments for more complex eye disorders such as diabetic retinopathy, macular degeneration, and various diseases that injure ganglion cells and the optic nerve. There is a plan to move these studies to a potential human clinical trial.

Nanobiotechnology for Treatment of Glaucoma

Glaucoma involves abnormally high pressure of the fluid inside the eye, which, if left untreated, can result in damage to the optic nerve and vision loss. Human carbonic anhydrase (hCAII), a metalloenzyme that catalyzes the reversible hydration of carbon dioxide to bicarbonate, is associated with glaucoma. High pressure occurs, in part, because of a buildup of carbon dioxide inside the eye. Drug therapy is aimed at blocking hCAII. Carbonic anhydrase inhibitors such as acetazolamide, methazolamide, ethoxzolamide, and dichlorophenamide were and still are widely used systemic antiglaucoma drugs. Their mechanism of action consists in inhibition of CA isozymes present in ciliary processes of the eye with the consequent reduction of bicarbonate and aqueous humor secretion, and of elevated intraocular pressure.

However, barely 1–3% of existing glaucoma medicines penetrate into the eye. Earlier experiments with nanoparticles have shown not only high penetration rates but also little patient discomfort. The miniscule size of the nanoparticles makes

them less abrasive than some of the complex polymers now used in most eye drops. A specialized cerium oxide nanoparticle has been bound with a compound that has been shown to block the activity of an hCAII (Patil et al 2007). Carboxybenzenesulfonamide, an inhibitor of the hCAII enzyme, was attached to nanoceria particles using epichlorohydrin as an intermediate linkage. Along with the CA inhibitor, a fluorophore (carboxyfluorescein) was also attached on the nanoparticles to enable the tracking of the nanoparticles in vitro as well as in vivo. X-ray photoelectron spectroscopic studies carried out at each reaction step confirmed the successful derivatization of the nanoceria particles. The attachment of carboxyfluorescein was also confirmed by confocal fluorescence microscopy. Preliminary studies suggest that carboxybenzenesulfonamide-functionalized nanoceria retains its inhibitory potency for hCAII.

Dendrimers and other nanotechnology devices are being used at the University of Michigan (Ann Arbor, MI) for ophthalmic genetics and genomics as applied to glaucoma research. Apart from the study of the disease, there is a search for improved methods of drug delivery. The investigators are interested in devices that will enable: (1) genotyping in real time in clinical or point-of-care situations; (2) assaying levels of gene expression in situ to enable evaluation of effects during attempted interventions; and (3) methods to deliver gene therapy to specific cell types without using viral vectors.

One of the problems in treating glaucoma is to get the drug into the cells rather than in the surrounding space or on cell surface. Trabecular meshwork (TM) cells have phagocytic properties and could be induced to take up a variety of carrier particles. A latrotoxin analogue can be used to direct the dendrimer to the latrotoxin receptor on the surface of the TM cells. The presence of a relatively specific protease, PCSK1, in TM cells offers the possibility of genetically engineering the protein to be attached to the particle. This can be done via a cleavable peptide attachment that will result in release from the dendrimer once it enters the cell in which the protease is located.

Chapter 13
Regenerative Medicine and Tissue Engineering

Nanobiotechnology in Tissue Engineering

Tissue engineering is an interdisciplinary field, which applies the principles of engineering and the life sciences to the development of biological substitutes that restore, maintain, or improve tissue function. Tissue engineering is an emerging field between traditional medical devices and regular pharmaceuticals. It faces many challenges and is also a field that is extremely interdisciplinary requiring the efforts of physicians, cell biologists, material scientists, chemical engineers, and chemists. Apart from the use of nanoparticles for diagnostic and therapeutic purposes, nanotechnology has applications for the development of tissue engineering as indicated by some of the studies in life sciences (Emerich and Thanos 2003). Potential areas for application of nanobiotechnology in regenerative medicine include CNS injury, neurogenerative disorders, diabetes, cartilage injuries, and bone fractures.

The response of cell motility and metabolism to changes in substrates has been thoroughly studied in the past decade. Size, structure, geometry, integrin-binding, and other factors have all been investigated. Various techniques have been employed to create micropatterned surfaces of different materials to study cell behavior. In the presence of patterned stripes of bovine serum albumin and laminin, Schwann cells aggregate preferentially on the laminin regions. Osteogenic cells have been cultured in 3D nanohydroxyapatite/collagen matrix, which is precipitated in such a manner that hydroxyapatite crystals are uniformly distributed in a matrix of collagen, seemingly ideal for bone construction.

Microfluidic devices enable the study of methods for patterning cells, topographical control over cells and tissues, and bioreactors. They have not been used extensively in tissue engineering but major contributions are expected in two areas. The first is growth of complex tissue, where microfluidic structures ensure a steady blood supply, thereby circumventing the well-known problem of providing larger tissue structures with a continuous flow of oxygen as well as nutrition and removal of waste products. The second, and probably more important function of microfluidics, combined with micro/nanotechnology, lies in the development of in vitro physiological systems for studying fundamental biological phenomena.

K.K. Jain, *The Handbook of Nanomedicine*,

Three-Dimensional Nanofilament-Based Scaffolds

Ideally the tissue-engineering scaffolds should be analogous to native extracellular matrix (ECM) in terms of both chemical composition and physical structure. Polymeric nanofiber matrix is similar, with its nanoscaled nonwoven fibrous ECM proteins, and thus is a candidate ECM-mimetic material (Ma et al 2005). Scaffolds for tissue engineering are typically solid or porous materials with isotropic characteristics and present regenerative cues such as growth factors or ECM proteins but these do not explicitly guide tissue regeneration. Scientists at the Georgia Institute of Technology (Atlanta, GA) have developed novel 3D nanofilament-based scaffolds for tissue regeneration. This invention mimics the strategy used by collagen and other fibrillar structures to guide cell migration or tissue development and/or regeneration in a guided, direction-sensitive manner. The critical advantage of this technology is that it provides directional cues for cell and tissue regeneration. This strategy not only can be used to guide the migration of endogenous or transplanted cells and tissues to damaged tissues of the peripheral and central nervous systems to restore function, but could also be applied to tissue engineering.

The development of effective biological scaffold materials for tissue engineering and regenerative medicine applications hinges on the ability to present precise environmental cues to specific cell populations to guide their position and function. Natural ECMs have an ordered nanoscale structure that can modulate cell behaviors critical for developmental control, including directional cell motility. Scientists at Harvard Medical School (Boston, MA) have described a method for fabricating fibrin gels with defined architecture on the nanometer scale in which magnetic forces are used to position thrombin-coated magnetic microbeads in a defined 2D array and thereby guide the self-assembly of fibrin fibrils through catalytic cleavage of soluble fibrinogen substrate (Alsberg et al 2006). Time lapse and confocal microscopy confirmed that fibrin fibrils nucleate near the surface of the thrombin-coated beads and extend out in a radial direction to form these gels. When controlled magnetic fields were used to position the beads in hexagonal arrays, the fibrin nanofibrils that polymerized from the beads oriented preferentially along the bead–bead axes in a geodesic (minimal path) pattern. These biocompatible scaffolds supported adhesion and spreading of human microvascular endothelial cells, which exhibited coalignment of internal actin stress fibers with underlying fibrin nanofibrils within some membrane extensions at the cell periphery. This magnetically guided, biologically inspired microfabrication system is unique in that large scaffolds may be formed with little starting material, and thus it may be useful for in vivo tissue-engineering applications in the future.

In addition to fabricating 3D microfabricated scaffolds as templates for cell aggregate formation, nanoscale technologies can be used for controlling the features such as shape and pore architecture, as templates for microtissue formation or as improved bioreactors (Khademhosseini et al 2006). The nanoscale control of cellular environments can also be used to probe the influence of the spatial and temporal effects of specific cell–cell, cell–ECM, and cell–soluble factor interactions.

Electrospinning Technology for Bionanofabrication

Jet-based technologies are increasingly being explored as potential high-throughput and high-resolution methods for the manipulation of biological materials. Previously shown to be of use in generating scaffolds from biocompatible materials, electrospinning technology has been used to deposit active biological threads and scaffolds comprised of living cells (Townsend-Nicholson and Jayasinghe 2006). This has been achieved by use of a coaxial needle arrangement where a concentrated living biosuspension flows through the inner needle and a medical-grade poly(dimethylsiloxane) medium with high viscosity and low electrical conductivity flows through the outer needle. Cells cultured after electrospinning were shown to be viable with no evidence of having incurred any cellular damage during the bionanofabrication process. This demonstrates the feasibility of using coaxial electrospinning technology for biological and biomedical applications requiring the deposition of living cells as composite nanothreads for forming active biological scaffolds. The process could enable significant advances to be made in technologies ranging from tissue engineering to regenerative medicine. Perhaps in the future such living nanothreads might be spun directly into wounds.

Nanomaterials for Combining Tissue Engineering and Drug Delivery

A variety of organic and inorganic nanostructures have been developed for scaffolds in tissue regeneration as well as drug delivery. These nanostructures provide favorable biological integration of implants and have applications in many areas, including orthopedics, cardiovascular medicine, and ophthalmology. Additionally, these nanostructures are capable of delivering drugs in a localized and controlled manner, accounting for the short biological half-life, lack of long-term stability and tissue selectivity, and potential toxicity of many therapeutic compounds.

Elastin-like polypeptides (ELPs) are artificial polypeptides, derived from Val-Pro-Gly-Xaa-Gly (VPGXG) pentapeptide repeats found in human tropoelastin. The potential of ELPs to self-assemble into nanostructures in response to environmental triggers is another interesting feature of these polypeptides that promises to lead to a host of new applications (Chilkoti et al 2006). Genetically encodable ELPs are monodisperse, stimuli responsive, and biocompatible, properties that make them attractive for combining drug delivery and tissue engineering.

In sophisticated tissue-engineering strategies, the biodegradable scaffold is preferred to serve as both a 3D substrate and a growth factor delivery vehicle to promote cellular activity and enhance tissue neogenesis. A novel approach has been described for fabrication of tissue-engineering scaffolds capable of controlled growth factor delivery whereby growth factor containing microspheres are incorporated into 3D scaffolds with good mechanical properties, well-interconnected macroporous and nanofibrous structures (Wei et al 2006). The microspheres were uniformly

distributed throughout the nanofibrous scaffold and their incorporation did not interfere the macro-, micro-, and nanostructures of the scaffold. The release kinetics of platelet-derived growth factor-BB (PDGF-BB) from microspheres and scaffolds was investigated using poly(lactic-co-glycolic acid) (PLGA50) microspheres. Incorporation of microspheres into scaffolds significantly reduced the initial burst release. Sustained release from several days to months was achieved through different microspheres in scaffolds. Released PDGF-BB was demonstrated to possess biological activity as evidenced by stimulation of human gingival fibroblast DNA synthesis in vitro. The successful generation of 3D nanofibrous scaffold incorporating controlled-release factors indicates significant potential for more complex tissue regeneration.

Nanobiotechnology for Organ Replacement and Assisted Function

Nanobiotechnology techniques are very relevant for the development of artificial tissues and organs. The process of exocytosis using liposomes and nanotubes has been cleverly characterized (Cans et al 2003). In an effort to understand and reproduce neuronal transmission, this group has effectively simulated natural physiology using a system composed of liposome–nanotube networks controlled by electroinjection. A small vesicle is introduced into a liposome that is connected to a nanotube, which together resembles a neurotransmitter vesicle and elongated fusion pore and forms the liposome–nanotube network. The system can be further controlled to investigate individual phases of this process.

Several devices are used to repair, replace, or assist the function of damaged organs such as kidneys. The technologies range from those for tissue repair to those for device to take over or assist the function of the damaged organs. The following sections include some examples of these applications.

Exosomes for Drug-Free Organ Transplants

Exosomes are nanovesicles shed by dendritic cells. They may hold the key to achieving transplant tolerance, i.e., the long-term acceptance of transplanted organs without the need for drugs (Morelli et al 2004). Exosomes are no larger than 65–100 nm; yet each contains a potent reserve of major histocompatibility complex (MHC) molecules - gene products that cells use to determine self from nonself. Millions of exosomes scurry about within the bloodstream, and while their function has been somewhat of a mystery, researchers are beginning to surmise that they play an important role in immune regulation and response.

Because certain dendritic cells have tolerance-enhancing qualities, several approaches under study involve giving recipients donor dendritic cells that have been modified in some way. The idea is that the modified donor cells would convince

recipient cells that a transplanted organ from the same donor is not foreign. MHC-rich vesicles, siphoned from donor dendritic cells, are captured by recipient dendritic cells and processed in a manner important for cell-surface recognition. Thus one can efficiently deliver donor antigen using the exosomes as a magic bullet. The exosomes are caught by the dendritic cells of the spleen, the site where dendritic cells typically present antigens as bounty to T cells. However, these dendritic cells internalize the exosomes instead of displaying them to T cells despite the exosomes' rich endowment of donor MHC molecules. Once internalized, the exosomes are ushered inside larger vesicles, special endosomes called MHC-II enriched compartments, where they are processed with the dendritic cell's own MHC molecules. This hybrid MHC-II molecule, now loaded with a peptide of donor MHC, is then expressed on the cell's surface. As one family of MHC molecules, MHC-II serves as a beacon for a specific population of T cells called CD4+ T cells. Such cells are activated during chronic rejection in a process associated with the indirect pathway of immune recognition.

This finding is significant because current immunosuppression therapies used in the clinical setting are not able to efficiently prevent T-cell activation via the indirect pathway. Perhaps the CD4+ T cells normally involved in this pathway would retreat from attack if they encountered a cell-surface marker that is of both donor and recipient origin, such as that observed following the dendritic cell's internalization of the donor-derived exosomes. The process of internalizing the donor exosomes does not affect maturation of the dendritic cell. Only immature dendritic cells can capture antigens efficiently and are believed to participate in the induction of transplant tolerance. By contrast, once mature, dendritic cells are capable of triggering the T-cell activation that leads to transplant rejection. Additional research will be required to determine whether donor-derived exosomes will enhance the likelihood that an organ transplant from the same donor will be accepted. Only a few research groups are engaged in active study of exosomes with most of the research taking place in Europe.

Nanobiotechnology and Organ-Assisting Devices

Organ-assisting devices (OAD) is an emerging area for application of nanobiotechnology. This includes implants and other devices to assist or replace the impaired function of various organs. One example of this is restoration of function of the tympanic membrane of the ear by magnetically responsive nanoparticles. Other examples would be given in the later sections of this chapter.

Superparamagnetic iron oxide nanoparticles (SNP) composed of magnetite (Fe(3)O(4)) were studied preliminarily as vehicles for therapeutic molecule delivery to the inner ear and as a middle ear implant capable of producing biomechanically relevant forces for auditory function (Kopke et al 2006). Magnetite SNP were synthesized, then encapsulated in either silica or poly(D,L-lactide-co-glycolide) or obtained commercially with coatings of oleic acid or dextran.

Permanent magnetic fields generated forces sufficient to pull them across tissue in several round window membrane models (in vitro cell culture, in vivo rat and guinea pig, and human temporal bone) or to embed them in middle ear epithelia. Biocompatibility was investigated by light and electron microscopy, cell culture kinetics, and hair cell survival in organotypic cell culture and no measurable toxicity was found. A sinusoidal magnetic field applied to guinea pigs with SNP implanted in the middle ear resulted in displacements of the middle ear comparable to 90 dB SPL.

Nanotechnology-Based Human Nephron Filter for Renal Failure

Approximately 1 million patients worldwide suffer from end-stage renal disease and require treatment through dialysis or transplantation. The number is expected to more than double by 2010, placing considerable stress on healthcare systems throughout the world. Despite the availability of various forms of renal replacement therapy for nearly four decades, mortality and morbidity is high and patients often have a poor quality of life.

A human nephron filter (HNF) development could eventually enable a continuously functioning, portable or implantable artificial kidney (Nissenson et al 2005). The HNF is the first application in developing a renal replacement therapy to potentially eliminate the need for dialysis or kidney transplantation in end-stage renal disease patients. The HNF utilizes a unique membrane system created through applied nanotechnology. The ideal renal replacement device should mimic the function of natural kidneys, continuously operating, and should be adjustable to individual patient needs. No dialysis solution would be used in this device. Operating 12 h a day, 7 days a week, the filtration rate of the HNF is double that of conventional hemodialysis administered three times a week. The HNF system, by eliminating dialysate and utilizing a novel membrane system, represents a breakthrough in renal replacement therapy based on the functioning of native kidneys. The enhanced solute removal and wearable design should substantially improve patient outcomes and quality of life. Animal studies using this technology are scheduled.

Blood-Compatible Membranes for Renal Dialysis

Scientists at the Rensselaer Polytechnic Institute (Troy, NY) have fabricated a novel heparin- and cellulose-based biocomposite membrane with nanopores by exploiting the enhanced dissolution of polysaccharides in room temperature ionic liquids (Murugesan et al 2006a). Using this approach, it is possible to fabricate the biomaterials in any form, such as films or membranes, nanofibers, nanospheres, or any shape using templates. Surface morphological studies on this biocomposite film showed the uniformly distributed presence of heparin throughout the cellulose matrix. Activated partial thromboplastin time and thromboelastography demonstrate that this

composite is superior to other existing heparinized biomaterials in preventing clot formation in human blood plasma and in human whole blood. Membranes made of these composites allow the passage of urea while retaining albumin, representing a promising blood-compatible biomaterial for renal dialysis, with a possibility of eliminating the systemic administration of heparin to the patients undergoing renal dialysis.

EVM Technologies is developing a new ceramic filter that has the potential to make kidney dialysis much more efficient and to reduce by 30 min–1 h the time required for a dialysis treatment. Specifically, the new filter promises to double the amount of toxins removed during dialysis and to double the glomulellar filtration rate (GFR) or rate of toxin removal. GFR is 100% in a normal person but only 15% at best for a dialysis patient, a rate that has changed little in the past 30 years. The ceramic filter's secret lies in its pores, which are organized in orderly rows and columns and which measure mere nanometers in diameter. These nanopores correspond more closely to the nanosized toxins in the blood than do the larger pores of the standard dialysis filter.

Chapter 14
Miscellaneous Applications

Nanodermatology

Nanotechnology-Based Products for Skin Disorders

Nanoparticles for Improving Targeted Topical Therapy of Skin

Long-term topical glucocorticoid treatment can induce skin atrophy by the inhibition of fibroblasts. Therefore, investigators have looked for the newly developed drug carriers that may contribute to a reduction of this risk by an epidermal targeting. Prednicarbate (PC, 0.25%) was incorporated into solid lipid nanoparticles of various compositions and studies were conducted where conventional PC cream of 0.25% and ointment served as reference (Santos Maia et al 2002). Local tolerability as well as drug penetration and metabolism were studied in excised human skin and reconstructed epidermis. With the latter drug recovery from the acceptor medium was about 2% of the applied amount following PC cream and ointment but 6.65% following nanoparticle dispersion. Moreover, PC incorporation into nanoparticles appeared to induce a localizing effect in the epidermal layer which was pronounced at 6 h and declined later. Dilution of the PC-loaded nanoparticle preparation with cream did not reduce the targeting effect while adding drug-free nanoparticles to PC cream did not induce PC targeting. Therefore, the targeting effect is closely related to the PC-nanoparticles and not a result of either the specific lipid or PC adsorbance to the surface of the formerly drug-free nanoparticles. Lipid nanoparticle-induced epidermal targeting may increase the benefit/risk ratio of topical therapy.

Topical Nanocreams for Inflammatory Disorders of the Skin

Inflammatory skin diseases, including atopic dermatitis and psoriasis, are common. The current treatment is unsatisfactory although several topical and systemic therapies, including steroids and immunomodulators, are available. The efficacy is not durable and they are associated with adverse effects. Efforts continue to develop safer alternative treatment for these disorders.

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Nanocrystalline silver has been demonstrated to have exceptional antimicrobial properties and successfully used in wound healing. Studies conducted by Nucryst Pharmaceuticals have revealed that topical application of nanocrystalline silver cream (0.5 and 1%) ointment produced significant suppressive effects on allergic contact dermatitis in a guinea pig model. There was a clear concentration–response relationship to the decrease of inflammation as lower concentrations were not effective. The effects were equivalent to the immunosuppressant tacrolimus ointment. This study suggests that nanocrystalline silver cream has therapeutic potential for treating inflammatory skin diseases.

Nanoparticle-Based Sunscreens

Zinc oxide offers the best broad-spectrum protection from the sun. Unlike titanium dioxide, another commonly used inorganic sunscreen, zinc oxide offers protection from both UVB and the more harmful UVA rays. In spite of the advantage of zinc oxide as a natural UV filter, its marketability was lacking because of its whiteness. This has been overcome with the use of nanoparticle technology to create an invisible screen. ZinScreen (Advanced Powder Technologies, Perth, Western Australia) is such a product. It has been marketed successfully in Australia since 2003.

NanoGard® zinc oxide (Nanophase Technologies Corporation), which is produced under cGMP conditions and is FDA approved for use as an active ingredient in personal care products, can also provide effective UV attenuation. The application of solid lipid nanoparticles as physical sunscreens and as active carriers for molecular sunscreens has been investigated. The amount of molecular sunscreen could be decreased by 50% while maintaining the protection level compared to a conventional emulsion (Wissing and Muller 2003).

OPTISOL™ (Oxonica Ltd) is a photostable UV absorber with enhanced action that has applications in skin-care products and other materials. The OPTISOL technology works by absorbing UVA radiation without the concurrent formation of free radicals. Furthermore, while providing balanced UVA and UVB protection, OPTISOL also provides additional benefits in absorbing free radicals that may be generated by other components of the sunscreen formulation and also enhances formulation stability. OPTISOL is based on ultrafine titanium dioxide with the inclusion in the crystal of a small amount ($<1\%$) of manganese. This causes a reconfiguration of the crystal's internal electronic structure that allows absorbed UV energy to be dissipated, virtually eliminating the generation of free radicals (Wakefield et al 2004). Secondly, manganese near the crystal surface can catalyze free radicals that have been generated by other sunscreen components into harmless chemical species. A reduction in free radical load has benefits for both the skin and the sunscreen formulation. Free radicals are implicated in photoaging of the skin, photocarcinogenesis, and organic component degradation. OPTISOL is currently under evaluation by a number of leading global sunscreen formulators. The first products containing OPTISOL are expected to be in the market early in 2006.

Cubosomes for Treating Skin Disorders of Premature Infants

Procter & Gamble is exploring the manufacture of cubosomes to create new treatments for skin disorders of premature infants. The cubosomes permit a "breathing layer" for skin at the nano level, which is due to their bicontinuous structure of oil and water interweaved together but never crossing each other. Unlike vaseline, which forms a protective barrier layer over the skin, cubosomes can protect skin from outside elements and at the same time let the skin "breathe" and exchange moisture with its environment. P & G, in collaboration with scientists at the Skin Science Institute of the University Children's Hospital (Cincinnati, OH), is developing an "artificial vernix"—cubosome-based outer protective layer that will help premature babies born without a fully developed outer skin layer. P & G is trying to find a way to make large-scale manufacture of cubosomes more efficient. The only way known to manufacture cubosomes initially was to use very high energy processes like ultrasound to fragment bulk cubic phase into cubosomes for use in cosmetic products for the skin. With a change in focus to applications in pediatric dermatology, P & G handed over their IP, patents, and research notes to University of Cincinnati in 2002. Less than 2 years later, the university's medical researchers filed for a patent for their cubosome-based "artificial vernix," putting forth the idea of blending man-made and natural structures to protect babies with undeveloped skin layers.

Nanopulmonology

Nanobiotechnology is expected to find potential applications in the treatment of pulmonary disorders. Currently the most important use appears to be in the delivery of therapeutics to the lungs. Nanoparticle-based diagnostics and imaging will also be useful for assessment of pulmonary diseases.

Nanoparticles for Drug Delivery to the Lungs

It is generally accepted in the field of pulmonary delivery that particles must be in the range of 1–3 μm to be delivered effectively to the deep lung. Larger particles have too much energy and hit the walls of the branching upper airways. Smaller particles do not have enough energy and tend to drift and adhere to the walls of the upper airways. However, as particle size is reduced further still, into the nanometer range, an increase in deep lung deposition is seen.

The suitability of nanoparticles, synthesized from porcine gelatin, human serum albumin, and polyalkylcyanoacrylate, as drug and gene carriers for pulmonary application was investigated in vitro on primary airway epithelium cells and the cell line 16HBE14o (Brzoska et al 2004). Confocal laser scan microscopy and flow cytometry experiments showed that the nanoparticles were incorporated into bronchial

epithelial cells provoking little or no cytotoxicity and no inflammation as measured by IL-8 release. Based on their low cytotoxicity and the lack of inflammatory reaction in combination with an efficient uptake in human bronchial epithelial cells, protein-based nanoparticles are suitable drug and gene carriers for pulmonary applications.

Nanoparticle Drug Formulations for Spray Inhalation

Scientists at the Welsh School of Pharmacy of Cardiff University (Cardiff, UK) are working on new nanoparticle drug formulations to improve the effectiveness of drugs such as insulin taken through spray inhalers. Drugs delivered through inhalers are usually either in a suspension (as particles dispersed in liquid) or in a solution (when the drug is dissolved in the liquid). However, there are problems with both methods: a suspension can lead to sediment in the inhaler and less of the drug reaching the target area of the lung, while solutions present problems in dissolving the drug in the inhaler propellant liquid and can make the drug itself less stable. The Cardiff team's approach is to prepare the drug in nanoparticle form, ensuring the correct dosage reaches the lung and the drug retains its stability, and providing the possibility of slowing the release of the drug in the lung for longer therapeutic effect. This could lead to the possibility of more drugs being administered effectively by inhaler, rather than by tablet or injection. Meanwhile, the team is also developing a process which uses a naturally occurring substance to enhance the absorption of insulin. Initial studies suggest insulin is absorbed three to four times more effectively by this process. The Pulmonary Research Group at the university aims to combine the two innovations to prolong and maximize the absorption effect. Patients suffering from conditions as diverse as asthma and diabetes could benefit from this research.

In Vivo Lung Gene Transfer Using Compacted DNA Nanoparticles

Scientists at Copernicus Therapeutics Inc have shown that nanoparticles consisting of single molecules of DNA condensed with PEG-substituted lysine 30-mers efficiently transfect lung epithelium following intrapulmonary administration (Fink et al 2006). Nanoparticles formulated with lysine polymers having different counterions at the time of DNA mixing have distinct geometric shapes: trifluoroacetate or acetate counterions produce ellipsoids or rods, respectively. Based on intracytoplasmic microinjection studies, nanoparticle ellipsoids having a minimum diameter less than the 25-nm nuclear membrane pore efficiently transfect nondividing cells. This 25-nm size restriction corresponds to a 5.8-kbp plasmid when compacted into spheroids, whereas the 8–11 nm diameter of rod-like particles is smaller than the nuclear pore diameter. In mice, up to 50% of lung cells are transfected after dosing with a rod-like compacted expression plasmid, and correction of the CFTR chloride

channel was observed in humans following intranasal administration. To further investigate the potential size and shape limitations of DNA nanoparticles for in vivo lung delivery, reporter gene activity of ellipsoidal and rod-like compacted luciferase plasmids ranging in size between 5.3 and 20.2 kbp was investigated. Equivalent molar reporter gene activities were observed for each formulation, indicating that microinjection size limitations do not apply to the in vivo gene transfer setting. This technique has potential applications in gene therapy of cystic fibrosis.

Nanomedical Aspects of Oxidative Stress

Free radical reactions involving reactive oxygen species (ROS) and reactive nitrogen species (RNS) contribute to the pathogenesis and progression of several human diseases. Antioxidants, such as vitamins C and E, 21-aminosteroids, and other free radical scavengers, have met with only limited success in clinical applications. This is partly due to our inability to design efficient antioxidants with site-directed, controlled activity. Nanotechnology has provided dramatic improvement in controlling or eliminating oxidation reactions in materials applications, which may provide a new basis for pharmacological treatment of diseases related to oxidative stress.

Nanoparticle Antioxidants

Nanotechnology has made significant advances in the reduction of free radical damage in the field of materials science. Cross-disciplinary interactions and the application of this technology to biological systems has led to the elucidation of novel nanoparticle antioxidants. Three of the most studied nanoparticle redox reagents at the cellular level are rare earth oxide nanoparticles (particularly cerium), fullerenes, and carbon nanotubes.

Antioxidant Nanoparticles for Treating Diseases Due to Oxidative Stress

The prospects for the use of nanoparticles for free radical scavenging in diseases due to oxidative stress are promising. However, further studies in animals and clinical trials will be needed to ascertain this beneficial effect. Other nanoparticles such as fullerenes also show biological antioxidant activity and potent neuroprotective effects, which need to be investigated. Some studies indicate that there may be an optimal level of free radical scavenging above which antioxidant nanoparticles may interfere with the beneficial roles of free radicals within the cell and have harmful effects. This is important for establishing the safety and proper doses of antioxidant nanomedicines.

Nanogeriatrics

The human life expectancy has nearly doubled during the last century. Aging is not a disease but certain diseases are associated with aging. The incidence of these diseases, many of which are incurable at the present state of knowledge, has spurred research activity. Geriatrics is the branch of medicine dealing with disorders of aging, and application of nanobiotechnology to this could be termed nanogeriatrics. In the academic sector there is an increase in the research activity to unravel the biology of ageing and several companies are developing products for managing disorders associated with aging. Several factors play a role in aging process. These include mitochondria and telomeres.

Telomeres are proteins that function like caps on the ends of chromosomes and ensure successful DNA replication when a cell divides. However, every time a cell divides, the telomeres shorten and eventually become exhausted. In general, aging cells become progressively less able to form and maintain tissue. This dysfunction plays a key role in a variety of presently incurable age-associated diseases such as macular degeneration, arteriosclerosis, atherosclerosis, osteoporosis, skin atrophy, progeria, and others.

Synthetic DNA nanocircles (Telomolecular Corporation), consisting of multiple complements of telomere repeat sequences, can be used to elongate telomeres and thereby rejuvenate tissues. They can be used as templates for polymerases to make or to extend new telomere sequences on an existing DNA or RNA molecule. They can be used to extend the lifespan of normal cell populations and can also induce apoptosis and death in cancerous cells. Therapeutic applications such as the treatment of macular degeneration, effects of skin aging, liver degeneration, and cancer are under investigation. Telomere-encoding nanocircles are easier to produce and store than telomerase enzyme. Because they are much smaller than plasmids, they can be readily synthesized in large quantities and in any sequence using a DNA synthesizer. Because they can be constructed to contain only telomere sequence, they can uniquely catalyze the extension of purely natural telomeric sequences. In addition, they can be designed to add nonnatural sequences to a telomere or to a telomere sequence primer. They can be used to extend the length of telomeres on natural or artificial chromosomes. PLGA nanoparticles (Telomolecular Corporation), loaded with vTRT (telomerase reverse transcriptase) or nanocircles, can be used to treat age-associated diseases. By combining DNA nanocircles and oTRT with its rights in the field of large-molecule delivery, Telomolecular believes it will be possible to develop breakthrough pharmaceutical products, and is working presently on the regeneration of human tissues in vivo in age-related disorders.

Nanoimmunology

Allergic and immune disorders are leading cause illness. Although various treatments have been developed to control allergy, no cure has yet been found. Nanobiotechnology is now being applied to tackle allergic and immune

disorders to advance the emerging field of medicine known as nanoimmunology. The immune system can protect as well as cause harm, so there is need to help manage the harmful effects.

Mast cells are responsible for causing allergic response and are stuffed with granules containing histamine. They are present in nearly all tissues except blood. When mast cells are triggered, inflammatory substances such as histamine, heparin, and a number of cytokines are quickly released into the tissues and blood, promoting an allergic response. Fullerenes (buckyballs) are able to interrupt the allergy/immune response by suppressing a fundamental process in the mast cells that leads to the release of histamine. Human mast cells and peripheral blood basophils exhibit a significant inhibition of IgE-dependent mediator release when preincubated with C60 fullerenes (Ryan et al 2007). Protein microarray demonstrated that inhibition of mediator release involves profound reductions in the activation of signaling molecules involved in mediator release and oxidative stress. Follow-up studies demonstrated that the tyrosine phosphorylation of Syk was dramatically inhibited in Ag-challenged cells first incubated with fullerenes. In addition, fullerene preincubation significantly inhibited IgE-induced elevation in cytoplasmic ROS levels. Furthermore, fullerenes prevented the in vivo release of histamine and drop in core body temperature in vivo using a mast cell-dependent model of anaphylaxis. These findings identify a new biological function for fullerenes and may represent a novel way to control mast cell-dependent diseases including asthma, inflammatory arthritis, heart disease, and multiple sclerosis.

Nanotechnology for Wound Healing

Several nanotechnology-based products have been used for wound care. Polyurethane membrane, produced via electrospinning (a process by which nanofibers can be produced by an electrostatically driven jet of polymer solution), is particularly useful as a wound dressing because of the following properties: it soaks fluid from the wound so that it does not build up under the covering and does not cause wound desiccation (Khil et al 2003). Water loss by evaporation is controlled, there is excellent oxygen permeability, and exogenous microorganism invasion inhibited because of the ultrafine pores size. Histological examination of the wound shows that the rate of epithelialization is increased and the dermis becomes well organized if wounds are covered with electrospun nanofibrous membrane. This membrane has potential applications for wound dressing.

In 2005, Uhru was awarded a US Army SIBIR grant relating to the development of a wound dressing using its hydrogel nanoparticle aggregate technology, which can be placed over burns to enhance the rate of wound closure. Such a dressing should be capable of absorbing wound fluids, require fewer changes of dressings, provide antibacterial action, deliver nutrient substances to the wound, and not require special storage conditions.

Nanotechnology-Based Management of Diabetes Mellitus

Nanosensors for Glucose Monitoring

One of the main reasons for developing in vivo glucose sensors is the detection of hypoglycemia in people with insulin-dependent (type 1) diabetes. It is possible to engineer fluorescent micro/nanoscale devices for glucose sensing. Deployment of nanoparticles in the dermis may allow transdermal monitoring of glucose changes in interstitial fluid. Using electrostatic self-assembly, an example of nanotechnology for fabrication, two types of sensors are being studied: (1) solid nanoparticles coated with fluorescent enzyme-containing thin films and (2) hollow nanocapsules containing fluorescent indicators and enzymes or glucose-binding proteins. Nanoengineering of the coated colloids and nanocapsules allows precision control over optical, mechanical, and catalytic properties to achieve sensitive response using a combination of polymers, fluorescent indicators, and glucose-specific proteins. Challenges to in vivo use include understanding of material toxicity and failure modes and determining methods to overcome fouling, protein inactivation, and material degradation. Noninvasive glucose sensing will maximize acceptance by patients and overcome biocompatibility problems of implants. Near infrared spectroscopy has been most investigated but the precision needs to be improved for eventual clinical application.

The nanotube-based optical biosensor could free people with diabetes from the daily pinprick tests now required for monitoring blood sugar concentrations. Carbon nanotubes are coated with glucose oxidase, an enzyme that breaks down glucose molecules. Then ferricyanide, an electron-hungry molecule, is sprinkled onto the nanotubes' surfaces. Ferricyanide draws electrons from the nanotubes, quenching their capacity to glow when excited by infrared light. When glucose is present, it reacts with the oxidase, producing hydrogen peroxide. In turn, the hydrogen peroxide reacts with ferricyanide in a way that reduces that molecule's hunger for electrons. The higher the glucose level, the greater the nanotube's infrared fluorescence.

A technique has been reported for micromechanical detection of biologically relevant glucose concentrations by immobilization of glucose oxidase (GOx) onto a microcantilever surface (Pei et al 2004). The enzyme-functionalized microcantilever undergoes bending due to a change in surface stress induced by the reaction between glucose in solution and the GOx immobilized on the cantilever surface.

Researchers at the University of Arkansas have fabricated and tested a novel biosensor that detects glucose close to real time and with much greater sensitivity than other comparable, biocompatible sensors (Xie et al 2007). The sensor is made of multiwalled carbon nanotubes, which are coated with platinum nanoparticles between 1 and 5 nm in diameter. The researchers tested sensors with and without the platinum nanoparticles and discovered that the carbon nanotubes with platinum exhibited a higher sensitivity than those without platinum. Tests revealed that for every square centimeter tested, a typical platinum-coated nanotube-based glucose sensor had a sensitivity of around 50 μamps/mmol. Microamps

refer to levels of electrical current. In this case, millimoles are units that describe molecular concentrations of glucose. The goal of researchers is to further increase the sensitivity value of 52.7 μamps/mmol. The biosensor has a response time of 15–30 s, which renders it capable of providing glucose screenings close to real time. The improved sensibility is attributed to various factors related to the application of platinum to the multiwalled nanotubes. Most importantly, the platinum nanoparticles create a larger electroactive surface area on the carbon nanotubes. The larger surface area enables the carbon nanotubes to act as a glucose oxidase reservoir, which helps to create uniform immobilization and high loading of glucose oxides for sensing. In addition, the platinum nanoparticles enhance electron transfer and facilitate better physical and chemical bonding between glucose oxides and carbon nanotubes.

Nanotechnology-Based Methods for Delivery of Antidiabetic Agents

The need for daily injections and blood glucose measurements are a problem in compliance among millions of patients with diabetes mellitus leading to serious and costly complications. Development of a glucose nanosensor that allows noninvasive monitoring of the blood glucose level is an important contribution to improve patient compliance.

The development and clinical introduction of better delivery methods, both for insulin and also for the newly developed incretin agents, is therefore needed. Nanotechnology should also improve on this by developing noninjectable forms of drug-carrying nanoparticles capable of feedback-modulated release of therapeutic agents to control glucose homeostasis according to the current physiological needs of the patient.

All-*trans* retinoic acid (atRA) is known as a member of retinoid, and the atRA nanoparticle coated with $CaCO_3$ (nanoegg-atRA) has been developed as a new drug delivery system. This nanoparticle stimulated insulin secretion from islets occurs in a glucose-dependent manner in streptozotocin-induced diabetes and shows not only the expression of PDX-1 (pancreatic duodenal homeobox1) but also the presence of β-cells in the islet of Langerhans. (Yamaguchi and Igarashi 2006). These data indicate that nanoegg-atRA might contribute to the regeneration of β-cells in vivo and provide useful information for future therapy of diabetes mellitus.

Nanotechnology-Based Device for Insulin Delivery

One of the main aims of insulin therapy for diabetes is to appropriately mimic physiological insulin secretion levels and their correlation with glucose concentration in healthy individuals. A possible nanoscale device with channels and insulin monomers/dimers enclosed has been proposed that will sense the increase in glucose levels and release monomeric insulin through channels in the nanocapsule

(Koch et al 2006). Ideally, insulin dimers would be blocked from passage, which will provide physiologically relevant insulin monomers to bind to the insulin receptor. Upon return of glucose levels to basal levels, the channels will close and insulin passage is stopped. Developments in functionalized nanocontainers will enable glucose-sensitive receptacles to be engineered. Such devices could be used to provide new therapeutic approaches in insulin treatment.

Application of Nanotechnology to Pain Therapeutics

Nanotechnology offers the potential to address multiple, major unmet problems in the diagnosis, treatment, and symptom management of a large variety of diseases and conditions, including cancer. Nanobiotechnology will contribute to improvement of cancer pain therapeutics through facilitation of drug discovery for pain. A more immediate application is in facilitating drug delivery for pain. A transbuccal transmucosal system, Buccal Patch®, has been developed for the administration of remifentanil for the management of breakthrough cancer pain (Sprintz et al 2005). The nanochannel size of the device permits the diffusion of the drug from its reservoir to the target tissue at a consistent and controlled rate, minimizing the risk of overdosing the patient. Intravenous administration of ibuprofen lipid nanocapsules has an advantage as an analgesic over oral preparations as described in Chapter 4.

Nanodentistry

Nanodentistry will make possible the maintenance of comprehensive oral health by involving the use of nanomaterials, biotechnology (including tissue engineering), and, ultimately, dental nanorobotics. The first dental nanorobots could be constructed by the year 2015 and will enable precisely controlled oral analgesia, dental replacement therapy using autologous cell teeth, and rapid nanometer-scale precision restorative dentistry.

Bonding Materials

Nano-Bond Universal Bonding System (Pentron Clinical Technologies) is based on Hybrid Plastics' POSS® technology (Polyhedral Oligomeric Sil sesquioxanes). It results in strengthened resin while it infiltrates the etched surface and provides strong interface between the tooth and the restorative material. The system consists of a uniquely formulated self-etch primer and adhesive system that are said to work together for great bonding to dentin and cut enamel. The kit also contains a dual cure activator that promotes reliable bonding to self- and dual-cured materials.

The Nano-Bond system greatly alleviates the problem of postbonding sensitivity by keeping tubules occluded during the self-etching step.

Adper™ Single Bond Plus Adhesive (3M ESPE) is a high bond strength dental adhesive. The improved adhesive incorporates a nanofiller technology that contributes to higher dentin bond strength performance. Adper is ideal for bonding all classes of direct composite restorations, as well as root surface desensitization and porcelain veneers. The nanofiller particles in Adper are added in a manner that does not allow them to cluster together. The particles are stable and will not settle out of dispersion. Therefore, no shaking is needed prior to use.

Dental Caries

The conventional treatment of dental caries involves mechanical removal of the affected part and filling of the hole with a resin or metal alloy. However, this method is not suitable for small early cavities because a disproportionate amount of healthy tooth must be removed to make the alloy or resin hold in place. Scientists at FAP Dental Institute (Tokyo, Japan) have produced a dental paste of synthetic enamel that rapidly and seamlessly repairs early caries lesions by nanocrystalline growth, with minimal wastage of the natural enamel (Yamagishi et al 2005).

The application of surfactants as reverse micelles or microemulsions for the synthesis and self-assembly of nanoscale structures is one of the most widely adopted methods in nanotechnology. The resulting synthetic nanostructure assemblies sometimes have an ordered arrangement. These developments in nanotechnology have been used to mimic the natural biomineralization process to create dental enamel—the hardest tissue in the human body. This is the outermost layer of the teeth and consists of enamel prisms, highly organized microarchitectural units of nanorod-like calcium hydroxyapatite (HA) crystals arranged roughly parallel to each other. The hydroxyapatite nanorods surface was synthesized and modified with monolayers of surfactants to create specific surface characteristics, which enable the nanorods to self-assemble into an enamel prism-like structure at a water/air interface (Chen et al 2005b). The size of the synthetic hydroxyapatite nanorods can be controlled and nanorods similar in size to human enamel were synthesized. The prepared nanorod assemblies were examined using TEM and AFM and were shown to be comprised of enamel prism-like nanorod assemblies with a Ca/P ratio between 1.6 and 1.7. It is possible that an enamel-like composite would be available within a year and crowns suitable for repairing decayed teeth within about 4 years. Application of synthetic enamel will not be limited to filling cavities. It has potential for use in bone repair and bone augmentation.

New treatment opportunities based on nanobiotechnology may include detection of dental decay spots prior to formation of cavities and repair of these, improved nanomaterials for covering dental enamel, and continuous oral health maintenance through the use of mechanical dentifrobots.

Nanospheres for Dental Hypersensitivity

Dental hypersensitivity, a painful condition due to exposure of the dentine of the tooth, affects millions of people worldwide. The dentine contains tiny fluid-filled channels which radiate outwards from the nerve terminals at the center of the tooth. Heat or cold and some chemicals can cause the fluid in these channels to move in or out irritating the nerve endings and causing sharp pain. According to a presentation by scientists at the Institute of Physics of the Leed University, United Kingdom, at EMAG-NANO Conference on 2 September 2005, creating nanospheres of a hydroxyapatite, a ceramic material, could be a long-term solution or cure for sensitive teeth. Commercially available silica nanospheres are approximately 40 m in diameter. Nanospheres could help dentists fill the tiny holes in the teeth that make them incredibly sensitive. If these channels are fully or partially blocked, the flow can be reduced and the pain stopped or significantly reduced. The next stage of the research will be to synthesize nanospheres combining hydroxyapatite and fluorine, which would fill the holes and encourage remineralization at the same time and also provide a powerful repair tool for dentists.

Nanomaterials for Dental Filling

The standard composite resin filling, a natural-looking restoration, is the method of choice when appearance is an issue. A dentist creates the filling by mixing the pure liquid resin with a powder that contains coloring, reinforcement, and other materials, packing the resulting paste into the cavity, and illuminating the tooth with a light that causes the paste to polymerize and harden. For decay-fighting composite fillings, the problem arises from an additive that is included in the powder to provide a steady release of calcium and phosphate ions. These ions are essential to the long-term success of the filling because they not only strengthen the crystal structure of the tooth itself, but buffer it against the decay-causing acid produced by bacteria in the mouth. Yet the available ion-releasing compounds are structurally quite weak, to the point where they weaken the filling as a whole.

Scientists at the American Dental Association's Paffenbarger Research Center (Gaithersburg, MD) have shown that nanotechnology has the potential to produce tooth restorations that are both stronger and more effective at preventing secondary decay than any decay-fighting fillings available currently. The new spray-drying technique yields particles of several compounds, one of which being dicalcium phosphate anhydrous (DCPA), that are about 50 nm across, 20 times smaller than the 1-μm particles in a conventional DCPA powder. Because these nanoscale particles have a much higher surface-to-volume ratio, they are much more effective at releasing ions, which means that much less of the material is required to produce the same effect. That, in turn, leaves more room in the resin for reinforcing fibers that strengthen the final filling. To exploit that opportunity, the Paffenbarger researchers

have also developed nanoscale silica-fused fibers that produce a composite resin nearly twice as strong as the currently available commercial variety.

Nanobiotechnology and Nutrition

Nanotechnologies will have an impact on nutrition research in many ways. Nanodevices can be used for real-time optical intracellular sensing (Ross et al 2004). These technologies may be particularly useful in obtaining accurate spatial information and low-level detection of essential and nonessential bioactive food components (nutrients) and their metabolites and in enhancing the understanding of the impact of nutrient/metabolite and biomolecular interactions. Nanobiotechnology will have an impact on food production as well as improved nutrition. Nanotechnologies can provide many benefits as shown in Table 14.1.

Nanobiotechnology and Food Industry

A major challenge in food production lies in the translation of established technologies into food production for delivering the optimal health and sensory benefits such as taste and smell. The major company involved in this area is Nestle SA. The

Table 14.1 Applications of nanotechnologies in food and nutrition sciences

Food manufacture
Nanoparticles and nanocrystals of essential nutrients to improve bioavailability
Use of self-assembly in nature and materials on a nanoscale with bottom-up approach
Use of nanoparticles to increase material strength barriers
Product research and innovation
Development of new products based on nanoscience research of natural foods
Control of bioavailability
Products based on simulation of customer preferences based on taste and smell nanosensing
Study of molecular physiology and genomics of taste cells
Testing of food effects via biomarkers
Product marketing
Unique nanobarcodes on proprietary products
Shelf life indicators
Quality control and testing
Nanodiagnostics for food contaminants and microorganisms
Nanosensors for quality control of food
Nutrition
Development of personalized nutrition based on metabolic needs of individual
Development of foods based on personalized sensory needs of the individual
Development of nutraceuticals
Development of nutricosmetics

Source: Jain PharmaBiotech.

company believes that nutrition, consumers, and use of new technologies in food science will be key drivers for future product innovation.

One example is that of lycopene, the carotenoid that gives tomatoes and other fruits and vegetables their red color. Health benefits of lycopene are well recognized. Lycopene has been of particular interest recently as regards its role in prostate cancer. Lycopene from fresh and unprocessed tomatoes is poorly absorbed by humans. Absorption of lycopene is higher from processed foods such as tomato paste and tomato juice heated in oil. Nestle has developed a food-grade lycopene formulation that is bioavailable in humans. Called "lactolycopene," it is made by trapping lycopene with whey proteins. However, bioavailability of lycopene in lactolycopene is no higher than that of tomato paste. Lycopene crystallizes in aqueous solution and forms nanocrystals. Micelles provide a convenient, inexpensive, and nontoxic vehicle for dissolving and stabilizing lycopene in tissue culture media and then delivering it to cells growing in culture. Nestle is researching this area for improving bioavailability of lycopene.

BioDelivery Sciences International has applied nanotechnology to food processing to encochleate sensitive and easily degraded nutrients (β-carotene, antioxidants, and others) for addition to processed food and beverages. Nanoencochleation's all-natural process encochleates and preserves essential nutrients like antioxidants into a protected "shell" for high-temperature/pressure canning and bottling applications.

Role of Nanobiotechnology in Personalized Nutrition

Nutrition plays a crucial role in health as well as disease. With advances in molecular biology, there is a shift in focus from epidemiology and biochemistry to an understanding of how nutrients act at molecular level. Advances in genomics have led to recognition of the importance of genes in human nutrition. Genetic predisposition is an important factor in mortality linked to diet such as cardiovascular disease.

Technologies such as high-density microarrays enable the simultaneous study of the whole transcriptome relevant to nutrition. Advances in proteomic and metabolomic technologies will also enable the analysis of the whole system at proteomic and metabolomic levels as well. Introduction of nanotechnologies will further improve and enable practical personalization of nutrition.

Nanobiotechnology for Public Health

There will be an increasing emphasis on preventive medicine and public health. High-technology medicines will benefit a limited number of population and mostly in the developed countries. Measures to improve public health will have a much larger impact on the future healthcare for most of the people on this earth. One of the major problems in developing countries is sanitary water supply. Nanostructured

water-filtration membranes could solve a lot of the world's drinking water problems. These are basically filters with pores so small that they let some molecules pass through, e.g., water, while keeping out larger particles, such as bacteria. Currently, these membranes are made using different materials, but nano-based materials may be more effective and ultimately cheaper.

Role of Nanobiotechnology in Biodefense

Nanobiotechnology provides several devices for the diagnosis of agents used in biological warfare and bioterrorism. Because of its ability to create structures of nanoscale dimension with large aggregate particle surface area-to-volume ratios, nanotechnology offers new opportunities to treat drug poisonings. Some examples from experimental studies support this potential.

Nanosuspension Formulations for Treating Bioweapon-Mediated Diseases

Several concepts of targeting of nanosuspension dosage forms for treatment of bioweapon-mediated diseases have been developed at the Baxter Healthcare Corporation. Alterations of pharmacokinetic profiles of existing antibiotics can lead to enhanced efficacy with reduced side effects. This has been shown for a nanosuspension formulation of the antifungal agent itraconazole. Secondly, viral sanctuaries breed resistance and often include the brain and lymphatics. These may be targeted by loading nanoparticulate drug into macrophages which target these organs, increasing antiviral drug concentration in these typically inaccessible regions. Finally, a strategy for dendritic cell vaccines has been developed for use against bioweapons.

Use of Antidotes as Nanoparticulate Formulations

Currently antidotes are not available to treat many harmful, even life-threatening reactions. Drug removal differs from drug delivery in that some drugs are given in encapsulated form to prolong action whereas drug removal has to be accomplished rapidly. Removal agents must reduce the available drug concentration to below-the-toxicity threshold and they must be biocompatible.

In one study, emulsion-based nanoparticles with diameter of 118.4 nm extracted bupivacaine from the aqueous phase in a physiological salt solution and attenuated the drug's cardiotoxicity in guinea pig heart to a greater extent than did a macroemulsion with particle diameter of 432 nm (Morey et al 2004). Additionally, nanoparticles sequestered bupivacaine from the aqueous phase of human blood and merit further investigation in animal models of intoxication.

Researchers at University of Florida (Gainesville, FL) have used oil-filled capsules to absorb an antidepressant, amitriptyline, from rat heart cells. Amitriptyline overdose is used in attempted suicide and produces cardiac complications. The study was carried out with nanocapsules, each measuring 150–600 nm and consisting of polymer-based silica shell surrounded by a droplet of ethyl butyrate oil, which is used in pharmaceutical preparations (Underhill et al 2002).

Removal of Toxins from Blood

An important part of treatment of poisoning is removal of toxins circulating in the blood. An even more critical need is for removal of biological and radiological toxic material from the blood after exposure to bioterrorist attacks or biochemical warfare. The Argonne National Laboratory and the University of Chicago are collaborating to develop a magnetic nanoparticle-based technology that removes biological, radiological, and in some cases chemical toxins from blood. Their goal is to devise a portable system that is fast and thorough, using magnetic nanoparticles coated with antibodies or chemicals that complement toxins. Once injected into the patient, the particles latch onto toxins and are later removed when the blood is pumped through tubing into a magnetic separator. Cleansed blood is then returned into the body. The approach is particularly safe because the closed loop system never exposes blood to the outside environment. Regardless of the type of exposure, if the toxins are removed before they accumulate in tissues, then organs will not fail and the patients will survive. This method may not be effective in case of rapidly acting agents such as nerve gas where the time between exposure and death is a matter of minutes. However, some chemical and many biological and radiological agents need hours or even days to cause fatal damage, allowing a wide enough window for the particle treatment to be effective. Some requirements for the development of this technology are as follows:

- The particles, which start with magnetic cores in the 8–12 nm range, must be the right size to navigate within the body. If they are too small, they may pass out of the kidneys; and if they are too large they may get trapped.
- The particles also need to be biocompatible so the body accepts them
- They should be biodegradable in case some remain after treatment.

Several companies are already developing magnetic nanoparticles for medical applications. Their use of FDA-approved antibodies, reagents, and off-the-shelf medical components could remove some regulatory hurdles. That could pave the way for not only military and civilian defense applications but clinical treatments such as overdoses. However, it will take a couple of years for this development.

Blood Substitutes

Artificial Red Cells

The artificial mechanical red blood cell, called respirocyte, measures about 1 μm in diameter and just flows along the bloodstream (Freitas 1998). It is a spherical nanorobot made of 18 billion atoms. The respirocyte is equipped with a variety of chemical, thermal, and pressure sensors and an onboard nanocomputer. This device is intended to function as an artificial erythrocyte, duplicating the oxygen and carbon dioxide transport functions of red cells, mimicking the action of natural hemoglobin-filled red blood cells. It is expected to be capable of delivering 236 times more oxygen per unit volume than a natural red cell. Specially installed equipment enables this device to display many complex responses and behaviors. Additionally, it has been designed to draw power from abundant natural serum glucose supplies, and thus is capable of operating intelligently and virtually indefinitely, whilst red blood cells have a natural lifespan of 4 months.

Chapter 15
Ethical, Safety, and Regulatory Issues of Nanomedicine

Introduction

Nanomedicine is not a medical specialty but involves the use of nanobiotechnologies. As has happened with all new technologies, there are ethical, safety, and regulatory issues. Currently there are no FDA regulations in force regarding nanobiotechnology products. There are only a few products, which use nanomaterials. But as more nanobiotechnology-based products are developed, the FDA will probably look into it. Eventually regulations are anticipated in this industry. The development of pharmaceuticals and methods of drug delivery, however, will be regulated by the FDA like any biopharmaceutical product. Meanwhile there is a move to set standards for nanotechnology in general.

Ethical, Legal, and Social Implications of Nanomedicine

Nanotechnology's impact in the past was mostly in engineering, communications, electronics, and consumer products. This has been rather undramatic, producing tennis balls that leak less air and polymer bottles that give beer a longer shelf life. Now that nanobiotechnology is being applied to human medicine, there is awareness of the consequences, good or bad, that are still not well understood. Communication with the public is important at this stage. This will involve education of the public and consideration of their opinion. Legal aspects of nanobiotechnology in healthcare are discussed in a separate article (Jain and Jain 2006).

University of South Carolina Nanocenter (Columbia, SC) with 20 faculty members and 10 student researchers has been awarded $2.8 million in federal grants to research ethical, legal, and social questions nanotechnology might pose. The team includes researchers in medicine, law, journalism, engineering, chemistry, and biology. There is plan to add an expert on religion and spiritual life. The mission of this project is in part to imagine any possible outcome of nanotechnology and to study the implications. No idea is too far-fetched for these researchers.

K.K. Jain, *The Handbook of Nanomedicine*,

Nanoethics

Ethical aspects are important for all new technologies and nanobiotechnology is no exception. Although nanotechnology has not raised any new ethical issues, it is worthwhile to keep this consideration in mind while developing and applying nanobiotechnologies to medicine. Ethical, social, and legal issues arising from the application of nanotechnology to medicine have been reviewed recently (Resnik and Tinkle 2007).

In the ethical debate on nanotechnology (nanoethics) there has been a strong tendency to strongly focus on extremes, either the upside or the downside, with the result that ethical assessments tend to diverge radically. Many of the extreme views are based on simplified and outdated visions of a nanotechnology dominated by self-replicating assemblers and nanomachines. There is a need for development of more balanced and better-informed assessments (Gordijn 2005). Various pitfalls of nanoethics are (1) the restriction of ethics to prudence understood as rational risk management; (2) the reduction of ethics to cost/benefit analysis; and (3) the confusion of technique with technology and of human nature with the human condition (Dupuy 2007). Once these points have been clarified, it is possible to take up some philosophical and metaphysical questions about nanobiotechnologies.

On 26 September 2006, the Nanoethics Group (http://www.nanoethics.org/), located in Santa Barbara, CA, announced that its core members have been awarded two grants, totaling approximately $250,000, by the National Science Foundation to study ethical issues related to human enhancement and nanotechnology. The grants will fund collaborative research between Dartmouth College and Western Michigan University for the next 3 years. The role of nanotechnology in enhancement procedures is not clear. Enhancement may mean use of hormones or cosmetic surgeries or other procedures to enhance human performance (mental or physical) or appearance. The group is concerned that the accelerating pace of new technology may lead to some fantastic scenarios such as advanced cybernetic body parts and computers imbedded in the brains, which will raise ethical issues.

On 17 January 2007, the European Group of Ethics (EGE) issued a draft report that recognizes the potential of nanomedicine in terms of developing new diagnostics and therapies (http://ec.europa.eu/european_group_ethics/activities/index_en.htm). The group proposes that measures be established to verify the safety of nanomedical products and devices, and calls on the relevant authorities to carry out a proper assessment of the risks and safety of nanomedicine. EGE recommends that there should be an EU web site on ethics and nanomedicine, where citizens can find information and pose questions to researchers. Academic and public debates should be held on the issues raised by forthcoming developments in nanomedicine. The report also places a strong emphasis on the importance of carrying out more research into the ethical, legal, and social implications (ELSI) of nanomedicine. They recommend that up to 3% of the nanotech research budget be set aside for ELSI research. They also call on the EC to set up a dedicated European Network on Nanotechnology Ethics. This network would bring together experts from a range of fields, promote deeper understanding of the ethical issues arising from

nanotechnology and nanomedicine, promote education in these fields, and work to ensure that ethics become embedded in research practices in nanomedicine and nanotechnology. The group also suggests that the EC fund a study on the social effects on nanomedicine in developing countries. On the legal front, EGE does not believe that structures set up specifically to deal with nanomedicine are needed right now. However, they suggest monitoring existing regulatory systems to ensure they do address all nanomedical products.

Safety Concerns About Nanobiotechnology

The success of nanomaterials is due to their small size, which enables us to get them into parts of the body where usual inorganic materials cannot enter because of their large particle size. There is an enormous advantage in drug delivery systems or cancer therapeutics. Current research is trying to find simple ways to control the degree of a particle's toxicity. This control means that the particle will be toxic only under certain desirable circumstances, such as for curing cancer. This also raises questions about unintentional effects of such powerful agents on the human body. This, however, would not be an issue for the use of nanoparticles for in vitro diagnostics.

Effects of particles on human health have been studied by toxicologists previously. Effects of larger particles generated by wearing down of implants in the body and aerosolized particles of all sizes have been studied. However, there is little information on health impacts of very small, nanoengineered particles under 20 nm. The main concern will be about particles <50 nm, which can enter the cells. There are still many unanswered questions about their fate in the living body. Because of the huge diversity of materials used and the wide range in size of nanoparticles, these effects will vary a lot. It is conceivable that particular sizes of some materials may turn out to have toxic effects. At this stage, no categorical statement can be made about the safety of nanoparticles, i.e., one cannot say that nanoparticles are entirely safe or that they are dangerous. Further investigations will be needed.

Toxicity of Nanoparticles

The biological impacts of nanoparticles are dependent on size, chemical composition, surface structure, solubility, shape, and aggregation. These parameters can modify cellular uptake, protein binding, translocation from portal of entry to the target site, and the possibility of causing tissue injury. Effects of nanoparticles depend on the routes of exposure that include gastrointestinal tract, skin, lung, and systemic administration for diagnostic and therapeutic purposes. Nanoparticles interactions with cells, body fluids, and proteins play a role in their biological effects and ability to distribute throughout the body. Nanoparticle binding to proteins may generate complexes that are more mobile and can enter tissue sites that are normally

inaccessible. Accelerated protein denaturation or degradation on the nanoparticle surface may lead to functional and structural changes, including interference in enzyme function. Nanoparticles also encounter a number of defenses that can eliminate, sequester, or dissolve them.

Testing for Toxicity of Nanoparticles

A mouse spermatogonial stem cell line has been used as a model to assess nanotoxicity in the male germ line in vitro (Braydich-Stolle et al 2005). The effects of different types of nanoparticles on these cells was evaluated using light microscopy, cell proliferation, and standard cytotoxicity assays. The results demonstrated a concentration-dependent toxicity for all types of particles tested, while the corresponding soluble salts had no significant effect. Silver nanoparticles were the most toxic while MoO_3 nanoparticles were the least toxic. These results suggest that this cell line provides a valuable model to assess the cytotoxicity of nanoparticles in the germ line in vitro.

Experiments have been conducted to test silica, silica/iron oxide, and gold nanoparticles for their effects on the growth and activity of *E. coli* (Williams et al 2006). TEM and dynamic light scattering were used to characterize the morphology and quantify size distribution of the nanoparticles, respectively. TEM was also used to verify the interactions between composite iron oxide nanoparticles and *E. coli*. The results from DLS indicated that the inorganic nanoparticles formed small aggregates in the growth media. Growth studies measured the influence of the nanoparticles on cell proliferation at various concentrations, showing that the growth of *E. coli* in media containing the nanoparticles indicated no overt signs of toxicity. Although the in vitro study has its limitations, it does indicate the relative safety of the nanoparticles tested under certain conditions.

Quantum Dot Safety Issues

To increase the stability, quantum dots (QDs) are made from cadmium selenide (CdSe) and zinc sulfide for use as fluorescent labels. These QDs may release potentially toxic cadmium and zinc ions into cells. While cytotoxicity of bulk CdSe is well documented, CdSe QDs are generally cytocompatible, at least with some immortalized cell lines. Using primary hepatocytes as a liver model, scientists at the University of California (San Diego, CA) found that CdSe-core QDs were acutely toxic under certain conditions (Derfus et al 2004), although previous in vitro studies had not shown significant toxicity as the cell line used in these studies were not sensitive to heavy metals or exposed to short-time QD labeling. The authors found that the cytotoxicity of QDs was modulated by processing parameters during synthesis, exposure to ultraviolet light, and surface coatings. These data further suggest that cytotoxicity correlates with the liberation of free Cd^{2+} ions due to deterioration of the CdSe lattice. When appropriately coated, CdSe-core QDs can be rendered nontoxic and used to track cell migration and reorganization in vitro. These results provide information for design criteria for the use of QDs in vitro and especially in

vivo, where deterioration over time may occur. Capping QDs with ZnO effectively prevented Cd^{2+} formation upon exposure to air but not to ultraviolet radiation, and attempts have continued to find better coating materials.

To solve this problem, scientists at the US Department of Energy's Lawrence Berkeley National Laboratory have coated QDs with a protective layer of polyethylene glycol (PEG), which is a very nonreactive and stable compound that is used extensively by the pharmaceutical industry in drug formulation. This layer is designed to prevent the dots from leaking heavy metal ions into cells once they are inside. The tool used to test the safety of QDs is a gene chip packed with 18,400 probes of known human genes and it is a comprehensive method to measure the toxicity of nanoscale particles. This chip is designed to enable the researchers to expose the human genome QDs and determine the extent to which the compound forces the genes to express themselves abnormally.

A high-throughput gene expression test determined that specially coated QD fluorescent nanoprobes affect only 0.2% of the human genome, dispelling the concern that the mere presence of these potentially toxic sentinels disrupts a cell's function (Zhang et al 2006b). The number of genes affected is very small given the large dose of QDs used in the study, which is up to 1,000 times greater than the dose that would typically be used in human applications. Moreover, the affected genes are not related to heavy metal exposure, which would be the case if the cells had been exposed to cadmium or zinc ions. Because of their protective coating, QDs have minimal impact on cells; the only gene changes are in transporter proteins, which is expected because the dots have to be transported into and within the cell.

Gold Nanoparticle Toxicity

Toxicity has been observed at high concentrations using gold nanoparticles. Studies using 2-nm core gold nanoparticles have shown that cationic particles are moderately toxic, whereas anionic particles are quite nontoxic (Goodman et al 2004). Concentration-dependent lysis mediated by initial electrostatic binding was observed in dye release studies using lipid vesicles, providing the probable mechanism for observed toxicity with the cationic particles.

Safety of Carbon Nanotubes in the Body

In contrast to the use of nanoparticles, the use of carbon nanotubes (CNTs) in life sciences is more recent. Toxicity of multiwalled CNTs, carbon nanofibers, and carbon nanoparticles was tested in vitro on lung tumor cells and clearly showed that these materials are toxic while the hazardous effect was size-dependent (Magrez et al 2006). Moreover, cytotoxicity is enhanced when the surface of the particles is functionalized after an acid treatment.

Water-soluble, single-walled CNTs (SWCNTs) have been functionalized with the chelating molecule diethylentriaminepentaacetic acid (DTPA) and labeled with

indium (^{111}In) for imaging purposes (Singh et al 2006b). Intravenous administration of these functionalized SWCNTs (f-SWCNTs) followed by radioactivity tracing using gamma scintigraphy indicated that f-SWCNTs are not retained in any of the reticuloendothelial system organs (liver or spleen) and are rapidly cleared from systemic blood circulation through the renal excretion route. The observed rapid blood clearance and half-life (3 h) of f-SWCNTs has major implications for all potential clinical uses. Moreover, urine excretion studies using both f-SWCNT and functionalized multiwalled CNT followed by electron microscopy analysis of urine samples revealed that both types of nanotubes were excreted as intact nanotubes. The next steps for this research is to prolong the blood circulation of CNTs in order to give them enough time before excretion to get to a target tissue. The researchers will also consider pharmaceutical development of functionalized CNTs for drug delivery.

In one study, carbon nanoparticles including CNTs, except C60 fullerenes, stimulated platelet aggregation and accelerated the rate of vascular thrombosis in rat carotid arteries (Radomski et al 2005). All particles resulted in upregulation of GPIIb/IIIa in platelets. In contrast, particles differentially affected the release of platelet granules, as well as the activity of thromboxane-, ADP, matrix metalloproteinase- and protein kinase C-dependent pathways of aggregation. Furthermore, particle-induced aggregation was inhibited by prostacyclin and *S*-nitrosoglutathione, but not by aspirin. Thus, some carbon nanoparticles have the ability to activate platelets and enhance vascular thrombosis. These observations are of importance for the pharmacological use of carbon nanoparticles and support the safety of C60 fullerenes.

Manufactured SWCNT usually contain significant amounts of iron as impurities that may act as a catalyst for oxidative stress. Because macrophages are the primary responders to different particles that initiate and propagate inflammatory reactions and oxidative stress, interaction of SWCNT (0.23wt% of iron) with macrophages has been studied (Kagan et al 2006). Nonpurified SWCNT more effectively converted superoxide radicals generated by xanthine oxidase/xanthine into hydroxyl radicals as compared to purified SWCNT. Iron-rich SWCNT caused significant loss of intracellular low molecular weight thiols (GSH) and accumulation of lipid hydroperoxides in macrophages. Catalase was able to partially protect macrophages against SWCNT-induced elevation of biomarkers of oxidative stress (enhancement of lipid peroxidation and GSH depletion). Thus, the presence of iron in SWCNT may be important in determining redox-dependent responses of macrophages.

Fate of Nanoparticles in the Human Body

Following inhalation, ultrafine and fine particles can penetrate through the different tissue compartments of the lungs and eventually reach the capillaries and circulating cells or constituents, e.g., erythrocytes. These particles are then translocated by the circulation to other organs including the liver, spleen, kidneys, heart, and brain, where they may be deposited.

Smaller particles apparently circulate for much longer and in some cases can cross the blood–brain barrier (BBB) to lodge in the brain. They can leak out of capillaries and get into the fluids between cells. So they can go to places in the body that an average inorganic mineral cannot. Such effects may not be a concern in case of targeted delivery of nanoparticle-based therapy in cancer. The eventual decision to use nanoparticle-based therapy may depend on a risk-versus-benefit assessment.

Pulmonary Effects of Nanoparticles

Modern humans breathe in considerable numbers of nanoparticles on a daily basis in traffic fumes and even from cooking. Nanoparticles are used increasingly in industrial processes and have been hypothesized to be an important contributing factor in the toxicity and adverse health effects of particulate air pollution. Small size, a large surface area, and an ability to generate reactive oxygen species play a role in the ability of nanoparticles to induce lung injury. In some individuals they can trigger asthma by setting off an inflammatory response from the body's immune system. In one study, rats were instilled with fine and ultrafine carbon black and titanium dioxide (Renwick et al 2004). Ultrafine particles induced more polymorphonuclear recruitment, epithelial damage, and cytotoxicity than their fine counterparts, exposed at equal mass. Both ultrafine and fine particles significantly impaired the phagocytic ability of alveolar macrophages. Only ultrafine particle treatment significantly enhanced the sensitivity of alveolar macrophages to chemotact towards C5a. It was concluded that ultrafine particles of two very different materials induced inflammation and epithelial damage to a greater extent than their fine counterparts. In general, the effect of ultrafine carbon black was greater than ultrafine titanium dioxide, suggesting that there are differences in the likely harmfulness of different types of ultrafine particle. Epithelial injury and toxicity were associated with the development of inflammation after exposure to ultrafines. Increased sensitivity to a C5a chemotactic gradient could make the ultrafine exposed macrophages more likely to be retained in the lungs, so allowing dose to accumulate.

The experience of researchers at DuPont, who tested CNTs, was different (Warheit et al 2004). When the researchers injected nanotubes into the lungs of rats, the animals unexpectedly began gasping for breath and 15% of them quickly died. Yet surprisingly, all the surviving rats seemed completely normal within 24 h. What initially looked like disaster pointed to a possible safety feature: the nanotubes' tendency to clump rapidly led to suffocation for some rats exposed to huge doses, but it also kept most tubes from reaching deep regions of the lung where they could not be expelled by coughing and could cause long-term damage. Now researchers see the clumping of CNTs and other nanomaterials as a new field for inquiry. Other findings of Dupont scientists were as follows:

- Exposures to quartz particles produced significant increases versus controls in pulmonary inflammation, cytotoxicity, and lung cell parenchymal cell proliferation indices.

- Exposures to CNTs produced transient inflammatory and cell injury effects. They produced a nondose-dependent series of multifocal granulomas, which were evidence of a foreign tissue body reaction and were nonuniform in distribution and not progressive beyond 1 month following exposure.

In further studies, DuPont scientists observed that exposures to the various alpha-quartz particles produced differential degrees of pulmonary inflammation and cytotoxicity, which were not always consistent with particle size. The results of their studies demonstrate that the pulmonary toxicities of alpha-quartz particles appear to correlate better with surface activity than particle size and surface area (Warheit et al 2007).

It has been suggested that the nanoparticulate component of 10μM (PM10) is capable of translocating into the circulation with the potential for direct effects on the vasculature and is a potential risk factor for cardiovascular disease. A study was conducted in healthy volunteers to determine the extent to which inhaled technetium-99m (99mTc) labeled carbon nanoparticles (Technegas) were able to access the systemic circulation (Mills et al 2006). Technegas particles were 4–20 nm in diameter and aggregated to a median particle diameter of approximately 100 nm. Radioactivity was immediately detected in blood, with levels increasing over 60 min. Thin layer chromatography of whole blood identified a species that moved with the solvent front, corresponding to unbound 99mTc-pertechnetate, which was excreted in urine. There was no evidence of particle-bound 99mTc at the origin. Gamma camera images demonstrated high levels of Technegas retention in the lungs, with no accumulation of radioactivity detected over the liver or spleen. Thus the majority of 99mTc-labeled carbon nanoparticles remain within the lung up to 6 h after inhalation. In contrast to previously published studies, thin layer chromatography did not support the hypothesis that inhaled Technegas carbon nanoparticles pass directly from the lungs into the systemic circulation.

The physiological relevance of various findings should ultimately be determined by conducting an inhalation toxicity study. As yet no one has created a realistic test for the effects of inhaled nanoparticles; such a test could easily cost more than $1 million to design and carry out. Some studies have been planned to study any possible adverse effects of nanoparticles on the lungs. One of those studies is a $3-million multiyear project led by the US National Institute of Environmental Health Sciences that will examine the potential toxic and carcinogenic effects of inhalation exposure to nanomaterials.

Blood Compatibility of Nanoparticles

Given that the majority of nanoparticles are intended to travel to tumors through the bloodstream, the effects of nanoparticles on blood cells are of particular concern to those developing nanoparticle-based therapeutic and imaging agents. The blood compatibility of nanoparticles depends on the material used.

Carbon Nanoparticle-Induced Platelet Aggregation

To determine the potential for blood platelet–nanoparticle interactions, the effects of engineered and combustion-derived carbon nanoparticles were studied on human platelet aggregation in vitro and rat vascular thrombosis in vivo (Radomski et al 2005). Multiple-wall and single-wall nanotubes, C60 fullerenes, and mixed carbon nanoparticles were compared with standard urban particulate matter (average size 1.4 μm). Carbon particles, except C60 fullerenes, stimulated platelet aggregation and accelerated the rate of vascular thrombosis in rat carotid arteries. All particles resulted in upregulation of GPIIb/IIIa in platelets. The particle-induced aggregation was inhibited by prostacyclin and *S*-nitroso-glutathione, but not by aspirin. It is concluded that some carbon nanoparticles and microparticles have the ability to activate platelets and enhance vascular thrombosis. These observations are of importance for the pharmacological use of carbon nanoparticles and pathology of urban particulate matter.

Compatibility of Lipid-Based Nanoparticles with Blood and Blood Cells

Pegylated and nonpegylated cetyl alcohol/polysorbate nanoparticles (E78 NPs) are being tested as drug carriers for specific tumor and brain targeting. Because these nanoparticle formulations are designed for systemic administration, the compatibility of these lipid-based NPs with blood and blood cells was tested with a particular focus on hemolytic activity, platelet function, and blood coagulation (Koziara et al 2005). E78 NPs did not cause in vitro red blood cell lysis at concentrations up to 1 mg/ml. In addition, under conditions tested, E78 and polyethylene glycol (PEG)-coated E78 NPs (PEG-E78 NPs) did not activate platelets. In fact, both NP formulations very rapidly inhibited agonist-induced platelet activation and aggregation in a dose-dependent manner. It was concluded that PEG-coated and nonpegylated E78 NPs have potential blood compatibility at clinically relevant doses. Based on the calculated nanoparticle-to-platelet ratio, the concentration at which E78 NPs could potentially affect platelet function in vivo was approximately 1 mg/ml.

Transfer of Nanoparticles from Mother to Fetus

The toxicopathology research group at the University of Liverpool (Liverpool, United Kingdom) has investigated the fate of injected gold nanoparticles into pregnant rats to determine whether they can be transferred across the placenta to the fetus. The findings, reported in January 2004, show unidentified particles in the fetus but follow-up results have not been reported. This research on possible transfer of nanoparticles to the fetus could indicate a new, particular, hazard of nanoparticles that would be a cause for concern. Scientists at the Center for Biological and Environmental Nanotechnology of Rice University are of the opinion that it is still too early to establish the transfer of nanoparticles to the fetus. They believe that first

they have to establish whether nanoparticles can be accumulated in the body and then they can investigate chronic effect. This question of transplacental transfer of nanoparticles thus remains unanswered.

Cytotoxicity of Nanoparticles

Cytotoxicity refers to toxic effects on individual cells. In cytotoxicological studies, identical cell cultures are exposed to various forms and concentrations of toxins. In order to compare the toxicity of different compounds, scientists determine the concentration, measured in parts per million or parts per billion, of materials that lead to the death of 50% of the cells in a culture within 48 h.

A particle's surface chemistry may determine how it interacts with the tissues of the body. AFM was used to show that aqueous solutions of poly(amidoamine) dendrimers cause the formation of holes 15–40 nm in diameter in previously intact lipid bilayers (Mecke et al 2004). In contrast, carboxyl-terminated core–shell tectodendrimer clusters do not create holes in the lipid membrane but instead show a strong affinity to adsorb to the edges of existing bilayer defects. Multiwalled CNTs, not derivatized nor optimized for biological applications, are capable of both localizing within and initiating an irritation response in human epidermal keratinocytes, which may occur in the skin of workers as occupational exposure during manufacture of nanotubes (Monteiro-Riviere et al 2005).

Nanoparticle Deposits in the Brain

Passage of nanoparticles to cross the BBB to enter the brain has already been documented (see Chapter 6). There is a possible risk in inhaling nanoparticles that are so small that they can slip through membranes inside the lungs, enter systemic circulation, and lodge in the brain. Research on rats has shown nanoparticles deposited in the nose can migrate to the brain and move from the lungs into the bloodstream. They can also change shape as they move from liquid solutions to the air, making it harder to draw general conclusions about their potential impact on living things.

There is the potential for neurodegenerative consequence of nanoparticle entry to the brain. Histological evidence of neurodegeneration has been reported in both canine and human brains exposed to high ambient particulate matter levels, which may be caused by the oxidative stress pathway. Thus, oxidative stress due to nutrition, age, genetics among others may increase the susceptibility for neurodegenerative diseases (Peters et al 2006). More experiments are needed to establish the impact of nanoparticles on the brain if they remain there.

Fullerenes (buckyballs) are lipophilic and localize into lipid-rich regions such as cell membranes in vitro, and they are redox active. Other nanosize particles and soluble metals have been shown to selectively translocate into the brain via the olfactory bulb in mammals and fish. A preliminary study found rates of brain damage

17 times higher in largemouth bass exposed to a form of water-soluble buckyballs than unexposed fish (Oberdorster 2004). Significant lipid peroxidation was found in brains of largemouth bass after 48 h of exposure to 0.5 ppm uncoated nano-C60. Buckyballs are also toxic in vitro, causing 50% of the cultured human cells to die at a concentration of 20 ppb (Sayes et al 2004). With the addition of an antioxidant, L-ascorbic acid, the oxidative damage and resultant toxicity of nano-C60 was completely prevented (Sayes et al 2005).

Measures to Reduce Toxicity of Nanoparticles

Scientists at the Rice University's Center for Biological and Environmental Nanotechnology are able to significantly lower the toxicity level of buckyballs when exposed to liver and skin cells in a petri dish. They have accomplished this by attaching other molecules to the surface of buckyballs. This simple chemical modification could lower potential exposure risks during disposal of a product like a fuel cell or within a manufacturing plant. Removing attached molecules and enhancing toxicity could also be useful in chemotherapy treatments, for instance.

Water-soluble single-wall carbon nanotubes (SWCNTs) are significantly less toxic to begin with and can be rendered nontoxic with minor chemical modifications. SWNTs can be rendered soluble via the attachment of the chemical subgroups hydrogen sulfite, sodium sulfite, and carboxylic acid. The cytotoxicity of undecorated SWNTs is 200 ppb, which compares to the level of 20 ppb for undecorated buckyballs (Sayes et al 2006). The research is a continuation of pioneering efforts to both identify and mitigate potential nanotechnology risks. For medical applications, it is encouraging to see that the cytotoxicity of nanotubes is low and can be further reduced with simple chemical changes.

Despite the huge potential for a new generation of gold nanoparticle (AuNP)-based nanomedicinal products, nontoxic AuNP constructs and formulations that can be readily administered site-specifically through the intravenous mode, for diagnostic imaging by CT scan or for therapy via various modalities, are still rare. The use of gum arabic (GA) was explored as it has been used for a long time to stabilize foods such as yogurt and hamburgers. It has unique structural features, including a highly branched polysaccharide structure consisting of a complex mixture of potassium, calcium, and magnesium salts derived from arabic acid. GA can be used to absorb and assimilate metals and create a "coating" that makes gold nanoparticles stable and nontoxic. A study has described the synthesis and stabilization of AuNPs within the nontoxic phytochemical gum arabic matrix (GA-AuNPs) and has presented detailed in vitro analysis and in vivo pharmacokinetics studies of GA-AuNPs in pigs to gain insight into the organ-specific localization of this new generation of AuNP vector (Kattumuri et al 2007). X-ray CT contrast measurements of GA-AuNP vectors were carried out for potential application in molecular imaging. The results demonstrate that naturally occurring GA

can be used as a nontoxic phytochemical excipient in the production of readily administrable biocompatible AuNPs for diagnostic and therapeutic applications in nanomedicine.

Concluding Remarks on Safety Issues of Nanoparticles

There is no consensus on the real risks of nanomaterials. Risk evaluation presents challenges due to a lack of data, the complexity of nanomaterials, measurement difficulties, and undeveloped hazard assessment frameworks. There is a paucity of published material on this topic, which could provide scientific guidance; less than 400 journal articles on health risks of engineered nanomaterials have been published as of July 2006. Until the risk assessment is evaluated further, some precautionary measures should be considered to reduce risks, such as exposure control. It is recommended that manufacturers of nanomaterials should inventory all products and applications to potential exposures across the product life cycle. The risk of each application should be characterized based on exposure and available knowledge about hazard. The risk of exposure should be mitigated through additional testing and product redesign.

Research into Environmental Effects of Nanoparticles

Research strategies for safety evaluation of nanomaterials have been planned in the United States, Europe, and Japan. An important component of these programs is the development of reliable risk and safety evaluations for these materials to ensure their safety for human health and the environment. The scope of each of these programs includes efforts to assess the hazards posed by nanomaterials in realistic exposure conditions (Thomas et al 2006a). The University of Wisconsin-Madison's Nanotechnology in Society Project has published a summary of some of the key data gaps, uncertainties, and unknowns that need to be addressed to develop adequate risk assessments for nanomaterials and to take timely and appropriate public health precautions (Powell and Kanarek 2006).

Work at NanoSafety Laboratories Inc, UCLA

UCLA (University of California, Los Angeles, CA) has developed a new testing method that would help manufacturers monitor and test the safety and health risks of engineered nanomaterials (Nel et al 2006). UCLA is establishing NanoSafety Laboratories Inc in association with the California NanoSystems Institute at UCLA to help manufacturers assess the safety and risk profiles of engineered nanomaterials. The testing model developed at UCLA is based on toxicity testing for occupational and air pollution particles, which include nanoparticles. The strong scientific

foundation of air pollution particle testing is used to help understand the health impact of engineered nanoparticles and ensure safe manufacturing of nanoproducts. The impact of nanoparticle interactions with the body is dependent on their size, chemical composition, surface structure, solubility, shape, and how the individual nanoparticles amass together. Nanoparticles may modify the way cells behave and potential routes of exposure, include the gastrointestinal tract, skin, and lungs. The three key elements of the toxicity screening strategy include (1) the physical and chemical characterization of nanomaterials; (2) tissue cellular assays; and (3) animal studies.

A mature toxicological science has emerged from the study of these particles, providing a framework for a predictive testing strategy applicable to engineered nanomaterials. A predictive strategy is one in which a series of simple but high-quality tests can be employed to predict which materials could be hazardous, and therefore speed up the process of classifying materials into those that are safe and those that could pose toxicity problems. This type of approach is similar to that used by the National Toxicology Program for evaluation of chemical agents. The UCLA model predicts toxicity according to the ability of some nanoparticles to generate toxic oxygen radicals that can cause tissue injury, including inflammation and other toxic effects. For air pollution particles, this injury can translate into asthma and atherosclerotic heart disease. Using this model, the UCLA laboratory has developed a series of tests to assess nanoparticle toxicity in nonbiological environments as well as in tissue cultures and animal models. Funding for the research on air pollution particles that contributed to this paper came from the National Institute of Environmental Health Sciences and the US Environmental Protection Agency.

Center for Biological and Environmental Nanotechnology

Rice University's Center for Biological and Environmental Nanotechnology (CBEN) has played an active role in informing the public, lawmakers, and industry about potential unintended environmental consequences of nanotechnology. CBEN's research aims to understand how nanomaterials function in water-based environments such as living organisms and ecosystems. In its first 5 years since its founding in 2000, CBEN helped produce groundbreaking research in nanomedicine, nanobiotechnology, nanotoxicology, and nanoscale methods for environmental remediation. On 14 September 2006, the National Science Foundation extended funding for CBEN with a 5-year renewal worth $12 million. The renewal ensures CBEN programs will continue through 2011.

Further details of CBEN can be viewed at its web site (http://cben.rice.edu/). Projects that are addressing the interactions of nanoparticles with biological and environmental systems include:

- *activity of bionanoconjugates*, where the basic interactions of nanoparticles with specific biomolecules is under investigation;
- *biomedical applications of SWNT*, where the impacts of SWNTs on cells are being studied;

- *all of the Theme 2 projects*, where understanding the interactions of nanoparticles with cells, tissues, and whole organisms are vital to successful development of the technologies;
- *polymer flow on the nanoscale*, where nanoscale fluid dynamics, important for understanding how nanoparticles move in the environment, is being studied;
- *sorption of contaminants onto engineered nanomaterials*, where the impact of nanoparticles on the transport of other types of environmental contaminants is being investigated;
- *nanocell interactions*, where the cytotoxicology of nanoparticles is under direct investigation; and
- *environmental exposure routes*, where the transport of nanoparticles through groundwater systems is being studied.

On 19 October 2006, the International Council on Nanotechnology (ICON)—a coalition of academic, industrial, governmental, and civil society organizations administered by CBEN—issued a comprehensive review of existing efforts to develop "best practices" for handling nanomaterials in the workplace. The work was performed by researchers at the University of California (Santa Barbara, CA) as part of a two-phase project to catalogue how industry is managing the potential occupational safety risks posed by nanomaterials. The report can be found on ICON's web site: http://icon.rice.edu/.

The phase I report, "Current Knowledge and Practices Regarding Environmental Health and Safety in the Nanotechnology Workplace," offers a review and analysis of existing efforts to develop best practices. The report found that efforts to catalogue workplace practices have not systematically documented current environment, health, and safety practices in a variety of workplace settings and geographies. Moreover, it finds that some existing documents are not publicly available.

In the second phase of this project, the researchers interviewed a range of US and international firms to produce an international snapshot of workplace practices in nanotechnology industries. ICON plans to issue a report of those findings in November 2006.

Efforts by Nanotechnology Companies to Establish Safety of Nanoparticles

In addition to the academic and government-sponsored research on safety of nanoparticles, some nanotechnology companies are also contributing to this effort. On 7 March 2007, QuantumSphere, the manufacturer of nanometals and alloys markets demanding advanced materials, completed a baseline assessment and facility review on potential nanomaterial exposure in the workplace as part of a voluntary collaboration with the National Institute for Occupational Safety and Health (NIOSH). As part of this project, the NIOSH Nanotechnology Field Team was invited to visit QuantumSphere's manufacturing facility in Santa Ana, CA, to perform

a baseline assessment of potential workplace emissions and materials/chemical handling techniques. This visit was requested by QuantumSphere under the ongoing NIOSH research program to visit multiple nanomaterial-handling facilities in an effort to better understand and learn of the possible occupational safety and health implications, as well as general applications of nanotechnology. NIOSH recommended only one minor engineering control improvement consisting of the addition of a portable exhaust system to augment the efficiency of clean-in-place processes. Partnerships with manufacturer as QuantumSphere aim at generating the data required for a better understanding of the nature of nanomaterials and developing best practices for their safe use and effective handling. These data will be utilized as the basis for a two pronged approach to environmental health and safety excellence: eliminate the risk of material loss from operational processes through the voluntary use of OSHA's Process Safety Methodology and continuously identify and eliminate potential worker exposure opportunities by the application of appropriate engineering controls, administrative controls, and the use of appropriate personal protective equipment for process operations.

Public Perceptions of the Safety of Nanotechnology

Past and current experience in biotechnology has shown how environmentally concerned public react to new technologies. One can predict antinanotechnology groups in the future similar to the antibiotechnology groups. Apart from the unknown long-term effects of nanomaterials in the human body, a much greater concern is expressed about the environmental effects of release of nanoparticles from the industry. One of the questions that is being already asked is about the possibility of accumulation of nanomaterials in water or the earth and the risk if this takes place. Some of these issues are already under investigation. So far the public's outlook on nanotechnology remains positive despite a lack of knowledge, but press coverage and agitation from various groups indicate that nanotechnology industry will not be able to dodge these questions much longer. Instead of remaining silent on this issue, companies need a communications strategy to share their safety studies, collaborate with trusted partners, and explain the benefits nanotechnology can bring. The public relations departments of research institutes and companies involved in nanotechnology will have a major task of educating the public about the safety of nanotechnology.

As an example, it was anticipated that exploration of the human genome could result in public concerns - ethical, legal, and cultural. So 3–5% of federal research money was set aside to fund the study of these issues and to communicate with the public and encourage lots of openness and transparency. This is now the model for a proactive approach to new technology development.

The largest and most comprehensive survey of public perceptions of nanotechnology products finds that US consumers are willing to use specific nano-containing products—even if there are health and safety risks—when the potential benefits

are high (Currall et al 2006). The study, which was conducted by researchers at Rice University's Center for Biological and Environmental Nanotechnology (Houston, TX), University College London, and the London Business School, also finds that US consumers rate nanotechnology as less risky than everyday technologies like herbicides, chemical disinfectants, handguns, and food preservatives. The research was based on more than 5,500 survey responses. One survey polled consumers about how likely they would be to use four specific, nano-containing products: a drug, skin lotion, automobile tires, and refrigerator gas coolant. This is the first large-scale study to experimentally gauge the public's reaction to specific, nano-containing products, and the use of scenarios about plausible, specific products yielded results that challenge the assumption that the public focus narrowly on risk. The greater the potential benefits, the more risks people are willing to tolerate.

Evaluation of Consumer Exposure to Nanoscale Materials

Although there are numerous likely consumer advantages from products containing nanoscale materials, there is very little information available regarding consumer exposure to the nanoscale materials in these products or any associated risks from these exposures. The products include cosmetics, sunscreen, textiles, and sporting goods. An important component in addressing potential health risks is the potential exposure to the consumer. The presence of a toxic substance in a consumer product does not constitute a health hazard if the product design or use prevents the consumer from being exposed to the substance. For consumer product applications, if the nanomaterial is attached to the product in a manner that minimizes its release, the exposure potential will be minimal. If the nanomaterials are released in significant quantities during reasonably foreseeable product use or misuse, exposure may result via dermal contact, ingestion, or inhalation.

Toxic Substances Control Act gives the US Environmental Protection Agency (EPA) authority to regulate the manufacture, use, distribution in commerce, and disposal of chemical substances. This act authorizes the agency to regulate both new and existing compounds and is currently undergoing scrutiny by the agency to determine to what extent it can incorporate engineered nanomaterials (Thomas et al 2006b). FDA regulates very few materials but many types of products. Cosmetics do not require premarket approval from the FDA, but if the FDA considers that there is a safety concern resulting from the use of any cosmetic ingredient, including nanoparticles, then it has several options to prohibit the marketing and sale of those products. Sunscreens are considered to be cosmetics in Europe; in the United States they are considered to be drugs. Currently, the FDA is involved in studies of marketed sunscreens in collaboration with the National Toxicology Program, Rice University, and the National Institute for Standards and Technology. These studies will help identify those sunscreens that contain nanoscale particles of titanium dioxide and zinc oxide and characterize the size ranges for these nanoscale particles.

US Consumer Product Safety Commission (CPSC) is charged with protecting the public from unreasonable risks of serious injury or death from over 15,000 types of consumer products under the agency's jurisdiction. Nanotechnology-derived products entering commerce, containing materials with novel chemical, physical, biological, optical, and electronic properties, will require assessment to determine if there may be exposure to a potential health risk that might negatively impact consumer safety. The potential health risk of nanomaterials can be assessed with existing CPSC statutes, their administering regulations, and interpretative guidelines.

FDA Regulation of Nanobiotechnology Products

The FDA regulates a wide range of products, including foods, cosmetics, drugs, devices, and veterinary products, some of which may utilize nanotechnology or contain nanomaterials. The FDA has not established its own formal definition, though the agency participated in the development of the National Nanotechnology Initiative (NNI) definition of nanotechnology. Using that definition, nanotechnology relevant to the FDA might include research and technology development that both satisfies the NNI definition and relates to a product regulated by FDA.

The first generation of nanomedicines (liposomal preparations) were approved more than a decade ago before a real awareness existed about a number of issues related to safety concerns of nanomaterials and with a demonstrable relative success, in terms of their clinical safety assessment and safe use in cancer. However, nanomaterials such as phospholipids or biodegradable/bioerodible polymers are of a completely different nature from other anticipated materials that will be produced in the near future from the research pipeline. CNTs, quantum dots, and other nonbiodegradable and potentially harmful materials should be given different and more closer attention, looking at their toxicological potential impact in a number of different applications. By the same standards and in the new context, already existing nanopharmaceuticals, when administered for the same or new therapeutic indications making use of different administration routes (e.g., pulmonary), should not be waived of a full assessment of their differential potential toxicology impact, particularly in the proinflammatory area (Gaspar 2007).

The FDA approval is essential for clinical applications of new technologies, and substantial regulatory problems may be encountered in the approval of nanotechnology-based products. Previously approved products with particles in the nanosize range were not considered to be nanotechnology products and were subject to the same testing requirements as all other products reviewed by the agency. But some of the novel platforms being developed, such as the multifunctional dendrimers, may require a multifaceted approach toward their review and evaluation. There is every expectation that some novel products utilizing nanotechnology will be combination products (i.e., drug–device, drug–biologic, or device–biologic) and will likely undergo a relevant review process. In order to insure that nanotechnology products are regulated in a coordinated fashion across all product types, the FDA has

established a NanoTechnology Interest Group on which all FDA centers and offices that report to the Office of the Commissioner participate. Centers have established multidisciplinary working groups in order to share information and help coordinate the review for the various product types. The groups are charged with identifying and defining the scientific and regulatory challenges in the various review disciplines and to propose a path forward. However, the appropriate review divisions will conduct the review of nanotechnology applications submitted to FDA. The Working Group on Nanotechnology has discussed the need for a central resource to acquire basic safety data such as biodistribution, pharmacokinetics, efficacy, and toxicity for nanoparticles and other macromolecules (Buxton et al 2003). Information on FDA and its regulation of nanotechnology products can be viewed at the FDA web site (*http://www.fda.gov/nanotechnology/regulation.html*). Some of the questions that FDA considers internally are the following:

- What are the standard tools used to characterize nanoparticle properties?
- How to determine the short- and long-term stability of nanomaterials in various environments?
- What are the critical physical and chemical properties of nanomaterials including residual solvents, impurities, and excipients and how do these affect product quality and performance?
- What are the critical steps in the scale-up and manufacturing process of nanotechnology products?
- What is the residence time of nanoparticles in body tissues, their clearance from the body, and their effects on cell and tissue function?
- Are current methods used for measuring drug levels in blood and tissues adequate for assessing nanoparticle levels?
- What methods would identify the nature, quantity, and extent of nanoparticle release in the environment and what would be the impact of this on other species?

While the significant impact of nanotechnology and its applications is expected to be in the future, FDA has already approved many products such as imaging agents and nanoparticle ingredients in sunscreens. There are also cosmetics currently on the market that claim to contain nanoparticles. However, cosmetics do not undergo premarket approval, as do drugs and devices. Finally, there are products on the market that are reformulated to contain nanoparticles of previously approved products, in order to improve product performance.

While sponsors of nanotechnology products will be subject to the same testing requirements as for nonnanotechnology products, there likely will be certain challenges prior to commercialization. Specifically, there will need to be an understanding of the physical and chemical parameters that are crucial to product performance. Additionally, appropriate test methods and specifications to control the product or the manufacturing processes may need to be developed.

Finally, because much of the data currently available on nanotechnology products result from pilot batches produced in universities or small laboratories, very little is known about what might be the challenges of scale-up to mass production. While testing during the investigational phase of the product may be conducted with pilot

batches, bridging the investigational data to cover the scaled-up batches that will be commercialized may pose challenges for some of the novel nanotechnology formulations. However, these challenges are not considered to be insurmountable.

FDA's Nanotechnology Task Force

On 9 August 2006, the FDA announced the formation of an internal Nanotechnology Task Force. The new task force is charged with determining regulatory approaches that encourage the continued development of innovative, safe, and effective FDA-regulated products that use nanotechnology materials. The task force will identify and recommend ways to address any knowledge or policy gaps that exist so as to better enable the agency to evaluate possible adverse health effects from FDA-regulated products that use nanotechnology materials. FDA will continue to address product-specific nanotechnology-related issues on an ongoing basis. Specifically, the task force will:

Chair a public meeting to help FDA further its understanding of developments in nanotechnology materials that pertain to FDA-regulated products, including new and emerging scientific issues such as those pertaining to biological interactions that may lead to either beneficial or adverse health effects. This public meeting was scheduled for 10 October 2006 in order to

- assess the current state of scientific knowledge pertaining to nanotechnology materials for purposes of carrying out FDA's mission;
- evaluate the effectiveness of the agency's regulatory approaches and authorities to meet any unique challenge that may be presented by the use of nanotechnology materials in FDA-regulated products;
- explore opportunities to foster innovation using nanotechnology materials to develop safe and effective drugs, biologics, and devices, and to develop safe foods, feeds, and cosmetics;
- continue to strengthen FDA's collaborative relationships with other federal agencies, including the agencies participating in the National Nanotechnology Initiative such as the NIH, the Environmental Protection Agency (EPA), and the US Department of Agriculture (USDA), as well as with foreign government regulatory bodies, international organizations, healthcare professionals, industry, consumers, and other stakeholders to gather information regarding nanotechnology materials used or that could be used in FDA-regulated products;
- consider appropriate vehicles for communicating with the public about the use of nanotechnology materials in FDA-regulated products; and
- submit its initial findings and recommendations to the Acting Commissioner within 9 months of the public meeting.

At the meeting on 10 October 2006, witnesses agreed that nanotechnology holds promise for a vast range of products, including new medicines to treat diseases or delivery systems to get drugs to body parts now hard to reach. But some complained

that dozens of cosmetics and a handful of drugs made with nanomaterials already have made it to the market while regulators have done little to track their use or safety. The FDA has treated products made with nanotechnology the same way it handles others. For drugs with nanomaterials, that means companies must provide evidence of safety and effectiveness before they reach the market. But cosmetics, foods, and dietary supplements are not subject to FDA oversight before they are sold—with or without nanoparticles. As they called for close FDA oversight, many experts said they felt the agency was ill-equipped to regulate the new technology in the midst of other responsibilities. New nanoenabled drugs and medical devices seem to have placed burdens on an oversight agency that is already stretched extremely thin. The FDA internal task force on nanotechnology is due to report to the commissioner in 9 months.

FDA Collaboration with Agencies/Organizations Relevant to Nanotechnology

With the advent of nanotechnology, the regulation of many products will involve more than one center, for example a "drug" delivery "device." In these cases the assignment of regulatory lead is the responsibility of the Office of Combination Products. To facilitate the regulation of nanotechnology products, the agency has formed a NanoTechnology Interest Group (NTIG), which is made up of representatives from all the centers. The NTIG meets quarterly to ensure there is effective communication between the centers. Most of the centers also have working groups that establish the network between their different components. There are also a wide range of products involving nanotechnologies, which are regulated by other federal agencies. The breadth of products regulated by FDA and the other agencies is shown below.

The only viable approach to providing the public with innovative and beneficial novel therapies is to maintain an open dialogue with the developers of such products. As such, the FDA has partnered with NIST and NCI. However, this partnership does not create a "fast track" through the back door to product approval. It is intended to create a straight track or an efficient and direct track through the front door.

By working together (FDA, academia, and industry) during the early stages of product development and evaluation, the appropriate test methodologies can be identified to insure that the correct tests are done at the outset. These early discussions also are critical to insure that the most efficient and predictive testing is done on the final commercial form of the product. If the right questions can be asked early, then the process can move forward. Additionally, if some of the test methods used can be standardized, then many of the regulatory hurdles may be overcome.

Within FDA, the Office of Science and Health Coordination (OC/OSHC) coordinates regular discussions on nanotechnology among the major experts from every organizational entity within the agency. In addition the centers within FDA, e.g., Drugs and Medical Devices, have organized similar regular discussion groups.

The purpose of these meetings is to share experiences with the review of the products, insure that each center is aware of product guidance that may be developing elsewhere within the agency, and generally educate staff and policy makers about nanotechnology. Safety issues are identified and studied.

In a similar manner, FDA coordinates knowledge and policy with the other US Government agencies as a member of the Nanoscale Science and Engineering Technology (NSET) Subcommittee of the National Science and Technology Council (NSTC) Committee on Technology. Also, FDA and NIOSH cochair the NSET Working Group on Nanomaterials Environmental and Health Implications (NEHI) to define new test methods/protocols to define safety of these products. Finally, FDA is a direct contributor to the evaluations of the toxicity of materials supported by the NIEHS and the National Toxicology Program (NTP).

In 2005, the National Institute of Standards and Technology, the FDA, and the National Cancer Institute established the Nanotechnology Characterization Laboratory to perform preclinical efficacy and toxicity testing of nanoscale materials.

Regulation of Nanotechnology in the European Union

The current impression is that the European Union (EU) will adopt a cautious approach to regulation of nanotechnology. Like in the United States and elsewhere, there is no existing regulatory framework for nanotechnology in the EU. Some fear that Registration, Evaluation, Authorization, and restriction of Chemicals (REACH), the new EU's chemical policy, may be used as a source of reference for the regulation of nanotechnology, which might imply a qualified shift of the burden of proof with regard to safety, from the authorities to the manufacturer. Product liability law is less likely to play a preponderant role, at least at the EU level (as opposed to within the individual member states) because the EU's harmonization in this field of practice is limited. An overview of current and future EU regulation of nanotechnology, with some comparisons between the EU and US regulatory frameworks, has been published (Geert van Calster 2006).

The scope of nanotechnology-based medicinal products for human use reflects current thinking and initiatives taken by the European Medicines Agency (EMEA) following recent development of nanotechnology-based medicinal products. Nanotechnology is an emerging scientific research field with wide applicability and, in the context of medical science, is expected to contribute in developing a more proactive paradigm for the diagnosis and therapy of disease. Medicinal products containing nanoparticles have already been authorized both in EU and the United States under existing regulatory frameworks.

Although nanosizing does not necessarily imply novelty, it is expected that nanotechnology will yield innovative products. Such products could span the regulatory boundaries between medicinal products and medical devices, challenging current criteria for classification and evaluation. Appropriate expertise will need to be mobilized for the evaluation of the quality, safety, efficacy, and risk manage-

ment of nanomedicinal products, and the need for new or updated guidelines will be reviewed in the light of accumulated experience. EMEA has created the Innovation Task Force (ITF) to ensure EMEA-wide coordination of scientific and regulatory competence in the field of emerging therapies and technologies, including nanotechnologies, and to provide a forum for early dialogue with applicants on regulatory, scientific, or other issues that may arise from the development.

UK Government Policy on Safety of Nanoparticles

In October 2006, the UK government published a progress report on the government's direction and details of progress made on research into possible risks posed by engineered nanoparticles to human health and the environment. This report can be viewed at: http://www.defra.gov.uk/environment/nanotech/research/reports/index.htm. A full progress report, updating both current knowledge and research objectives, is scheduled to be published by the end of 2007. The report demonstrates the work already in hand across government to ensure that fundamental elements of understanding, such as how to measure and detect nanoparticles, is known. Further results from the research are intended to help inform decisions on appropriate control within the development of nanotech-based products and throughout their lifecycle. The interim report follows the publication in November 2005 of the first UK government research report "Characterising the potential risks posed by engineered nanoparticles." The 2005 report committed the government to a program of research to help address recognized gaps in knowledge about the health and environment-related risks and identified 19 research objectives.

The new report is intended to contribute evidence for regulators and provide a source of information both for applicants for research funding and for managers of research funding bodies. The area is being looked at by five task forces, reporting to the Nanotechnology Research Coordination Group, addressing the following subjects/areas:

- Metrology, Characterisation, Standardisation, and Reference Materials
- Exposure—Sources, Pathways, Technologies
- Human Health Hazard and Risk assessment
- Environmental Hazard and Risk assessment
- Social and Economic Dimensions of Nanotechnologies

Safety Recommendations of the Royal Society of UK

The Royal Society of UK has issued a report "Nanoscience and nanotechnologies: opportunities and uncertainties" (http://www.nanotec.org.uk/report/summary.pdf), containing a section on the safety issues of nanotechnology. This study is the first of its kind and responses are expected from organizations within the United Kingdom

as well as from other countries. Some comments and recommendations in the report are as follows:

- Most nanotechnologies pose no new risks to health and almost all the concerns relate to the potential impacts of manufactured nanoparticles and nanotubes that are free rather than fixed to or within a material.
- It is very unlikely that new manufactured nanoparticles could be introduced into humans in doses sufficient to cause the health effects that have been associated with nanoparticles in polluted air.
- Until more is known about the environmental impacts of nanoparticles and nanotubes, the release of manufactured nanoparticles into the environment should be avoided as far as possible.
- The chemicals in the form of nanoparticles or nanotubes should be treated as new substances under the existing Notification of New Substances (NONS) regulations and in the "Registration, Evaluation, Authorization, and Restriction of Chemicals."
- Overall, given the appropriate regulation and research along the lines just indicated, there is no need for the moratorium, which some have advocated on the laboratory or the commercial production of manufactured nanomaterials.

European Commission and Safety of Nanocosmetics

The European Commission has requested the Scientific Committee on Consumer Products (SCCP) to prepare an opinion on "Safety of Nanomaterials in Cosmetic Products." The preliminary version of the opinion can be found online (http://ec.europa.eu/health/ph_risk/committees/04_sccp/docs/sccp_o_099.pdf). The results obtained with nanosized delivery systems were not consistent. The following list of potential properties was considered:

- Nanomaterials constituents (such as lipids or surfactants) may act as penetration enhancers by penetrating individually into the stratum corneum (after particle disruption on skin surface) and subsequently altering the intercellular lipid lamellae within this skin layer.
- Nanomaterials may serve as a depot for sustained release of dermally active compounds.
- Nanomaterials may serve as a rate-limiting membrane barrier for the modulation of systemic absorption, hence providing a controlled transdermal delivery system.

TiO_2 used as a mineral UV filter in sunscreen cosmetic product does not penetrate through the stratum corneum of healthy skin. It poses no local or systemic risk to human health from cutaneous exposure (Borm et al 2006, Gamer et al 2006). Little information is available concerning other nanoparticles. Current investigations of nanoparticle penetration into the skin using static imaging technology are unable to detect small fractions of nanoparticles reaching the dermis, vascular bed

of the dermis, and hence, the blood stream. However, if the administered dose of nanoparticles is very large, as for instance could be the case for TiO_2 in sunscreens, a possible minute uptake of nanoparticles may be of relevance. A specific feature of nanoparticles is that not only the dose to the intake organ but also the dose in secondary target organs as a result of nanoparticle biokinetic distribution needs to be considered. In addition, nanoparticles may affect more cell types than larger particles because of use of endocytotic and nonendocytotic pathways.

Although cosmetic products are meant to be used on normal skin, it is known that they are also applied on nonhealthy skin where the barrier properties may be impaired. There is no published information yet available on the potential penetration of nanomaterials through atopic or sunburnt human skin. The possible uptake of nanosized materials from cosmetics via inhalation has also been considered.

The SCCP adopted a preliminary report on the risk assessment of nanomaterials on 19 June 2007. The report provides a review of the applicability of currently available risk assessment methods to nanomaterials in cosmetic products, recommends a general approach in order to assess the health risks of nanomaterials in cosmetic products, and identifies data and methodological gaps where further research and development is needed.

Chapter 16
Worldwide Development and Commercialization of Nanomedicine

Introduction

Nanotechnology originated in the United States but research and development of nanobiotechnology for applications in healthcare are being pursued worldwide. Besides the United States, significant developments are taking place in Europe, Israel, Australia, Japan, South Korea, India, and China.

Markets for Nanomedicine

Markets in nanomedicine will cover all clinical applications, current as well as potential. There were few products using nanotechnology and the total market in the year 2003 was approximately $500 million. With more indications opening during the year 2006, the market expanded to $800 million. It will grow to $3 billion by the year 2010 based on the new technologies that will be applied in nanomedicine. This market will be worth $17 billion by the year 2015 when biomedicine will be routine with use of nanorobots (nanobots), which will be expensive. However, since the term nanomedicine is being increasingly applied to all healthcare applications of nanobiotechnology, this market will be much larger incorporating diagnostics and drugs. The total market for healthcare applications of nanobiotechnology was $9 billion in 2006 and is projected to grow to $20 billion in 2010 and $70 billion in 2015. A more detailed analysis of this market is described elsewhere (Jain 2007e).

Impact of Nanobiotechnology on Markets for Current Pharmaceuticals

Along with its impact on healthcare, there will be changes in the patterns and values of pharmaceutical markets. Breast cancer would be a good example to illustrate this. If nanobiotechnology innovations in diagnosis, drug delivery, and therapeutics are applied to breast cancer treatment, the following results are anticipated:

K.K. Jain, *The Handbook of Nanomedicine*,

- Early diagnosis will mean that more patients would be under treatment.
- There will be expansion of the nanodiagnostic market and nanoscreening but some of the older technologies such as mammography will have a set back.
- One of the technologies to replace mammography and breast biopsies will be nano-enabled MRI contrast agents.
- Use of nano-reformulations of existing chemotherapy agents would mean that lesser quantities of the drugs would be needed.
- New treatments such as nanoparticle-induced ablation of the tumor might replace surgery.

All of these would mean increased survival from breast cancer and improvement in quality of life. It would be difficult to place a monetary value on these.

Nanobiotechnology in the European Union

In 2004, the European Commission adopted the communication "Towards a European Strategy for Nanotechnology" (COM 2004). It sought to bring the discussion on nanosciences and nanotechnologies to an institutional level and proposed an integrated and responsible strategy for Europe that takes into account the following issues:

- Increase investment and coordination of R&D to reinforce the industrial exploitation of nanotechnologies whilst maintaining scientific excellence and competition.
- Develop world-class competitive R&D infrastructure that takes into account the needs of both industry and research organizations.
- Promote the interdisciplinary education and training of research personnel together with a stronger entrepreneurial mindset.
- Ensure favorable conditions for technology transfer and innovation to ensure that European R&D excellence is translated into wealth-generating products and processes.
- Integrate societal considerations into the R&D process at an early stage.
- Address any potential public health, safety, environmental, and consumer risks upfront by generating the data needed for risk assessment, integrating risk assessment into every step of the life cycle of nanotechnology-based products, and adapting existing methodologies and, as necessary, developing novel ones.
- Complement the above actions with appropriate cooperation and initiatives at international level.

The European Commission believes that only a proactive European approach will enable the EU to acquire an autonomous command of the opportunities created by nanotechnology, which it refers to as the third industrial revolution taking place over the next two to three decades. Currently a budget of €1.3 billion ($1.5 billion) has been allocated with the following objectives:

- To help provide Europe with the critical mass of capacities to develop and exploit those high technologies on the basis of products, services, and production processes of the future, which are essentially knowledge based
- To develop intelligent materials for applications in sectors such as transport, energy, electronics, and biomedicine representing a potential market of several billion Euros
- To develop flexible, integrated, and clean systems requiring a substantial research effort in the application of new production and management technologies

Community action will concentrate on three major fields:

1. Nanotechnologies and nanosciences
   - Long-term interdisciplinary research: understanding phenomena, command of processes and the development of research tools
   - Supramolecular and macromolecular architecture
   - Nanobiotechnologies
   - Nanometric-scale engineering techniques for creating materials and components
   - Development of manipulation and control devices and instruments
   - Applications in fields such as health, chemicals, energy, and the environment
2. Knowledge-based multifunctional materials
   - Development of fundamental knowledge
   - Technologies associated with the production and transformation of knowledge-based multifunctional materials and biomaterials
   - Support engineering
3. Production processes and methods
   - Flexible and intelligent production processes and systems using advances in virtual production technologies, decision-aiding interactive systems, high-precision engineering, and innovative robotics
   - Systemic research (including on biological processes) for sustainable waste management, risk control, reduced consumption of basic products, and less pollution
   - Concepts for life cycle optimization of industrial systems, products, and services, in particular with a view to ecoefficiency and reduced emission of substances which are harmful to the environment.

Nano2Life

Nano2Life is the first European Network of Excellence supported by the European Commission under the Sixth Framework Program. Its objective is to merge existing European expertise and knowledge in the field of nanobiotechnology in order to

keep Europe as a competitive partner and to make it a leader in nanobiotechnology transfer. Nano2Life is tackling the fragmentation of European nanobiotechnology by joining 23 previously unconnected dynamic, highly specialized, and competent regions and centers with experience in initiating and running nanobiotech programs. A pool of 21 high-tech companies is associated with the network.

Nano2Life aims at setting the basis for a virtual European Nanobiotech Institute, focused on the understanding of the nanoscale interface between biological and non-biological entities and its possible application in the area of complex and integrated novel sensor technologies for health care, pharmaceuticals, environment, security, food safety, etc.

The partners have agreed on a Joint Program of Activity with actions in

- joint research projects;
- education and training;
- sharing of resources; and
- communication and dissemination

Nano2Life will contribute to ensure the development of nanobiotech devices, material, and services in agreement with international social and ethical standards and according to the needs of European industry. The network started operation in 2004 and has integrated over 170 researchers from 12 countries since then.

European Technology Platform on Nanomedicine

This is an industry-led consortium, bringing together the key European stakeholders in the sector (European Commission 2006). In 2005 it delivered a common vision of this technologically and structurally multifaceted area and defined the most important objectives in this Strategic Research Agenda (SRA) that addresses the member states of the European Union, its candidate countries, and associated states to the EU Framework Programs for research and technological development, as well as the European Commission itself. Its main aim is to put forward a sound basis for decision-making processes for policy makers and funding agencies, providing an overview of needs and challenges, existing technologies, and future opportunities in nanomedicine. The SRA also takes into consideration education and training, ethical requirements, benefit/risk assessment, public acceptance, regulatory framework, and intellectual property issues, thus representing a possible reference document for regulatory bodies. The proposed disease-oriented priority setting of this SRA is based on several parameters such as mortality rate, the level of suffering that an illness imposes on a patient, the burden put on society, the prevalence of the disease, and the impact that nanotechnology might have to diagnose and overcome certain illnesses. The scientific and technical approach is horizontal and exploits the benefits of interdisciplinarity and convergence of relevant technologies via breakthrough developments in the areas of diagnosis, targeted delivery systems, and regenerative

medicine. The effective implementation of the SRA is expected to provide a major step forward in patient-oriented affordable healthcare.

Nanobiotechnology in Australia

The Australian government is backing leading-edge nanotechnology. Combined with state and territory governments and private entities, up to A$100 (US $77) million per annum is being invested for research and commercialization purposes. Australia has a vibrant and dynamic nanotechnology sector. Ten of the companies profiled in this report are located in Australia. Significant numbers of multinational corporations and foreign government agencies are investing in the development and commercialization of breakthrough Australian nanotechnologies. Results are being delivered to manufacturing, raw materials, and environmental sectors. Australian nanotechnology expertise is broad, and it is retaining and attracting outstanding, internationally recognized researchers who are supported by a high-quality domestic science education base.

Australia has well-recognized capabilities, both in the development of medical diagnostic and therapeutic systems and in the preparation of novel nanoparticles. The activities are most marked in the state of Victoria where Nanotechnology Victoria Ltd. is the key organization for delivering nanotechnology research outcomes to industry. A number of teams have already developed important technologies which will allow development of next-generation imaging, for example the encapsulated nanoparticle systems at the University of Melbourne, the multifunctional polymer particles at Monash University, and synthesis of fluorescent nanoparticles (quantum dots). Nanotechnology Victoria has recognized the importance of these technologies and is preparing to launch a major initiative in the field of in vivo imaging, which captures some of the major recent developments, and could ultimately result in a commercial product for global markets.

One particular focus area for these emerging technologies is for diagnosis of early stage cardiovascular disease, which affects approximately 4 million Australians; this figure is predicted to double by 2015. It is anticipated that the use of biofunctionalized nanospheres loaded with contrast agents will enable early detection of diseased blood vessels. The use of effective preventive measures for patients at high risk of developing cardiovascular diseases could reduce the incidence of acute events such as stroke and heart failure and has major impact on associated healthcare costs.

Nanomedicine in the Developing World

Nanomedicine is developing in China and India and other developing countries, such as Thailand, the Philippines, Chile, Argentina, and Mexico, are also pursuing healthcare applications of nanobiotechnology. Several researches in this area

are needed to combat some of the serious medical problems facing the developing world. To expand on this idea, an initiative, called "Addressing Global Challenges Using Nanotechnology," to accelerate the use of nanotechnology to address critical sustainable development challenges has been proposed, modeled on the Foundation for the NIH/Bill and Melinda Gates Foundation's Grand Challenges in Global Health (Salamanca-Buentello et al 2005). The goal is to use nanobiotechnology in a meaningful way to generate sustainable healthcare benefits for the 5 billion people in the developing world. One of the important shortcomings of healthcare is lack of modern laboratory diagnostics. The developing world does not have access to many of the best medical diagnostic technologies; they were designed for laboratories with refrigerated storage of chemicals, a constant supply of calibrators and reagents, stable electrical power, highly trained personnel, and rapid transportation of samples. Microfluidic and nanofluidics systems enable miniaturization and integration of complex functions, which could move sophisticated diagnostic tools out of the developed-world laboratory (Yager et al 2006). These systems must not only be inexpensive, but also reliable and well suited to the medical and social contexts of the developing world.

Big Pharma and Nanomedicine

Although the NIH considers nanomedicine as one of the top five priorities and a big chunk of nanotechnology venture capital goes to life sciences start-ups, none of the major pharmaceutical companies has allocated adequate resources to nanobiotechnology research. Currently, the major interest of pharmaceutical companies is in technologies that facilitate drug discovery and target validation. Although nanobiotechnology plays a role in drug discovery, the major immediate applications are in drug delivery. Nanobiotechnology deserves greater attention of the pharmaceutical industry for the following reasons:

- Nanobiotechnology enables new formulations of existing drugs.
- By extending the limits of drug delivery, nanobiotechnology can rescue drugs that have failed in clinical trials due to lack of efficacy because of inadequate delivery.
- Nanodiagnostics, e.g., quantum dots for cancer, enable integration of diagnostics and therapeutics.
- Nanobiotechnology will facilitate development of personalized medicine, which already has the support of several major pharmaceutical companies.

Drivers for the Development of Nanomedicine

Drivers for the development of nanomedicine are shown in Table 16.1.

Table 16.1 Drivers for the development of nanomedicine

Driver	Example
Molecular diagnostics is a growing field	Early detection of disease
	Point-of-care diagnostics
	Non-PCR methods
Growing importance of drug delivery	For improving drug therapy, e.g., delivery across blood–brain barrier facilitated by nanobiotechnology.
Nanobiotechnology facilitates understanding of disease mechanism	Important component of personalized medicine along with pharmacogenomics
Future trends in medicine for minimally invasive procedures and correction of disease pathology	Nanobiotechnology will facilitate the development of nanoscale devices for performing such procedures
Regenerative medicine	Improved materials for tissue reconstruction and implants

Source: Jain PharmaBiotech.

Collaboration of Chemical Industry and the Government

For the successful application of nanotechnology, the chemical industry, the government, and academia should work together to leverage the expertise of chemical manufacturers. The participation of the chemical industry is essential because the industry has broad experience in manufacturing small-scale products. The chemical industry can help solve manufacturing problems. At the same time, the chemical industry must pay close attention to the potential health and environmental problems that could result from the dispersion of nanoparticles. The industry is attuned to what happened to the biological engineering of agricultural foodstuffs. It poses a risk to this entire area. The industry, along with the government and academia, should get involved early in shaping public opinion about nanotechnology, which would involve paying for studies to examine the health, safety, and environmental affects of nanoparticles. Government assistance is by way of funding for studies in this area.

Companies Using Nanotechnology for Healthcare

More than 230 companies are involved in applications of nanobiotechnology relevant to healthcare. These are profiled in a special report (Jain 2007e). A selection of companies using nanotechnology for healthcare and therapeutics are shown in Table 16.2.

Table 16.2 Companies using nanotechnology for healthcare and therapeutics

Company	Technology	Application
Ablynx (Ghent, Belgium)	Nanobodies are naturally highly homologous to human antibodies	Oncology, inflammation, CNS, and cardiovascular disease
Advance Nanotech Inc (New York, NY)	Iron oxide nanoparticles UV absorbers, transparent magnetic coatings, catalysts, and medical imaging	To diagnose and treat a wide variety of medical conditions
Advanced Magnetics Inc (Cambridge, MA)	Superparamagnetic iron oxide nanoparticles used in diagnostics and pharmaceutical products	Ferumoxytol for anemia of chronic renal disease is in phase III clinical trials
Alnis BioSciences Inc (Research Triangle Park, NC)	MagNaGel™: nanoscopic hydrogels, made of polymers, bioactives, and targeting molecules	Diagnosis and treatment of cancer
Asklepios BioPharmaceutical (Durham, NC)	Biological Nano Particle (BNP) is a synthetic vector derived from AAV	Muscular dystrophy, congestive heart failure, hemophilia B, bone graft
Copernicus Therapeutics (Cleveland, OH)	Nonimmunologic and nonviral DNA nanoparticle gene transfer technology	Gene therapy for cystic fibrosis and retinal degeneration
Durham Scientific Crystals (Sedgefield, United Kingdom)	Semiconductor nanocrystals	Medical x-ray imaging
Focal Point Microsystems (Atlanta, GA)	Two-photon 3D lithography fabricates any 3D structure at nanoscale	Tissue engineering
Hemoteq (Wuersellen, Germany)	Thromboprotective camouflage: a biomimicry ultrathin coating	Prevents thrombosis on devices placed in the cardiovascular system
ImaRx Therapeutics Inc (Tuscon, AZ)	NanoSurgery™ and NanoInjection™: ultrasound to perform site-specific drug and gene delivery with nanoparticles	Stroke
MagForce Nanotechnologies (Berlin, Germany)	Iron oxide nanoparticles and magnetic field for thermoablation	Cancer

Table 16.2 (continued)

Company	Technology	Application
Molecular Insight Pharmaceuticals Inc (Cambridge, MA)	Ultratrace™ radiolabeling technology using only nanograms of active material	Safe and targeted radiotherapy
Nanobac Life Sciences Inc (Tampa, FL)	Pathogenic role of calcifying nano-particles (CNPs) or "nanobacteria" in human disease	Treatment of urological and cardiovascular diseases where CNP involved in pathogenesis
Nanotrope Inc (Washington, DC)	Stem cells grown in self-assembling 3D biodegradable scaffolds of nanofibers	Neural repair
Nano Interface Technology (Lorton, VA)	Hydroxyapatite nanoparticles to coat the implants for the human body	To decrease the failures of the implants
NanoMech (Fayetteville, AR)	Electrostatic spray coating followed by laser treatment	Improvement in hydroxyapatite dental implants
Nanopeutics (Liberic, Czech Republic)	Nanospider™ technology based on nanofibers	Wound care
NanoScale Materials Inc (Manhattan, KS)	NanoActive materials: reactive nanoparticles	Neutralization of chemical warfare agents
Nanospectra Biosciences (Houston, TX)	Nanoshells (AuroShell™): optically tunable nanoparticles and external laser	To deliver light to nanoshells in tumor for thermal destruction
NanoViricides Inc (West Haven, CT)	TheraCour technology: nanotechnology-based antiviral therapy	Treatment of HIV and HCV
PlasmaChem GmbH (Mainz, Germany)	BioDiamond stents: biocompatible and nanolayered DLC coating	Stents with reduced thrombogenicity for arteries
pSivida Com Ltd. (Perth, Australia)	BioSilicon™: nanostructured silicon implants	Various implants in the human body for tissue engineering
QinetiQ Nanomaterials Ltd. (Hampshire, United Kingdom)	Antiviral nanoparticles	Targeted initially at pandemic viruses such as H5N1 Potential as broad-spectrum antiviral material

Table 16.2 (continued)

Company	Technology	Application
Signalomics GmbH (Steinfurt, Germany)	Study of faulty signal transduction in tumor cells by fluorescent nanocrystals	Personalized therapy of cancer (diagnosis + therapeutics)
Spire Corporation (Bedford, MA)	Nanostructured silicon surfaces will be used as neural growth scaffolds and to reduce glial scarring from electrode implantation	Electrode implantation for neurological disorders such as epilepsy, Parkinson's disease, and for relief of central pain
Starpharma Holding (Melbourne, Australia)	Dendrimers for polyvalent interaction with biological targets in diseases	Cancer, HIV, and respiratory diseases
TDA Research Inc (Wheat Ridge, CO)	Fullerenes: imaging agents, radiopharmaceuticals, nanoencapsulates	Radiology: diagnostic and therapeutic
Velbionanotech (Bangalore, India)	Short fragments of DNA as drugs assembled in nanochips for delivery into the human body	Neurological, cardiac, and urological disorders
Xintek Inc (Chapel Hill, NC)	Carbon nanotubes for x-rays	Diagnostic imaging, e.g., CT scan

Source: Jain PharmaBiotech.

Chapter 17
Research and Education in Nanomedicine

Introduction

Education of Healthcare Professionals

Most practicing physicians know little about nanotechnology beyond what they read in the popular press. There is not much information about nanobiotechnology in the common physician's journals. Physicians are barely catching up with the genomic revolution and learning the rudiments of genomic medicine. At this stage, it is important to educate physicians about the potential for nanomedicine. Medical trends take a few years to change and medical education should start a few years before the products are expected to enter the market. Companies can take an active part in dissemination of scientific information about the basics and potential of nanomedicine. Although there are several restrictions on the role of pharmaceutical companies in organizing educational symposia for the physicians, it should be easier with new technologies which do not have high-value products for sale at this stage.

Although a large work force of nanobiotechnology experts is available both in the academia and the industry, there are no courses in nanobiotechnology or nanomedicine in medical curricula. It will be important to introduce courses in basics of nanomedicine in medical curricula so that the next generation of physicians is well informed about the technologies and products that they will encounter as they start practice of medicine.

Education of the Public

According to the findings of an international team of researchers from Penn State University, University of Vermont and University of Waikato (New Zealand) released in February 2007, business leaders view nanotechnology with cautious optimism whereas government and quasi-official organizations find nanotechnology important. Many antinanotechnology activists predict the creation of destructive, uncontrollable life forms from nanobiotechnology. The general public, however, is only vaguely aware of nanotechnology. The public sees nanotechnology as having

K.K. Jain, *The Handbook of Nanomedicine*,

some benefits, but is concerned with how business and industry develop the field. However, the majority is unaware of exactly what nanotechnology is and of the potential problems in its development.

Experience with other biotechnologies has shown that provision of proper information to the public is essential for the broad support during development phase. Misconceptions can hinder the progress and create unnecessary opposition to new technologies. Direct public engagement such as citizen juries or panels should identify at an early stage broad "desired futures" for nanobiotechnologies, significant ethical concerns, or the acceptability of key applications and options. The quality of scientific and other input to such public engagement activities is critical to their success. Research should be conducted into public attitudes and concerns towards nanotechnologies. Information about the possible hazards of nanoparticles and the research results in environmental impact of biotechnology should be openly shared with the public. Scientists should respond to concern expressed about the safety of nanotechnology in lay press.

Nanobiotechnology Research in the Academic Centers

Nanobiotechnology research in the commercial sector is described in profiles of companies in Chapter 11. Noncommercial institutes, where research is conducted in nanobiotechnology, are shown in Table 17.1.

Table 17.1 Noncommercial institutes/laboratories involved in nanobiotechnology

Center/program	Parent institutes	Areas of interest
Applied NanoBioscience Center at Biodesign Institute	State University of Arizona (Tempe, AZ)	Nanoscale processing technologies for improving molecular diagnostics
Australian Institute for Bioengineering and Nanotechnology	University of Queensland (Brisbane, Australia)	Cell and tissue engineering Systems biotechnology Biomolecular nanotechnology
Biomedical Engineering Center	Industrial Technology Research Institute (Taiwan)	In vivo nanodevices, biomimetic sensing, nanobiolabeling/diagnosis
Bio-molecular Engineering Group	University of Missouri (Columbia, MO)	Engineered membrane protein channels used to make single molecule biosensors
BioSecurity and NanoSciences Laboratory	Lawrence Livermore National Laboratory (Livermore, CA)	Nanoscience to detect even the single smallest molecule of harmful substances
Birck Nanotechnology Center	Purdue University (West Lafayette, IN)	Nanocantilever biosensors for detection of microorganisms and use of bacteria for delivery of nanoparticles into the cell
Burnham Institute for Medical Research	University of California (Santa Barbara, CA)	Nanoparticles that target tumors and bind to their blood vessels to destroy them

Table 17.1 (Continued)

Center/program	Parent institutes	Areas of interest
California Nanosystems Institute	UCLA, University of California (Santa Barbara), industry, and the state of California	Molecular-level diagnosis and treatment of disease
Center for Bio/Molecular Science and Engineering	US Naval Research Laboratory (Washington, DC)	FRET-derived structure of a quantum dot-protein bioconjugate nanoassembly
Center for Functional Nanomaterials	Brookhaven National Laboratory (Upton, NY)	Study of interaction of nanomaterials with biosystems at level of single molecules
Center for Molecular Imaging Research	Massachusetts General Hospital (Boston, MA)	Nanoparticles for in vivo sensing and imaging of molecular events
Center for Nanotechnology	Wake Forest University (Winston-Salem, NC)	Controlling cellular function through nanoscale engineering, e.g., insertion of complex nanostructures into human monocytes
Center for Nanotechnology	University of Washington (Seattle, WA)	Bionanotechnology for cancer diagnostics and therapeutics
Center for Nanotechnology	NASA Ames Research Center (Moffett Field, CA)	Carbon nanotubes and nanowires for biological sensing
Center for Photonics and OptoElectronic Materials	Princeton University (Princeton, NJ)	Interphase of nanotechnology and biological systems
Cornell NanoScale Science & Technology Facility	Cornell University (Ithaca, NY)	Biosensors, drug delivery systems, microarrays
Centre for Nanoscale Science & Technology	Queen's University (Belfast, Ireland)	Nanostructured materials as templates for tissue engineering
Center for Nanoscience & Nanotechnology	Georgia Institute of Technology (Atlanta, GA)	Nanodevices and nanosensors for biotechnology
Clinatec (a clinic specializing in nanotechnology-based treatment)	University of Grenoble (France)/Minatec	Nanoneurosurgery of degenerative neurological disorders
FIRST (Frontiers in Research, Space and Time)	Swiss Federal Institute of Technology (Zurich, Switzerland)	AFM as a nanolithography tool
Heath Group	California Institute of Technology (Pasadena, CA)	Nanobiology: Nanolab combines several assays on a square-centimeter silicon chip resembling a miniature cell farm with rows of cells
IMTEK—Institute of Microsystem Technology	University of Freiburg, (Freiburg, Germany)	Nanoparticles for biosensors
INSERM	Paris, France	Nanodetection, drug delivery
Institute of NanoScience and Engineering	University of Pittsburgh (Pittsburgh, PA)	Nanotubes for molecular diagnostics and nanocarpet to detect and destroy bacteria
Institute for Nanotechnology	Northwestern University (Evanston, IL)	Nanoparticles and biosensors; nanobarcode for detection of proteins; nanofibers for neuroregeneration

Table 17.1 (Continued)

Center/program	Parent institutes	Areas of interest
Institute of Microtechnology	University of Neuchatel, Switzerland	Biological applications of nanotechnology
Institute of Micro- and Nanotechnology	Technical University of Denmark, Denmark	Study of nanoscale structures with in situ scanning tunneling microscopy
Institute of Physical Chemistry	National Centre for Scientific Research (Athens, Greece)	Polymer-based nanosponges, nanotubes, drug delivery
Laboratory for Micro- and Nanotechnology	Paul Scherrer Institute (Villigen, Switzerland)	Nanopore membranes, biosensors, and artificial noses
Laboratory for Photonics and Nanostructures	CNRS (Marcoussis, France)	Separation methods for DNA sequencing Protein analysis and on-chip detection Microfluidic systems for cell sorting.
Lerner Research Institute	The Cleveland Clinic (Cleveland, OH)	Nanometer-scale tissue engineering, diagnostics, nanosensors for surgery
London Center for Nanotechnology	University College (London, United Kingdom)	Use of nanotechnology to develop low-cost diagnostics and drug delivery systems and personalized medicine
MacDiarmid Institute, BioNanotechnology Network	University of Canterbury (Christchurch, New Zealand)	Development of biochip for AFM imaging Biosensors by splicing polymers with QDs
Michigan Nanotechnology Institute	University of Michigan (Ann Arbor, MI)	Nanoemulsions as antimicrobial agents Dendrimers for drug delivery in cancer Magnetic nanoparticle MRI agents Dendritic polymer-based nanosensors
Micro and Nano Biosystems Laboratory	Boston University (Boston, MA)	Application of nanotechnology to tissue engineering and cell/drug encapsulation
Nanotechnology Research Institute	University of Ulster (Jordanstown, United Kingdom)	In vivo nanobiosensors
National Center for Competence in Research Nanoscale Science	Biozentrum, University of Basel (Basel, Switzerland)	To bring nanotechnology from the bench to the patient by developing new tools
Nanobioengineering Laboratory	National University of Singapore (Singapore)	Nanohydroxyapatite/chitosan as resorbable bone paste
NanoBiomolecular Engineering Group	University of California (Berkeley, CA)	BioCOM cantilever chip for cancer diagnosis, DNA-based self-assembly/replication of inorganic nanostructures, and electrophoretic separation microchip
Nano-Mechanical Technology Lab	Massachusetts Institute of Technology (Cambridge, MA)	Study of changes in human cells for research projects on infectious diseases like malaria and sickle cell anemia, and cancers of the liver and pancreas

Table 17.1 (Continued)

Center/program	Parent institutes	Areas of interest
Nanomedicine Center	University of North Carolina (Chapel Hill, NC)	To measure the spatiotemporal activity of complex, dynamic signaling networks (nanosystems) in living cells; and development of engineered nanosystems, which upon sensing a dysregulated signaling network is able to reverse the harmful aspects of the dysregulation
Nanomedicine Development Center	Emory University/Medical College of Georgia, Atlanta	Focus on DNA damage repair by protein complexes
NanoRobotics Lab	Carnegie Mellon University (Pittsburgh, PA)	Nano-enabled imaging capsule to look inside the small intestine
NanoBioTechnology Initiative	Ohio University (Athens, OH)	Diagnosis/treatment: cancer and diabetes
Nanoscale Research Team	University of California (Davis, CA)	Artificial cell membrane to study single protein interaction with cell membrane
Nano-Science Center	University of Copenhagen (Copenhagen, Denmark)	Boron carbide nanoparticles for boron neutron capture therapy of cancer
NanoSystems Biology	California Institute of Technology (Pasadena, CA)	Nanowire biosensors for early detection of cancer biomarkers
Nanosystems Laboratory	University of Washington (Seattle, WA)	Nanoprobes based on thin film technology for rapid and cheap sequencing DNA
National Institute of Nanotechnology of Canada	University of Alberta (Edmonton, Canada)	X-ray scattering beamline to determine the size of biomolecules on nanoscale
Pharmaceutical Bioengineering & Nanotechnology Group	University of London (London, United Kingdom)	Bridging the gap between nanomaterials engineering and pharmaceutical science to develop nanomedicines
Purdue Nanomedicine Development Center	Purdue University (West Lafayette, IN)	NIH-supported center for research on phi29 nanomotor for potential use in the diagnosis and treatment of diseases
Richard E. Smalley Institute for Nanoscale Science & Technology	Rice University (Houston, TX)	Carbon nanotechnology Improved delivery of bioactive molecules, nanoscale sensory systems, biochips
Roukes Group	California Institute of Technology (Pasadena, CA)	Nanotechnology for neurophysiology Nanodevices for molecular biosensing
Sandia National Laboratories (Albuquerque, NM)	Department of Energy, US Government	Nanodevices: biosensors to detect biological agents
Siteman Center of Cancer Nanotechnology Excellence	Washington University School of Medicine (St. Louis, MO)	Molecular imaging using nanoparticle tags and MRI, combined with therapy
Swiss Nanoscience Institute	University of Basel, Switzerland	Rapid and sensitive detection of disease- and treatment-relevant genes Toxicity of nanoparticles

Table 17.1 (Continued)

Center/program	Parent institutes	Areas of interest
USC Nanocenter	University of South Carolina (Columbia, SC)	Nanomedicine as well as social and ethical implications of nanotechnology
Winship Cancer Institute	Emory University (Atlanta, GA)	Cancer nanotechnology: nanoparticles for molecular and cellular imaging.
Yale Institute for Nanoscience & Quantum Engineering	Yale University (New Haven, CT)	Smart nanoparticles: a new class of nanomaterials with properties that mimic biological vectors like bacteria and viruses, for vaccine delivery

Source: Jain PharmaBiotech.

Chapter 18
Future of Nanomedicine

Future Potential of Nanomedicine

Disease and other disturbances of function are caused largely by damage at the molecular and cellular level but current surgical tools are large and crude. Even a fine scalpel is a weapon more suited to tear and injure than heal and cure. It would make more sense to operate at the cell level to correct the cause of disease, rather than chop off large lesions as a result of the disturbances at cell level.

Nanotechnology will enable construction of computer-controlled molecular tools that are much smaller than a human cell and built with the accuracy and precision of drug molecules. Such tools will be used for interventions in a refined and controlled manner at the cellular and molecular levels. They could remove obstructions in the circulatory system, kill cancer cells, or take over the function of subcellular organelles. Instead of transplanting artificial hearts, a surgeon of the future would be transplanting artificial mitochondrion.

Nanotechnology will also provide devices to examine tissue in minute detail. Biosensors that are smaller than a cell would give us an inside look at cellular function. Tissues could be analyzed down to the molecular level, giving a completely detailed "snapshot" of cellular, subcellular, and molecular activities. Such a detailed diagnosis would guide the appropriate treatment.

It is expected that within the next few years, we will have a better understanding of how to coat or chemically alter nanoparticles to reduce their toxicity to the body, which will allow us to broaden their use for disease diagnosis and for drug delivery. Biomedical applications are likely to be some of the earliest. The first clinical trials are anticipated cancer therapy.

US Federal Funding for Nanobiotechnology

Companies developing micro and nanotechnology-related products will receive more than $20 million from the federally funded Advanced Technology Program (ATP), beginning October 2004. The US Senate Appropriations Committee reinstated the funding and added another $24 million in 2004, for a total of $203 million

K.K. Jain, *The Handbook of Nanomedicine*,

for 2005. The National Institute of Standards and Technology announced its latest round of ATP awards at the end of September 2004, providing $80.1 million in awards with industry matches equaling $56.9 million. Nanobiotechnology companies receiving awards include the following:

- Dow Chemical Co, in partnership with Veeco Instruments Inc, will receive $6.5 million to develop an AFM platform for nanomechanical measurements.
- Nanospectra Biosciences will receive $2 million to develop an integrated approach for detecting and destroying cancer cells using gold nanoshells.
- Quantum Dot Corp will receive $2 million to develop quantum dots without the use of the heavy metal cadmium for imaging in medical diagnostics and treatment.

Nanomedicine Initiative of NIH

The NIH started a nanomedicine initiative in 2004 by soliciting comments from the scientific community to help shape the research project aimed at developing new tools to improve human health. Further information about this initiative is available at the following web site: http://nihroadmap.nih.gov/nanomedicinelaunch/.

The initiative, which could last a decade, is a broad program that seeks to catalog molecules and understand molecular pathways and networks. Nanomedicine is one of nine initiatives that make up NIH's roadmap, a long-term plan for improving and accelerating biomedical research. This is a program oriented towards addressing biological issues of health and clinical applications in a context of the overall mission of the National Institutes of Health.

Unlike many research projects, NIH is not predetermining specific areas of study. Instead, it is calling for proposals aimed at helping to fulfill the project's goals. To start with there will be a debate to find the best way to proceed with the nanomedicine initiative. Much of the initial research will take place at three or four Nanomedicine Development Centers to be established by the initiative. NIH is inviting applications from those interested in starting a center. It is the first step in a three-part process and specifically calls on applicants to outline their vision for the content and structure that will be examined at a center.

From these outlines, NIH will award about 20 grants for concept development plans and will select the centers from those plans by September 2005. The centers will be staffed by teams of scientists from many different areas, such as biologists, mathematicians, biochemists, and engineers. NIH is setting aside $6 million for the centers in 2005 and funding in 2006 may allow for creation of additional centers while also providing money for those already established. Some examples of possible areas of study are as follows:

- A probe of molecular events inside cells on biologically relevant time scales that may be on the order of milliseconds or microseconds or even nanoseconds.
- One goal of the initiative is to design systems for engineering within living cells.

- To ensure the biocompatibility of some nanodevices in humans and develop devices that may eventually reduce the cost of health care.

NIH Nanomedicine Center for Nucleoprotein Machines

In October 2006, the NIH awarded Georgia Institute of Technology, Emory University and Medical College of Georgia a grant to partner on the Nanomedicine Center for Nucleoprotein Machines. The new center will initially focus on understanding how the body repairs damage to DNA, a problem that lies at the heart of many diseases and illnesses. As cells replicate, mistakes are created in the DNA that, if not repaired, cause defects that lead to illness. DNA breakage can also occur from ionizing radiation, which is found in the environment, cosmic rays, radon gas, and even the soil, as well as in our bodies, primarily from potassium and carbon. Learning how protein complexes repair DNA damage could be the key to understanding structure–function relationships in the cell nucleus' protein machines, called nucleoprotein machines, that synthesize, modify, and repair DNA and RNA. This could someday be used to reverse genetic defects, cure disease, or delay aging. By studying the way natural machines are engineered by the body, researchers will develop the general principles that will enable engineering of artificial machines that could carry out these processes for therapeutic purposes, e.g., to fix genetic defects.

The center will receive between $6 million and $10 million from the NIH over the next 5 years and almost $3 million from the Georgia Research Alliance, a public–private partnership of Georgia universities, businesses, and government created to build the state's technology industry.

NCI Alliance for Nanotechnology in Cancer

One of the most important applications of nanotechnology will be in cancer. On 13 September 2004, the National Cancer Institute (NCI) launched a $144-million, 5-year plan to apply nanoscale technology to researching and treating cancer. This will bring together researchers, clinicians, and public as well as private organizations to translate cancer-related nanotechnology research for the benefit of the patient.

To carry out the effort, the institute is forming the NCI Alliance for Nanotechnology in Cancer, which will bring together researchers, clinicians, and organizations to develop and translate cancer-related nanoresearch into clinical practice. The alliance will create nanoresearch centers within existing public facilities and a laboratory for preclinical testing that will help boost regulatory review and translation of nanomaterials and devices into the clinical realm. More detailed information on the NCI Alliance for Nanotechnology in Cancer is available on the web site (http://nano.cancer.gov).

The alliance is designed as one of the first steps in crafting a Cancer Nanotechnology Plan, which will include milestones to measure success over two time periods. Within the first 3 years, the plan calls for accelerating projects promising

for near-term clinical application. After 3 years, the plan will focus on solutions to more difficult technological and biological problems that could affect detection and treatment.

Research in Cancer Nanotechnology Sponsored by the NCI

On 3 October 2005, NCI (National Cancer Institute, a part of the NIH) made first year awards totaling $26.3 million to help establish seven Centers of Cancer Nanotechnology Excellence. The eighth center was announced on 1 March 2006. These centers are as follows:

1. Carolina Center of Cancer Nanotechnology Excellence at the University of North Carolina (Chapel Hill, NC): This center will focus on the fabrication of "smart" or targeted nanoparticles and other nanodevices for cancer therapy and imaging.
2. Center of Nanotechnology for Treatment, Understanding, and Monitoring of Cancer at University of California (San Diego, CA): This center will focus on a smart, multifunctional, all-in-one platform capable of targeting tumors and delivering payloads of therapeutics.
3. Emory-Georgia Tech Nanotechnology Center for Personalized and Predictive Oncology (Atlanta, GA): This center will aim to innovate and accelerate the development of nanoparticles attached to biological molecules for cancer molecular imaging, molecular profiling, and personalized therapy.
4. MIT-Harvard Center of Cancer Nanotechnology Excellence (Cambridge, MA): This center will focus on diversified nanoplatforms for targeted therapy, diagnostics, noninvasive imaging, and molecular sensing.
5. Nanomaterials for Cancer Diagnostics and Therapeutics at Northwestern University (Evanston, IL): This center plans to design and test nanomaterials and nanodevices to improve cancer prevention, detection, diagnosis, and treatment.
6. Nanosystems Biology Cancer Center at California Institute of Technology (Pasadena, CA): This center will focus on the development and validation of tools for early detection and stratification of cancer through rapid and quantitative measurement of panels of serum and tissue-based biomarkers.
7. The Siteman Center of Cancer Nanotechnology Excellence at Washington University (St. Louis, MO): This center has a comprehensive set of projects for the development of nanoparticles for in vivo imaging and drug delivery, with special emphasis on translational medicine.
8. Stanford University School of Medicine (Palo Alto, CA): It will aim its efforts at imaging diseases in vivo and determining what is going on within patients' bodies through blood or tissue sample analysis.

On 17 October 2005, the NCI awarded 12 Cancer Nanotechnology Platform Partnership awards totaling $35 million over 5 years, with $7 million awarded in the first year. The teams granted these awards are linked to NCI-designated cancer centers and will create and implement new nanotechnology-based materials in six important areas spanning all key steps from prevention to detection to diagnosis to treatment to

monitoring of efficacy. In addition to the Platform Partnerships, the NCI's Alliance for Nanotechnology in Cancer encompasses three other programs, including Centers of Cancer Nanotechnology Excellence; multidisciplinary research training and team development awards; and the Nanotechnology Characterization Laboratory. Thus, in just over 1 year since launch, the Alliance for Nanotechnology in Cancer has put forward one of the largest efforts in the world to apply nanotechnology to biomedical goals. Brief descriptions of the 12 award are as follows:

Nanotherapeutic strategy for multidrug-resistant tumors. This partnership, which includes researchers from Northeastern University, the Roger Williams Medical Center, Massachusetts General Hospital, and Massachusetts Institute of Technology, will develop multifunctional targeted nanoscale devices to deliver therapeutic agents and tumor resistance modulators directly to cancer cells as a means of overcoming multiple-drug resistance. Preliminary work by this team has already produced biodegradable, tumor-targeted drug nanocarriers, and this team is now ready to begin translational efforts to move this research along a development path to the clinic. The initial oncology focus of this project will be breast and ovarian cancers. The Northeastern University, has expertise in nanoparticle design, pharmaceutical chemistry, cancer biology, and clinical oncology.

DNA-linked dendrimer nanoparticle systems for cancer diagnosis and treatment. This partnership at the University of Michigan will develop multicomponent, dendrimer nanoparticles that will target, image, and treat cancer. The team will first refine technology designed to assemble the various dendrimer components into a multifunctional device and then begin preclinical testing of the resulting formulations and make extensive use of the NCI's Nanotechnology Characterization Laboratory to generate the preclinical safety and pharmacokinetic data needed to move these nanoparticles to the clinic. The initial focus of this project will be epithelial tumors. The team has expertise in dendrimer development, immunology, cancer biology, and clinical oncology.

Metallofullerene nanoplatform for imaging and treating infiltrative tumor. This partnership at the Virginia Commonwealth University will develop metal-based fullerenes to simultaneously deliver imaging agents and anticancer therapeutics to brain tumors. The initial focus of this project will be brain cancer. The team has expertise in experimental and clinical imaging, chemistry, neurosurgery, oncology, and tumor targeting.

Detecting cancer early with targeted nanoprobes for vascular signatures. This partnership, involving researchers from the Comprehensive Cancer Center at University of California, San Francisco, and the Burnham Institute, will develop highly specific molecular imaging probes that will enable noninvasive early detection of incipient cancer. These targeted probes will serve as platforms for testing the benefits of new nanotechnology-based imaging agents with improved properties (e.g., higher signal output) forthcoming from the new Centers of Cancer Nanotechnology Excellence and the Cancer Nanotechnology Platform Partnership programs. The team has expertise in angiogenesis and mouse models of cancer; vascular profiling; and clinical and experimental molecular imaging.

Photodestruction of ovarian cancer: ErbB3 targeted aptamer-nanoparticle conjugate. This partnership at the Massachusetts General Hospital is focused on

developing multifunctional nanoparticles that can deliver light-activated anticancer compounds specifically to ovarian cancer cells. Once bound to the target cells, the nanoparticles are activated using a miniature endoscopic laser to illuminate only the tumors, providing a second means of ensuring that healthy tissue is spared damage during therapy. The team has expertise in photodynamic therapy, fiber-optic procedures, and nanoparticle design and synthesis.

Hybrid nanoparticles in imaging and therapy of prostate cancer. This partnership at the University of Missouri-Columbia will use its established expertise in nanomaterial design to create gold nanoparticles capable of imaging molecular abnormalities associated with the earliest stages of prostate cancer. By incorporating gold nanoparticles on cancer-specific peptides, the partnership's investigators hope to create agents that can both image and treat small prostate tumors. The team has expertise in chemistry, radiology, veterinary sciences, pathology, physics, and biocompatible nanoparticle development.

Near-infrared fluorescence nanoparticles for targeted optical imaging. This partnership, a collaboration between the researchers at The University of Texas M. D. Anderson Cancer Center and Eastman Kodak, aims to develop novel nanoparticles for targeted molecular optical imaging of early-stage tumors. The fluorescent nanoparticles, developed at Kodak, emit near-infrared fluorescence light that can penetrate deep into tissues. The nanoparticles will be targeted to tumor-associated antigens, reporting their presence or absence in the tumors. Nanoparticles are also designed to respond to enzymatic action which light up only when first activated by enzymes found exclusively on the surface of certain types of cancer cells. The partnership will focus on studies to fully characterize the biological behavior of these particles and target them to a wide variety of cancer cells. The initial oncology focus of this project will be brain, breast, and skin cancers. The team has expertise in nanoparticle formulation, imaging science, neurosurgery, and molecular biology.

Integrated system for cancer biomarker detection. This partnership at the Massachusetts Institute of Technology will develop microfluidic devices whose nanochannels are capable of concentrating rare proteins from biospecimens. These devices will then be integrated with another chip-based device to detect and quantify panels of proteins that may serve as early signs of cancer. The devices will be fabricated in such a way as to enable widespread and low-cost distribution for use in the healthcare setting. The initial oncology focus of this project will be prostate cancer. The team has expertise in nanofabrication, clinical oncology, and cell biology.

Novel cancer nanotechnology platforms for photodynamic therapy and imaging. This partnership, which includes team members from the Roswell Park Cancer Institute, the University of Buffalo, and the University of Michigan, will develop targeted nanoparticle platforms for detecting and imaging cancers and selectively delivering light-activated anticancer compounds for guided photodynamic therapy (PDT). Because of the team's extensive experience with the systems they are developing, this partnership expects to validate the usefulness of their nanoparticles both for imaging tumors and then killing them with PDT, using models for breast, lung, prostate, and colon cancers. The team has expertise in nanoparticle design, animal models of human cancer, photodynamic therapy, imaging, and clinical oncology.

Multifunctional nanoparticles in diagnosis and therapy of pancreatic cancer. Investigators from the State University of New York at Buffalo and Johns Hopkins School of Medicine have combined forces in this partnership to develop and test multifunctional, hybrid ceramic–polymeric nanoparticles that will deliver imaging and therapeutic agents to pancreatic tumors. This group has a strong history of developing novel, biocompatible nanomaterials, including nontoxic quantum dots, that have the capacity to be targeted to specific types of cancer cells. Based on the prior work by members of this partnership, they expect to begin translating their work into preclinical and clinical studies in the near-term. The team has expertise in materials design and clinical oncology.

Nanotechnology platform for targeting solid tumors. This partnership at the Sidney Kimmel Cancer Center will build on extensive experience in nanoparticle development and blood vessel biology to create nanodevices that will target specific cells lining blood vessels in order to improve transit out of the bloodstream and into tumors. Miniaturized probes can be injected into the bloodstream to go throughout the body and not only report back the state of each organ, but actually seek out and treat cancer. This technology has application for imaging and therapy of a wide variety of solid tumors, both primary and metastatic (or disseminated disease) including breast, prostate, kidney, colon, and lung. The team includes chemists, molecular imagers, tumor biologists, and molecular biologists.

Nanotechnology platform for pediatric brain cancer imaging and therapy. A collaborative effort among researchers at the University of Washington, the Fred Hutchinson Cancer Research Center, Children's Hospital and Regional Medical Center, and Philips Medical Systems, this partnership will develop imaging agents and multifunctional nanoscale drug delivery vehicles targeted to medulloblastoma, the most common brain tumor in children. This partnership will focus on building on its previous research and developing translational efforts to bring this technology into the clinic. The team has expertise in pediatric brain cancer, tumor molecular biology, magnetic resonance imaging, and materials science.

Global Enterprise for Micro-Mechanics and Molecular Medicine

In October 2005, leaders of 10 research universities from around the world gathered at the Massachusetts Institute of Technology (Cambridge, MA) to launch an international collaboration to use nanotechnology tools for global health and medical research. The members include the National University of Singapore and Institut Pasteur of France. The collaboration, called GEM4, or Global Enterprise for Micro-Mechanics and Molecular Medicine, represents an ambitious effort to apply global sourcing principles to research at the intersection of engineering and life sciences. The initiative could herald a new model for international research, with far-flung researchers sharing their expertise in person, online, and through teleconferencing. GEM4 will use tools like atomic force microscopes, laser tweezers, and nanoscale plate stretchers to study changes in human cells for research projects on infectious diseases like malaria and sickle cell anemia, cancers of the liver and pancreas, and

cardiovascular diseases. One of the aims will be to discover connection between the development of the disease and the ability of a cell to change shape, move through the body, and stick to a blood vessel wall.

Role of Nanobiotechnology in Personalized Medicine

As mentioned in various chapters of this book nanobiotechnology will play an important role in the development of personalized medicine. Personalized medicine simply means the prescription of specific therapeutics best suited for an individual. It is usually based on pharmacogenetic, pharmacogenomic, and pharmacoproteomic information but other individual variations in patients are also taken into consideration. A detailed account of personalized medicine is given elsewhere (Jain 2007b). Apart from refinements in molecular diagnostics, an important basic of personalized medicine, nanobiotechnology also helps in the discovery of biomarkers that are crucial for the development of personalized medicine. A good example of application of nanobiotechnology for personalized medicine is that of cancer.

Nanobiotechnology for Personalized Management of Cancer

In case of cancer the variation in behavior of cancer of the same histological type from one patient to another is also taken into consideration. Personalization of cancer therapies is based on a better understanding of the disease at the molecular level and nanotechnology will play an important role in this area (Jain 2005e). Various components of personalized therapy of cancer that are relevant to nanobiotechnology are shown in Fig. 18.1.

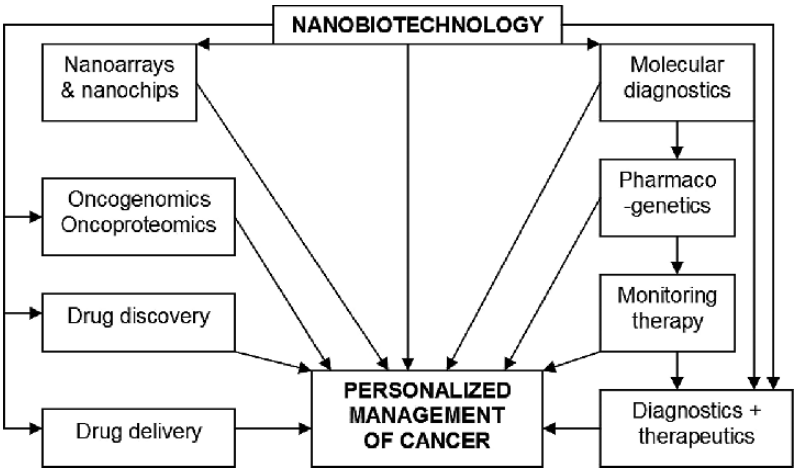

Fig. 18.1 Role of nanobiotechnology in personalized management of cancer
Source: Jain PharmaBiotech.

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Index