Sougata Jana
Subrata Jana *Editors*

Particulate Technology for Delivery of Therapeutics

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Preface

Particle technology is a term used to refer the science and technology related to the handling and processing of particles. The production of particulate materials, with controlled properties is of major interest to a wide range of applications, including chemical, food and pharmaceutical industries.

Nanoparticle technology appears to be promising nanotechnology in many engineering and industrial fields including nanomedicine in drug delivery, biotechnology and tissue engineering.

This book focuses on novel particulate carriers for the purpose of drug delivery to humans. Nowadays, macro- and nanoscale particles are being investigated for targeted delivery of small and large biological macromolecules. The targeting of drugs can minimize the dosage regimen and reduce dose-related potential toxicity of drug molecules, which in turn may lead to increased patient compliance. Various types of organic, inorganic and polymer particles are currently being investigated and attracted a lot of attention of the research workers in the field of drug delivery science and technology.

The purpose of this book is to discuss state of the art of some novel particle technology for the delivery of therapeutics.

In the introductory chapter, the need for the development of polymer-based micro- and nanoparticulate systems and other particulate systems designed so far have been described along with their fabrication technology and an overview of their therapeutic applications.

Cancer is one of the leading causes of death worldwide. Though a number of anticancer drugs are available, the development of effective delivery strategies still poses a problem for the pharmaceutical scientist. Therefore, the delivery of anti-cancer therapeutics represents an important area of current research. The scientists are putting their utmost effort to deliver effective anti-cancer therapeutics using particulate carriers. Therefore, Chapters "The Development and Achievement of Polymeric Nanoparticles for Cancer Drug Treatment" and "Nanotechnology Based Immunotherapeutic Strategies for the Treatment of Cancer" have been devoted to the discussion on polymeric nanoparticles and immunotherapeutic strategies for cancer drug treatment.

Because cancer stem cells (CSCs) can initiate and repopulate tumours, failure to control CSCs can potentially lead to tumour regrowth, even though the bulk tumour

may have been treated successfully. Nanocarriers have recently been employed to deliver therapeutics for reducing the population of CSCs at the tumor site with great success. In Chapter "Nano-therapeutic Approaches for Targeting Cancer Stem Cells", various nanotherapeutic strategies meant for targeting signalling pathway and subsequently the cancer stem cells have been discussed.

The core–shell structures of dendrimer have enabled to entrap numerous of various drugs in their core and thus provides an opportunity to deliver drugs by designing different nanoscale polymer-based dendrimers. An overview of dendritic nanostructure towards application in drug delivery is given in Chapter "Dendrimers as Nanostructured Therapeutic Carriers".

Nowadays a great deal of attention has been paid to the development of natural polymer-based particulate systems due to their biodegradable, biocompatible and nontoxic nature. Chitosan is the most widely studied biomaterial in drug delivery. Hence, Chapter "Chitosan Based Nanoparticulate Systems: Implication Towards Therapeutics Application" has been dedicated to illustrate the updated information emphasizing its potential therapeutic applications. Sometimes, native natural polymers are not found suitable for designing hydrogel particles in an aqueous media. Hence, chemical modifications of their structures are required for hydrogel preparation. In particular, carboxymethyl derivatives have been investigated in details. Hydrogel are 3D polymer network that are being considered as an effective delivery devices. The hydrogel allows controlling the release of drug in response to variable pH of gastrointestinal tract. Chapter "Carboxymethyl Polysaccharide-Based Multiunit Hydrogel Systems for Drug Delivery" has been introduced to cover this aspect.

Lipids possess a solid lipid core matrix that can solubilize lipophilic molecules. Colloidal lipid carrier-based strategies have been developed for the delivery of drugs to intestinal lymphatic. Chapter "Lipid Carriers: Role and Applications in Nano Drug Delivery" highlighted the design of lipid nanoparticles as controlled drug release carriers.

More than 40% of the drugs suffer from poor water solubility and thereby cause erratic absorption of drug. In recent days, nanocrystal technology has been emerged as a viable option for improving their solubility and bioavailability. Chapter "Nanocrystals for Delivery of Therapeutic Agents" discusses the recent development in nanocrystals technology.

In Chapter "Inorganic Nanocomposites—A New Paradigm in Drug Delivery", the preparation, characterization and drug delivery aspects of inorganic nanocomposites such as mesoporous silica, Quantum dots, carbon nanotube, Iron oxide nanoparticles, etc., are described.

Currently, a popular area in nanomedicine is the implementation of plasmonic gold nanoparticles for cancer diagnosis and photothermal therapy, attributed to the intriguing optical properties of the nanoparticles. Gold nanoparticles have emerged as promising agents for cancer therapy and are currently being investigated as drug carriers and other medical applications. The biomedical applications of gold nanoparticles have been detailed in Chapter "Green Synthesized Gold Nanoparticles for Future Biomedical Applications".

With the advent of biotechnology a number of macromolecules are available to their unprecedented level of purity, which are believed to be non-immunogenic, but their cellular targeting is a challenge for the pharmaceutical scientist. Cationic polymers have been found useful as vector for gene targeting. Hence, this subject entitled "cationic polyelectrolyte vectors in gene delivery" is discussed in Chapter "Cationic Polyelectrolyte Vectors in Gene Delivery". In the last chapter, the impact of nanoparticulate system to our defense system has been included in Chapter "Nanoparticulate Immunotherapy: An Intelligent Way to Tailor Make Our Defense System".

The book is useful for the students, researcher scholars, industry personnel and scientists in the area of pharmaceuticals, biological and material science.

We express our sincere gratitude to all the authors for their contribution to edit this book. We also thank to the publisher for their continuous support for completion of this reference book.

Asansol, India Prof. Sougata Jana
Amarkantak, India Prof. Subrata Jana

Contents

About the Editors

Prof. Sougata Jana is working at the Department of Pharmaceutics, Gupta College of Technological Sciences, under Maulana Abul Kalam Azad University of Technology, West Bengal, India. He is an M. Pharm (Pharmaceutics) from Biju Patnaik University of Technology (BPUT), Odisha, India. He is engaged in research for 10 years and that of teaching for 9 years. He qualified GATE examination in the year 2005. He received "Gold Medal" from West Bengal University of Technology (WBUT), Kolkata for standing first at UG level. IPA Bengal branch, Kolkata, India conferred him "M.N Dev Memorial Award" for securing the highest marks in the state of West Bengal in the year 2005. He bragged 'Best Poster Presentation Award' at 21st West Bengal State Science and Technology Congress-2014, Burdwan, and "Outstanding Paper Award" 1st Regional Science and Technology Congress-2016, Bardhaman Division. Organized by DST, Govt of West Bengal & Bankura Christan College, West Bengal, India. He published 30 research and review papers in different national and international peer reviewed journals. He is edited in four books in Elsevier, Springer and Pharmamedix India Publication Pvt. Ltd. More than 25 book chapters are also in his credit in Elsevier, Wiley VCH, CRC Press, Taylor & Francis group. He is reviewer of various international journals such as Elsevier, Wiley, Springer, Taylor and Francis, Dove Press etc. He is a life member of Association of Pharmaceutical Teachers of India (APTI) and Associateship (AIC) from Institution of Chemists, India. He successfully guided 16 postgraduate students for their research projects. He is working in the field of drug delivery science and technology including modification of synthetic and natural biopolymers, microparticles, nanoparticles, semisolids and interpenetrating network (IPN) system for controlled drug delivery.

Prof. Subrata Jana obtained his PhD in organic chemistry from Indian Institute of Engineering Science and Technology (IIEST), Shibpur, India. His doctoral work was based on design and synthesis of abiotic receptors for the recognition of biologically active neutral molecules and ions along with development synthetic methodologies. He is also studied the recognition process in solution phase as well as in solid state. Overall he extensively studied on supramolecular behaviour of the host-guest interaction. Besides, he has worked on the development of synthetic methodologies for substituted heterocyclics like pyrimidines, naphthyridines, quinoline and diazepines etc. by exploiting microwave protocol for green chemical synthesis. After that he moved to University of Victoria, Canada, to work with Dr Fraser Hof on supramolecular and medicinal chemistry as a post-doctoral fellow, where he worked on the synthesis of different receptors targeting N-methylated protein residue along with anions. He then worked further with Dr Kenneth J Woycechowsky at University of Utah, USA, on protein engineering and enzyme catalysis as post-doctoral research associate. He studied of enzyme activity when it is encapsulated inside the capsid which is a nano carrier and an excellent delivery vehicle for important biological substrate including drug molecules. Presently, he is working as Associate Professor at Department of Chemistry, Indira Gandhi National Tribal University, Amarkantak, Madhya Pradesh, India and his current research focuses on design and synthesis of artificial receptors for the recognition of anions, cations and N-methylated protein residue. He is also working on biodegradable polymeric based carrier systems for the delivery of drug molecules by collaboration with pharmaceutical scientist. So far he has published $\sim$40 research paper in peer reviewed international journals and contributed more than 10 book chapters in different edited books published by internationally renowned publishers. He also serve as editorial board member for the Journal of PharmaSciTech (ISSN: 2231-3788) and International Journal of Scientific and Engineering Research (ISSN: 2229-5518) as well as reviewer for International Journal of Biological Macromolecule (Elsevier), Journal of PharmaSciTech and Current Pharmaceutical Design (Bentham).

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Introduction to Novel Therapeutic Carriers

Sougata Jana, Suma Oommen Sen and Kalyan Kumar Sen

Abstract

The scope of drug delivery has developed immensely in the past few decades by introducing a wide range of advanced drug delivery systems. Conventional forms of drug delivery system are generally based on pills, tablets, capsules, eye drops, ointments, and parenteral formulations. In recent times, different novel drug delivery methods have been studied. Some of the methods are chemical modification of drug, liposome that are administered into the bloodstream, and drug incorporated within pumps or polymeric materials those are administered orally, or through parenteral route or implanted in desired bodily compartments (for example, the eye or beneath the skin). This development causes increased therapeutic activity compared to the intensity of side effects, decreasing the required dose during treatment, or eliminating the need for frequent injections. Thus the newer types of delivery systems improve human health and patience compliance, and continuous research on this may transform the way many drugs are delivered.

Keywords

Drug delivery systems · Methods of preparation · Kinetic model · Microparticles · Nanoparticles

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1 Introduction

Pharmacotherapy means the treatment and prevention of sickness and disease by means of drugs of chemical or natural origin. It includes various methods of medical treatment, along with surgery, physical treatment, radiation, and psychotherapy. Pharmacotherapy, in combination with better sanitation, diet, and housing, has improved general health of the people, so the life expectancy and quality of life are also increased. The tremendous developments of genomics and molecular biology today also present many new drug targets. The use of in silico drug design and fast advances in immunology, cell science, and microbiology enable the synthesis of a large number of new drug molecules, biologics, and vaccines in a very short span of time. Though development of appropriate dosage forms or drug delivery systems for all these new drug molecule and vaccine candidates are required to administer these bioactive compounds to the patient effectively and safely, the drug delivery system significantly manipulates the pharmacological effect of the drug, the pharmacokinetic profile, the rate of drug release, and subsequently the adverse effect profile.

Conventional dosage forms include compressed tablet, capsules, solutions, suspensions, semisolids such as creams ointments, etc. But these types of dosage forms have several limitations. They require higher dosage; they often show lower effectiveness, toxicity, and adverse side effects.

An optimal drug delivery system ensures the availability of the drug at the site of action at optimum concentration for correct time and longer duration. The optimum drug concentration at the appropriate site should be within the therapeutic window of the drug that means the plasma concentration should be more than the minimal effective concentration (MEC) and less than the minimal toxic concentration (MTC). Throughout the world scientists are working on various types of drug delivery systems to overcome the limitation of the conventional dosage forms and improve their potential. Researchers are also focusing on the interaction between microenvironment of the cells and these types of novel delivery system (Li et al. 2011a, b). These novel drug delivery systems encouraged the old concept of the magic bullet as projected by Paul Ehrich's vision (Haag and Kratz 2006). To bring new drug molecule in market is a very costly affair and also time consuming. But improving risk–benefit ratio of "old" drugs using newer technologies is explored to individualized drug therapy, decreasing dose selection, and therapeutic drug monitoring. Researchers have investigated different technologies to deliver drug at controlled rate, slow delivery rate, and targeted drug delivery system (Panchagnula 1997; Rao and Diwan 1997, 1998; Nayak et al. 2011; Jana et al. 2013a). The therapeutic benefits of these new systems include the following:

- More effectiveness of the drug
- Tissue or organ specific delivery
- Decreased toxicity/side effects
- Decreased healthcare costs—both short and long term
- Better patient agreement.

This new kind of dosage forms is developed by various polymeric materials and smart techniques. Microencapsulation has been important to the fabrication of new drug delivery systems and widely utilized to produce both hydrophilic and hydrophobic drugs entrapped microspheres within biocompatible polymers. These carriers controlled the release of drug and thus maintain therapeutic drug levels for a specific time period thus reducing systemic absorption (Thompson et al. 2007). Microparticles like microcapsules and microspheres can be made of polymers or lipids (liposomes) with sizes ranging from 1 to 250 μm (ideally <125 μm and exceptionally 1000 μm) (Allemann et al. 1998; Couvreur and Puisieux 1993).

Researchers throughout the world are investigating these various types of drug delivery systems to minimize the limitation of conventional dosage forms as mentioned above and increase the efficacy of the particular drug. On the other hand, microenvironment of the cells and their interaction with these new dosage forms are also widely studied (Li et al. 2011a, b).

2 Type of New Drug Carriers Systems

From the introductory discussion we can understand that drug delivery devices can modify drug release profile and pharmacokinetic parameters of the drug for the advantage of improving product effectiveness and safety, patient convenience, and conformity. Most common routes of administration include the preferred noninvasive peroral, topical (skin), transmucosal nasal, buccal/sublingual (Jana et al. 2010), vaginal, ocular, and rectal and inhalation routes (Bertrand and Leroux 2011). Because of their destruction due to enzymatic degradation or less absorption into the systemic circulation efficiently due to large molecular size and charge issues, drugs like peptide and protein, antibody, vaccine, and genetically engineered drug generally may deliver by injection or a nano-needle array. For example, many immunizations are based on the delivery of protein drugs and are often done by injection. Recent efforts in the vicinity of drug delivery include the development of targeted delivery, sustained release formulations, and smart drug deliveries. So the set of most relevant new drug delivery systems is deduced as follows:

(a) Transdermal Delivery Systems
(b) Carrier-Based Delivery Systems
    - Liposomes
    - Monoclonal Antibodies
    - Nanoparticles
    - Microspheres

(c) Variable Release Delivery Systems

- Osmotic Pump
- Reservoir based
- Matrix based

The purpose of these controlled drug delivery systems is at least one of the following:

- Sustain drug action at predetermined rates.
- Localize drug action by placing a rate-controlled system near or at the desired tissue or organ.
- Target drug action by using carriers or chemical derivatization to deliver drug to a particular site.

2.1 Transdermal Drug Delivery System

Transdermal drug delivery systems are layered patches which stick to the skin and allow permeation of drugs to the skin surface through its different layers into the systemic circulation, at steady rates, ensuing in sustained blood levels. These patches are used to fabricate new applications for presently available drugs that reduce the first-pass drug degradation effects. Transdermal drug delivery systems also decrease untoward side effects, for example, oestradiol patches do not cause liver damage (Cramer and Saks 1994); clonidine, nitroglycerin, and fentanyl patches demonstrate decrease adverse effects as compared to conventional oral dosage forms. In some studies it has been observed that solid lipid nanoparticles can permeate human and pig skin ex vivo more fast and to a higher extent than existing dosage forms and a nanoemulsion due to an initial burst release followed by water evaporation (Korting and Schafer-Korting 2010). Studies also exposed that permeation through skin expected to be increased in association with dendrimers because of their ability to interact with lipid bilayers in the skin (Kaminskas et al. 2011). Transdermal transport of hydrophilic drugs is more challenging than hydrophobic drugs due to their low permeabilities. However, researchers are investigating using penetration enhancers to increase the permeability of hydrophilic drugs through skin (Peck et al. 1994; Mitragotri 2003). Permeation of hydrophilic drugs through skin surface can also take place through hair follicles and sweat ducts (that is, shunt pathways) that could permit diffusion of solutes not only across the stratum corneum, but also across the epidermis. Recently, scientists investigated arrays of microscopic needles for transdermal drug delivery (McAllister et al. 2000). Transferosomal gel was studied for delivery of peptide insulin through transdermal route (Malakar et al. 2012). Needles of micron dimensions are used to pierce into the skin surface to produce holes large enough for molecules to enter without producing pain or significant damage. Studies have

revealed that insertion of microneedles into skin surface can augment the permeability in the orders of magnitude for small drugs, large macromolecules, and nanoparticles (Henry et al. 1998; McAllister et al. 2003).

2.2 Carrier-Based Delivery Systems

A wide variety of drug carrier systems have been fabricated and studied; everyone has unique merits and demerits. Some of the widely studied drug carriers include liposomes, polymeric micelles, microspheres, and nanoparticles (Svenson 2004; Jana et al. 2014). Different methods of tailoring the new carrier-based dosage form have been implemented, including adsorption of drug on the carrier molecule, integration into the bulk matrix structure, encapsulation, and covalent bonding (Zhang et al. 2013).

The use of biocompatible and biodegradable polymer to fabricate microparticles is advantageous because they are easily cleared by physiological systems thus avoiding the possible cytotoxicity caused by accumulation in cells and tissues. Drugs may be either adsorbed at the surface of the polymer or encapsulated within the particle. In addition, smart drug delivery systems can be fabricated using pH-sensitive (especially useful in intravenous delivery) and/or thermo-sensitive polymers (Gandhi et al. 2015). Microparticles have been used to encapsulate several peptides (e.g., calcitonin and insulin), anesthetics, anti-viral drugs, antihypertensive, anticancer drugs, vaccines, etc. (Allemann et al. 1998; Freiberg and Zhu 2004). But nanoparticles offer marked advantages over microparticles (Panyam and Labhasetwar 2003; Pinto Reis et al. 2006).

For example, PLGA micro- and nanoparticles were compared for their uptake in caco-2 cells and revealed a higher uptake from nanoparticles (Desai et al. 1997). Moreover, targeting to specific tissues such as inflamed and cancerous tissues may be limited only to nanoparticles but microparticle-based systems are also thoroughly investigated for targeting different parts of GI tract (Avnir et al. 2011). There are a number of factors inspiring interest to engineer these new devices, concepts, and techniques. Microparticle-based drug delivery system may tackle the shortcomings of the current oral drug delivery systems by combining the approaches like the use of protective coatings (Saffran et al. 1990), targeted delivery (Fara et al. 1985), permeation enhancers (Fasano and Uzzau 1997), protease inhibitors (Schwarz et al. 2000), and bio-adhesive agents (Arangoa et al. 2000; Lehr 2000; Bies et al. 2004) in a single drug delivery platform. Unlike other spherical drug delivery particles, microparticulate systems may be fabricated to be flat, thin, and disk-shaped within the range of diameters of 1–500 μm to maximize contact area with the intestinal mucin lining and reduce the contact to the liquids flow in intestine (Tao and Desai 2003; Jana et al. 2015) and large enough to avoid endocytosis of the entire particle. Permeation enhancers, such as bile salts and metal chelating agents, can be added to slacken the tight junctions of the intestinal epithelium. Enzyme inhibitors can also be added or protective coatings can be made to protect the macromolecule from intestinal degradation. Thiolation of the delivery

system using relatively simple surface chemical modification can provide the system more bio-adhesive. Microparticles or nanoparticles may be used to target specific cell types and tissues for therapy as well as imaging by attaching specific markers. This helps to deliver the drug at high concentration locally while maintenance the systemic concentration at a low level. Again these devices can have several reservoirs of desired size to contain not just one, but also many drugs or biomolecules of interest (Martin and Grove 2001). Khandai et al. (2010) studied the preparation of algino-sericin mucoadhesive microspheres and also evaluate their mucoadhesiveness. They concluded that this system can be used for sustained drug delivery. Studies have demonstrated an increased understanding of the pharmacokinetic and pharmacodynamic principles that influence the action and disposition of potent opioid analgesics, inhalation anesthetic agents, sedative/hypnotics, and muscle relaxants. Studies are also performed to develop delivery systems those utilize skin and buccal and nasal mucous membranes as alternate routes of drug delivery with controlled release technology (Thacharodi and Rap 1995; Krishna and Pandit 1996; Bhat et al. 1995).

Biodegradable polymeric nanoparticles are extensively studied to encourage a new idea of chemotherapy, which may comprise sustained, controlled, and targeted chemotherapy. For last 30 years there has been a substantial research attention in the area of drug delivery using particulate delivery systems as carriers for small and large molecules. Nanoparticulate systems have been utilized as a physical method to alter and increase the pharmacokinetic and pharmacodynamic properties of various types of drug molecules (Jana et al. 2013b). The major goals in developing nanoparticles as a delivery system are to regulate the particle size, surface properties, and release of drugs in order to attain the site-specific action of the drug at the therapeutically optimal rate and dose schedule. Targeted nanoparticle to deliver chemotherapeutic agents in cancer therapy is a very useful strategy that offers many advantages to advance drug/gene delivery and to minimize many problems associated with conventional cancer treatment (Koo et al. 2011; Ali et al. 2011; Heidel and Davis 2011). Based on their composition, function, and morphology, nanoparticles are classified as nanocrystals, polymeric, liposomal, nanosuspensions, and solid lipid nanoparticles (SLN). Recently poly(ethylene glycol) (PEG)-coated biodegradable polymeric nanoparticles, known as long circulating particles, have been studied as prospective drug delivery strategy because of their ability to circulate for a prolonged period of time(Larsen et al. 2009), ability to target a specific organ, use as carriers of DNA in gene therapy, and their low cytotoxicity (Anbarasu et al. 2015). Studies have shown that due to their unique nanosize structure, nanoparticles behave differently both in vitro and in vivo in comparison to the conventional microparticles. It has been observed that large proportion of solid drugs have poor aqueous solubility, and so poor bioavailability. Nanoparticles provide significant higher surface area to volume ratio that results in increased solubility of poorly water-soluble drug. Noyes–Whitney equation illustrates that the surface area available for dissolution influences the dissolution rate of API in proportionate manner and an increase in the solubility of nanosized API is also anticipated according to the Ostwald–Freundlich equation (Müller et al. 1998).

Drugs can be entrapped into nanoparticles by several methods: (1) entrapped in the polymeric matrix; (2) encapsulated in a nanoparticle core; (3) chemically conjugated to the polymer; (4) surrounded by a shell-like polymer membrane; and (5) adsorbed on particle surface.

Various materials such as proteins, polysaccharides, and synthetic polymers are used to fabricate nanoparticles. Several factors like size of the nanoparticles, intrinsic properties of the drug, e.g., aqueous solubility and stability, surface characteristics such as charge and permeability, degree of biodegradation, biocompatibility, and toxicity and antigenicity of the final product affect the selection of matrix materials. Studies revealed that the membrane stability and drug leaching problems associated with liposomes and emulsions can be overcome by using polymeric nanoparticles and they also facilitate delayed drug release. Polylactic acid and polylactide co-glycolides based polymeric nanoparticles have excellent biodegradability and low toxicity. Polymeric nanoparticles prepared by the dispersion of drugs in an amorphous form within a polymer matrix could be fabricated as nanospheres that consist of dispersion of the drug uniformly throughout the matrix or as nanocapsules.

2.3 Variable Release Delivery Systems

2.3.1 Osmotic Pump-Based Drug Delivery Systems

Osmotic systems operate on the principle of osmotic pressure for the drugs release in the body after administration. The principle for osmotic pumps utilizes the tendency of a liquid to equalize the concentration of dissolved substances on both sides of a semi-permeable membrane. The release of drug from osmotic pump-based dosage forms is independent of body pH and other physiological parameters to a large extent. It is possible to alter the release characteristic by optimizing the properties of drug and systems and it is a variable release system (Verma et al. 2002; Xing-Gang et al. 2006). It can also be utilized to deliver drugs at a restricted and programmed rate. The oral osmotic pumps have been developed in various forms, and products are available on this technology and number of patent granted in the last few years that makes its presence felt in the market (Higuchi and Leeper 1973, 1976). The osmotic drug delivery system offers several advantages. The release rates of drug from this system provided are higher than diffusion-controlled systems. The biological environmental influences like pH, gastro-intestinal motility, etc., on the system are very less. Drug properties also are not significantly affecting this system. The delivery rates can be predicted and programmed. These systems provide satisfactory in vitro and in vivo release profiles. The side effects of drugs administered can be lowered. The frequency of dosing is reduced so better patient compliance. The device is tamper resistant and provides controlled delivery of drugs utilizing osmotic pressure as driving force. Elementary osmotic pump consists of an osmotic core (containing drug with or without an osmogent) coated with a semi-permeable membrane which permits the aqueous fluids to enter at a rate influenced by the fluid permeability of the

membrane and osmotic pressure of core formulation (Jerzewski and Chien 1992). This causes the release of the drug from the system at a controlled rate. Push–pull osmotic pumps are utilized for administration of drugs having extremes aqueous solubility. This system is composed in the form of bilayer tablet coated with a semi-permeable membrane. The upper compartment contains drug along with osmogen and the lower compartment contains a polymeric osmotic agent. The upper drug compartment has a fine orifice to attach the outer environment. When this system comes in contact with body fluids, the polymeric compartment swells and pushes the drug compartment to release the drug dispersion at controlled rate (Cortese and Theeuwes 1982). Controlled porous core osmotic pumps are made of water-soluble additives in the coating membrane, which in contact with body water, dissolves resulting in an in situ microporous membrane formation. The resulting membrane is largely permeable to both water and dissolved solutes and the mechanism of drug release from these systems was observed primarily as osmotic, and the simple diffusion is playing a minor role (Zentner et al. 1985). Controlled release delivery of drugs using osmotic pump-based systems may decrease the side-effect profile. Since plasma levels are remaining above the Minimum Effective Concentration longer in osmotic systems, trough plasma levels over the dosing interval can be avoided. However, use of this system is limited due to a complicated manufacturing process and high cost as compared to existing dosage forms. Yet once-daily formulations based on osmotic principles are playing an increasingly significant role in improving patient compliance.

2.3.2 Reservoir-Based System

The reservoir-based system is one of the widely utilized controlled drug delivery systems to date and provide unique advantages. In these systems, a drug core is coated with a polymeric film, its properties (e.g., polymer composition and molecular weight), the thickness of the coating, and the physicochemical properties of the enclosed drug, such as solubility, drug particle size, and molecular weight control the drug release rate (Langer 1990; Freiberg and Zhu 2004). Reservoir-based systems may be either external to the body or implanted and they also allow a well-controlled environment for a drug formulation with increased drug stability and prolonged delivery times. Various novel delivery systems, including zero order, pulsatile, and on demand dosing, as opposed to a standard sustained release profile are developed using reservoir systems. Reservoir-based systems are beneficial for one of the following two applications: (1) a mid-/long-term administration of a medication that is localized to a specific region (i.e., organ, body cavity, etc.). This practice is usually utilized if it is difficult to reach the targeted area via systemic administration (i.e., eye, ear) and/or the drugs administered are toxic and may need a long-term therapy (i.e., cancer treatments). (2) A drug depot for long-term systemic administration. This is generally administered as an intramuscular or subcutaneous injection or implantation.

2.3.3 Matrix-Based Drug Delivery System

The greater part of oral drug delivery systems are matrix-based. Swellable monolithic matrices are fabricated by compression of a powdered mixture of a hydrophilic polymer and a drug. The fabrication of superior dosage form is strictly influenced on the selection of an appropriate carrier that is able to activate control of the drug delivery. Mainly three types of polymers, biocompatible, biodegradable, and smart or stimuli responsive polymers, are used as carrier to develop matrix-based system. Swellable polymers although may not respond to the presence of triggering stimuli such as pH, ionic strength, and temperature, but act in response to the presence of water or biological fluids, which change the polymer structure, allowing the drug to be released from the dosage form at controlled rate. Particularly the high-consistency grade of hydroxypropyl methyl cellulose (HPMC) is a polymer that is every now and again utilized as a part of swellable networks manufacturing (Alderman 1984; Hogan 1989). Different polymers, for example, Carbopol or characteristic gums, for example, xanthan and guar, that show practically identical performance have been proposed for use as a transporter in swellable matrices. Every one of these polymers is generally nonreactive to the pH of the medium. However, a reliance on pH could be viewed as very useful in certain delivery applications, for example, colon delivery. Swellable drug delivery system must be separated from genuine swelling-controlled delivery systems (Peppas 1987). Genuine swelling-controlled delivery systems are nonporous matrices in which the drug is basically made immobile when the polymer is in dry glassy state, yet generally versatile when the polymer is wet and in the rubbery state. In pharmaceutical practice, swellable matrices are solely permeable dosage form prepared by compacting a powdered blend of a drug and a hydrophilic polymer. After that in the system the glassy–rubbery transition of the polymer makes a gel layer that goes about as a hindrance restricting water and medication transport. At the point in the presence of body fluid a matrix that contains a swellable glassy polymer change from the glass to the rubbery state due to assimilation of water so that their end-to-end separation and radius of gyration rise to solvated state (Lee and Peppas 1987). This occurs due to fall of transition temperature of the polymer (Tg), which influenced by concentration of the body fluid, temperature, and thermodynamic interactions of the polymer–water system. A sharp change from glassy state to rubbery state increases the volume of swelling slowly. These results an anomalous non-Fickian transport of the drug related to the relaxation process of the polymer chain. So drug in the outermost layer exposed to the body fluid entrapped is dissolved first and then diffuses out of the matrix system. That causes this system to be diffusion controlled; the rate of dissolution of drug particles inside the matrix base should be more rapidly than the diffusion rate of dissolved drug that exit from the matrix system (Brahmankar and Jaiswal 2009).

3 Preparation of Particulate Drug Delivery Systems

Particulate drug delivery systems (DDS), like particulate carriers, are composed chiefly of lipids and/or polymers, and their related drug. This novel technique is used to prepare controlled and tailored drug delivery. They are advantageous over other systems, including reduced toxicity, predictable bioavailability, decrease in dose dumping, decrease in fluctuations in plasma concentration of drug and high dose-strength administration. Particulate systems demonstrate more repeatable pharmacokinetic behavior and less intra- and inter-subject inconsistency than conventional (i.e., monolithic). Pelleted tablets decrease the esophageal residence time, compared with capsules, and increase physicochemical stability, compared with suspensions (Abdul et al. 2010). Particulate carrier system generally covers less than 10 nm to a few millimeters. Generally, drug attachment with a carrier causes decreasing drug clearance (the half-life increases), so the volume of distribution lowers, and increase in the area under the time-versus-concentration curve occurs (Gabizon et al. 2003). For a big particulate carrier, such as liposome, polymer–drug conjugate, and nanosphere, the size of the carrier (normally 50–200 nm in diameter) restricts it mainly to the systemic circulation, and the volume of distribution of the carrier associated drug will come near that of the plasma volume when slower release rate of the drug. In a study it has been observed significant lowering of toxicity occurred in case of liposome-encapsulated paclitaxel, in addition to improvement in plasma AUC and half-life (Cabanes et al. 1998). Most polymer–drug conjugates and liposomes follow an intermediate position between these two extremes; the PK and PD of a system are a combination of the PK and PD of the free drug and the PK and PD of the carrier, with the equilibrium depends on the rate of drug release from the delivery system.

According to size they are classified as

(1) Microparticulate drug delivery system and
(2) Nanoparticulate drug delivery system.

Particulate drug delivery systems are prepared by several methods. Method selection is influenced by several factors like particle size, thermal and chemical stability of the drug, repeatability of the release kinetic profiles, stability and residual toxicity associated with the final product, the nature of the active molecule, as well as the type of the delivery device.

Micro/Nanoparticles can be prepared by several methods which are very useful techniques.

1. Dispersion method

   a. Solvent evaporation method
   b. Emulsification or solvent diffusion method

2. Coacervation and coalescence method
3. Polymerization method
4. Spray drying
5. Emulsion cross-linking method
6. Reverse micellar method
7. Supercritical method.

3.1 Dispersion Method

Nanoparticle can be fabricated by the uniform dispersion of the drug in biodegradable polymers like PEG, PLA, and PLGA. There are various ways to utilize this method which is given below (Kompella et al. 2001; Jana et al. 2013c).

3.2 Solvent Evaporation Method

Polymers or matrix are dissolved in organic volatile solvent like chloroform, dichloromethane, ethyl acetate, and hydrophobic drugs are also dissolved in that solvent. Then emulsification of this solution of polymer and drug with aqueous solution of surfactant or emulsifying agents is done to form oil in water emulsion. Organic solvents are removed by evaporation under reduced pressure and continuous stirring. Size of particles is influenced by various factors like concentration of the stabilizers, homogenizer speed, and nature of polymers (Zambaux et al. 1998).

3.3 Emulsification or Solvent Diffusion Method

It is a new form of solvent evaporation method; it includes water miscible solvent along with water immiscible solvent. Tiny nanoparticles are developed due to spontaneous diffusion of solvent and development of interfacial turbulence activity between the two phases. If the concentration of water miscible phase is increased, then particle size is reduced and obtained the good nature of nanoparticle. Solvent evaporation and solvent diffusion methods are used for both hydrophilic and lipophilic drugs (Niwa et al. 1993).

3.4 Coacervation Method

For the preparation of microparticle and nanoparticle, many natural and synthetic polymers like chitosan, gelatin, eudragit, and sodium alginates are used in coacervation method. This was the first reported process to be adapted for the industrial fabrication of microcapsules. Right now, two strategies for coacervation are accessible, to be specific simple and complex procedures. The principle of microcapsule and nanocapsule development for both procedures is identical, aside from

the way in which the polymer is separated. In simple coacervation a desolvation agent is included for phase partition, while complex coacervation includes complexation between two oppositely charged polymers (Huang et al. 2012).

There are three fundamental phases in complex coacervation: (i) formation of three immiscible phases, (ii) deposition of coating material, and (iii) rigidization of the coating material. The initial phase incorporates the formation of three immiscible stages: fluid accumulating vehicle, core material, and coating material. The core material is dispersed in a solution of the coating polymer. The coating material part is generally an immiscible polymer in liquid state, which causes coat formation by (i) change of temperature of polymer solution, (ii) addition of salt, (iii) addition of non-solvent, (iv) addition of incompatible polymer to the polymer solution, and (v) inducing polymer–polymer interaction. The second step includes deposition of liquid polymer upon the core material. Finally, the prepared microcapsules are stabilized by cross-linking, desolvation, or thermal treatment. Aspirin-loaded albumin nanoparticles were prepared by coacervation process followed by cross-linking with glutaraldehyde (Das et al. 2005). Merodio et al. (2002) had developed the ganciclovir-loaded albumin nanoparticles. In a study by Chen et al. (2007) it was experientially proved that chitosan–DNA nanoparticles had been formed readily by coacervation between the positively charged amine groups on the chitosan and negatively charged phosphate groups on the DNA. Oppositely charged polyelectrolyte used in complex coacervation process has been widely utilized to develop and strengthen natural polysaccharides-based particles. The modification of one polysaccharides with other polymers can manipulate pore size and network complexity of the particles in order to enhance its performance as drug carrier system. Studies revealed that incorporation of other polymers such as gelatin and chitosan in alginate has been found to decrease the problem of drug leaching during preparation (George and Abraham 2006).

3.5 Polymerization Method

Polymerization occurs between the monomers in aqueous solution and formed polymerized nanoparticles. Drugs can be dissolved during the polymerization or adsorbed after polymerization on the surface of polymers. Before the polymerization, nanoparticle suspension is prepared and then purified to remove the surfactants and stabilizers, and after that polymerization will start by ultracentrifugation and re-suspending the particle into the ionic surfactant solution (Boudad et al. 2001). Zhang et al. (2010) developed a simple but effective method to prepare well-defined core–shell nanoparticles using simultaneous polymerization and self-assembly between the components. Hydroxyethyl cellulose was chosen as the template macromolecule and they developed core–shell nanoparticles composed of hydroxyethyl cellulose and poly(meth acrylic acid) successfully via template polymerization. Reis et al. (2006) reviewed different methods of preparation of nanoparticles. They observed that development of nanoparticle preparation methods is marked by three aspects: need for less toxic reagents, simple and economic procedure, and

optimization to increase yield and entrapment efficiency. Competent drug entrapment efficiency and transition to large scale are the two major important factors to be considered to industrial applicability. Now simpler, nontoxic and reproducible methods are widely used to prepare drug-loaded nanospheres and nanocapsules.

3.6 Spray Drying and Congealing

Spray drying is a low-cost commercial process which is mostly used for the formulation of microcapsules. Core materials are dispersed in a polymeric solution and sprayed into a hot chamber. The shell material rigidized onto the surface of the core particles as the solvent evaporates such that the microcapsules obtained are of polynuclear or matrix type. In one study chitosan microspheres were formulated by spray drying technique and crosslinked with three different cross-linking agents viz tripolyphosphate (TPP), formaldehyde (FA), and glutaraldehyde (GA). The effect of these cross-linking agents on the properties of spray-dried chitosan microspheres was widely studied. It was observed that the type (chemical or ionic) and extent (1 or 2% w/w) of cross-linking agents have influenced the surface morphology, percentage erosion, percentage water uptake, and drug release properties of the spray-dried chitosan microspheres (Desai and Park 2005). Spray drying equipment is used in spray congealing method where protective coating will be applied as a melt. Here coating material melts rather than a coating solution is used to disperse the core. Coating solidification is done by spraying the hot mixture into cool air stream. The coating materials used are waxes, fatty acids, and alcohols, polymers which are solids at room temperature but meltable at reasonable temperature. Albertini et al. (2009) developed mucoadhesive microparticles to design an innovative vaginal delivery system for econazole nitrate to improve the anti-fungal activity of the drug.

3.7 Emulsification-Cross-Linking Method

In this method nanoparticles are prepared by emulsification and simultaneous cross-linking by different cross-linkers. Li et al. (2011a, b) demonstrated a method to prepare a kind of alginate microspheres encapsulated protein by a novel emulsification/internal gelation technique for mass production of microspheres with small size high encapsulation efficiency. Emulsification-Cross-Linking method is used for the preparation of novel surfactant–polymer nanoparticles for optimum encapsulation and extended release of water-soluble drugs. The nanoparticles were fabricated using dioctyl sodium sulfosuccinate (Aerosol OT; AOT) and sodium alginate. AOT is an anionic surfactant that is approved as oral, topical, and intramuscular excipient (US Food and Drug Administration's Inactive Ingredients Database). Sodium alginate is a naturally occurring polysaccharide polymer, widely studied for drug delivery and tissue engineering purposes.

3.8 Reverse Micellar Method

Reverse micellar method is used to prepare inorganic nanoparticles. Reverse micelles are self-assembled nanostructures with hydrophilic head of surfactant molecules leaning inward and hydrophobic tails outward the matrix. O'Connor et al. (2001) studied the synthesis of core–shell structured iron–metal complex coated with gold nanoparticles with a total diameter of 12 nm by the reversed micelle method and anticipated many applications. Reverse micelle method is suitable to prepare core–shell nanoparticles having controlled shape using particular surfactants, for example Igepal® series (Tamil Selvan et al. 2007), AOT (Tago et al. 2002), Brij-07 or Triton X-100 (Santra et al. 2001).

3.9 Supercritical Method

Nanoparticle can be prepared by the use of supercritical fluid. This is the alternative choice for the biodegradable polymers and safe for the environment. Conventional methods like solvent evaporation, solvent diffusion, and organic phase separation create the hazardous to the environment due to the organic solvents (Jung and Perrut 2001). Supercritical fluid is defined as a solvent at temperature above its critical temperature which is remained in a single phase regardless of pressure are called supercritical fluid. Carbon dioxide (CO_2) is used as supercritical fluid which is most often used in pharmaceutical industries and has advantages of low price, nontoxic in nature, and noninflammable. For the formation of supercritical fluids many processes are involved like supercritical anti-solvent and rapid expansion of critical solution. The process of supercritical anti-solvent methanol is used and it is completely miscible with the supercritical fluid to dissolve the solute at the process condition. Solutes are insoluble in supercritical liquid and extract with supercritical fluid after that precipitation occurs and nanoparticles are formed (Thote and Gupta 2005).

4 Release Kinetics from Nondegradable Polymeric Matrices

Peroral dosage forms, transdermal films, and implant devices are widely fabricated using nondegradable polymers (Pillai and Panchagnula 2001). These polymers include polyurethanes, silicone rubber, poly (ethylene vinyl acetate), just to name a few. Materials for long-term use, such as orthopedic and dental implants, require water-repellent surfaces to avoid degradation or erosion processes. Nondegradable polymers which can serve that purposes are characterized by tissue/blood compatibility, durability, robust structure, and mechanical strength during in vivo application (Katz et al. 2004). Major mechanism of solute moves from nondegradable polymeric systems is due to diffusion. Nondegradable polymers can be fabricated into "reservoir-" and "matrix-" type devices (Fung and Saltzman 1997).

By definition, reservoir-type devices defined as those devices composed of inner core material having an inert coating material, which functions as a rate-limiting membrane. The rate of drug release remains relatively constant and does not depend on concentration gradient, but is mostly related to the thickness and permeability of polymeric membrane. In contrast for matrix-type devices, drug release is more likely to be Fickian diffusion driven, which is affected by concentration gradient, diffusion distance, and the degree of swelling (Siepmann and Siepmann 2008). Salehi et al. (2016) demonstrated a model that followed both Fickian and non-Fickian transport including anomalous and Case-II drug release profiles.

5 Release Kinetics from Biodegradable Polymeric Matrices

Biocompatible and biodegradable macromolecular materials are extensively utilized in the development of new drug delivery systems that can be advantageous to enhance patient compliance and decrease adverse effects through sustained dosing and targeting. A number of pharmaceutical products including hormones, antitumour drugs and antibiotics prepared with biodegradable polymeric systems have been marketed (Dorati et al. 2007). In general, surface degradation and bulk degradations through hydrolysis or enzymatic degradation are major cause of degradation of biodegradable polymers. Degradation in a surface degrading polymer occurs from the superficial surface of the system (Heller 1980). In a bulk degrading polymer, though, degradation takes place constantly throughout the material. One of the major causes of degradation is hydrolysis and water is the major cause of this and diffusion of water into the device is of considerable importance for the study of degradation kinetics as well as release kinetics. The degradation of semi-crystalline polymers occurs in two stages: (i) Infusion of water into the amorphous regions with arbitrary hydrolytic breakdown of susceptible bonds, such as ester bonds, occurs in the first stage; (ii) After degradation of most of the amorphous regions the second stage starts. Degradation causes the decrease of polymer chain, and hence the quantification of the degradation process over time can be measured by the change in the average molecular weight of the polymer.

Generally the gradual erosion-type dissolution of the polymer causes the drug release from soluble polymers. So dissolution of the polymer and drug diffusion may be on the whole dual mechanism of release. Drug release from insoluble hydrogels generally follows Fickian or non-Fickian diffusion kinetics (Lee 1986). The release mechanism from the dosage form may be calculated by modeling the drug release (first 60%) to the following empirical formula:

$$\frac{M_t}{M_\infty} = kt^n, \tag{1}$$

where M_t/M_∞ = fraction of drug released, k = kinetic constant, t = time, and n = diffusional exponent.

Dasgupta et al. (2016) observed this empirical model is unsuitable for drug release from an eroding matrix due to break down of the polymer backbone. They explained the release of the drug modeled as

$$-\frac{\mathrm{d}Ct}{\mathrm{d}t} = K_d C_t (M - M_{\lim})^n. \tag{2}$$

In Eq. (2), C_t is the amount of drug released at time t and k_d is the rate constant of drug release. Equation (2) considers that the release of the drug is first order with respect to drug concentration and nth order with respect to polymer degradation. M is the mass of the polymer, and n is the order of degradation. Since these polymers attain a limiting mass after a certain period of time, this mass ($M_{\lim}$) has been taken as the weight of polymer after long hour of degradation.

Diffusion is the process by which the mass of individual molecules of a substance is transferred by haphazard molecular motion and it is related with the concentration gradient. The mechanism of transfer of solute molecule is probably by simple molecular permeation or by movement through pore and channels. Diffusion is depended with the moving of solute molecule. Fick's first law of diffusion is related with the diffusive flux to concentration under assumption of steady state. It postulates that the flux goes from region of higher concentration to region of lower concentration, which is proportional to the concentration gradient. In one dimension, the law is

$$J = -D(\mathrm{d}c/\mathrm{d}x), \tag{3}$$

where J = amount of substance passing perpendicularly through a unit surface area per time, D = diffusion coefficient, and $\mathrm{d}c/\mathrm{d}x$ = concentration gradient. Negative sign indicates that diffusion occurred from higher to lower concentration.

Fick's second law of diffusion illustrates that the rate of concentration is changed in the volume with the diffusional field proportional to the rate of change in spatial concentration gradient at that point in the field. Proportionality constant is the diffusion coefficient. The law is as follows:

$$\mathrm{d}c/\mathrm{d}t = D\, d^2c/\mathrm{d}x^2. \tag{4}$$

There are some limitations in diffusion of drugs having heterogeneous structure, moving boundary condition, non-Fickian diffusion, and ionic species. In case of heterogeneous structure, each layer is made up by different materials. In such type of condition, diffusion coefficient cannot be considered to be constant throughout the system.

Fick's first and second laws are applicable to fluid flux and concentration gradient across the membrane.

The mechanism of drug release may be Fickian diffusion when the value of n in Eq. (1) is 0.5, anomalous (non-Fickian) transport when n = 0.5–1.0, and case-II transport when n = 1.0. A value of n above 1 signifies super case-II transport as the mechanism of drug release.

Higuchi's mathematical equation (Higuchi 1963) to illustrate drug release kinetics from matrix system is the probably most renowned and widely applied mathematical model which is applicable to the different geometrics and porous systems.

The extended model is based on the following hypothesis, viz…

- Initial concentration of drug in the matrix is much higher than the drug solubility.
- Diffusion of drug occurs only in one dimension (edge effect negligible).
- Drug particles much smaller than system thickness.
- Swelling of matrix and dissolution is negligible.
- Drug diffusivity constant.
- In the release environment perfect sink conditions are maintained.

The basic equation of Higuchi model is

$$C = [D(2q_t - C_s)C_s t]^{1/2}, \tag{5}$$

where

C total amount of drug release per unit area of the matrix (mg/cm^2)
D diffusion coefficient for the drug in the matrix (cm^2/h)
q_t total amount of drug in a unit volume of matrix (mg/cm^3)
C_s dimensional solubility of drug in the polymer matrix (mg/cm^3)
t time (h).

Data obtained were plotted as cumulative percentage of drug release verses square root of time.

The other release models as proposed by Kopcha et al. (1991) (Eq. (6) and **Hixson–Crowell** (Ratsimbazafy et al. 1999) [Eq. (7)] are as follows:

$$M = At^{1/2} + Bt \tag{6}$$

$$100^{1/3} - (100 - Q)^{1/3} = ct + d. \tag{7}$$

In the above equations, M ($\leq 70\%$) and Q ($\leq 90\%$) are the percentages of drug released at time, t, while c and d are constants; A and B are, respectively, diffusion and erosion terms.

Sequential Model (Siepmann 2001)

Sequential model is applied to study the swelling of hydrophilic matrix tablet and release behavior of drug from it. The effect of the device geometry on the drug release pattern can also be determined. In this model, it is considered that layer-by-layer swelling occurs; initially the swelling of first exposed layer to the medium took place followed by neighboring inner layer. This model is useful for prediction of molecules release from swelling and release behavior from tablet containing hydrophilic matrix and also useful for determination of shape and dimensions of the tablet.

Other empirical model and semi-empirical models are as follows:

Models developed by Peppas and Sahlin (1989) are

$$\frac{M_t}{M_\infty} = K_1 t^m + K_2 t^{2m}, \tag{8}$$

where k_1 and k_2 are model constants. In this equation, M_t/M_∞ is the fraction of drug released; the first term of the right-hand side of the equation explains the Fickian contribution, while the second term illustrates the Case-II relaxational contribution. The coefficient m is the only Fickian diffusion exponent for a device of *any* geometrical shape which exhibits controlled release

Weibull Model (Costa 2001)

This model has been described for different dissolution processes as the following equation:

$$C = C_0 \left[1 - \exp \frac{-(t - T)^b}{a} \right], \tag{9}$$

where

C amount of dissolved drug as a function of time "t"
C_0 total amount of drug being released
T lag time measured as a result of dissolution process parameters
a scale parameter that describe the time dependence
b shape of dissolution curve.

Due to its empirical his model cannot provide any kinetic fundamental about drug release, it has some deficiencies and limitations, such as it does not effectively exemplify the dissolution of drug kinetic properties, intrinsic dissolution rate of the drug cannot be described, and it is not able to establish in vivo/in vitro correlations.

6 Concluding Remarks

This chapter has illustrated and reviewed a variety of drug delivery strategies. We discussed several mechanisms of drug release from various types of dosage forms these may be at play in a particular release system, especially when more than one stage is involved. But the drug transportation inside pharmaceutical system and its release sometimes involve multiple steps forced by different physical or chemical phenomena making it hard or even unfeasible to get a mathematical model describing in the accurate way. Particulate drug delivery systems offer substantial value to a drug. However, it should be recognized that development of a particulate drug delivery system product can be expensive. Nanoparticulate drug delivery will presume an essential place in drug delivery and human therapeutics. Although the development of drug delivery systems is just promising, it shows a hopeful future. Nanotechnology, which is still in its early years, may be investigated to develop delivery system of rapidly eliminated drugs from the body, drugs whose effectiveness would be enhanced by targeting, and drugs that are susceptible to degradation inside the body before absorption. Molecules that possess these attributes are fabricated at the time of the discovery and development stages with understanding of these technologies and we shall be able to increase the number of drug molecules those can be fabricated fully into suitable dosage forms.

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The Development and Achievement of Polymeric Nanoparticles for Cancer Drug Treatment

Wing-Hin Lee, Ching-Yee Loo, Paul M. Young, Daniela Traini and Ramin Rohanizadeh

Abstract

Limited aqueous solubility, stability, and bioavailability are common characteristics of hydrophobic drugs which impedes the delivery of drugs to intended site of action. In recent years, polymeric nanoparticles as drug delivery vehicle are widely used in various chronic disease therapies owing to their favorable features such as versatility, biocompatibility, biodegradability, and biomimetic characters. In the field of smart drug delivery, polymeric nanoparticles have improved specificity toward targeted site of action, reduced toxicity, and prolonged residency time in vivo. To date, various successful marketed clinical use products of polymeric nanoparticles include Genexol-PM and Abraxane® (cancer), Capaxone® (multiple sclerosis), Peglntron® (hepatitis C), Cimzia™ (rheumatoid arthritis), and Micera® (kidney failure). Ideal requirements of polymeric nanoparticle systems often include effectively controlled particle size, alterable surface chemistries, enhanced solubility, and therapeutic index of active drugs at predetermined rate and time. For instance, conjugation of polymeric nanoparticles with ligands is beneficial to avoid macrophage clearance and achieve long-circulation in vivo. This chapter will provide an overview of the achievement of polymeric nanoparticles which exhibit passive and active

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targeting, stimuli-triggered releases of chemotherapeutic drugs and therapeutic implications both in vitro and in vivo. In addition, the scope of successful translation polymeric nanoparticle system into clinical practice and future considerations will be outlined in the review.

Keywords
Polymeric micelles · Cancer · Nanoparticles · Nanomedicines

1 Introduction

Cancer is the leading cause of death in developed countries and is projected to surpass cardiovascular disease in terms of number of cases in near future. Statistics revealed that in 2008 more than 12 million new cancer patients were diagnosed and a staggering 8 million of deaths in the same year (Bray et al. 2013). From the socioeconomic point of view, due to escalated unhealthy lifestyles and aging population, cancer disease has contributed to significant economic burden to countries. For example, the National Institutes of Health in United States has provided $227 billion to cost of cancer treatment in 2007 (Popat et al. 2013). Cancer is characterized as group of cells which does not adhere to normal cell growth cycle processes. These phenomena was contributed by external factors that mainly related to the lifestyle such as smoking, unhealthy diet, and lacking of physical activities; however, internal factors are highly affected by the variation of genetic or epigenetic which related to genetic mutations and interruption of expressing inflammations markers (Doll and Peto 1981; Willet 2001; Calle et al. 2003; Hanahan and Weinberg 2000, 2011). Current standard protocols for cancer treatment include size reduction of tumor, surgery, radiotherapy, and chemotherapy. For nonmetastatic, primary cancers, surgery, and radiotherapy are most appropriate and effective treatment method (Brannon-Peppas and Blanchette 2012). However, these methods lack responsiveness from patients harboring multidrug-resistant, metastatic cells. At metastatic stage, chemotherapy is the preferred method in order to ensure the circulation of chemotherapeutic drugs to reach other parts of body via bloodstream. It is undoubtedly that chemotherapeutic drugs are effective to inhibit rapid proliferation of cancer cells (Brannon-Peppas and Blanchette 2012). However, the inherent toxicity inflicted by these drugs impacted the survival of healthy cells simultaneously during treatment course. More specifically, some of these compounds are not compatible in maintaining bone marrow and intestinal epithelium. This has significantly contributed to the failure to improve patients' survival. The ballooning cancer incidence has increased the pressure to develop effective methods to deliver chemotherapeutic drugs to the patient. However, it is important to understand the limitation factors of drug delivery that contributed to the failure in treating cancer disease (Iyer et al. 2013). Despite the advances in cancer research, our understanding

in cancer treatment is still limited. Current urgent need for cancer treatment is the discovery of novel biocompatible and side-effect free therapeutics with singular ability to eliminate cancer or solid tumor while leaving the healthy cells untouched. To further understand or design better cancer treatment method, we should understand the role of physiological barriers in tumor/cancerous cell and the effect of physicochemical properties of the chemotherapeutic drugs in cancer treatment. These two factors were identified as the main barrier in cancer treatment by lowering the cellular concentration of chemotherapeutic drugs.

1.1 Physiological Conditions of Tumor Tissues

Tumor tissues exist in abnormal microenvironments such as vascular abnormalities, oxygenation, perfusion, pH, and metabolic states (Vaupel et al. 1989). During initial growth of solid tumor (<1–2 mm^3), both supplies of nutrients and oxygen are readily accessible to tumor cells via diffusion and they are dependent on blood supply (Vaupel et al. 1989). However, once the tumor sizes have exceeded 2 mm^3, the supply of oxygen and nutrients becomes limited and the cells fall into realm of hypoxia. As a result, tumor cells respond by recruiting new blood vessels (known as angiogenesis) via activating the transcription of cellular hypoxia-inducible factor, thereby activating expression of cascade inducible growth factors such as the vascular-endothelial growth factor (VEGF), the platelet-derived growth factor (PDGF), and the tumor necrosis factor-α (TNF-α) (Folkman 1995; Bates et al. 2002). During the formation of new blood vessels, these immature vasculatures are subjected to various remodeling process, however, this processes are normally not complete and result in dilated, tortuous, and heterogeneous blood vessels (Jain and Stylianopoulos 2010). In addition, they are disorderly branched, have irregular capillary bed and pore size from 200 to 2000 nm (Hobbs et al. 1998). Owing to the heterogeneous blood vessels, the flows of blood through tumor vessels are highly depended on the type of tumors and their microenvironment conditions (Jain 1989; Hobbs et al. 1998). This has directly resulted in poor drug penetration into tumor. In addition, poorly organized structure of solid tumors is also another factor which limits the delivery of drugs into the larger tumors.

In addition, due to the abnormal structure of lymphatic vessel in solid tumor, the level of tumor interstitial fluid pressure (IFP) are relatively higher compared to normal tissue which is approximately 0 mm/Hg (Swartz and Fleury 2007). The increment of IFP has served as a barrier for delivery of chemotherapeutic drugs into solid tumor and the situation is worsened especially dealing with high molecular weight drugs. The increase of IFP results in the reduction of transcapillary transport into tumor, thus contributing extremely low concentration of drugs in tumor region (Jain 1989). In addition, IFP is relatively higher in the central of tumor and this IFP activity is likely to create a mass flow movement of fluid out from the central of tumor. Thus this once again diminishes the optimal concentration of drugs inside the central of tumor (Padera et al. 2004).

For healthy cells, the extracellular pH (pH_e) and intracellular pH (pH_i) are stringently maintained at pH 7.4 and 7.2, respectively (Wike-Hooley et al. 1984).

However, the pH is reversed ($pH_i > pH_e$) in most tumor tissues. It should be noted the variation of pH_e depends on the tumor histology, volume and location of the tumor. The low extracellular pH is primarily owing to rapid growing and angiogenesis of cancer cells which cause rapid depletion of oxygen and shift toward anaerobic glycolysis leading toward buildup of lactic acid in the tumor interstitium (Mura et al. 2013; Wike-Hooley et al. 1984; Biswas et al. 2016).

1.2 Rationale of Cancer Nanotechnology

Cancer nanotechnology is believed to provide cutting-edge advances in cancer detection, diagnosis, imaging, and treatment, which involve the integration of various disciplines including biology, chemistry, engineering, and medicine. Nanotechnology is typically defined as materials with sizes in the 1–1000 nm for at least one dimension and synthesized using both top-down and bottom-up methods (Mura et al. 2013). It is established that nanoparticles exhibit unique properties based on the size, shape, or functionality that are not seen when existing as bulk sold solids. Recent trends have emerged on the use of superparamagnetic iron oxide nanoparticles as both contrast and hyperthermia agents for cancer detection (Schleich et al. 2013; Schweiger et al. 2011; Zhao et al. 2015a, b; Wu et al. 2015; Thomas et al. 2015; Quinto et al. 2015). Meanwhile, other semiconductor and metal nanoparticles are under intense scrutiny as potential diagnostic biomarkers and molecular profiling studies (Devi et al. 2015; Vidotti et al. 2011). Effective early detection of neoplastic lesions is crucial to improve the survival of cancer patients and yet remains a far-fetched goal. Current clinical imaging techniques lack the sufficient resolution for early detection (Devi et al. 2015; Vidotti et al. 2011). The use of multifunctional nanoparticles comprising of contrast agents covalently linked to targeting agents or recognition molecules such as antibodies in conjunction with existing imaging technologies may significantly improve the chances of early cancer detection (Taleat et al. 2014). In addition, nanoparticles also offer the possibility of simultaneous tumor imaging and treatment, as various functional groups could be chemical conjugated on the surface of nanoparticles (Han et al. 2016; Bwatanglang et al. 2016; Ma et al. 2015; Li et al. 2015a, b, c, d, e). Another significant impact of nanotechnology is optimized drug delivery in which nanocarriers are adaptable to encapsulate various drugs including hydrophobic/hydrophilic molecules, peptides, antibodies, and nucleic acids (Tangsangasaksri et al. 2016; Jones et al. 2016; Corbet et al. 2016; Zhao et al. 2015; Yin et al. 2015). In addition, functional nanoparticles could be covalently decorated with specific biological molecules to enhance the targeting and uptake by specific tissue (Gao et al. 2015a, b; Liu et al. 2015a, b; Baek et al. 2014). Meanwhile, surface coating of nanoparticles with stealth-like polymer results in increased blood circulation and retention of drugs (Leal et al. 2015; Kooijmans et al. 2016). Additionally, therapeutic molecules existing within the nanoparticles are usually protected from degradation, hence, the significantly higher stability of drugs in vivo. Nanoparticles could be engineered to either release drugs in a sustained release manner or stimuli-triggered release manner (Scheeren et al. 2016; Gao et al. 2015a, b).

1.3 Important Considerations in Nanoparticle-Based Delivery for Cancer Treatment

In the context of nanoparticle-based drug delivery for cancer therapeutics, several important aspects have to be considered. They are passive and active targeting in which the former exploits the enhanced permeability and retention (EPR) effect while the latter is focused on targeting specific overexpressed receptors and surface molecules in tumors (Fig. 1). Other factors include physicochemical characteristics of nanoparticles (particle size, charge, and shape) and subtle microenvironments variations between healthy and cancer cells (pH, redox potential, temperature, and enzyme expression) (Bertrand et al. 2014).

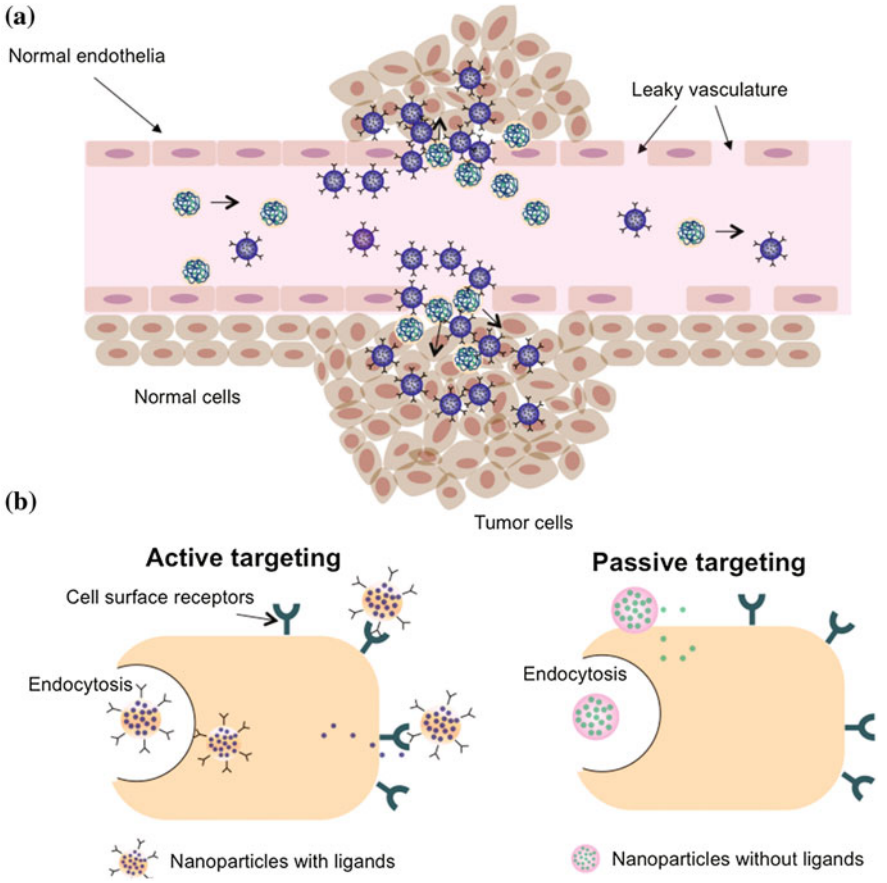

Fig. 1 **a** Schematic representative showing the exploitation of enhanced permeability and retention (EPR) effect by nanoparticles (with or without targeting ligands) to exit blood vessels through leaky vasculature, accumulate within tumor tissues, and subsequently internalized into cells via endocytosis. **b** Differences in the internalization mechanisms of nanoparticle through passive and active targeting. Adapted and modified from Lee et al. (2014)

1.3.1 Passive Targeting of Nanoparticles: Enhanced Permeability and Retention (EPR) Effect

Most nanoparticle-based delivery systems available in the market employ passive targeting approach by exploiting unique tumor microenvironments, which include leaky tumor microvessels and malfunctioned lymphatic drainage systems. The former results in enhanced permeability toward macromolecules relative to healthy tissues whereas the latter causes higher fluid retention in tumor interstitial space (Bertrand et al. 2014). These characteristics, otherwise known as enhanced permeation and retention (EPR) effect has been capitalized to foster at least 100× higher accumulation of nanoparticles in perivascular tumors' microenvironment compared to soluble drugs. The fundamental principles and factors contributing to EPR effect were comprehensively reviewed recently (Bertrand et al. 2014).

Tumor vasculatures are often disorderly branched, and are dilated with large interendothelial junctions and leaky vessels (Hobbs et al. 1998). These result in large capillary pore size ranging from 100–2000 nm in diameter (Hobbs et al. 1998). In contrast, the pore size for normal vessels is 5–10 nm in diameter. These unusually large pores allow higher permeability in tumor thus enabling nanoparticles to pass into tumor. This phenomenon forms the enhanced permeation of the EPR effect. Secondly, solid tumors are generally characterized with impaired lymphatic systems as rapidly proliferating cancer cells compress lymphatic cells causing the collapse of most vessels. These compromised lymphatic vessels lead to poor drainage of interstitial fluid in tumor. This forms the enhanced retention aspect of the EPR effect.

Physicochemical characteristics of nanoparticles such as size, shape, and surface charges have been instrumental to influence the extent of nanoparticles extravasation. As a rule of thumb, the particle sizes should be smaller than the cut-off diameters of the fenestrations in the neovasculature, which ranges between 100 and 2000 nm depending of type of tumor. Cabral and coworkers compared the accumulation of sub-100 nm polymeric micelles particles on both hyperpermeable and poorly permeable tumors (Cabral et al. 2011). These micelles were found to localize and accumulate evenly in hyperpermeable tumor (murine colon adenocarcinoma) irrespective of particle sizes (30, 50, 70, and 100 nm). However, in poorly permeable tumor (human pancreatic adenocarcinoma), only particles with diameters smaller than 70 nm could be internalized in the tumor (Cabral et al. 2011). Besides size, surface charges of a nanoparticle could modify recognition, opsonization, internalization, and plasma circulation kinetic (Alexis et al. 2008; Levchenko et al. 2002; Xiao et al. 2011).

Positively charged liposomes were also found to possess stronger affinities toward negatively charged phospholipid groups found in tumor endothelial cells. Results from animal model suggested that positively charged nanoparticles with sizes ranging from 50 to 100 nm enhanced penetration and accumulation of drugs in tumor (Davis et al. 2008). Steric stabilization with PEGylation on the surface of nanoparticles imparts stealth properties by helping them to evade opsonization and clearance by RES; thus making these nanoparticles long circulating (Lin et al. 2012). Nevertheless, it has been suggested that EPR effect is much more complex

than initially thought as other biological process such as angiogenesis, vascular permeability, hemodynamic regulation, heterogeneities in tumor genetic profile, and tumor microenvironments are involved in tumor growth and vary according to cancer patients. Therefore, the designing of nanocarriers have to take into consideration these biological processes rather than just focusing on EPR alone.

1.3.2 Active Targeting of Nanoparticles

Active targeting also known as ligand-mediated targeting is achieved by peripheral conjugation of targeting moieties to surface of nanoparticles for specific delivery systems. In most cases, ligands decorated nanoparticles are chosen for specific recognition to overexpressed receptors and surface molecules in tumors. The first active targeted delivery was described in late 1970s based on the concept of coating antibodies to nanoparticles' surface to allow interactions with target antigens. To date, representative ligands include peptide, transferrin, aptamers, growth factor, folate, antibodies, or antibody fragments (Liu et al. 2016; Zhou et al. 2013; Singh et al. 2016; Guo et al. 2011; Shargh et al. 2016; Yamamoto et al. 2014). Two common approaches are used in ligands-mediated targeting: (i) to target tumor cell surface receptors and (ii) to target tumor microenvironments. Targeting the cell surface receptors present in tumors is beneficial to increase cell–drug association while eliminating nonspecific interactions with healthy tissues. Meanwhile, tumor microenvironment such as the extracellular matrix and intracellular signaling genes is often targeted to activate immune response and angiogenesis. For effective active targeting, ligand moieties should be in close proximity with target receptors. Again, systemic clearance of ligands-mediated nanoparticles also affects the bioavailability of therapeutics in bloodstream to tumor. As the blood flow in tumor is low, the loss of nanoparticles via clearance sometimes overwhelms the increased affinities of nanoparticles toward tumors.

2 Polymeric Nanocarriers for Therapeutic Applications in Cancer

Polymeric micelles are self-assembled amphiphilic block or graft copolymer chains in aqueous conditions. Polymeric micelles are typically nano-sized, spherical, and exist as supramolecular colloidal particles with hydrophobic core and hydrophilic corona (Attia et al. 2011). The hydrophobic core serves to encapsulate and solubilize anti-cancer drugs while the hydrophilic compartment provides particles' stability against aggregation in aqueous microenvironment. Polymeric micelles are versatile and could accommodate different classes of hydrophobic molecules such as therapeutics, imaging and contrast agents for wide ranges of application. The most commonly used hydrophilic polymers for the preparation of micelles is poly (ethylene glycol), PEG (Li et al. 2014a, b, c; Liu et al. 2015a, b; Ruiz-Hernandez et al. 2014). To date other hydrophilic polymers include chitosan, poly(*N*-vinyl pyrrolidone) (PVP), and poly(*N*-isopropylacrylamide) (pNIPAAm) (Bailly et al.

2012; Movagharnezhad and Moghadam 2016; Abbasi et al. 2016; Yang et al. 2016). Meanwhile the polymers used to form the core of micelles include poly (lactide-*co*-glycolic acid) (PLGA), poly(ε-caprolactone) (PCL), poly(β-aminoesters)P(bAE), distearoyl (phosphatidylethanolamine) (DSPE), and polyamino acids (Lee et al. 2015; Diao et al. 2010; Jin et al. 2007; Zhang et al. 2016a, b; Alavizadeh et al. 2016).

2.1 Polymeric Micelles for Therapeutic Applications in Cancer

2.1.1 Doxorubicin (DOX) Conjugated Polymeric Micelles

It is accepted that the encapsulation efficiency of drugs is dependent on the hydrophobic core of micelles; which in turn determines anti-cancer therapeutic efficacy (Table 1). In a recent study by Sun and coworkers, the extent of hydrophobicity of amphiphilicdi block copolymer containing PEG and polyphosphoester (PPE) on anti-cancer efficacy was investigated (Sun et al. 2014a, b). PPEs with different alkyl side chain length (butyl, hexyl, and octyl) were synthesized to fine-tune the hydrophobicity of micellar core (Sun et al. 2014a, b). The length of alkyl groups influenced release rate of doxorubicin and in vitro anti-cancer activity. Micelles with the least hydrophobicity demonstrated the highest killing effect of breast cancer cell line (MDA-MB-231), which might be attributable to the higher release rate of drug in vitro. Owing to prolonged circulation time and enhanced doxorubicin retention in tumors, PEG-PPOE (most hydrophobic core) micelles were considered as one the most effective micelles material to reduce tumor burden in vivo (Sun et al. 2014a, b).

Lee and coworkers developed a self-assembled, biocompatible triblock PEG-poly(amino acid)-based micellar system where doxorubicin was loaded via dialysis method. The poly(ethylene glycol)-poly(L-asparticacid)-poly(L-phenylalanine) [PEG-PAsp-PPhe] micelles could selectively release entrapped DOX at low intracellular pH, in endosomal compartments of MCF-7 (breast adenocarcinoma) cells (Lee et al. 2011). In a recent work, DOX was conjugated to methyl poly (ethylene glycol)-distearoylphosphatidylethanolamine (MPEG-DSPE) via thin film hydration method to produce near spherical micelles with particles size of 20 nm, which was the ideal size range lymph node metastasis targeting (Li et al. 2015a, b, c, d, e). These micelles efficiently evaded macrophage clearance and demonstrated higher uptake by A375 cells in vitro in comparison to MPEG-DSPE/Rhodamine B micelles. Additionally, subcutaneous injection of MPEG-DSPE/DOX micelles to lymphatic metastatic mice model bearing A375 cells resulted in decrease of popliteal and iliac lymph nodes, an indication that the drug-loaded micelles were accumulated in the lymphatic drainage system (Li et al. 2015a, b, c, d, e). A multifunctional micellar system consisting of pH-responsive and bone targeting behavior was conceived to increase the efficacy of DOX in bone metastasis treatment (Ye et al. 2015). DOX was conjugated to PEG-alendronate (ALN) via acid cleavage hydrazine linkages and spontaneously self-assembled to form micelles

Table 1 Examples of ligands targeted polymeric micelles for active targeting of chemotherapeutic drugs

Polymeric materials	Drug	Active ligand	Targeting receptor	Remarks	Reference
Poly(ethylene glycol)–poly(aspartate hydrazoneadriamycin) [PEG–P (Asp-Hyd-ADR)]	Adriamycin (ADR)	Folate	Folate receptor	Significant cytotoxicity effect toward KB cells with folate decorated ADR-loaded micelles. This was contributed by the higher cellular uptake of ADR via receptor and ligand interaction	Bae et al. (2005)
Oligoethylene glycol methylethermethacylate and methyl acrylic acid	Cisplatin and oxoplatin	Folate	Folate receptor	Folate conjugated micelles encapsulating with drugs were only effective against folate-overexpressed OVCARS cells. In non-folate expressing cells such as A549, no significant enhancement was demonstrated	Scarano et al. (2013)
Poly(ethylene glycol)-*b*-poly(acryloyl carbonate)-*b*-poly(D,L-lactide) [PEG-PAC-PLA]	PTX	Folate	Folate receptor	PTX-loaded micelles (either cross-linked or non-cross-linked) were highly toxic toward KB cells. The toxicity effect of folate decorated PTX-loaded micelles was contributed by higher cellular uptake via folate receptor-mediated endocytosis	Xiong et al. (2011)
PHIS–PEG and Fol–PEG–PLA	DOX	Folate	Not mentioned	Folate decorated DOX-loaded micelles with pH-sensitive characteristics were superior to suppression MCF-7ADR proliferation. In vivo, this micellar formulation was not only effective to reduce tumor volume; it was also nontoxic to mice. The survival of mice receiving DOX-loaded micelles was 100% compared to only 60% receiving free DOX	Li et al. (2015a, b, c, d, e)
Polyethylene glycol-dextran (PEG-HMD)	PTX	Folate	Not mentioned	PAX loaded folate decorated PEG-HMD micelles significantly decreased the IC_{50} values of HeLa and M109-HiFR cells line compared to Taxol. The prepared dry powder formulation	Rosiere et al. (2016)

(continued)

Table 1 (continued)

Polymeric materials	Drug	Active ligand	Targeting receptor	Remarks	Reference
				(void micelles, without PAX) was contributed nonstatistically differences in pro-inflammatory cytokine expression (IL-1β, IL-6 and TNF-α from mice lung tissue	
Poly(ethylene oxide)-*b*-poly(e-caprolactone) [PEO-*b*-PCL] or Poly(ethylene oxide)-*b*-poly(a-benzyl carboxylate-ε-caprolactone) [PEO-*b*-PBCL]	PTX	c(RGDfK) or p160 peptides	Not mentioned	Both p160 and c(RGDfK) modified polymeric micelles were highly selective toward MDA-MB-435 and HUVEC cell lines. p160 functionalized PTX-loaded micelles demonstrated more selective targeting toward tumor cells	Shahin et al. (2011)
Poly(lactic acid-*co*-glycotic acid) and poly (ethylene glycol)	PTX	H7K(R2)2 peptide	Not mentioned	The peptide conjugated PTX micelles were densely accumulated in tumor sites compared to non-peptide conjugated PTX micelles. At the same time, peptide modified formulation possessed the highest antitumor activity against MCF-7 tumor-bearing mice	Zhao et al. (2012)
Poly(2-ethyl-2-oxazoline)-poly(D,L-lactide) [PEOz-PLA]	PTX	Cyclic RGDyK peptide	$\alpha_v\beta_3$	Cyclic RGDyK conjugated PTX micelles: (i) increased cellular uptakes in PC-3 cells via $\alpha_v\beta_3$ interaction on cell surface, (ii) rapidly release PTX in pH microenvironment mimicking endosomal-lysosomal compartments (iii) conferred negligible systemic toxicity and (iv) were remarkably effective in the suppression of tumor growth PC-3 xenograft bearing mice	Gao et al. (2015a, b)
Poly(ethylene glycol)-poly(D,L-lactic acid) PEG-PLA	DOX	AP (CRKRLDRN) peptides	IL-4 receptor	The confocal images revealed that the presence of AP peptides promoted higher cellular uptake. More than 4-fold decrease in tumor volume and weight in MDA-MB-231 tumor-bearing mice compared was noted 15 days of posttreatment	Wu et al. (2010)

(continued)

Table 1 (continued)

Polymeric materials	Drug	Active ligand	Targeting receptor	Remarks	Reference
Poly(ethylene glycol)-phosphatidylethanolamine (mPEG-PE)	R547	Transferrin	Transferrin receptor	R547 loaded transferrin targeted micelles showed higher toxicity compared to free R547 and nontargeted R547 micelles	Sawant et al. (2014)
Poly(lactic acid-*co*-glycolic acid) and poly (ethylene glycol) PLGA-PLA	Doxorubicin (DOX)	Monoclonal antibody; Hab18 $F(ab')_2$	Antigen	The toxicity of targeted micelles was proven to be effective in tumor cells (in vitro and in vivo). The survival fraction of HepG2 and Huh7 were reduced to approximately 0.2 after 24 h of treatment. In nude mice bearing HepG2 xenograft, the bioavailability of DOX was mostly found in tumor tissue when targeted micelles were used to deliver DOX. Thus, this contributed to higher efficiency in controlling the tumor growth in the mice model	Jin et al. (2010)
Poly(lactic acid-*co*-glycolic acid) and poly (ethylene glycol) PLGA-PLA	Gefitinib (GNB)	Mannose	Lectin	Mannose decorated GND loaded micelles resulted in higher cellular internalization, and subsequently caused higher apoptotic and necrotic activities in A549 cells. In addition, the stability of GNB in micelles was also improved for about 5-fold. GNB accumulations in in vivo tumor were increased 7-fold compared to free GNB	Wang et al. (2015a, b, c)
Methoxypoly(ethylene glycol)-grafted-poly (β-amino ester)	DOX	Biotin	Not mentioned	Biotin conjugated micelles improved the cellular uptake and effectively regulated the release of DOX in acidic conditions	Kim et al. (2012)

with particle size and loading of 114 nm and 24.3%, respectively (Ye et al. 2015). Intravenous administration of PEG-ALN/DOX micelles to tumor-bearing mice exhibited higher drug retention in bone metastatic tumor tissue, delayed tumor growth, decreased bone loss, and reduced cardiac toxicity in vivo (Ye et al. 2015). Kim and coworkers have successfully synthesized DOX-loaded poly (ethylene oxide)-poly [(R)-3-hydroxybutyrate]-poly (ethylene oxide) (PEO-PHB-PEO) triblock polymeric micelles with excellent drug penetration and distribution in an in vitro 3D spheroids tumor culture model (Kim et al. 2010). The PEO-PHB-PEO/DOX micelles were rapidly diffused into the core of spheroids within 30 min while free DOX was mostly sequestrated in acidic endosomes of cells close to vasculature. The enhanced penetration characteristic of micelles was seemingly due to the ability of PHB to form channels in cell membrane. Furthermore, PEO-PHB-PEO/DOX micelles induced apoptosis and inhibited tumor growth without significant systemic toxicity in tumor-bearing mice (Kim et al. 2010).

To improve the loading of DOX to PEG-PCL micelles, three different molecules with π-conjugated moieties (cinnamic acid, 7-carboxymethoxy coumarin, and chrysin) were functionalized to the terminal hydroxyl groups of PCL blocks (Liang et al. 2015). More than twofold increase in DOX loading was noted after the modification. Among the three modified PEG-PCL micelles, the presence of chrysin exhibited the most potent anti-cancer activity both in vitro and in vivo (Liang et al. 2015). In another study, chemical conjugation of DOX to succinic anhydride activated pluronic F68 was synthesized via linkage of primary amine group of DOX to carboxyl group of pluronic F68 (Zhao et al. 2011). Similar to other published literature, DOX-pluronic F68 micelles demonstrated higher cytotoxicity effect against A549 cells and could successfully circumvent the MDR of A549/DOX cells (Zhao et al. 2011).

Folate (FA)-receptor delivery of DOX-loaded poly(*N*-isopropylacrylamide-*co*-*N*, *N*-dimethylacrylamide-*co*-2-aminoethyl methacrylate)-*b*-poly(10-undecenoic acid) (P(NIPAAm-*co*-DMAAm-*co*-AMA)-*b*-PUA) polymeric micelles was synthesized in which FA was conjugated to the hydrophilic block of AMA (Liu et al. 2007). These FA-DOX-loaded polymeric micelles demonstrated higher internalization, and cytotoxicity in cancer cells with overexpressed FA receptors. For instance, the IC_{50} of DOX-loaded micelles with FA against 4T1 and KB cells were significantly lower than that of DOX-loaded micelles without FA (3.8 vs. 7.6 μg/ml for 4T1 and 1.2 vs. 3.0 μg/ml for KB) (Liu et al. 2007).

A novel cell penetrating peptide (CPP) mediated polymeric micelles as nanocarrier for DOX was recently synthesized through the introduction of octa-arginine (PArg) into the reduction-cleavable copolymers (Cui et al. 2016). The synthesized polyamide amine-*g*-polyethylene glycol/polyarginine/DOX (PAA-PEG/PArg/DOX) micelles were monodisperse, spherical with sizes ranging from 20 to 80 nm in diameter and displayed enhanced EPR effect in tumor sites. The in vivo distribution data of DOX-loaded micelles delivered via intravenous injection to 4T1 bearing mice revealed stronger fluorescence signals and directional aggregation at tumor sites. Meanwhile, free DOX solution was systemically distributed throughout the whole body with weaker fluorescent signal (Cui et al. 2016).

The PAA-PEG/PArg/DOX micelles exhibited excellent tumor growth suppression in tumor-bearing Babl/c mice model compared to PEG-PAA/DOX micelles (Cui et al. 2016).

FA-decorated PEG5 k-EB2/DOX micelles for active targeting and tumor inhibition with particle sizes of 20 nm demonstrated sustained release kinetics of DOX profile over a period of 80 h. In addition, intracellular uptake of FA-decorated-DOX-loaded micelles was facilitated in breast cancer cells (4T1.2) and drug-resistant cells (NCI/ADR-RES) and simultaneously inhibited the function of P-gp efflux pump. (Lu et al. 2014). To overcome MDR in colorectal adenocarcinomas, co-delivery of DOX and small interfering RNA (siRNA) with active targeting was employed (Amjad et al. 2015). The authors reported that FA-functionalized cholic acid-polyethylenimine (CA-PEI) loaded with DOX/siRNA micelles were positively charged (+12 mV), moderately sized (150 nm), and showed high affinities toward DOX (Amjad et al. 2015). Both FA-functionalized CA-PEI/DOX micelles and FA-functionalized CA-PEI/DOX/siRNA micelles enhanced overall in vivo antitumor activity through inhibition of tumor growth and induction of cancer cell death (apoptosis and necrosis). However, no significant differences between the two micelles were observed, thus indicating that the siRNA did not act synergistically to improve anti-cancer activity (Amjad et al. 2015).

2.1.2 Paclitaxel (PTX) Conjugated Polymeric Micelles

PTX is one the most common and effective chemotherapeutic agents to treat a variety of cancers including ovarian, breast, non-small lung cancer, etc., (Rowinsky et al. 1989; Crawford et al. 2013; Razi et al. 2015; Bernabeu et al. 2016; Lam et al. 2014). The commercial PTX (under the tradename of Taxol®) is currently formulated in the mixture of Cremophor® EL and ethanol. One main disadvantage of Taxol® is that the vehicle agent Cremophor® EL induces significant side effects which include severe hypersensitivity, neurotoxicity, nephrotoxicity, and myelosuppression (Kumar et al. 2010). This section describes current designs of polymeric micelles systems proposed as nontoxic alternative vehicle to overcome the shortcomings of Cremophor® EL. Recently, a novel self-assembling diblock copolymer comprised of CL, trimethylene carbonate (TMC), and PEG_{750} has been found to form micelles spontaneously in water. The encapsulation of anti-cancer drugs such as PTX into the micellar core requires simplistic approach without needing organic solvent, dialysis, or evaporation step (Liu et al. 2007). Owing to the high hydrophobic nature of PCL/TMC block segments, significantly higher (threefold higher) solubilization of PTX into PEG-PCL-TMC micelles was achieved (Danhier et al. 2009a, b, c). Danhier and coworkers elaborated that PTX-loaded PEG-PCL-TMC micelles exhibited higher tolerance in mice compared to Taxol® irrespective of administration route. The maximum tolerated dose (MTD) of PTX-loaded PEG-PCL-TMC micelles delivered via intraperitoneal and intravenous route was 45 and 80 mg/kg, respectively. Meanwhile the MTD for Taxol® was 13.5 and 13.5 mg/kg, respectively (Danhier et al. 2009a, b, c). As expected due to higher dose tolerance, PTX-loaded PEG-PCL-TMC micelles were more effective to induce tumor growth retardation (Danhier et al. 2009a, b, c).

In another study, Zhang and coworkers proposed the use of PCL-PEG-PCL triblock copolymer as nano-vehicle for delivery of PTX via intravenous administration (Zhang et al. 2012a, b). PTX was incorporated into polymeric micelles via thin hydration methods and these resultant PTX-loaded micelles exhibited core-shell like morphology with average diameter of 93 nm, which fitted into the particle size window for passive targeting via EPR effect (Zhang et al. 2012a, b). The authors also compared the pharmacokinetic of PTX-loaded micelles with Taxol® after intravenous administration to rats (PTX dose: 7.5 mg/kg). PTX-loaded micelles had longer systemic circulation time and slower plasma elimination rate compared to Taxol® as evident from the area-under-curve (AUC) and mean residence time (MRT). Encapsulated PTX were 4.3-fold and 12.0-fold higher, respectively, than free PTX (Zhang et al. 2012a, b). Histopathological analyses, behavioral and body mass changes in EMT6 breast tumor-bearing rats revealed that PTX in the form of micelles enhanced therapeutic efficacy (reduced tumor size and evident coagulative necrosis) while simultaneously reduced its systemic toxicity (no significant behavioral changes and body mass reduction in rats) (Zhang et al. 2012a, b).

Linear-dendritic block copolymers using cholic acid (CA) as the building block have been reported to self-assemble into stable micelles in water. However, drug loading capacities of linear CA-PEG conjugates and star-shaped CA-PEG micelles were limited. Recently, telodendrimer PEG-CA, with a dendritic oligomer of CA conjugated on one end of linear PEG was designed which formed stable micelles in water with PTX loading efficiency of 36.5% (Xiao et al. 2009). In vivo efficacy of PEG-CA/PTX micelles were evaluated in both subcutaneous and intraperitoneal ovarian cancer mouse models and demonstrated superior antitumor activity with respect to Taxol® and Abraxane® (Xiao et al. 2009).

Another class of common amphiphilic block copolymers used as nano-micellar carrier is pluronic (PEO-PPO-PEO). In selected studies by the Fang's group, mixed or single pluronic polymeric micelles were chosen as carrier of PTX to achieve thermodynamically stable particles, increased drug loading rate, low immunogenicity relatively longer systemic circulation time, slower plasma elimination rate and higher PTX accumulation in tumor-bearing mice (Zhang et al. 2010a, 2011a, b). They have demonstrated the MRT of PTX-loaded pluronic F127/P123 micelles in the lung was 3-fold higher than commercial Taxol® (Zheng et al. 2010). More significantly, the tumor volume of A549 tumor-bearing rats 28 days after receiving PTX-loaded mixed micelles was only 31.8% that of Taxol® (Zheng et al. 2010). In addition, PTX-loaded pluronic F127/P123 micelles were also significantly effective against metastatic tumors (Zhang et al. 2011a, b).

Apart from synthetic polymers, natural polysaccharides such as hyaluronic acid (HA) and cell membrane like phospholipids (PE) are also considered as potential micellar carrier of PTX (Saadat et al. 2014a, b) (Table 1). In a recent study, two different PEs including dimiristoyl (phosphatidylethanolamine) (DMPE) and DSPE were investigated as hydrophobic block segments in combination with HA for encapsulation of PTX (Saadat et al. 2014a, b). HA-DMPE copolymers or HA-DSPE copolymers were linked via amide bonds using carbodiimide chemistry conjugation, and was subsequently sonicated in water to form mixed micelles (Saadat et al. 2014a,

b). The PTX loading and release rate for HA-DSPE micellar system 59.9% and 34 h, respectively, whereas, in comparison for HA-DMPE/PTX micelles, PTX was released at a higher rate (23 h) (Saadat et al. 2014a, b).

It is well established that transferrin receptor (TfRs) are overexpressed in human cancer and have been studied in detail in breast cancer, prostate cancer, myeloid leukemia, etc. (Table 1). In a recent work, surface transferrin-conjugated micelles were constructed to introduce the specific binding to TfR in cell membrane. The hybrid micelles were synthesized by mixing amphiphilicmal-PEG-*b*-PLA, with a maleimide group at the end of the PEG segment, and m(PEG-*b*-P(LA-*co*-MCC)/PTX followed with transferrin functionalization on outer surface of micelles (Yue et al. 2012). Coupled with PTX, these transferrin-modified micelles were accumulated at higher extent in nude mice bearing subcutaneous TfR-overexpressing cancer and also demonstrated stronger antitumor effect in SGC-7901 gastric carcinoma model (Yue et al. 2012).

2.1.3 Other Chemotherapeutic Drugs Conjugated Polymeric Micelles

Cabazitaxel (CBZ) is a second-generation taxane designed specifically to treat taxane-resistant tumors and has been approved by the US Food and Administration (FDA) for use in prostate cancer (Paller and Antonarakis 2011). Owing to a decreased propensity for P-gp mediated drug resistance, CBZ has been shown to be clinically effective in taxane-resistant metastatic breast cancer, advanced lung cancer, and metastatic prostate cancer (Mita et al. 2009). Like most taxane, CBZ is highly hydrophobic, water-insoluble; thus commercial CBZ under the tradename Jevtana® contains large amount of excipients (polysorbate 80 and ethanol) which leads to hypotension, bronchospasm, and erythema in patients (Mita et al. 2009). Zhuang and coworkers investigated the potential of CBZ-loaded mPEG-PCL micelles to overcome these disadvantages (Zhuang et al. 2016). Solid dispersion was used to encapsulate CBZ into monomethoxy polyethylene glycol-poly (ε-caprolactone) (mPEG-PCL) copolymers pre-synthesized via ring opening polymerization method. The mPEG-PCL/CBZ micelles had high drug loading (10.52%) with average size of 30 nm and sustained release kinetics in vitro. The apoptosis and cell cycle arrest was more pronounced for lung carcinoma cell line and LLC bearing mice when mPEG-PCL/CBZ micelles were used. This was in tandem with prolonged life span and tumor growth suppression in LLC bearing mice (Zhuang et al. 2016). Supramolecular pseudo-block copolymeric micelles with multiarmed star polymer as micellarexterior were recently investigated as potential CBZ carrier to treat drug-resistant A2780/T cell lines (Xie et al. 2015). Multiarmed β-cyclodextrin (βCD) grafted PVP was synthesized using combination of reversible addition–fragmentation chain transfer polymerization (RAFT) and click reaction and was non-covalently linked to linear PCL. PVP7-PCL micelles demonstrated high particle stability, low protein corona adsorption, and high CBZ encapsulation (Xie et al. 2015). The IC_{50} of free CBZ and CBZ-loaded PVP7–PCL micelles against A2780/T cells is 274 and 269 ng/ml, respectively. The authors noted that the cytotoxic effect CBZ-loaded PVP7–PCL micelles was much stronger compared

to free CBZ by taking into account that only approximately 65% of the loaded CBZ was released in 48 h (Xie et al. 2015).

Methotrexate (MTX) is amongst the first anti-metabolite drugs for cancer therapy and acts as a folate metabolite via competitive inhibition of dihydrofolatereductase to suppress conversion of folic acid to folate cofactors (Kaasgaard et al. 2009). MTX is commonly used in various cancer treatments such as childhood acute lymphocytic leukemia, osteosarcoma, non-Hodgkin's lymphoma, Hodgkin's disease, head and neck cancer, lung cancer, and breast cancer (Seo et al. 2009). Pluronic F127-P105/MTX mixed micelles with pH-dependent characteristics was shown to have markedly increased blood circulation time of MTX and enhanced accumulation in tumor tissues of xenograft KBv bearing mice (Chen et al. 2013a, b). The IC_{50} values of Pluronic F127-P105/MTX mixed micelles and free MTX against KBv resistant cells were 0.073 μg/ml and >50 μg/ml, respectively. In addition, this micellar system induced much higher cell cycle arrest at phase G2/M compared to control (38.35% vs. 3.71%) (Chen et al. 2013a, b). The therapeutic activity of pluronic F127/P105-MTX micelles was further strengthened with the incorporation of a homing ligand receptor, FA on the outer surface of micelles (Chen et al. 2015). In addition to both caveolae- and clathrin-mediated endocytosis, FA-F127/P105-MTX micelles were also internalized via folate receptor-dependent pathways. Therefore, compared to nonspecific F127-P105/MTX micelles, the presence of homing receptor increased the accumulation in tumor regions by 1.5-fold. Nonspecific F127-P105/MTX micelles, however, were more localized in liver, spleen, and kidney (Chen et al. 2015). Other recent studies using variants of polymeric micelles as carrier of MTX also showed similar enhanced anti-cancer efficacy and higher drug's stability (Ren et al. 2015; Fattahi et al. 2015).

To increase the stability of 9-nitro camptothecin (9-NC), novel polymeric micelles encapsulating the drug were synthesized using cinnamic acid as the lipophilic moiety and mPEG as the hydrophilic block (Liang et al. 2013). The increase of lipophilic moiety in their micelles reduced the burst release effect of drugs from the micellar as well as enhanced the potency against liver cancer (Liang et al. 2013).

Docetaxel (DTX) is a member of taxane family and binds to tubulin, stabilizing spindle microtubules, and blocks mitosis (Bhalla 2003). In 2015, Hu and coworkers developed DTX-entrapped core-cross-linked polymeric micelles (CCL/DTX micelles) through covalent conjugation of DTX and CCL via hydrolysable ester bond to achieve sustained release of drug in vitro (Hu et al. 2015). The CCL consists of poly(ethylene glycol)-*b*-poly[N-(2-hydroxypropyl)methacrylamidelactate] (mPEG-*b*-p(HPMAm-Lac) copolymers. Single dose intravenous administration of CCL/DTX micelles (30, 60, or 125 mg/kg) demonstrated superior therapeutic efficacy in mice bearing MDA-MB-231 tumor xenografts compared to Taxotere®. Complete regression of tumor leading to 100% survival was observed for mice receiving single dosing of 125 mg/kg CCL/DTX micelles. This extraordinary efficacy could be perhaps explained by the enhanced accumulation of DTX, prolonged systemic circulation of CCL/DTX micelles, and suppression of angiogenesis (Hu et al. 2015). Treatment with CCL/DTX micelles reduced expression of

tumour stromal components such as pericytes, fibroblasts, and extracellularmatrix (collagen-1). These components are essential to contribute to increase in interstitial fluid pressure, tumor rigidity, and maturation of blood vessels in tumor microenvironment (Hu et al. 2015). Co-delivery of DTX and an autophagy inhibitor chloroquine (CQ) with enhanced drug stability, reduced toxic effect, and higher therapeutic efficacy was achieved using mixed micelles based on D-α-tocopherylpoly(ethylene glycol) (TPGS) and PEO–PPO–PCL with different length of hydrophobic chains (Shi et al. 2015). The size and drug loading of polymeric micelles was increased in conjunction with hydrophobicity of the copolymers. Irrespective of the hydrophobic chain lengths, the dual-drug-loaded PEO-PPO-PCL/TPGS micelles induced low hemolysis rate (<5%), exhibited sustained drug release and were significantly more effective than free DTX against MCF-7/ADR cells in vitro. Up to 190-fold decrease in IC_{50} were detected when cells were treated with drug-loaded micelles (Shi et al. 2015). Other recent findings have also demonstrated that the DTX delivery using polymeric micelles are suitable for treatment of metastatic and multidrug-resistant tumors (Li et al. 2014a, b, c).

Singh and coworkers recently developed transferrin-targeted novel polymeric micelles encapsulating DTX for enhanced treatment of lung cancer (Singh et al. 2016). The polymeric micelles were composed of poly(lactic acid)-D-α-tocopherylpoly(ethylene glycol) 1000 succinate (PLA-TPGS) and assembled with TPGS-Tf conjugate. The loading of DTX (39.69 ± 2.4 μg/mg) into micellar core was achieved using solvent displacement method. Unmodified PLA-TPGS/DTX micelles and PLA-TPGS-Tf/DTX micelles achieved approximately 7.7- and 70.34-folds decrease in IC_{50} value (stronger cytotoxicity) against A549 cell line compared with clinical Docel™ (Singh et al. 2016).

2.2 Polymeric Nanoparticles for Therapeutic Applications in Cancer

2.2.1 PTX-Loaded Polymericnano Particles

Several studies have shown that the encapsulation of PTX in PLGA nanoparticles improved the stability of the drug and anti-cancer activities compared to commercial Taxol® and free PTX (Yu et al. 2016; Averineni et al. 2012) (Tables 2 and 3). In a recent study, PTX-loaded PLGA nanoparticles with sizes ranging between 49 and 95 nm and alterable surface charges were produced via nanoprecipitation without the use of detergents (Le Broc-Ryckewaert et al. 2013). In the presence of serum, negatively charged PTX-loaded PLGA nanoparticles were relatively stable with slight increase in size. Meanwhile, for positively charged PTX-loaded PLGA nanoparticles, a serum effect which corresponds to altered size and zeta potential as a result of serum protein adsorption, occurred. PTX-loaded PLGA nanoparticles with positive charges were more cytotoxic against PC3 human prostate cancer cells compared to negatively charged nanoparticles. The authors have illustrated that positively charged nanoparticles had higher interaction with PC3 cells and tend to be localized in the cell membrane (Le Broc-Ryckewaert et al. 2013).

Table 2 Recent examples of polymeric nanoparticles as delivery vehicle for chemotherapeutic drugs

Polymeric material	Physicochemical properties (size, zeta potential)	Drug	Targeting cancer	Reference
Poly(butyl cyanoacrylate)	With poloxamer 188 stabilizer (246 nm, −23 mV); with dextran stabilizer (202 nm, −13.5 mV)	DOX	Glioblastoma	Petri et al. (2007)
Methoxy polyethylene glycol-poly (lactic-*co*-glycolic acid)	120–140 nm	DOX	Breast cancer	Alibolandi et al. (2015)
Methoxy polyethylene glycol-poly (caprolactone)	144 nm, −0.99 mV	Ursolic acid	Gastric cancer	Zhang et al. (2013a, b)
Chitosan-graft-D-α-tocopheryl polyethylene glycol	121–183 nm, 19.9–29.6 mV	DOX	Multidrug resistant liver and breast cancer	Guo et al. (2014)
Chitosan and β-glycerophosphate	23–24 nm, 0.03 to −14.1 mV	Cisplatin	Colon cancer	Abdel-Bar et al. (2016)
Polyethylene glycol-carboxymethylcellusose	118–134 nm	DTX	Prostate, breast, lung, pancreas cancer	Ernsting et al. (2011)
Polyethylene glycol-*b*-poly (D,L-lactide)	112 nm, neutral	Bortezomib	Triple negative breast cancer	Shen et al. (2015)
Poly(L-lysine)-carboxylate and hyaluronic acid	Depend on the ratio between carboxylic acid to primary amine: particle size range from 217-38,118 nm and zeta potential values range −23 to 0.36 mV	PTX and gemcitabine	Biliary cancer	Noh et al. (2015)
Poly(L-lactic acid) and Poly(L-lactic acid)-blend polyethylene glycol	283–294 nm	5-fluorouracil	Throat cancer	Mattos et al. (2016)
Poly(lactic-*co*-glycolic acid)	62–120 nm, −20.3 to −25.2 mV	5-fluorouracil and curcumin	Throat cancer	Masloub et al. (2016)
Poly(lactic-*co*-glycolic acid)	318 nm	PTX		

(continued)

Table 2 (continued)

Polymeric material	Physicochemical properties (size, zeta potential)	Drug	Targeting cancer	Reference
			Breast and cervix cancer	Jin et al. (2009)
Poly(lactic-*co*-glycolic acid)	133–160 nm, −23 to −33 mV	PTX	Lung cancer	Fonseca et al. (2002)
Poly(lactic-*co*-glycolic acid)	98.8 nm, −0.8 mV	Vincristine and verapamil	Drug-resistant breast cancer	Song et al. (2009)
Poly(lactic-*co*-glycolic acid)	250–280 nm, −17 to 19 mV	CPT	Colon cancer	Xiao et al. (2015a, b)
Poly(ethylene glycol)-poly(lactic-*co*-glycolic acid)	112–190 nm, 6.2–6.5 mV	PTX	Cervix cancer	Danhier et al. (2009)
Poly(lactic-*co*-glycolic acid)-D-α-tocopheryl polyethylene glycol	200–300 nm	PTX	Breast cancer	Zhao and Feng (2010)
Poly(lactic-*co*-glycolic acid)-D-α-tocopheryl polyethylene glycol	120 nm, −15 to −22 mV	PTX	Lung cancer	Sun et al. (2014a, b)
Mannitol-core-poly(D,L-lactic-*co*-glycolic acid)-D-α-tocopheryl polyethylene glycol	91–138 nm, −11 to −21 mV	DTX	Breast cancer	Tao et al. (2013)
Poly(L-lactide)-glycolic acid)-D-α-tocopheryl polyethylene glycol	130–270 nm	DOX	Drug-resistant breast cancer	Li et al. (2010)
Poly(ε-caprolactone)-D-α-tocopheryl polyethylene glycol	237 to 444 nm, −30 to −37 mV	PTX	Breast cancer	Bernabeu et al. (2014)
Poly(methyl methacrylate)	92 nm, −45 mV	Zinc (II) phthalocyanine	Leukimia cancer	Feuser et al. (2016)
Glycol chitosan	220–416 nm	PTX	Breast cancer	Kim et al. (2006)

Table 3 Examples of ligands targeted polymeric nanoparticles for active targeting of chemotherapeutic drugs

Type of ligands	Polymeric material	Physiochemical properties (size, zeta potential)	Drug	Targeted cells	Reference
Transferrin	Poly(lactic-*co*-glycolic acid)	93 nm, −11.3 mV	Methotrexate	Brain cancer	Jain et al. (2015)
Transferrin	Poly(lactic-*co*-glycolic acid)	187 nm, −17.3 mV	7α-(4′-amino) phenylthio-1,4-androstadiene-3,17-dione	Breast cancer	Zheng et al. (2010)
Peptide: RGD	PEGylated-poly(lactic-*co*-glycolic acid)	146 nm	PTX	Liver cancer	Danhier et al. (2009a, b, c)
Peptide: RGDfk	Polyglylutamic acid	7 nm	PTX	Brain cancer	Eldar-Boock et al. (2011)
Peptide: iNGR	Poly(ethylene glycol)-poly(lactic-*co*-glycolic acid)	127 nm, −18.6 mV	PTX	Brain cancer	Kang et al. (2014)
Peptide: LFC131	Chitosan	189 nm, −31 mV	DTX	Lung cancer	Wang et al. (2015a, b, c)
Peptide: LFC131	Poly(lactic-*co*-glycolic acid)	301 nm, −37 mV	DOX	Lung cancer	Chittasupho et al. (2014)
Peptide: Bombesin	Poly(lactic-*co*-glycolic acid)	111 nm, −26 mV	DTX	Breast cancer	Kulhari et al. (2014)
Peptide: low molecular weight protamine	Poly(lactic-*co*-glycolic acid)	Approximately 230 nm, +30 mV	DOX	Multidrug-resistant breast cancer	Wang et al. (2014)
Folate	Poly(ester-urethane) linked poly (ε-caprolactone) and polyethylene glycol	132 nm, −7.1 mV	DOX	Breast cancer	Kumar et al. (2015)
Folate	Chitosan-functionalized poly(lactic-*co*-glycolic acid)	206.9 nm, +21.7 mV	Bicalitamide	Prostate cancer	Dhas et al. (2015)

(continued)

Table 3 (continued)

Type of ligands	Polymeric material	Physiochemical properties (size, zeta potential)	Drug	Targeted cells	Reference
Folate	Gelatin	210 nm, −11.7 mV	Cisplatin	Cervix cancer	Dixit et al. (2015)
Folate and peptide	Poly(ethylene glycol)-poly(lactic-*co*-glycolic acid)	224–235 nm, −8.5 to −13 mV	Vincristine sulfate	Breast cancer	Chen et al. (2012)
Aptamer	Poly(ethylene glycol)-poly(lactic-*co*-glycolic acid)	156 nm, −32.9 mV	PTX	Brain cancer	Guo et al. (2011)
Epidermal growth factor	Gelatin	230 nm	Cisplatin	Lung cancer	Tseng et al. (2009)
Epidermal growth factor: anti-HER2	Poly (2-methyl-2-carboxytrimethylene carbonate-*co*-lactide) graft-poly (ethylene glycol)-furan	125 nm	DOX	Breast cancer	Shi et al. (2009)
Monoclonal antibody: Herceptin	Polyethylenimine and Poly(lactic-*co*-glycolic acid)	280 nm, 1 mV	PTX	Breast cancer	Yu et al. (2016)
Heparin	Gelatin	189 nm, −26.8 mV	Cisplatin	Breast cancer	Jain et al. (2012)
Bisphosphonate: alendronate	Poly(lactic-*co*-glycolic acid)	223–235 nm	Curcumin and bortezomib	Metastasis breast cancer	Thamake et al. (2012)
Bisphosphonate: alendronate	Poly(lactic-*co*-glycolic acid)	Approximately 180 nm	Cisplatine	Bone cancer	Yu et al. (2016)
Bisphosphonate: zoledronate	Poly(lactic-*co*-glycolic acid)	132 nm	DTX	Bone metastasis	Ramanlal Chaudhari et al. (2012)

In 2014, Gupta and coworkers described the synthesis of novel PLGA/poloxamer blend nanoparticles encapsulating PTX for intravenous administration using oil-in-water emulsion/solvent evaporation technique (Gupta et al. 2014). The spherical nanoparticles exhibited negative surface charge (−22.7 ± 5.7 mV) with particle size of 180 nm, stable without significant changes for three months and were non-hemolytic in vitro. The introduction of hydrophilic PEO chains of poloxamer acted as a steric barrier to prevent aggregation and adsorption of plasma protein onto the nanoparticle and subsequent clearance by phagocytic cells (Gupta et al. 2014). PTX-loaded PLGA/poloxamer blend nanoparticles showed significantly improved cytotoxicity against MCF-7 and colo-205 (human Caucasian colonadenocarcinoma cells). For example, PTX in the form of nanoparticles (IC_{50}: 0.53 nM) were approximately10-folds more cytotoxic toward Colo-205 compared to free PTX (IC_{50}: 5.34 nM) (Gupta et al. 2014). In another study, PTX blend nanoparticles consisting of poloxamer 188 and PCL were investigated to overcome MDR in a PTX-resistant human breast cancer cell line (MCF-7/TAX) (Zhang et al. 2010a, b). An increased internalization of PTX-loaded PCL/poloxamer nanoparticles by MCF-7/TAX was observed which was accompanied with stronger anti-cancer activity compared to Taxol® (IC_{50}: 0.65 μg/ml vs. 6.82 μg/ml) (Zhang et al. 2010a, b).

Using free radical dispersion polymerization to facilitate attachment of small molecules onto nanoparticles' surface, multifunctional PTX-loaded PLA-PEG nanoparticles with stealth properties were developed (Adesina et al. 2014). The long-circulating properties of spherical PTX-loaded PLA-PEG nanoparticles were confirmed using both qualitative and quantitative method. Through SEM observation, the nanoparticles were constructed from a lactide core and a corona of PEG. Quantitatively, complete PTX release from the nanoparticles was only observed after 7 days (Adesina et al. 2014). In vitro cytotoxicity data against breast and ovarian cancer cells suggested that the nanoparticle formulation had comparable effect with free PTX.

As mentioned earlier, PTX is an ideal chemotherapeutic agent as it could inhibit cell division via promoting the gathering and stabilization of microtubules. Owing to its intrinsic high hydrophobicity, PTX failed to penetrate the blood brain barrier (BBB) which in turn resulting in extremely low efficacy against brain tumor treatment.

To overcome this restriction, PTX has been encapsulated in mPEGylated PCL nanoparticles (Xin et al. 2010). The cytotoxicity effect of PTX-loaded mPEG-PCL nanoparticles against C6 cells was not significantly different compared to Taxol®, which could be attributable to the limited amount of PTX released from nanoparticles over incubation period (Xin et al. 2010). In vivo fluorescence imaging, however, revealed that mPEGylated nanoparticles displayed much stronger intensity in the brain of C6 bearing mice. The anti-glioblastoma activity evaluated with respect to survival of intracranial C6 bearing mice followed the decreasing trend: MPEG-PCL/PTX nanoparticles > PCL/PTX nanoparticles > saline ≈ Taxol®. The enhanced therapeutic activity of mPEGylated nanoparticle in vivo was based on the effect of passive tumor targeting and long-circulation time (Xin et al. 2010). In addition, PTX-loaded mPEG-PLA nanoparticles enhanced the chemo-radiotherapeutic efficacy against

non-small lung cancer cells (A549). Both in vitro clogenic assay and in vivo tumor survival assay demonstrated that PTX-loaded mPEG-PLA nanoparticles sensitized the tumor cells for radiation and simultaneous killing activities (Jung et al. 2012). Ranganath and coworkers proposed the use of novel submicron/nanoscale PLGA implants as delivery vehicle of PTX in the application of post-surgical local chemotherapy against glioblastoma (Ranganath et al. 2010). The authors have previously demonstrated that PLGA microfibers with the ratio of 50:50 showed promising potential for PTX delivery in vitro and in vivo (Ranganath and Wang 2008). It was postulated that better drug penetration into the brain could be achieved using electrospun PLGA 50:50 nanofibers (approximately 100 nm in size), hence increased local PTX bioavailability in vivo and superior anti-glioma activity (Ranganath et al. 2010). BALB/c nude mice with intracranial humanglioblastoma (U87 MG-luc2) randomized to receive implants of PLGA/PTX nanoparticles (10 mg/kg), placebo, and the intratumoral injection of Taxol® once a week (10 mg/kg). Enhanced PTX penetration into mice brain (up to 5 mm from implant site) was observed even after 42 days post-implantation and this was coupled with statistically significant tumor inhibition (30-folds) and low proliferation index (Ranganath et al. 2010).

2.2.2 DOX-Loaded Polymeric Nanoparticles

It is known that the site of action of DOX is within the nucleus. Therefore, nuclear localization signal (NLS) conjugated DOX/PLGA nanoparticles for intracellular trafficking within nucleus is a potentially effective drug delivery method for improved antitumor activity (Misra and Sahoo 2010). The NLS peptide was conjugated to the carboxylic group of the DOX/PLGA nanoparticles to yield relatively uniform particle size distribution (size: 226 nm and PI index: 0.15). Confocal observations revealed that the NLS conjugated DOX/PLGA nanoparticles were transported directly to nucleus of MCF-7 cells and acted as intracellular depot for prolonged DOX retention. This was reflected in the differences in IC_{50} values and G2/M arrest events for cells treated with NLS modified nanoparticles (2.3 μM, 60.2%) and free DOX (17.6 μM, 40.7%) (Misra and Sahoo 2010).

Another type of peptide with enzyme-sensitive feature, which ideally releases DOX at tumor site upon degradation by cathepsin B or papain, has been successfully developed recently (Yang et al. 2013). The enzyme-sensitive GFLG peptide was sandwiched between DOX and HPMA block copolymer in which the former was covalently bound to the peptide. Meanwhile, GFLG was introduced into the main chain of the copolymers with hydrophilic and hydrophobic blocks. The resultant MA-GFLG peptide-DOX nanoparticles exhibited significantly improvement in antitumor performance using 4T1 murine breast cancer model as evaluated using mice weight shifts, tumor growth curves, tumor growth inhibition, and immunohistochemical analyses (Yang et al. 2013). Owing to the high molecular weight of copolymer (90 kDa) coupled with enzymatic biodegradability characteristics, alteration of pharmacokinetics (prolonged blood circulation and accumulation) in conjunction with increased particles' stability in vivo was evident. The authors suggested that the presence of GFLG oligopeptide spacer, which is

susceptible to tumor-overexpressed lysosomal cysteine proteinase cathepsin B, provided additional stability in plasma and serum, altered microenvironment of tumor tissues and finally improved tumor suppression (Yang et al. 2013). A polyester-based hyperbranched dendritic-linear (HBDL)-based nanoparticles loaded with DOX was designed to overcome microsomal glutathione transferase 1 (MGST1)-mediated drug resistance in breast cancer cells (Zeng et al. 2014). HBDL/DOX nanoparticles was translocated across membrane of resistant cells via endocytic transport mechanisms involving macropinocytosis and clathrin-mediated endocytosis that are distinct from the uptake pathway of free DOX. In comparison, the internalization of free DOX is often via diffusion through the membrane where P-gp efflux pumps are located (Zeng et al. 2014).

2.2.3 Other Chemotherapeutic Drugs Loaded Polymericnano Particles

Tamoxifen (TAM), a nonsteroidal antiestrogen molecule, is used to treat patients with estrogen receptor (ER) positive breast cancer through inhibition of estradiol induced cell proliferation (Goetz et al. 2008). Oral therapy of TAM results in severe side effects such as oxidative stress mediated hepatotoxicity, hemolytic anemia, and possible high risk of endometrial cancer (Mocanu and Harrison 2004; Lee et al. 2010). Alternative nanoparticle-based drug delivery system to increase the bioavailability of TAM and reduced side effects to date include TAM/PCL nanoparticles, TAM/PLGA nanoparticles, TAM-loaded injectable microspheres or TAM-loaded alginate/chitosan nanoparticles. In a recent work, it was demonstrated that the presence of surfactants is crucial to obtain stable, non-aggregated TAM-loaded PLA nanoparticles with optimal loading efficiency and surface charges. The authors reported that the nanoparticle parameters were independent on the concentration of surfactants; rather influenced by the type of surfactant itself. For instance, PVA exhibited higher hydrophobic interactions and anti-aggregation properties compared to poloxamer-188 and polysorbate-80, hence promoted stronger steric stabilization in oil–water emulsion (Altmeyer et al. 2016). TAM-loaded PLA nanoparticles exhibited negligible hemolysis (independent of drug concentration or time of incubation) while maintaining the cytotoxicity over Hela cells. It was suggested that slow and prolonged TAM release from PLA nanoparticles is the reason causing low hemolytic potential and sustained cytotoxicity effect (Altmeyer et al. 2016). In vivo investigation on the effect of TAM-loaded PLA nanoparticles on DMBA induced mammary tumor in female Wistar rat showed marked reduction in both tumor size and hepatoxicity as compared to free TAM and control groups (Pandey et al. 2015). Simultaneous oral delivery of dual-drug-loaded PLGA nanoparticles (TAM and topotecan hydrochloride, TOP) was engineered to achieve synergistic effect in metastatic breast cancer treatment via enhancement of TOP permeation through the guts (Khuroo et al. 2014). Although both TAM and TOP act on DNA, their mechanism actions are different; hence synergistic action can be realized. The ability of TAM to inhibit P-gp like ABC transporters prevents the efflux of TOP and subsequently reduces the development of TOP resistance. These spherical TAM/TOP

loaded PLGA nanoparticles (average size: 150 nm) were highly lethal at small concentrations against MCF-7 cell lines compared to single or combination free drugs. Using ex vivo gut permeation study, the authors revealed that the cumulative TOP permeated from TAM/TOP nanoparticles showed a 2-fold increase compared to free TOP suspension alone (Khuroo et al. 2014).

Householder and coworkers have first reported an effective therapy of an intracranial gliomatumor with camptothecin (CPT)-loaded PLGA nanoparticles delivered using systemic route (Householder et al. 2015). CPT-loaded PLGA nanoparticles were more densely accumulated in the tumor core without exerting obvious adverse effect up to 20 days compared to healthy and peri-tumor brain regions. C57BL/6 albino mice bearing intracranial glioma tumor showed positive responses to the 119 nm-CPT nanoparticles in terms of retardation of tumor growth and mice survival (Householder et al. 2015). The median survival for mice receiving CPT nanoparticles, free-CPT, and saline were 36.5, 32, and 28 days, respectively (Householder et al. 2015). Hybrid cyclodextrin and chitosan nanoparticles encapsulating CPT intended for oral chemotherapy also resulted in enhanced drug permeability and anti-cancer activity (Unal et al. 2015). Both anionic and cationic CPT-hybrid nanoparticles, synthesized using wet nanoprecipitation method, had an average size of 187.5 and 204.2 nm, respectively, and were stable in both gastric and intestinal environments (i.e., minimal changes of size and zeta potential) (Unal et al. 2015). In another study, a series of cationic chitosan-functionalized polymeric nanoparticles (193–224 nm) were employed as co-delivery vehicles of CPT and CUR for effective colon cancer therapy (Xiao et al. 2015a, b). The cationic CPT/CUR nanoparticles exhibited clear synergistic patterns against colon-26 cells and the effect was drug ratio-dependent with CPT/CUR ratio of 4:1 being the most cytotoxic (Xiao et al. 2015a, b).

3 Stimuli-responsive Polymeric Nanocarriers

3.1 Endogenous Stimuli-responsive Polymeric Nanoparticles and Micelles

Stimuli-responsive polymeric micelles are a class of polymers that undergoes structural changes in response to both intrinsic and external stimuli for applications such as controlled drug release, enhanced internalization, and controlling the intracellular drug fate on the surrounding tumor tissue. The intrinsic stimuli in tumor microenvironment include pH, redox potential, and temperature (Mura et al. 2013). Among the different intrinsic stimuli in tumor microenvironments, the incorporation of a pH-triggered mechanism is the most common for delivery of various chemotherapeutic drugs such as DOX, PTX, DTX, and gemcitabine (GCT) (Scheeren et al. 2016; Zhao et al. 2012, 2013). Comprehensive reviews on the strategies used to design pH-responsive micelles and nanoparticles have been

published elsewhere (Biswas et al. 2016; Liu et al. 2014; Gao et al. 2013; Mura et al. 2013).

Tumor tissues possess lower extracellular pH (pH_e) compared to normal tissues as a result of excessive accumulation of lactic acid via anaerobic glycolysis. It should be noted the only subtle differences in pH exist between healthy tissues ($\sim$7.4) and the extracellular environment of solid tumors (6.5–7.2) (Engin et al. 1995). Acidic microenvironment benefits the survival of tumor cells because it slows down the internalization of basic drugs; hence, leading to reduced anti-cancer efficacy and higher tumor angiogenesis. Therefore, an effective pH-responsive nanosystem should demonstrate obvious signals to any slight changes in extracellular pH of tumor microenvironment. The exploitation of acidic pH as drug-release trigger can be loosely categorized into three strategies; (i) pH-responsive nanoparticles for rapid drug release; (ii) pH-responsive nanoparticles for cellular targeting and internalization; and (iii) pH-responsive nanoparticles for intracellular cytosolic drug release (Table 4; Fig. 2). Many studies have explored the use of amphiphilic copolymers with ionizable groups with higher solubility in acidic conditions (Bae et al. 2005). Anionic polymers with carboxyl groups such as poly (acrylic acid) (PAA), and poly(methacrylic acid) (PMA) most commonly used for this purpose (Biswas et al. 2016). Other pH-responsive polymers are polymers with sulfonamide groups or poly(amino acids) consisting of carboxylic acid group in the side chains (Biswas et al. 2016). Another method to incorporate pH-sensitive feature is via conjugation of polymeric systems (diblock, triblocketc) with acid-labile bonds (i.e., hydrazone, oxime, or acetals) in which the cleavage releases the chemotherapeutic drugs either encapsulated within the core of micelles or anchored on the polymer (Zhu et al. 2012; Jin et al. 2011; Du et al. 2012; Deng et al. 2015). Incorporating targeting ligands and altering surface charges of polymer (i.e., amine-containing polymers) have been used as well to escape endosomal compartment within cells (Zhang et al. 2012a, b; Kim et al. 2008).

In this context, Xu and coworkers had published an article on how to design and predict drug release in pH-responsive polymeric micelles (Xu et al. 2012). By employing citraconic amide as pH-sensitive precursor, Cao and coworkers have designed mPEG-pH-PCL and non-pH-sensitivemPEG-CA-PCL micelles to deliver DOX to treat HepG2 and 4T1 cell lines (Cao et al. 2014). The in vitro release study of DOX revealed that mPEG-pH-PCL has higher DOX release compared to mPEG-CA-PCL at acidic condition (pH 5.5), but the release rates were comparable at physiological condition (pH 7.4). Cell viability (IC_{50}) of HepG2 and 4T1 cell lines were more significantly affected when treated with loaded mPEG-pH-PCL/DOX compared to mPEG-CA-PCL/DOX micelles. However, 4T1 cell line was more susceptible to mPEG-pH-PCL/DOX micelles compared to HepG2 which was an indirect indication suggesting the variation of sensitivity of cancer cells of different origin (Cao et al. 2014). In addition Lee and coworkers have developed pH-sensitive poly(L-histidine) (PHis)-based loaded with DOX micelles specifically for targeting acidic tumor microenvironment (Lee et al. 2003). PHis has a pH-dependent solubility behavior in which its unsaturated nitrogen of imidazole ring is protonated at low pH (Lee et al. 2003; Gao et al. 2005a, b). As a

Table 4 Examples of pH-responsive polymeric micelles for delivery of chemotherapeutic drugs

Polymeric materials	Drug	Cancer cell line	Remarks	Reference
Poly(2-ethyl-2-oxazoline)	DOX	MCF-7	DOX-loaded polymeric micelles via conjugation exhibited lower IC_{50} values compared to free DOX. DOX-loaded micelles were present in nucleus and cytoplasm of cells The reduction of tumor volume on MCF-7 bearing athymic nude mice followed the decreasing order: Conjugated DOX-loaded micelles (72.4%) > free DOX (62.3%) (45.5%) > chemically bound DOX to polymer	(Li et al. 2015a, b, c, d, e)
PEGylated glucolipid-like conjugate with chitosan and stearic (CS) acid [PCCS]	DOX	BRL-3A and BEL-7402	Up to 87% of DOX was encapsulated into PCCS micelles. The release of DOX was more than 70% of DOX within 48 h at pH 5.0 pH-responsive PCCS/DOX micelles increased the cytotoxicity effect toward BEL-7402 by 2–3 fold compared to non-pH-responsive micelles (PCS) Due to the higher cellular uptake of PCCS/DOX micelles compared to PCS/DOX micelles and free DOX in tumor tissue, PCCS formulation was more effective to inhibit tumor growth and simultaneously reduced toxicity effect against tumor-bearing mice	Hu et al. (2012)

(continued)

Table 4 (continued)

Polymeric materials	Drug	Cancer cell line	Remarks	Reference
Poly(β-amino ester)-poly(ethylene glycol) [RPAE-PEG]	DOX	HepG2	The release rate of DOX was higher in acidic buffer (pH 6.5) compared to pH 7.4. The presence of DTT (reducing agent) triggered more DOX release at pH 6.5. RPAE-PEG/DOX micelles were rapidly internalized via endocytosis and exerted stronger cytotoxicity against HepG2 cells than free DOX	Chen et al. (2011)
Poly(ethylene oxide)-block-poly(ε-caprolactone) [PEO-*b*-PCL]	DOX and multidrug resistant (MDR)-1 siRNA	MDA-MB-435	Significantcellular uptake, improved DOX penetrationinto nucleus, and enhanced DOX cytotoxicity in DOX-resistant cells was demonstrated by the polymeric micelles harboring cell membrane translocation (cell penetrating TAT), pH-triggered (via hydrazone linkage) drug release and cancer therapeutics (DOX and siRNA) properties	Xiong and Lavasanifar (2011)
Polysialic acid	PTX	SGC-7901	More than 40% of PTX was released in mild acidic condition (pH 5.0) after 72 h of incubation. Although PTX-loaded micelles 53.8% of cell viability) was weaker than free PTX (35.3% of cell viability) in terms of cytotoxicity, but the micellar formulation was highly stable at physiological condition	Zhang et al. (2016a, b)
Methoxy poly(ethylene glycol)-poly(ε-caprolactone) (mPEG-PCL)	DTX	MCF-7/A, MCF-7 and A549	mPEG-PCL/DTX micelles exerted negligible hemolytic effect and higher cancer killing effect irrespective of concentration and treatment time	Zhang et al. (2016a, b)

(continued)

Table 4 (continued)

Polymeric materials	Drug	Cancer cell line	Remarks	Reference
Poly(ethylene glycol)-poly(L-histidine)-poly(D,L-lactide-*co*-glycolide) (PEG-pHis-PLGA) and D-α-tocopheryl polyethylene glycol 1000 (TPGS) (mixed micelles)	Gambogic acid (GA)	Drug-sensitive human breast MCF-7 and drug-resistant MCF-7/ADR cells	GA-loaded micelles improved the toxicity effect on both MCF-7 and MCF-7/ADR cell lines. More than 50% of apoptotic cells in MCF-7/ADR were observed when treated with GA-loaded mixed micelles. The caspase 3/7 activities and cleaved PARP level were significantly higher when the resistant cells were treated with GA-loaded mix micelles compared to free GA. This mixed formulation also effectively reduced the expression of anti-apoptotic proteins Bcl-2 and survivin	Wang et al. (2015a, b, c)
N-benzyl-*N*,*O*-succinyl chitosan (BSCS)	Curcumin (CUR)	HeLa, SiHa and C33a	CUR encapsulated in chitosan micelles was stable up to four months. The IC_{50} of CUR/chitosan micelles were 4.7, 3.6, and 12.2 folds lower than free CUR against HeLa, SiHa and C33 cell lines, respectively. In addition, higher apoptosis levels were observed in these three cell lines when treated with chitosan/CUR micelles	Sajomsang et al. (2014)
mPEG-*b*-polycarbonate	DOX	HepG2, HEK293, 4T1	Mussel-inspired copolymer design with catechol side chain for covalent conjunction of DOX to acid-sensitive linker (*p*-quinoneimines) was developed. Self-assembled mPEG-polycarbonate/DOX micelles were kinetically stable, released drug via	Chan et al. (2016)

(continued)

Table 4 (continued)

Polymeric materials	Drug	Cancer cell line	Remarks	Reference
			pH-triggered intracellular mechanism and possessed comparable toxicity to free DOX in vitro In vivo efficacy of DOX-loaded micelles was significantly higher than free DOX	
PEG	DOX	HEK-293, HeLa, 4T1	Micelles consisted of shell-forming PEG and core-forming DOX covalently conjunction through acid-sensitive hydrazone bonds to the sidechains of the norbornene block. In aqueous conditions, the block copolymer self-assembled into spherical nanostructure with well-shielded drug moieties. The micelles were capable to control intracellular environment-sensitive drug release and enhanced DOX delivery	Rao et al. (2012)
methoxy poly(ethylene glycol)-*b*-poly(ε-caprolactone-*co*-γ-dimethyl maleamidic acid-ε-caprolactone) mPEG-*b*-PCL	DOX	HepG2	PEG-*b*-PCL copolymer micelles exhibited pH-responsive characteristics by harboring different amount of acid-labile β-carboxylic amides on the polyester moiety Hydrolysis of β-carboxylic amides in acidic conditions rapidly released DOX and triggered higher cellular uptake via electrostatic endocytosis. IC_{50} values for free DOX and DOX-loaded micelles were 0.49 and 0.62 μg/ml, respectively	Deng et al. (2014)

(continued)

Table 4 (continued)

Polymeric materials	Drug	Cancer cell line	Remarks	Reference
D-α-Tocopheryl Polyethylene Glycol Succinate-Poly(β-amino ester) TPGS-*b*-PBAE	DTX	Drug-sensitive human ovarian A2780 and drug-resistant A2780/T cells	DTX-loaded TPGS-*b*-PBAE micelles possessed the ability to overcome multidrug resistance by the synergistic effect of the pH-sensitive behavior (PBAE) and P-gp inhibition (TPGS) The IC50 of DTX-loaded micelles were 100-fold lower than commercial DTX in against A2780/T cells	Zhao et al. (2013)
1,2-distearoyl-sn-glycero-3-phosphoethanolamine-polyethylene glycol DSPE-PEG	PTX	4T1	pH-responsive and rapid intracellular (endosomal) drug release of DSPE-PEG-based micellar system was achieved through introduction of antinucleosome antibody (mAb 2C5) and poly(l-histidine) (PHIS). Up to 95% PTX was released within 2 h at pH around 5.5 PTX-loaded micelles were more effective against 4T1 cells at acidic conditions b (pH 5.8 versus pH 7.4)	Wu et al. (2013)
Styrene-maleic acid (SMA)	Cisplatin	HeLa	A high-loading, water-soluble cisplatin-polymer complex was developed by formation of ionic complex with dicarboxylate residues of maleylgroups in the SMA chain Higher accumulation of SMA/cisplatin was found in tumor tissue after 24 h of intravenous administration compared to free cisplatin	Saisyo et al. (2016)

(continued)

Table 4 (continued)

Polymeric materials	Drug	Cancer cell line	Remarks	Reference
Stearoyl–PEG–poly-sulfadimethoxine methacrylate stearoyl–PEG–polySDM	PTX	MCF-7	PTX loading was 3.25 ± 0.25% (w/w%). The PTX-loaded micelles were stable at pH 7.4 and rapidly aggregated at pH 6.5 to release PTX, thus resulted in higher degree of association with cells	Ravazzolo et al. (2013)
poly(cholesteryl acrylate-*co*-methoxypoly(ethylene glycol) methacrylate) poly(CHOLy-*co*-mPEG)	CPT	MCF-7, HeLa	Sustained release of drug was triggered by hydrolysis of the ester linkages in acidic conditions. CPT-loaded micelles were non-hemolytic and could easily permeate into both MCF-7 and HeLa cells	Laskar et al. (2014)

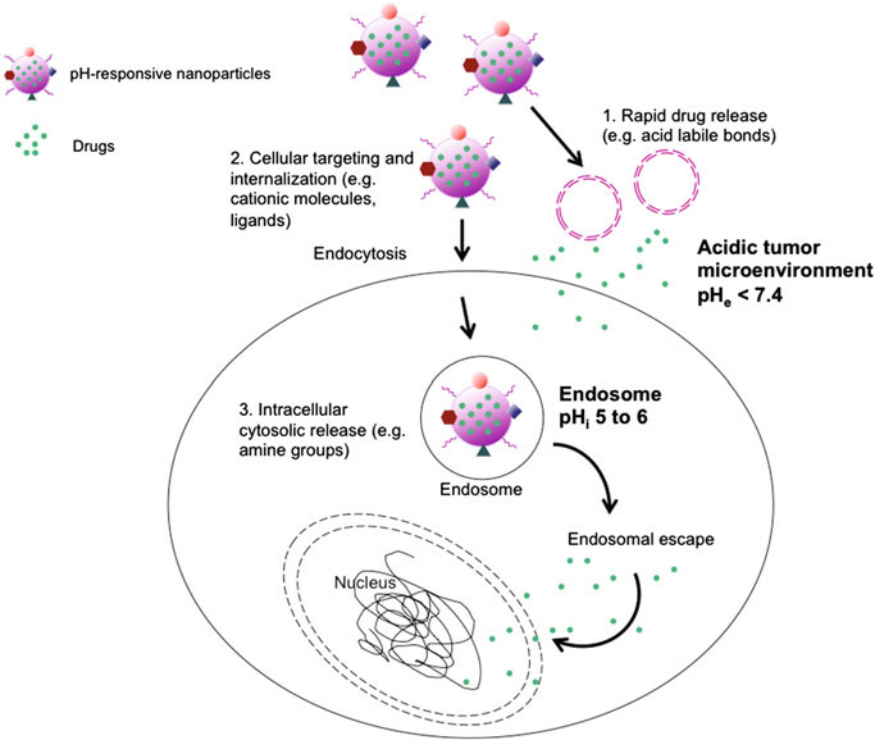

Fig. 2 Schematic diagram demonstrating the common strategies to exploit acidic tumor microenvironment as drug release trigger for pH-responsive nanoparticle systems

consequence, the destabilization of PHis-based micelles caused the higher DOX release rate at pH lower than 7.0. When A2780 cells were cultured with PHis-PEG/DOX micelles at different pH conditions (6.8, 7.4 and 8.0), higher intracellular DOX concentration was detected in acidic conditions (Lee et al. 2003). The authors believed that the rapid dissolution of DOX from polymeric micelles coupled with the stealth properties of PEG, the retention and availability of DOX was much increased compared to free DOX (Lee et al. 2003). PTX-loaded pH-sensitive polymeric micelles could be effectively delivered to prostate cancer cell lines (PC-3 and 4T1.2) as reported by Zhang and coworkers (Zhang et al. 2015). Besides the promotion of PAX release in acidic condition, the authors demonstrated that the regulation of carbon chain and different electron-withdrawing groups via stearic environments around the hydrazone linker modulated the pH sensitivity level of micelles (Zhang et al. 2015).

Nanoparticles with high DOX loading and pH-responsive behavior were synthesized through nanoprecipitation of DOX-polymer conjugate (Yu et al. 2014). The PLA-*g*-DOX nanoparticles was prepared using multistep reactions: (i) azide–alkyne click reactions to transform acetylene-functionalized PLA into PLA-*graft*-aldehyde (PLA-*g*-ALD); (ii) conjugation of amine functionality of DOX to ALD backbone of

PLA-*g*-ALD via acid-labile linker; and (iii) nanoprecipitation of graftedPLA-*g*-DOX in water containing distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[maleimide-PEG2000] (DSPE-PEG-MAL) (Yu et al. 2014). The resultant PLA-*g*-DOX nanoparticles were spherical, negatively charged, and high colloidal stability without noticeable aggregation in serum media. More than 3-folds increase of DOX release from PLA-*g*-DOX was achieved at pH 5.5. Irrespective of different concentration of DOX, PLA-*g*-DOX NP was the most potent to kill MCF-7 (Yu et al. 2014).

Two different compositions of shell cross-linked micelles (SCLM): SCLM-50 and SCLM-100 to deliver cabazitaxel (CTX) were developed for prostate cancer treatment (Aydin et al. 2016). Both prostate cell lines (PC-3 and C4-2B) were more sensitive toward SCLM-50/CTX micelles and non-shell cross-linked/CTX (NSCLM/CTX) micelles compared to free-CTX and SCLM-100/CTX micelles. This was contributed to the difference in uptake efficiency of these prostate cancer cell lines. It should be noted that the void micelles were nontoxic to HEK 293 cells (human embryonic kidney cells) even at high concentrations (50–100 folds higher than IC_{50} of CTX). Interestingly, the micelles formulation has lower recognition and uptake by macrophage (less than 10% of internalization) after exposure to serum proteins; thus suggesting that this micellar formulation could be ideal CTX delivery in regard to prostate cancer treatment (Aydin et al. 2016).

A star-shaped pH-sensitive polymeric nanoparticle based on [tetra-(methoxy-poly(ethylene glycol)-poly(2-(*N*,*N*-diaethylamino)ethyl methacrylate)-poly(ε-caprolactone) pentaerythritol) [mPEG-pDEA-PCL)4-PET], PDCP was developed for rapid PTX release and simultaneous prolonged blood circulation and rapid drug (Li et al. 2015a, b, c, d, e). Surprisingly, the cytotoxicity effect on MCF-7 cell lines and antitumor effect on in vivo mice model were nonstatistically different between free PTX and PTX-loaded PDCP. However, the tolerability and survival of mice toward PDCP nanoparticles was higher compared to free PTX solutions as demonstrated in increase body weight over time (Li et al. 2015a, b, c, d, e).

A pH-sensitive drug nanocarrier based on poly(styrene-*co*-maleic acid) [PSMAC]/PTX nanoparticles with accelerated drug release at pH mimicking lysosomes (pH 4.2) have been reported recently (Dalela et al. 2015). The maleic moiety of PSMAC was conjugated to PTX via ester linkage, using dicyclohexyl-carbodiimde (DCC) and 4-dimethylaminopyridine (DMAP). The conjugated PSMAC/DOX assembled into nanoparticles ranging from 165 to 185 nm in aqueous condition with negative surface charges (−22.2 mV). The longer incubation period of PSMAC/PTX nanoparticles in various cancer cell lines (MCF-7, MDA-MB 231, A549, HeLa, SKHEP 1 HepG2, CHO-K1, and L929) resulted in higher potency owing to slow, but sustained release of available PTX (Dalela et al. 2015). For determination of in vivo antitumor efficacy, weekly, multidosing regimen of either PSMAC/PTX (60 and 80 mg/kg), Taxol® (20 mg/kg) or saline (as control) were administered intravenously to Ehrlich Ascites Tumor (EAT)-bearing BALB/c syngenic mice. At day-15 of posttreatment, the tumor volume for mice receiving saline, Taxol®, PSMAC/PTX (60 mg/kg) and PSMAC/PTX (80 mg/kg) were 151, 103.6, 85.2, and 72.3 mm^3, respectively. At the completion of dosing regimen, the treatment with PSMAC/PTX (80 mg/kg) showed complete tumor

regression with no recurrence. The apoptotic index, measured in terms of caspase three activity, for PSMAC/PTX (80 mg/kg), PSMAC/PTX (60 mg/kg), Taxol®, and control (saline) was 1.88, 1.53, 0.79, and 0.11%, respectively. It should be noted that survival rate was significantly higher for PSMAC/PTX nanoparticles-treated group (64–87%) compared to that of Taxol® (49%) (Dalela et al. 2015).

To increase the internalization of drugs, functionalization of nanocarriers with cationic molecules could be employed. The cationic micelles are electrostatically attracted to negatively charged cell membrane followed by electrostatic-adsorptive endocytosis. A series of cationic poly(dimethysiloxane)-poly(2-(dimethylamino) ethyl methacrylate) [PDMS-*b*-PDMAEMA] block copolymer micelles with different molecular weight (M_w) of PDMAEMA was synthesized to deliver DOX intracellularly into HeLa cell line (Car et al. 2014). The cytotoxicity of HeLa cells was directly correlated to the M_w of PDMAEMA, which was due to higher degrees of association between cells' surface (negative charge) and PDMAEMA micelles (positive charge). The authors showed that PDMS-*b*-PDMAEMA/DOX micelles responded to intracellular pH changes via increased DOX release in acidic pH and were internalized through endocytosis to acidic cell compartments. The confocal images further supported the toxicity of PDMS-*b*-PDMAEMA/DOX micelles at late stage of incubation, whereby cell morphology changes and apoptotic cells were observed (Car et al. 2014).

A novel cRGDyK peptide modified pH-responsive nanoparticle based on poly (ethylene glycol)-poly(2,4,6-trimethoxy benzylidenepentaerythritol carbonate) [PEG-PTMBPEC] diblock copolymer loaded DOX was synthesized to kill tumor and inhibit angiogenesis simultaneously (Qiu et al. 2016). Modified DOX-loaded nanoparticles were more pronounced against B16 and HUVEC cell lines. The higher toxicity in both cell lines was correlated to the higher uptake modified DOX-loaded nanoparticles via receptor-mediated endocytosis and acidic-triggered drug release within the cells (Qiu et al. 2016).

The pH gradient, which exploits the acidified endosome and fusion with lysosome within cells, is gaining attention for intracellular drug release. Nanocarriers that disassemble or swell to achieve alterable drug release behavior in endosomal pH conditions (pH 5–6) have been designed via decoration with acid-sensitive bonds in the polymer backbone and acid-protecting groups, respectively. Recently copolymers of poly-l-lysine, P(bAE), and P(His) have been widely used to protect chemotherapeutic drugs from harsh endosomal–lysosomal environment (low pH and high enzymatic activity). In a study by Zhu and coworkers, lysosomal targeting polymeric micelles loaded with analog gemcitabine (GemC18) showed stronger antitumor activity against melanoma in both melanoma in vitro and in vivo conditions (Zhu et al. 2012). This activity was much pronounced when acid-responsive polymer was introduced owing to the higher hydrolysis rate of GemC18 to active GEM by lysosomal enzymes such as cathepsin B and D (Zhu et al. 2012). In a separate study, cross-linkers with different functionality (acid degradable and nondegradable) were used to prepare CPT-loaded micelles to target ovarian cancer (Huynh et al. 2012). Cellular uptake and release rate of CPT by OVCAR-3 cells were significantly varied in both micelles with and without the acid cross-linkers.

The conjugation of PCT micelles to acid cross-linker (2,2′-(propane-2,2-diylbis (oxy)) diethamine resulted in higher CPT release irrespective of the pH microenvironments. Owing to the pH-sensitive characteristic, acid cross-linked polymeric micelles were more toxic toward OVCAR-3 cells as a result of enhanced internalization using endocytosis and passive diffusion pathway (Huynh et al. 2012).

As mentioned earlier, the concept of endosomal pH targeting is based on the exploitation of differences of extracellular pH in tumor tissues and healthy tissues. A pH-responsive, poly(β-amino ester) [PbAE] nanoparticle which undergoes rapid dissolution in acidic microenvironment, was employed for PTX delivery to treat Jurkat acute lymphoblast leukimia T-cells (Lundberg 2011). The effectiveness of PbAE/DOX nanoparticles at lower concentration compared to non-pH-responsive PCL/DOX nanoparticles was attributable to higher intracellular DOX content in cytosol after ingestion of particles (pH-triggered dissolution of PbAE polymer in acidic endosomal-lysosomal compartment) (Lundberg 2011).

The exploitation of redox sensitivity owing to the differences in glutathione (GSH) concentrations and their affinity to cleave disulfide bonds is another strategy to trigger cytosolic release of drugs. Glutathione (GSH) is a thiol containing tripeptide, which is responsible for the reduction of disulfide linkages in cytoplasm. The intracellular cytosolic GSH concentrations (2–10 mM) are almost 1000-folds higher than extracellular GSH concentrations in physiological body fluids (2–10 μM)(Jia et al. 2014). Compared to healthy tissue, the levels of GSH are 4-fold higher which is attributable to drug resistance mechanism in cancer cells via GSH-mediated phase detoxification (Lale et al. 2015; Cheng et al. 2013; Biswas et al. 2016). Theoretically, polymeric micelles or nanoparticles carrying disulfide bonds are stable and could only be triggered to release drugs within cytosolic of cancer cells (high internal GSH). Disulfide bonds are generally linked between hydrophobic and hydrophilic domains in the amphiphilic block copolymers or conjugated to hydrophobic backbone of copolymer (Liu and Liu 2015; Wen et al. 2011; Zhang et al. 2013a, b; Sun et al. 2010; Li et al. 2012). In addition, GSH-sensitive agents could be cross-linked to the shell of micelles, leading to rapid disassembly of micelles and intracellular drug release inside the cells (Liu and Liu 2015; Samarajeewa et al. 2013).

A redox-sensitive poly(ethylene glycol)-poly(lactic acid) [MPEG-SS-PLA] loaded with PTX was synthesized using optimized oil-in-water emulsion/solvent evaporation to treat various cancer cell lines (A549, MCF-7 and HeLa) (Song et al. 2011). Multistep reactions were employed to synthesize MPEG-SS-PLA with disulfide linker between MPEG and PLA. The optimization of solvent, copolymer concentration, ratio of solvent to water produced MPEG-SS-PLA/PTX nanoparticles with rice-shaped like morphologies. The mean particle major axis length and minor axis length were 373.9 ± 4.8 nm and 129.5 ± 3.8 nm, respectively. Almost 90% of PTX was released from the nanoparticle in conditions with high intracellular GSH concentration. The PTX levels in the serum were almost negligible. Using standard MTT assay, PEG-SS-PLA/PTX nanoparticles exhibited the highest toxicity toward all three cell lines (Song et al. 2011). A novel pH-redox responsive micelle system that disintegrates in acidic conditions and exposes nitroxide radicals was effective to

enhance antitumor activity of DOX. This effect was due to the ROS scavenging activity of the released nitroxide radicals. The nitroxide radical-containing nanocarrier was a core-shell type of self-assembling polymeric micelles, with average diameter of 40 nm, consisting of poly(ethylene glycol-*b*-poly[4-(2,2,6,6-tetramethylpiperidine-*N*-oxyl)aminomethylstyrene] (PEG-*b*-PMNT) (Yoshitomi et al. 2013). The intravenous administration of radical-containing micelles at dosage of 100 mg/kg was found to decrease the ROS levels and TNF-α expressions. The cardiotoxicity effect of DOX was also reduced in vivo when colon-26 tumor bearing mice were pretreated with radical-containing micelles (Yoshitomi et al. 2013).

3.2 Exogenous Stimuli-Responsive Polymeric Nanoparticles and Micelles

Temperature-responsive drug delivery is one of the most studied strategies in particular for oncology as local tumor site demonstrate distinct hyperthermia (40–42 °C). An ideal temperature-responsive carrier should retain the drug load at normal body temperature (37 °C) and rapidly release the drug in heated pathological area. To date thermo-responsive liposomes are the most studied temperature-responsive carriers as demonstrated in several clinical trials. For polymeric micelles or nanoparticles, thermo-responsive polymers consist of temperature-responsive hydrophilic segments and a suitable hydrophobic segment. Such polymers are fully soluble below a certain temperature, more commonly known as lower critical solution temperature (LCST), via formation of hydrogen bond in aqueous conditions. Above the LCST, the disruption between hydrogen bonds of water and the polymer chains induce phase separation and precipitation in polymers, and subsequently destabilization of micelles. Various preferred polymers building blocks for temperature-responsive polymeric micelles or nanoparticles include poly(*N*-isopropyl acrylamide), PNIPAAM, poly(γ-2-(2-(2-methoxyethoxy)-ethoxy)ethoxy-ε-caprolactone)-*b*-poly(γ-octyloxy-ε-caprolactone). The LCST of PNIPAAM can be adjusted to slightly above physiological temperature for cancer applications through introduction of hydrophilic segments (Takeda et al. 2004; Biswas et al. 2016). For instance, copolymers of P(NIPAAM-*co*-acrylamide)-poly (lactide) and P(NIPAAM-*co*-*N*,*N*-dimethylacrylamide) poly(lactide) have LCST of 41 °C (Biswas et al. 2016; Nakayama et al. 2014). DTX-loaded PLA-conjugated PNIPAAM-based acrylamide showed thermo-responsive behavior by releasing high amount of drugs in hyperthermia condition (Liu et al. 2008). Significantly higher antitumor efficacy was observed in mice treated with thermo-responsive DTX-loaded micelles compared with the conventional DTX formulation (Liu et al. 2008). Hennink and coworkers reported the development of thermo-responsive PTX-loaded polymeric micelles based on pHPMAmDL-*b*-PEG block copolymers and evaluated the cytotoxicity, stability, and drug release kinetics in vitro (Soga et al. 2005).

Ultrasound-triggered drug delivery is effective to achieve spatiotemporal control of drug release at specific locations without exerting too much harmful effects on healthy tissues. Other attractive features of ultrasound-based delivery include noninvasive, zero ionizing radiations, and ease of tissue penetration depth by mere regulation of frequency, duty cycle, and time exposures. Both thermal and mechanical effect generated by radiation forces induced instability of polymeric micelles and subsequently triggered the release of drugs. Drug-loaded polymeric micelles coupled with ultrasound therapy could promote anti-cancer treatment via inducing thermal effects, enhanced drug diffusion into tumor region, and increase intracellular drug bioavailability. Gao and coworkers have developed mixed micelles of PEG-PLLA and PEG-PEG for encapsulation of DOX and perfluoropentane (PFP) (ultrasound imaging contrast agent) (Gao et al. 2008). The emulsion nanodroplets were formed by PFP and stabilized by PEG-PLLA and PEG-PEG polymeric micelles. As the boiling point of PFP was low (29 °C), at physiological temperatures the evaporation of PFP converted the nanodroplets into nanobubbles. Meanwhile, DOX was localized within the nanobubbles' wall. The authors showed that PFP/DOX-loaded micelles underwent conversion to nanobubbles in vivo, followed by coalescence into larger echogenic microbubbles and accumulated in tumor tissue via EPR effect (Gao et al. 2008). In another study, DOX-loaded perfluorocarbon (PFC)/mPEG-PLGA nanodroplets which could be transformed into nanobubbles at 37 °C showed higher rate of tumor inhibition (Du et al. 2011). The ultrasound-triggered drug release when sonicated for 0.5 min at 37 °C was pH-dependent whereby up to 9.59% DOX was released at pH 6.5 compared to that of pH 7.4 (2.22%). In vivo tumor reduction for mice receiving receiving DOX-loaded PFC/mPEG-PLGA nanodroplets coupled with ultrasound was 84.3% compared to 60.4% when ultrasound was not applied (Du et al. 2011). This approach was further proven to be versatile in delivering other chemotherapeutic drugs such as PTX and curcumin (Ji et al. 2014; Wan et al. 2012).

In another study, the intracellular accumulation of DOX/Pluronic 105 micelles was higher in HL-60, A2780, A2780/ADR, and MCF-7 cell lines when high frequency ultrasound was applied and this effect was correlated to the increase of membrane permeability of cells (Marin et al. 2002). Similar observation was also reported by Wan and coworkers in which the PTX uptake and subsequently cytotoxicity effect on drug-sensitive (MDCKII and MCF-7) and P-gp expressing cell lines (MDCKII-MDR and NCI-ADR) was enhanced by twofold in conjunction with ultrasound therapy (Wan et al. 2012). The expressions of P-gp, MRP, and lung resistance protein in HepG2/ADM were drastically reduced with the combination therapy of DOX-loaded polymeric micelles and ultrasound therapy (Gao et al. 2005a, b). In addition, the application of ultrasound is not only to attenuate the expression MDR proteins, it induced higher apoptotic level in HepG2/ADM cells (Wu et al. 2011). Park and coworkers have developed a multifunctional DOX-loaded PLGA-gold nanoparticles, which could trigger the release of DOX upon exposure to near infrared (NIR) (Park et al. 2009). Using HeLa cells as cytotoxicity model, higher therapeutic effect was obtained with PLGA-gold/DOX nanoparticles compared to monotherapy (Park et al. 2009).

3.3 Multiple Stimuli-Responsive Polymeric Nanoparticles and Micelles

Dual function polymeric micelles were developed to further improve the targeting delivery of DOX for cancer treatment (Chen et al. 2013a, b). Thermo-sensitive and pH-dependent polymeric micelles coupled with pH-sensitive phase transitions at mild acidic pH and body temperature were fabricated by functionalizing PEG-PCL copolymers using 6-aminocaproic acid (ACA) (Chen et al. 2013a, b). The ACA-PEG-PCL copolymers self-assembled into nanomicelles (66–75 nm) and could be triggered to release DOX in response to pH and temperature variation. For instance, to achieve 30% release of DOX, at least 70 h of incubation was required in neutral pH at 37 °C. However, ACA-PEG-PCL/DOX micelles released the same amount of DOX in acidic condition after incubation for 6 h. Using a tumor xenograft mice model with melanoma (MDA-MB-435), the tumor reduction activities after 32 days of drug administration followed the following decreasing trend: ACA-PEG-PCL/DOX micelles > free DOX > PEG-PCL/DOX micelles > empty micelles. The ratio of tumor size in mice normalized against untreated mice for ACA-PEG-PCL/DOX micelles, PEG-PCL/DOX micelles, and free DOX were 0.12, 0.72, and 0.38, respectively. In addition, side effects of DOX were also minimized, as the average body weight loss of mice was less than 5% (Chen et al. 2013a, b).

Kashyap and coworkers have designed dual thermo- and enzyme-responsive micelles consisting of 3-pentadecylphenol and oligoethylene glycol acrylate amphiphilic copolymers for DOX delivery to MCF-7 and HeLa cell lines (Kashyap et al. 2016). Hydrophobic acrylate monomer from 3-pentadecylphenol (PDP) was copolymerized tooligoethylene glycol acrylate (hydrophilic segment) via radical and RAFT methodologies to produce novel amphiphilic copolymers with thermo- and enzyme- responsive features. In water, the amphiphilic copolymers self-assembled into core-shell nanoparticle and had higher affinity toward DOX (loading of 3.78%). Significant differences in DOX release from the loaded micelles could be seen at 37 °C (normal body temperature) and 43 °C (cancer tissue temperature). Only 20% DOX was released at 37 °C while up to 90% of drugs were leached out within 2 h. In addition, the presence of esterase triggered the rapid release of DOX as well (Kashyap et al. 2016).

Amphiphilic (β-aminoester)-grafted-disulfide methoxy polyethylene glycol copolymer (PAE-*g*-DSMPEG) micelles with pH and redox-sensitive properties were recently reported (Bui et al. 2015). The higher release of DOX from the PAE-*g*-DSMPEG micelles was induced in both acidic condition and presence of DTT (reducing agent). The PAE-*g*-DSMPEG/DOX-micelles formulation was highly potent in killing HepG2 cell even at low concentration (30 μg/ml). Although in vitro uptake of DOX by HepG2 cells demonstrated that free DOX were favored compared to PAE-*g*-DSMPEG/DOX-micelles, the in vivo model of HepG2 tumor-bearing mice revealed that micelles formulation was far more superior to free DOX. More specifically, less tumor cells and higher number of apoptotic cells were observed after treatment with PAE-*g*-DSMPEG/DOX-micelles compared to free

DOX (Bui et al. 2015). Meng and coworkers incorporated pH- and light-responsive features into chitosan-based micelles harboring camptothecin (CPT) (Meng et al. 2013). When this CPT formulation was exposed to MCF-7 cells under UV irradiation, the killing of MCF-7 cells was improved almost twofold compared to without UV exposure (Meng et al. 2013).

Recently, a photochemically triggered cytosolic-drug-release nanoparticle (PHANs) was developed based on conjugation of acetylated backbone to hyaluronic acid (HA)amine-containing polypeptide pH-responsive moiety and a photosensitizer, chlorin e6 (Ce6) followed with formation of self-assembled nanoparticle encapsulating DOX (Lee and Na 2014). Under high laser irradiation (1.2 J/cm^2), the localization of DOX/PHANs into HCT-116 cell nucleus cells was improved drastically from 56.7 to 78.8%, as confirmed by the escape of DOX via endosome-lysomomal membrane disruption. In terms of viability of HCT-116 cells, the number of viable cells decreased from the 74.9 to 42.9% with increase in irradiation exposure from 0.6 to 1.2 J/cm^2. Additionally, treatment of CT-26 bearing mice with DOX/PHANs coupled with laser irradiation also displayed similar efficacy (Lee and Na 2014).

4 Conclusions

This review has summarized some recent trends in the development of polymeric nanoparticles for cancer treatment. Nanoparticle-based drug delivery for cancer treatment has progressed tremendously in the last decade. Nanoparticle-based cancer therapeutics has evolved from its original intention to overcome the toxicity and instability issues of chemotherapeutic drugs toward fingerprinting, personalized nanomedicine. The concept of nano-cancer-target is appealing as multifunctional smart nanoparticles could be designed to exploit the pathological, physiological, and microenvironment of tumors to achieve optimal therapeutic efficacy. Various new and improved nanoparticles have undergone preclinical and clinical investigation (Tables 5 and 6). In this aspect, surface-decorated nanoparticles with biological targeting (i.e., folate, aptamer) has enhanced the affinity toward cancer cells. In addition, prolonged circulation of drug nanoparticle is made possible with the incorporation of long-circulating polymers such as PEG. The ‘stealth-like’ properties of nanoparticles are attractive to reduce drug-associated toxicity. The development of nanoparticle to deliver combination chemotherapeutics is also extensively studied. To further improve the treatment, researchers should explore the use of chemosensitizer and radiosensitizer in conjunction with drugs nanoparticles. Examples include siRNA and curcumin that sensitize the cancer leading to lower drug dosage. Following the success of first generation of nanomedicine, novel nanoparticles with complex stimuli-responsive drug release such as light, temperature, and ultrasound are also under clinical trial investigations. With the current development of nanomedicine, it is entirely plausible that the use of nanoparticle-based cancer therapeutics will be mainstream in the near future.

Table 5 Chemotherapeutics drug delivery with polymeric micelles: marketed and under clinical evaluation

Product name	Type of polymer	Type of drug	Type of cancer targeting	Status	References
Genexol-PM	PEG-poly(D,L-lactide)	PTX	Breast cancer and lung cancer	Marketed	
Genexol-PM	PEG-poly(D,L-lactide)	PTX	Metastatic breast cancer	Phase 3	NCT01644890
NK105	mPEG-poly(aspartic acid)	PTX	Gastric cancer	Phase 2	Kato et al. (2012)
NK105	mPEG-poly(aspartic acid)	PTX	Metastatic breast cancer	Phase 3	NCT01644890
NK105	mPEG-poly(aspartic acid)	PTX	Advanced stomach cancer	Phase 2	Hamaguchi et al. (2007)
NK911	mPEG-poly(aspartic acid)	DOX	Solid tumor	Phase 1	Matsumura et al. (2004)
SP1049C	Pluronic L61 and F127	DOX	Advanced adenocarcinoma of the esophagus and gastroesophageal junction	Phase 2	Valle et al. (2011)
NC-6004	mPEG-poly(glutamic acid)	Cisplatin	Solid tumors	Phase 1	Plummer et al. (2011)
NC-6004	mPEG-poly(glutamic acid)	Combination therapy with NC-6004 and gemcitabine	Locally advanced pancreatic cancer Metastatic pancreatic cancer	Phase 3	NCT02043288
NC-6004	mPEG-poly(glutamic acid)	Combination therapy of NC-6004 (nanoplatin) and gemcitabine	Locally advanced and metastatic pancreatic cancer	Phase 1 and 2	NCT00910741
NK012	mPEG-poly(glutamic acid)	SN38	Lung cancer and triple negative breast cancer	Phase 1 and 2	Matsumura (2011)
NK012	mPEG-poly(glutamic acid)	SN38	Solid tumor	Phase 1	Hamaguchi et al. (2010)

Table 6 Chemotherapeutics drug delivery with polymeric nanoparticles: marketed and under clinical evaluation

Product name	Type of polymer	Type of drug	Type of cancer targeting	Status	References
CRLX101	Cyclodextrin-poly(ethylene glycol) copolymer	CPT	Various cancers	Phase 2	
CT-2103	Polyglutamate	PTX	Persistent ovarian epithelial cancer or primary peritoneal cancer	Phase 2	NCT00045682
CT-2103	Polyglutamate	PTX	Ovarian epithelial or fallopian tube cancer or Primary peritoneal cancer	Phase 2	NCT00017017
CT-2103	Polyglutamate	PTX	Lung cancer	Phase 3	Langer et al. (2008)
CT-2103 (Xyotax)	Polyglutamate	PTX	Breast and metastatic breast cancer	Phase 2	NCT00148707
BIND-014	PLGA/PLA-PEG	DTX	Urothelial carcinoma Cholangiocarcinoma Cervical cancer Squamous cell carcinoma of head and neck	Phase 2	NCT01574274
BIND-014	PLGA/PLA-PEG	DTX	Metastatic castration-resistant prostate cancer	Phase 2	NCT01812746
BIND-014	PLGA/PLA-PEG	DTX	Lung cancer	Phase 2	NCT01792479
Transdrug®	poly(isohexyl cyanoacrylate)	DOX	Advanced hepatocellular carcinoma	Phase 3	NCT01655693
	Polybutylcyanacrylate	Mitoxantrone	Hepatocellular carcinoma	Phase 2	Zhou et al. (2009)

However, there are still limited clinical data to fully understand the implications of nanoparticle-based therapeutics. In addition, the numbers of FDA-approved nanomedicine for clinical use are still very few. One of the contributing factors is the uncertain biosafety issue and nanoparticle-cell interaction in human bodyas well as lack of established clinical protocols for nanomedicine development. Therefore, it is crucial that clinical investigators should fully understand the characteristics of their nanoparticles for a well-designed clinical trial.

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Nanotechnology-Based Immunotherapeutic Strategies for the Treatment of Cancer

Rajeev Sharma, Nishi Mody and Suresh P. Vyas

Abstract

Cancer is a dreadful disease and presently the leading cause of death worldwide. Scientists are continuously exploring new treatment regimen for successful management of this disease. Advancement in the field of nanotechnology and its integration with the field of immunotherapy has paved new ways for improving the treatment of cancer. Immunotherapy refers to therapeutic approaches that treat cancer by using patient's own immune system. By using nanometric-sized particulate and vesicular carriers, tumor-associated antigen(s) and adjuvant(s) can be simultaneously administered which augment the immune system activation and this concept can be wisely used for designing nanotechnology-based cancer immunotherapy. Also nanotechnology-based immunotherapy confers certain benefits like enhanced therapeutic effect, targeted delivery to immune cells, and reduced adverse outcomes. Nanotechnology-based therapeutic cancer vaccine consists of antigen(s), delivery system, and adjuvant. This chapter comprises of the expected outcomes of simultaneous delivery of tumor-associated antigen(s) and adjuvant to dendritic cells using vesicular and particulate vaccine delivery system(s). It is also a summarized overview on the advancement of polymeric- and lipid-based delivery systems for the development of nanotechnology-based cancer immunotherapy.

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Cancer · Immunotherapy · Dendritic cells · Tumor-associated antigen(s) · Particulate and vesicular carriers

1 Introduction

Today, cancer is a major health concern and one of the most leading causes of death across the world after heart disease. Around 12 million people are being diagnosed as new cases every year and it is estimated that by 2020, the number will be 15 million (Cancer fact and figures 2016; Siegelm et al. 2013; Brannon and Blanchette 2004). One of the major reasons for poor survival rate is the conventional treatment, which affects both cancer as well as normal cells since they cannot deliver anti-cancer bioactive(s) selectively to the tumor cells. Cancer is a heterogeneous disease that results from a multistep process, characterized by uncontrolled proliferation of tumor cells, invasion and metastasis. Tumor cells have also the ability to escape immune system (Fernald and Kurokawa 2013) and to evade programmed cell death (Zitvogel et al. 2006). Although genetic abnormalities are involved in 5–10% of cancers, other causes include the viral infection (HBV, EBV and HPV), use of tobacco, lack of physical activity, ultraviolet (UV) radiation exposure, and pollution which may directly damage genes leading to carcinogenic mutations (Anand et al. 2008). Patients may be treated based on currently available protocol, which includes chemotherapy, radiotherapy, surgery or combination of two or all three treatments. Chemotherapy in conventional form of treatment that targets all proliferating cells indiscriminately, thus killing both tumor as well as healthy cells. The radiotherapy and surgery fail to treat metastases. Individuals who are not cured by surgery are usually given a combination therapy. Further, the composition of treatment is determined by the stage and type of cancer. Limitations of conventional cancer therapeutics have thus demanded for an effective and less harmful therapy. The main focus of the treatment of different cancers invariably remains a tumor-specific therapy, which addresses the problems associated with the conventional therapies (Singh and Bhaskar 2014; Goforth et al. 2009; Perez 2005). In the past two decades, remarkable attempts have been made in the diagnosis; prevention and therapy of cancer; however, effective treatment of cancer yet remains an unachieved goal. Thus, there is a need to develop some nanomedicine-based approaches, which may provide specific and precise targeting to the tumor cells with minimum undesirable toxic effects to healthy cells. In recent years, discernable efforts have been made applying nanotechnology-based approaches to specifically target the tumor cells and increase localized delivery by increasing serum residence time and site specificity (Stephanie et al. 2013; Zamboni et al. 2012; Caldorera and Peppas 2009). Although these approaches could improve the therapeutic index with minimum systemic toxicity of bioactive(s), more efforts are needed to explore and

develop effective delivery strategies. Recently, cancer immunotherapy is an emerging paradigm seemingly as an attractive option. The immunotherapy tends to manipulate the patient's own immune system enabling it to recognize and destroy the cancer cells. The advantages of cancer immunotherapy include its ability to induce specific killing of tumor cells with low toxicity to the healthy cells or elimination of tumor owing to the involvement of natural immune response, induction of systemic antitumor immune response that can control metastases, and an immunological memory, which could provide long-term protection (Sheng and Huang 2011).

- Immunotherapy/vaccination helps to recognize the non-self-biomolecules associated with the cancer cells and helps body to fight against variant cancer cells.
- Nanotechnology-based immunotherapeutic approaches are aimed to induce tumor-specific T cells for its destruction (Restifo et al. 2012).
- Professional antigen-presenting cells like dendritic cells (DCs), macrophages are known to initiate and modulate immune response against various tumor-associated antigens including self-antigen(s) (HYi and Appel 2013). Targeted delivery of immune components to APCs leads to its recognition MHC I/ MHC II restricted presentation by DCs/macrophages for subsequent immune reaction(s) (Cruz et al. 2012).
- Immunotherapy is thus expected to protect and improve the quality of life of cancer patients because of its low toxicity and also by preventing reemission of cancer (North and Butts 2005).

2 Immunotherapies

Immunotherapies are intended to involve and boost the immune response against the antigenic determinants associated with a cancer. For the development of effective cancer immunotherapy, certain limitations are to be addressed which include (a) identification of target antigen or antibody, (b) selection of appropriate adjuvant for immunity modulators, (c) provision to overcome immune suppression that operates and used by the tumor, or by previous therapy, (d) revoke immune suppression that operates in the case of cancer cells and (e) design and develop specific immune response *vis a vis* ensure for no possibility of an autoimmunity as may be caused by immunotherapy (Finn 2003). In addition, enhancement of adjuvant activity through the use of nanodelivery system(s) is particularly exciting, as synergistic effects are seen resulting in stronger immune responses. Targeting these immune suppressive populations as well as stimulating immune effect or cells against tumors is a major goal of cancer immunotherapy. In a study, Kwong and their co-worker observed that lipid-based nanocarriers(s) could deliver the immune

stimulatory CpG oligonucleotide and anti-CD40 antibody, inducing an enhanced antitumor response without release of inflammatory cytokines typically associated with cancer treatment (Kwong et al. 2011).

3 Cancer and Immune System

The immune system is a complex network of cells, tissues, organs, and the biosubstances they help recognize "foreign invaders" and fight against infections or diseases. However, the immune system can recognize, eliminate, and subsequently protect the body from viral, bacterial infections, and the transformed cells (pre-cancer cell) extension (Sheng and Huang 2011). The immune system can respond against the cancer cells by reacting against tumor-specific antigens (molecules that are unique to cancer cells). Cancer immunologists investigated that cancer cells have the ability to suppress the immunity systemically as well as in and around the tumor microenvironment. In addition to immunosuppressive molecules, the vesicular endothelial growth factor (VEGF), inhibitory cytokines-interleukin (IL-10), and transforming growth factor β (TGF-β) (Teicher 2007), various human cancer cells also synthesize and release the immunosuppressive enzyme indolamine-2,3-dioxygenase (IDO) (Uyttenhove et al. 2003; Muller et al. 2007). This enzyme was reported as a regulator of autoimmunity, which mediates and regulates inhibition of T cell activation (Munn et al. 2002). Murine tumors, which produced TGF-β that converts antitumor effect or T cells into regulatory T cells, could escape their own destruction by the immune cells. An increase in regulatory T cells population is observed in the peripheral blood of patients with head and neck cancer or melanoma (Bergmann et al. 2007; Chikamatsu et al. 2007; Fecci et al. 2006).

The process of prevention, elimination, and growth of tumors is known as "immune surveillance". Various immune cells, including B and T-lymphocytes, dendritic cells, natural-killer (NK) cells, macrophages, and poly-morphonuclear leukocytes, etc., are recruited to the tumor site (Whiteside et al. 1986). The cancer cells however could evade the immune surveillance. The phenomenon that explains the process of immune escape is referred to as "immune editing". The various phases of immune editing as explained by Schreiber et al. (2011) are briefly discussed as follows:

1. ***Elimination***—This relates to the phases of tumor immune surveillance where in a tumor is detected and eradicated by innate and adaptive immunity, through the secretion of IFN-γ, IFN-α/β, and perforins, natural-killer group 2D (NKG2D; receptor plays a significant role in protecting the host from infections and cancer) and tumor necrosis factor related apoptosis inducing ligands (TRAIL; a member of the TNF family of ligands capable of initiating apoptosis through engagement of its death receptors). When the elimination process is complete, tumor cells are cleared. If it is incomplete, surviving tumor cells will enter into an equilibrium phase.

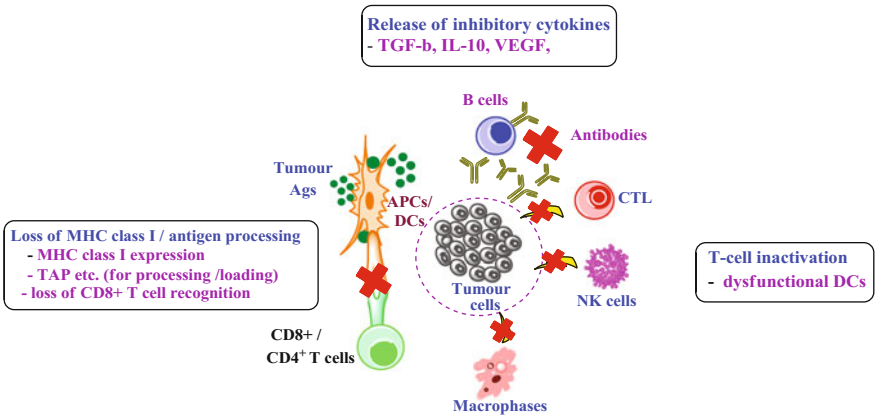


Fig. 1 Immune escape of tumor

2. ***Equilibrium***—In this phase, the tumor cells may continue to grow chronically with genetic variability and evading immune recognition thus generates a new population.
3. ***Escape***—In this phase, the new population may escape the immune system by various mechanisms including T cell inactivation, or generation of regulatory T (Treg) lymphocyte, corrupting MHC-I/ antigen processing, immune suppression/release of inhibitory cytokines such as IL-10, TGF-b, VEGF, etc., loss of adhesion molecules; blocking of NKG2D-mediated activation, expansion of myeloid derived suppressor cells (CD11b+ Gr-1+ cells, MDSCs), and induction of apoptosis of antitumor effect or cells. Schematically immune escape mechanisms of tumor are shown in Fig. 1 (Dunn et al. 2004a, b; Ugel et al. 2009; Gabrilovich et al. 2009; Poggi et al. 2006).

3.1 Limitations of Existing Cancer Therapies

The existing curative treatments for cancer, i.e., chemotherapy, surgery, and radiotherapy are generally successful if the cancer is detected at an early stage. The current treatments of cancer still have many major limitations as shown in Fig. 2. Once the tissue has metastasized, these therapies are less successful. Sometimes logical combinations of existing therapies are effective but only for limited period of time. Infact, it has been observed that single treatment regimens have limited ability to eliminate cancer cells owing to its heterogeneous nature (Hanahan and Weinberg 2000; Helmy et al. 2013). Although these approaches have improve the therapeutic efficacy of bioactive(s) with minimum systemic toxicity, much attention on development of effective approaches is still needed to reduce chemotherapy related side effects, prevent tumor evasion, drug resistance and to increase patients compliance (Dunn et al. 2002; Koebel et al. 2007; Chen et al. 2014; Xu et al. 2014).

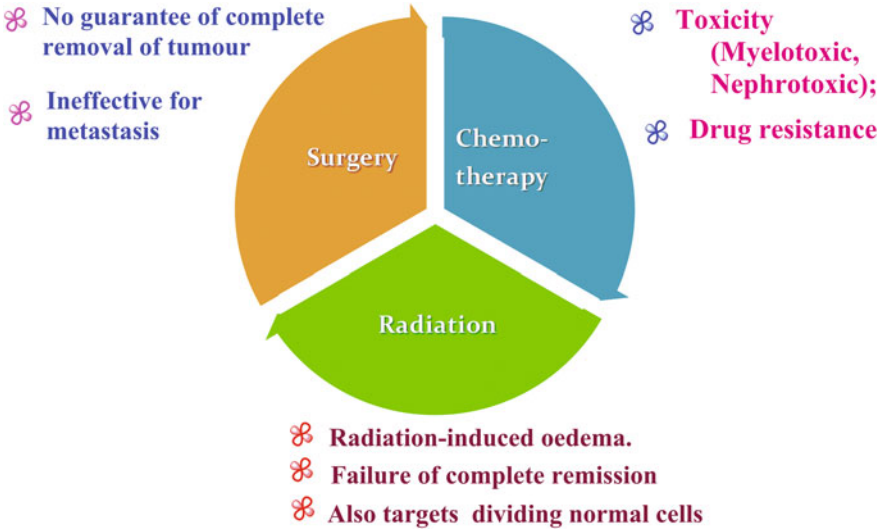


Fig. 2 Current approaches and major limitations of tumor therapy

4 Basics and Strategies of Cancer Immunotherapy

Cancer cells are imitative of normal cells and they possess similarity to the normal cell at molecular level. Initially immune cells and signaling molecules of the body attempts to control the growth of the tumor mass stimulating their destruction during the elimination phase. Nevertheless, the tumor mass is not eliminated completely by the immune cells and some of them become resistant to the immune attack and more into an equilibrium phase. These resistant tumor cells escape the defense mechanism and continue to grow in an uncontrolled manner leading to the progression of the cancer. At this stage, immune cells fail to recognize tumor cells as non-self. The intent of developing cancer immunotherapy is to teach and impart super ability to the immune system and to utilize its potential to recognize and wipe out cancer cells by educating them, ex vivo or in vivo. Also, the alterations in the extracellular matrix of the cancer cells influence the survival of cancer cells. Immunotherapy basically activates the soldiers of the immune system, i.e., macrophages, natural killer cell, cytotoxic T cells, and DCs to operate against the cancer cells (Dhake 2013). Macrophages and DCs perform the specialized function of antigen presentation and thus referred to as professional APCs of the immune system.

Macrophages and DCs process the foreign substance and present the antigen derived from them in association with MHC I/MHC II to the TH cells; hence they are called APCs. Such APCs then interact with the helper and cytotoxic T cell

followed by their activation and multiplication. These activated T cells further activate other phagocytes (Inactive macrophages and NK cells) and T cells which can directly target and destroy the tumor either by mass phagocytosis or by cellular toxins (Fig. 3). Apart from T cells, B-lymphocytes also play a role in fighting with the tumor cells. They produce antibodies specific to the tumor cells, which mark the cancer cells for destruction, and support the NK cells and macrophages. Additionally, an immune memory is also generated to suppress reformation and recurrence of the same tumor (Kumar et al. 2010). Immunotherapy is emerging as a promising option for the treatment of cancer that involves dynamic immunomodulation and active immune response. It is well established that to generate a lifelong immunity against tumor cells, priming of tumor-specific cytotoxic effecter as well as memory T cells is essential (Schuster et al. 2006). The following strategies have been explored for cancer immunotherapy. These include.

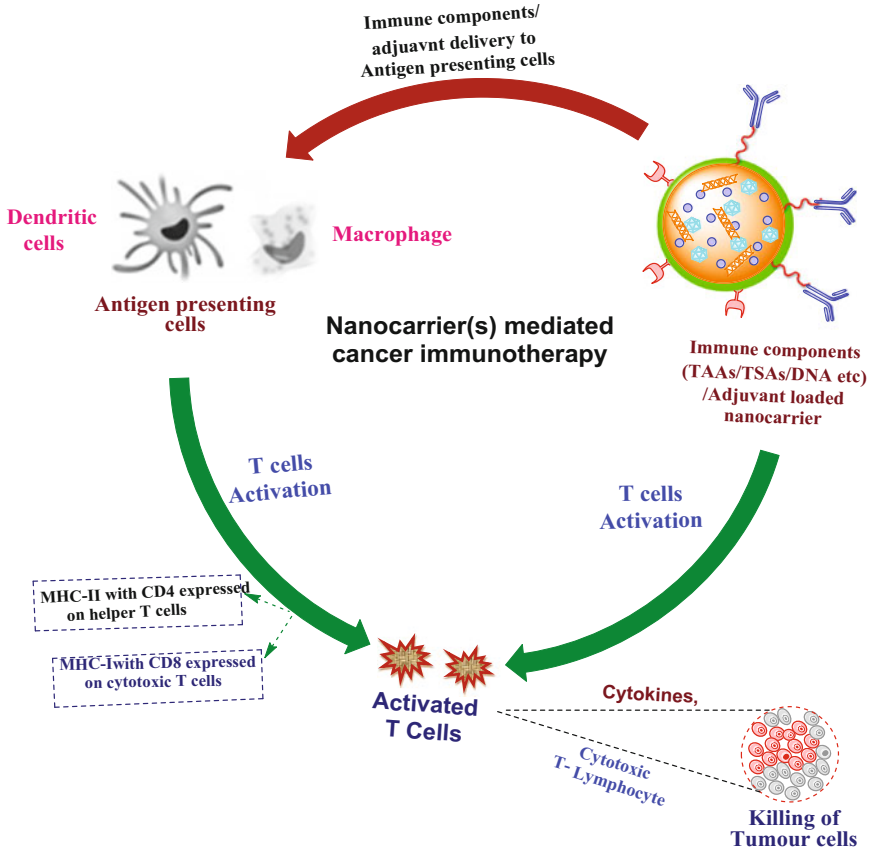


Fig. 3 Nanocarriers-based approaches for immunotherapy of cancer

4.1 Monoclonal Antibodies (mAbs)

Over past two decades, various mAbs have been investigated for their use in the treatment of cancer. mAbs are protein(s) that specifically target a specific antigen(s) against which they are raised, such as one found on cancer cells. mAbs can be very useful in treating cancer because they can be designed to attack the cancer cells in a very specific manner. The US Food and Drug Administration (FDA) has approved more than a dozen mAbs (cetuximab, catumaxomab, nimotuzumab, vivatuxin. etc.) to treat certain types of cancer (Table 1) (Weiner et al. 2012). Currently, a number of clinical trials on mAbs-based therapy of cancer are under way wherein some mAbs have attained a significant clinical success against different types of cancer (Scott et al. 2012a, b).

Table 1 Approved antibody-based cancer immunotherapies (reproduced with permission from Weiner et al. 2012)

Name	Antigen	Format	Isotype and subtype[a]	Indication(s)
Tumor antigen-targeted antibodies[b]				
Rituximab	CD20	Chimeric	IgG	Non-Hodgkin lymphoma
^{131}I-tositumomab	CD20	Humanized conjugate	Murine IgG2	Relapsed and/or refractory lymphoma
Ofatumumab	CD20	Human	IgG1	Chronic lymphocytic leukemia
^{90}Y-Ibritumomab tiuxetan	CD20	Mouse conjugate	Murine IgG1	Relapsed and/or refractory lymphoma
Alemtuzumab	CD52	Humanized	IgG1	Chronic lymphocytic leukemia
Brentuximab Vedotin	CD30	Chimeric	IgG1	Hodgkin lymphoma and anaplastic large cell lymphoma
Trastuzumab	HER2	Humanized	IgG1	Breast and gastric cancers
Cetuximab	EGFR	Chimeric	IgG1	Colorectal and head and neck cancer
Panitumumab	EGFR	Human	IgG2	Metastatic colorectal cancer
Bevacizumab A	VEGF-A	Humanized	IgG1	Metastatic colorectal, non-small cell lung, glioblastoma, and kidney cancers
Immune effector antigen-targeted antibodies				
Ipilimumab	CTLA-4	Human	IgG1	Metastatic melanoma

[a]Human isotype and subtype, unless otherwise specified
[b]Gemtuzumabozogamicin (Mylotarg) was withdrawn from the market in June, 2010

4.2 Cancer Vaccines

Vaccines are antigenic formulations, which are administered into the body, to boost the immune response against an antigen associated with a foreign infectious agent. Cancer vaccines belong to a class of substances that either treat existing cancer or prevent reemission of cancer by stimulating body's natural immune system to fight against cancer cells. Vaccine as a treatment is popularly referred as immunotherapy (Pardoll 1998).

4.3 Peptide Vaccines

An array of tumor-associated antigen(s) has been identified and explored for activation of immune system. These antigenic peptides possess immunogenic properties and are capable of initiating a cascade of events for the destruction of tumor. A number of tumor-specific antigens have been explored and found associated with certain tumors. They can be in the form of enzymes, which regulate cancer progression or in the form of peptides present on the tumor surface or as receptors for certain growth factors, etc. Nanotechnology can successfully be used to deliver these peptides specifically and selectively to the immune cells as these bio code(s)/signatures where they are engulfed by the APCs and are presented to immune cells for their recognition by T cells. Owing to structural variation or non-self to the body, they activate the immune system that eventually destroys them. The antigens which are present on cancer cells as well as histologically similar cells are called tumor-associated antigens (TAAs) while those which are confined to tumor cells only are termed as tumor-specific antigens (TSAs). Presently, numerous research groups are working on possible applications of immunogenicity of peptide(s) to design an effective cancer therapy. Either single or cocktail of peptides can be isolated and purified from cancer patient himself or can be artificially synthesized using recombinant DNA technology or chemical synthesis and can be amalgamated with adjuvants like antibodies, cytokines and other strong immunogenic peptides in order to generate an effective immune response (Bolhassani 2011).

4.4 Tumor Cell Based Vaccines

Similar to antigenic peptides, another approach for developing cancer vaccine is the use of tumor cell lysate. Tumor cells serve as finest source of those antigens, which are difficult to isolate and purify and hence are used to educate immune cells. Depending upon the type of isolated tumor cells, tumor cell based vaccines are of two types viz., (a) Autologous vaccines in which the tumor cell is isolated from the patient himself and (b) Allogenic vaccine in which the tumor cell is isolated from some other patient suffering from the same cancer. These cells are modified genetically in such a manner that they produce some immune molecules, which in turn generate the immune response in opposition to the tumor. The generation of

immune response is followed by inactivation of these immune molecules either by radiation or with the help of chemical treatment in order to avoid the generation of tumor after their administration into the patient. These cells can also be coupled with adjuvants so as to achieve better response. Tumor cells are engulfed by the APCs in the similar manner as discussed for peptide vaccines. Nevertheless, the specific cell types and molecules involved are not yet clear (Suckow 2013).

4.5 DNA Vaccines

With the advancement in the field of nanobiotechnology, it is now possible to isolate plasmid DNA and after recombination it can be incorporated some carrier(s) along with adjuvant to improve or to elicit the functions of immune system. A major advantage associated with DNA nanovaccines is their well-defined method of large-scale production, which makes it a useful strategy compared to others. A plasmid DNA that contains a regulatory sequence and the genetic code for TAA or TSA is selected. The regulatory sequence helps for the decoding of TAAs or TSAs sequence inside the human body. The nearby cells and APCs also take up DNA plasmid during its administration into the muscle or skin as nanovaccine. Once internalized, the genetic code is decoded followed by the synthesis of the peptide which then gets cross presented to the APCs and generating the immune response in the similar manner as in case of other strategies described earlier. To increase the effectiveness of the DNA vaccines, novel carrier-mediated strategies like use of nanoparticles, liposomes, nanogels, nanoemulisions, etc., are adapted. Also the addition of some pathogenic sequences next to the antigenic sequences may further improve the efficiency of these vaccines (Dhake 2013).

4.6 Vector-Based Vaccines

As the name suggests, these are the vaccines, which are developed with the help of vectors of nanometric size. Vectors are cargos that can deliver the genes into the selected cells. Bacteria, viruses, and yeast are most commonly used vectors for the development of vaccines. Vectors possess the innate ability to stimulate the immune responses and thus it is judicious to use them for designing of effective vaccines. An inert vector is loaded with the required DNA sequence, specific for a pre-identified tumor antigen, and is injected into the patient wherein it is internalized by normal body cells as well as immune cells. The DNA sequence so released is responsible for the expression of antigen which is subsequently presented onto the surface of immune cells in association with MHC-I/MHC-II and hence immune cells become alert for any subsequent exposure of such antigens. Once encountered with these tumor-specific antigens, the cascade of immune response is initiated to destroy the tumor cells, which bear the antigen on their surface (Bolhassani 2011).

4.7 Dendritic Cell Based Immunotherapy

Dendritic cells (a type of white blood cells) are professional APCs of mammalians immune system, which perform the function of antigen presentation in association with MHC to the T cells of the immune system and are supposed to play a major role in cancer immunity. They operate as messengers among the adaptive and the innate arms of the immune systems. Unlike other vaccination strategies, DCs can be educated ex vivo to distinguish and target the cancer cells. A group of immature white cells are collected from blood and cultured in nutritive medium. These immature cells, in the presence of certain macromolecules and cytokines get transformed from white blood cells into immature dendritic cells. This process is called as leukopheresis. These immature dendritic cells can be harvested with either inactive tumor cells or tumor lysate derived from patient or with vectors that contain code for antigenic peptide or antigenic peptides can be directly attached or directed to these cells. Any of the culture technique ultimately gives rise to group of mature DCs that present tumor antigen to the helper T cells and can now be used to activate other immune cells. These mature cells are injected into the patient where they are ready to discriminate and attack the tumor cells and strengthen the immune response by activating other immune cells to recognize and wipe out the tumor cells (Dhake 2013).

4.8 Immune Checkpoint Inhibitors

These are bioactive(s) often antibodies that essentially take the "brakes" off the body's immune system, which assists in recognizing and attacking on cancer cells. PD-1 or PD-L1 and CTLA-4 are checkpoint proteins on some immune cells called T cells that act as a type of "off switch" to keep the immune system in check (ACS 2015).

4.9 Nonspecific Immunotherapies

These therapies boost the immune system by using various approaches such as interferons(α), cytokines (IL2, IL-12) or toll-like receptors (TLRs) agonist treatment. These all strategies are used to fight against the tumor microenvironment and helps in immune system activation. A number of investigations using nonspecific immune activation have been reported to date (Sheng and Huang 2011).

4.9.1 Nano-immunotherapy

Nano-immunotherapy is nanotechnology-based approach to solve the problems encountered in immunotherapy. The approach mainly focuses on development of various nanostructures for controlled, sustained, and targeted delivery of loaded tumor antigens (TAs)/tumor-associated antigens (TAAs) to antigen-presenting cells. Nanostructures may include polymeric- and lipid-based nanoparticulate systems, nanocolloidal systems (liposomes, niosomes, etc.) micelles, carbon nanotubes, etc.

In this regard, TAs/TAAs antigens delivery through nanotechnology-based approach may be one of the promising approaches for effective immunotherapy of cancer owing to its prolonged antigen presentation, dose sparing, and immune-potentiation features. Furthermore, much stronger T cell mediated immune responses can be elicited. TAs-/TAAs-loaded nanostructure(s) have been investigated to deliver encapsulated antigens to professional APCs, also to prevent their proteolytic degradation, improving their stability and to regulate their release in a controlled/sustained manner. In addition, compared with soluble Ags/proteins or peptides, the antigens encapsulated in nanocarriers(s) are more efficiently cross presented on MHC class I molecules therefore can provoke both B and T cells mediated stronger immune responses against tumor (Krishnamachari et al. 2011; Zhang et al. 2012).

5 Challenges for Cancer Immunotherapy

At present several approaches are under investigation for development of successful cancer immunotherapy yet there are some major issues which demand for their redressed in order to increase their effectiveness and acceptability. These challenges include

(a) Aging immune system
(b) Host-derived immunosuppressive effects
(c) Tumor escape from immune effect or function

5.1 Aging Immune System

With the advancement of age, the thymus stops generating immature T cells, which results in compromised generation of primary immune response and its conversion to memory cells and thus the recognition probability decreases to an extent (Kapasi et al. 2002). Apart from poor recognition, the susceptibility of cancer increases at old age due to complexity in generation of effective immune response and changing pattern of T cell subsets. In order to address these issues, co-stimulatory molecule like 4-1BB (CD137) which enhances the T cell response or molecules like CTLA4 which are responsible for inactivation of negative regulators are tested over the mice (Egen et al. 2002), and it is expected that they may have similar effects on human. CpG-DNA is another such molecule, which seems to enhance humoral and cellular immunity and TH-1 type immune responses in aged mice. These types of studies are in progress. It is expected that they can lead to some fruitful outcomes. Age-associated immune studies illustrate that for successful vaccination therapy, the vaccination should be done at an early age of patient as well as at an early stage of disease and in the absence of any standard immunosuppressive therapy.

5.2 Tumor-Induced Immunosuppression and Immune Evasion

An immune response against the tumor is initiated only after it is detected by the immune system. At initial stages the rate of tumor growth is slow and it does not cause much damage to the nearby tissues. However as the molecules initiating the immune cascade fail to generate a potential immune response against the tumor, the tumor cells begin to grow and metastasize causing tissue destruction. The adaptive immune system of the patient activates DCs, which in turn present the tumor and tissue debris to T cells. Thus, the generated response is indicative of whether the tumor has been identified or it has bypassed the immune system. If the expression of MHC molecule and tumor-associated antigens is absent and tumor grows continuously, it indicates that the tumor has bypassed the defense system of the body. The whole phenomenon of immune surveillance wherein the cells of tumor are altered but not completely destroyed is termed as "cancer immunoediting". Tumor cells also produce transforming growth factor-β (TGF-β), IL-10 and other cytokines, which can affect the functions of immune system such as inhibition of maturation of DCs and inactivation of T cells. Treatment regimen involving reversal of immune suppression before the application of cancer vaccine can be a good and effective alternatives to enhance the effectiveness of vaccines. Nevertheless, understanding of the patient's immune system as whole is imperative, particularly when it is to be manipulated and modulated by using therapeutic cancer vaccines (Garcia-Hernandez et al. 2002).

6 Nanocarrier(s)-Based Delivery Approaches for Cancer Immunotherapy

Nano-sized particulate/colloidal carriers which deliver the encapsulated or entrapped immune bioactive(s) such as antigen(s), proteins, and DNA to their desired site (APCs) exhibited great potential and have revolutionized cancer immunotherapy. Owing to nano size, these delivery systems allow their efficient uptake by APCs and effectively deliver the loaded immune bioactive(s) to these cells. The immune response consequently boosted significantly to generate the robust immune response as compared to soluble antigen(s) (Xiang et al. 2008). Antigen(s) internalization into APCs (dendritic cells/macrophages) takes place through various pathways, as uptake of nanocarrier(s) increasing the immune response is also augmented in a proportional manner. Immune bioactive(s) loaded nanoparticulate carriers can provoke both T and B cells mediated immune response against tumor as shown in Fig. 4. In the last two decades, nanostructures-mediated cancer immunotherapy has been widely explored as an alternative approach for the effective cancer treatment. Development of nanostructures such as polymeric and lipid nanoparticles, carbon nanotubes, quantum dots, virus-like particles (VLPs), etc., offers a platform for advanced nanoimmunotherapy of cancer. However, over

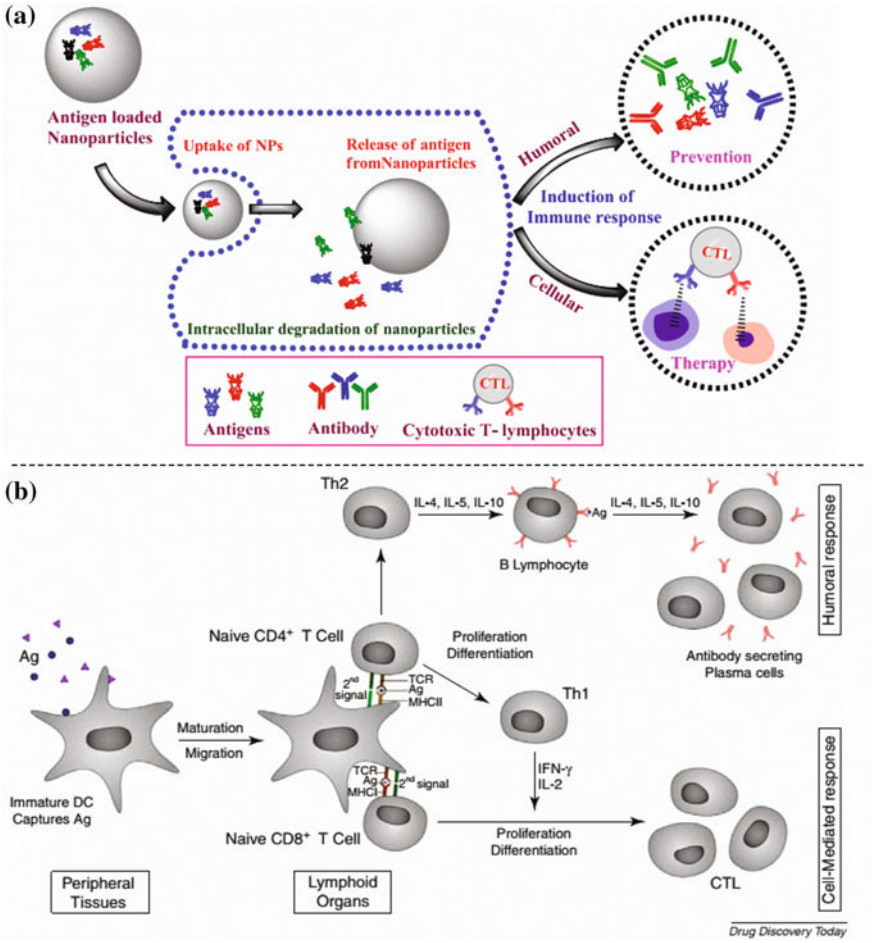


Fig. 4 Schematic overview of **a** induction of immune responses by nanoparticle-based vaccine and **b** antigen presentation and Th priming [Reproduced with permission of Elsevier ref. Sharma et al. (2015), De Temmerman et al. (2011)]

last 5 years major research advancements have been made for the development of new delivery candidates by designing multifunctional/targeted systems using ligands, PEGylation, adjuvants, immunomodulatory agents, etc. Such types of engineered nanostructures can be used to manipulate or deliver immunologically bioactive(s) more efficiently to professional APCs and harnessing the power and specificity of the immune system in the cancer therapy (Park et al. 2013). Concept of nanocarriers-mediated cancer immunotherapy depends on the fact that particulate carriers having size in nano range loaded with immune components (TSAs/TAAs, adjuvants) can be conveniently taken up by APCs and are capable to generate stronger cellular as well as humoral immune responses against cancer.

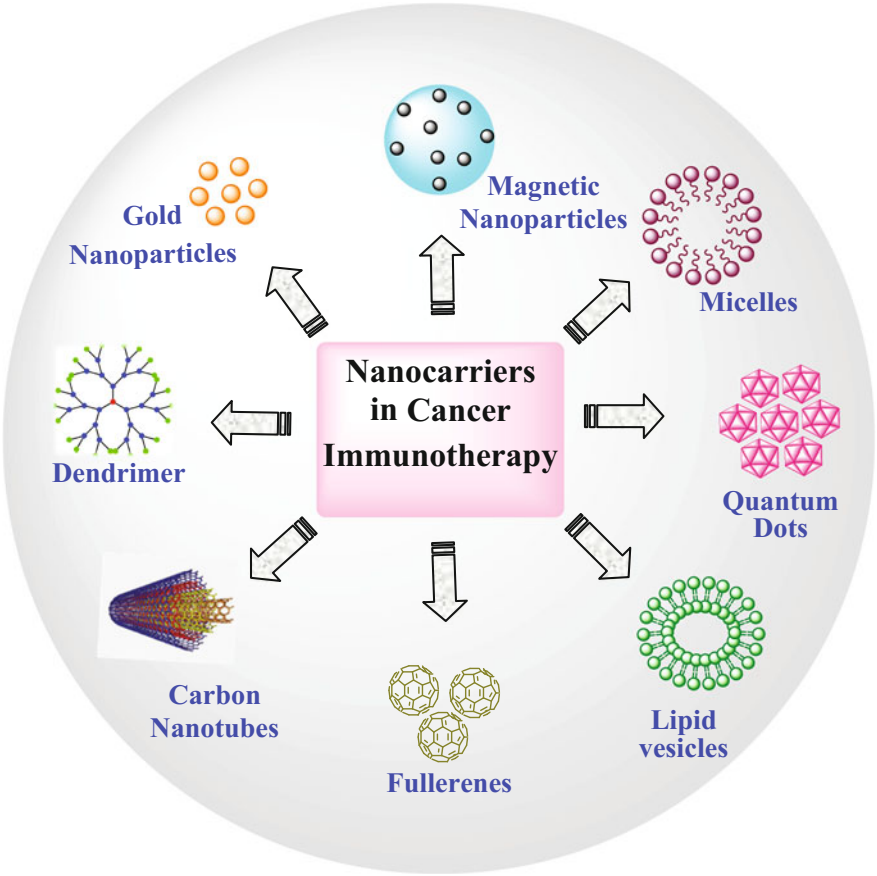
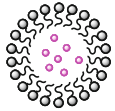
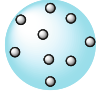


Fig. 5 Various nanodelivery systems for cancer immunotherapy

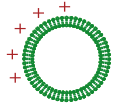
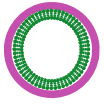
Nanoparticulate carriers serve as excellent delivery system *vis* a *vis* they can act as immunoadjuvant(s). Various nanocarriers investigated for cancer immunotherapy are illustrated in Fig. 5 and Table 2. Nanocarriers mainly serve three functions when used in cancer immunotherapy, i.e., they can specifically deliver the loaded immune component to the desired site (APCs) with greater efficiency; they themselves can act as adjuvant and co-deliver immunopotentiators along with antigens. In comparison to the cationic, anionic and neutral nanosystems, cationic system is rapidly internalized by DCs and macrophages (Conniot et al. 2014; Yeong et al. 2013; Thiele et al. 2003). Nanocarriers not only prevent the degradation of loaded immune bioactive(s) in harsh environments but also enhance the antigen persistence period which further helps in eliciting cytotoxic T cell responses in addition to the activation of B cells. It is also well investigated that such nanocarriers could increase the retention of loaded immune components in the local lymph nodes. Further, immune-potentiators can be included in the nanoparticulate formulations.

Table 2 Different nanocarrier delivery systems used in cancer vaccines

Nanocarrier	Description	Image	Pros	Cons	Reference
PBCA particles	(Polybutylcyanoacrylate); Commonly employed for brain targeting as possess the ability to cross BBB		Biodegradable/biocompatible Readily modified to allow delivery across the BBB	Negligible adjuvant properties	Schneider et al. (2008)
Gelatin-based nanoparticles	Gelatin, as a prominent biopolymer, is a generic name for the mixture of purified protein fractions obtained from collagen		Biodegradable/biocompatible ease of manufacture Readily modified Pyrogen free	Negligible adjuvant properties	Bourquin (2008)
Nano emulsions	Nanoemulsions are nano-sized emulsions, which are manufactured for improving the delivery of active pharmaceutical ingredients.		Thermodynamically stable Non-toxic Ease of manufacture Efficient antigen encapsulation Long circulatory times in vivo and increased uptake by APCs		Shi et al. (2005), Ge et al. (2009)
γ-PGA nanoparticles	Poly(γ-glutamic acid) (γPGA) nanoparticles (NPs) carrying antigens have been shown to induce potent antigen-specific immune responses		Biodegradable/non-toxic Ease of manufacture Intrinsic adjuvant properties		Yamaguchi et al. (2009)
Magnetite particles	Magnetite is a mineral and one of the three common naturally occurring oxides of iron		Enhances TAA presentation via MHC class I pathway Stimulates tumor-specific T cell activity	Non-biodegradable	Tanaka et al. (2005), Kikumori et al. (2009), Jimbow et al. (2008)

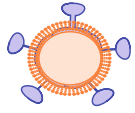
(continued)

Table 2 (continued)

Nanocarrier	Description	Image	Pros	Cons	Reference
Conventional liposomes	A minute spherical sac of phospholipid molecules enclosing a water droplet, especially as formed artificially to carry drugs or other substances into the tissues		I.V. administration targets APCs of spleen and liver macrophages Biodegradable/biocompatible	Suboptimal encapsulation of water-soluble proteins Readily cleared by RES Expensive to manufacture Not stably stored Potential for cellular toxicity	Worth et al. (1999), Nii and Ishii (2005)
Cationic liposomes-DNA complexes (CLDC)	Cationic liposomes are structures that are made of positively charged lipids		Increases immunopotency of CpG Strong inducer of innate immunity and nonspecific tumor or NK-mediated immunity	Potential for cellular toxicity	Lv et al. (2006), Bedikian and Del Vecchio (2008)
Stealth liposomes	Stealth liposomes are long-circulating liposomes with inclusion of the synthetic polymer poly-(ethylene glycol) (PEG) in liposome composition		Increased circulation time		Van Broekhoven et al. (2004)
Archaeosomes	Liposome made from polar ether lipids extracted from the *Archaea*		Biodegradable/biocompatible Increased cross-presentation Intrinsic adjuvant properties Stably stored (compared to other liposomes)	Suboptimal encapsulation of water-soluble proteins Rapidly cleared if administered i.v. or orally	Patel et al. (2002)

(continued)

Table 2 (continued)

Nanocarrier	Description	Image	Pros	Cons	Reference
Viruses	A virus is a small infectious agent that replicates only inside the living cells of other organisms		Highly immunogenic (capable of stimulating the adaptive and the innate arms of the immune response)	Can be excessively immunogenic Not suitable for repetitive use or gene therapy Potential danger of reversion to virulent form Potential contamination with replication competent virus	Dharmapuri et al. (2009)
Virus-like particles (VLP)	Versatile chemical and genetic modifications on the outer surfaces and inner cavities of VLPs facilitate the preparation of new materials that could meet the biocompatibility, solubility and high uptake efficiency requirements for drug delivery		Immunogenic–activate innate and adaptive arms of IR Safer than viruses Relatively easy, efficient and inexpensive to produce Prophylactically effective	Safety concerns with respect to degree of activation of innate arm Applicable only to cancers of viral origin	Villa et al. (2005), Garland et al. (2007)
Virosomes	Virosomes are reconstituted viral envelopes that can serve as vehicles		Preferentially target MHC class I presentation pathway Up regulate co stimulatory molecules on APCs/generate Th1-type response	Suboptimal loading capacity	Zurbriggen (2003), Amacker et al. (2005)

(continued)

Table 2 (continued)

Nanocarrier	Description	Image	Pros	Cons	Reference
PLGA	Poly(lactic-co-glycolic acid) (PLGA) is one of the most successfully developed biodegradable polymers		Biodegradable/biocompatible Easily scaled up for pharmaceutical manufacture Efficient passive DCs targeting Prolonged pulsatile release Can deliver antigens that are presented by both MHC class I and MHC class II pathways	Negligible adjuvant properties Potentially expensive to clean-up for clinical use. During microencapsulation select proteins can degrade	Waeckerle-Men and Groettrup (2005)

Advantages of nanocarrier(s) based immunotherapy:

- Act as strong adjuvant
- To elicit both cellular as well as humoral immune response
- Able to protect biological, functional, and structural integrity of loaded immune components
- Deliver encapsulated immune bioactive(s) to antigen-presenting cells
- Release loaded immune bioactive(s) in a controlled and sustained manner
- Reduce the frequency of dosages
- Owing to nano-size can be administered via various routes such as oral, intranasal, subcutaneous, intradermal, etc.
- Surface engineering such as ligand attachment, PEGylation, and varied compositions; can be further improve the denominators such as targetibility, stability, biocompatibility, release kinetics (Park et al. 2009; Wang et al. 2012).

6.1 Nanoparticles as a Versatile Platform for Cancer Immunotherapy

Cancer research institutes have recognized nanoparticles for their potential to overcome limitations associated with the conventional immunotherapy such as weaker immune response, lack of specificity, and low efficacy, etc.; hence nanoparticles present an efficient model that has a discernible impact on the immune diagnosis, immunotherapy, and a definitive role in the prevention of cancer. Recently, NPs are emerging as prime delivery system for immunotherapy of cancer owing to their nano size, strong adjuvant property, diverse composition, amenability for surface functionalization, and stability which offer unique opportunities to interact with the APCs (Hosseini et al. 2015; Park et al. 2009; Wang et al. 2008; Cho et al. 2008).

In past some decades, various nanoparticles-based delivery systems are being explored for cancer immunotherapy. The constructing materials properties also influence the immune component delivery to the APCs. For example, cationic NPs (positively charged surface) show better endocytosis and NPs with hydrophilic surfaces (PEGylation) can be used to provide stealth/long circulation property. Currently, various types of nanoparticles are being investigated including as polymeric NPs, solid lipid NPs (SLNs), nano-lipid constructs (NLCs), protein NPs, virus lipidNPs (VLPs), metallic NPs, AuNPs, magnetic NPs, etc. (Wang et al. 2012; Mark et al. 2008). The polymeric nanoparticles based formulations have received much attention as they serve as potential carriers for immune bioactive(s) delivery to APCs. Due to enormous beneficial physicochemical properties of synthetic and natural polymers, these appeared to be most admired choice for the development of NPs for cancer immunotherapeutics. A great variety of natural, synthetic, or biodegradable polymeric materials have been investigated for the nanoparticles synthesis. Synthetic polymeric materials used for the NPs formulations are

comprised of poly (D, L-lactide-co-glycolide) (PLGA), Poly (D, L-lactide) (PLA), poly(ε-caprolactone) (PCL), poly(ethylene glycol) (PEG), poly(g-glutamic acid) (ϒ-PGA), polyethylenimine (PEI). Natural polymers such as alginate, chitosan, polysaccharide, inulin, pollunan and gelatine are used as layout architectures for the designing of nanoparticles (Sharma et al. 2014; Li 2014; Li 2013). Delivering immunostimulatory and immunomodulatory bioactive(s) to the desired site is an extremely explored concept that substitutes the successful development of nanovaccine formulations (Leleux et al. 2013). It is desired to protect the loaded antigen/adjuvant from the surrounding biological milieu in order to increase its delivery to APC while minimizing the side effects and increasing its half-life. The objectives can be easily achieved by using of nanoparticles as carrier system. There are documentary evidences indicating in vivo applications of nanoparticles that activate(s) an adaptive immune response (Fig. 4). Encapsulated or surface-bound antigens and adjuvant trigger B and T cells response to a greater magnitude as compared to their soluble counterparts supporting these approaches for their therapeutic significance in cancer (Leleux et al. 2013; Kasturi et al. 2011; Gao et al. 2014). The engineering of nanoparticles for delivery of bioactive(s) and for the delivery of tumor-specific vaccine differs with each other since the former aims to bypass phagocytic cells including APCs while the later seeks professional APCs to deliver their payload. Nanoparticles can be smartly designed so that they can stimulate immune responses. These antigen-loaded nanoparticles can induce B and T cell mediated immune responses even in the absence of any exogenous adjuvant (Dwivedi et al. 2011). In order to produce antitumor immunity of therapeutic value, nanoparticles conjugated with model tumor antigens were injected into thymoma or lymphoma or OVA-expressing lymphoma bearing mice. A strong immune response was generated against colon adenocarcinoma and OVA-expressing lymphoma following administration of antigen-loaded nanoparticles. Also the survival time of animals was extended and delay in tumor growth was recorded (Cho et al. 2011). Biological activity of these compounds depends on the particle size. Small-size particles (<40 nm) drain out from the site of injection on their own and reach to the lymph nodes. Once they reach the lymph node, they are taken up by DCs. Peptides arising from the coated antigen are presented to MHC class I molecules followed by the activation of TAA-specific CD8+ T cells. Danger-sensing pathways are activated by the endocytosis of these particles by DCs promoting DC activation and maturation into immunogenic APCs (Thomas et al. 2014; Jeanbart et al. 2014), Antigens when conjugated to nanoparticles result into an enhanced structural integrity as well as enhanced immunogenicity. Furthermore, the rate of antigen release is sustained over a prolonged time period (Fig. 6).

6.2 Delivery of Nonspecific DC Stimuli (Adjuvants) to Tumor-Draining Lymph Nodes (TDLNs)

Tumor-associated antigen reaches to tumor-draining lymph node followed by their uptake by DCs and other antigen-presenting cells. Once taken up by APCs,

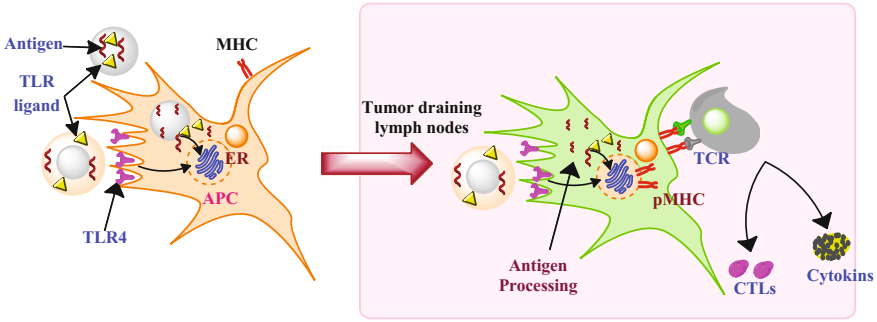


Fig. 6 Nanocarrier(s) mediated delivery of TAAs and/or immunopotentiator to tumor draining lymph nodes

tumor-associated antigen is presented to T cells. TDLNs consist of large number of tumor-specific T cells that are detected with the help of tumor-specific peptide-human leukocyte antigen (pHLA) tetramers (as marker reagents). However, DCs residing in TDLNs in general are immature/non-activated phenotypes, which have limited compromise their ability to activate antitumor T cell responses in a productive manner (Pinzon-Charry et al. 2005; Vicari et al. 2002; Van Mierlo et al. 2004). To overcome this limitation, nanocarriers-mediated delivery of antigens to DC was studied. The studies suggest an enhanced activation and maturation of DCs in TDLNs. The nanoparticles containing cytosine-phosphate-guanine (CpG) oligonucleotides as an adjuvant were administered subcutaneously in a melanoma model. It resulted into preferential accumulation of these molecules in the draining lymph nodes. CpG-nanoparticle complexes were rapidly taken up by APCs and consequently cytokines IL-12 and IL-6 were released resulting into activation of effector CD4+ T-helper-type 1 cells. Similar results were recorded with nanoparticles of different size range ranging from 25 to 270 nm in diameter. It was observed that on increasing the size of nanoparticle, the uptake was relatively low in case of nanoparticles greater than 100 nm. Likewise, anti-CD40/CpG-carrying liposomes could successfully trigger a robust anticancer response with minimal systemic side effects (Bal et al. 2011).

6.3 Co-delivery of Antigen and Adjuvant

Another extensively strategy used for the generation of immune response is simultaneous delivery of both antigens and adjuvants. Significant immunogenic activity was observed with lipid-based vesicles, which delivered OVA, or OVA-derived peptidases model tumor antigen. Studies with liposomes of different size concluded that with increase in the size of liposome, the immunogenic properties were also increased. The phenomenon of active phagocytosis could be a probable reason for this behavior (Macho et al. 2014; Fang et al. 2014). Recently, a

significant elicitation of immune response was recorded by Idoyaga and coworkers when they delivered OVA and CpG using 30 nm diameter micellar nanoparticles. The elicited immune response was several folds greater than that induced by nanoparticles that contained only OVA. The result supports the fact that nanoparticle type and size affect the extent of immune elicitation. A specialized subset of DC's is CD8+ cells which, possess augmented capacity of presenting antigenic peptides captured by DCs' in association with MHC class I molecules. The phenomenon is termed as cross-presentation of antigen (Idoyaga et al. 2009). Antigen-coated particles were specifically delivered to this subset of DC using endocytic C-type lectin receptor DEC205. Another study was performed with anti-CD205 antibody-coated PLGA nanoparticles loaded with OVA and α-GalCer, an antigenic ligand for invariant natural killer T cells (iNKT) iNKT cells represent a subset of T-lymphocytes other than conventional CD4+ and CD8+ T cells. Cytotoxic T cell response against the tumor was improved as a result of simultaneous presentation of α-GalCer and tumor antigen by DCs. Strong OVA-specific CD8+ T cell responses and decreased growth of OVA-expressing melanoma and EG7 was observed when anti-CD205 mAb-coated nanoparticles were administered. This could be due to the preferential uptake of anti-CD205 mAb-coated nanoparticles by CD8+ DCs. Immune stimulation activity of PLGA nanoparticles carrying both a TLR agonist and a STAT3-specific siRNA or OVA antigen and a suppressor of cytokine signaling 1 (SOCS1) using specific siRNA was evaluated by Heo and co-workers. The research group conducted ex vivo examination to study the effect on DCs maturation (Heo et al. 2014). Simultaneous administration of each of these molecules on separate small nanoparticles may be a potential option. This approach has been tested using tumor-specific peptide CpG 98 and Trp2 loaded lipid-calcium-phosphate (LCP) of 30 nm and alternatively, anisamide-coated anti-CD47-specific siRNA-loaded liposome-protamine-hyaluronic acid (LPH) of 40 nm. Even though administration of LCP nanoparticles elicit antigen-specific CTL responses that effectively eliminated Trp2 peptide-loaded splenocytes in tumor-bearing mice, this approach showed no significant effects on the growth of advanced tumors. Injection of LPH nanoparticles decreased TGF β expression by tumor cells, and augmented the anti-melanoma effects of LCP vaccination. Thus, combinations of different nanoparticles having complementary biological activities could be an approach that deserves consideration.

7 Inorganic Nanoparticles in Cancer Immunotherapy

7.1 Magnetite Nanoparticles (MNPs)

MNPs have gained considerable attention for number of biomedical applications because of the following advantages (Arruebo et al. 2007; Sun et al. 2008):

- their ability to be handled by an external magnetic field,
- their great potential in imaging,
- high uptake by the target tissue, and
- improving the treatment within the optimal dose range.

Normally, MNPs are made up of a magnetically active core covered by number of shells to affix with the target ligands or implement extra imaging modalities. Therapeutic drugs can then be loaded in the shell structure or could be chemically conjugated to their surface (Veiseh et al. 2010). Originally discussed for heat-based immunotherapy, magnetite nanoparticles are constantly reported to induce noteworthy degeneration of the tumor mass via induced heat shock protein (HSP) expression, which ultimately resulted in enhanced MHC class I-dependent TAAs presentation and the development of antitumor T-lymphocyte-mediated immunity. Magnetite nanoparticles either alone or in combination with added chemotherapeutics or immunotherapies like immunoliposomes and intratumoral cytokine or DC injection are well reported to study the hypothermic effect in numerous animal tumor models (Kikumori et al. 2009). This promising therapy might be more universally applicable in the clinical setting if it could be modified for systemic delivery with preferential targeting of tumor cells. A major drawback linked with magnetite and other inorganic nanoparticles is their non-biodegradable/inorganic nature which makes their systemic application little difficult because they are not biodegradable and can potentially accumulate in the body, which may lead to long-term toxicity (Krishnamachari et al. 2011).

7.2 Silica Materials

Another inorganic promising carrier reported for biomedical application is silica nanoparticles. Owing to inherited advantage of being biocompatible, ease of surface modification, and highly porous architect, they have been employed for delivering number of therapeutic drugs including anticancer drugs and antigen specific for cancer immunotherapy. Mesoporous silica nanoparticles were used to administer anti-CTLA-4 antibody via intratumoral route (Lei et al. 2010). High loading efficiency was recorded with silica nanoparticles, which further resulted in an enhanced antitumor efficacy as compared to the soluble antibody injected intraperitoneally in a murine melanoma model. The controlled release of antibody from an in situ depot could be the probable reason for the same. Another study was performed by Matsuo and co-workers in which they have formulated antigen-loaded PGA (poly glutamic acid) nanoparticles and the study concluded that DCs activated by these particles resulted in eventual release of TH_1 cytokines, elicited robust T cell activation in vitro, and enhanced protection against tumor challenge in mice (Matsuo et al. 2010).

7.3 Gold Nanoparticle (AuNPs)

Recently, AuNPs have emerged out as a promising carrier for immune therapies (Tao et al. 2014). Similar to other nanoparticles, they are accumulated easily in the immune system. Apart from their accumulation, they are biologically inert, easy to functionalize, and can be designed in desired size range. Additionally, AuNPs have unique optical properties which can subjugate the carcinoma, especially though their applications in light triggered drug delivery and photothermal ablation (Yang et al. 2013) AuNPs are considered as efficient nanocarriers as they are inert, chemically stable and able to protect the loaded cytokines and antigens from degradation. AuNPs can also be tagged with molecules that modulate dendritic cells and T cells activation, or induce humoral responses. Also, AuNPs can be fabricated to be less than 100 nm in diameter thus enabling for efficient passive delivery of conjugated biomolecules to DCs residing in the lymph nodes. Niikura and co-workers recently studied the effect of size and shape of gold nanoparticles on inflammatory responses in vitro and in vivo. The study reports the secretion of cytokines such as TNFα, IL-12, and IL-6 in bone marrow derived dendritic cells (BMDCs) was recorded in case of antigen-coated 40 nm spherical and cubic gold nanoparticles but with 20 nm spherical particles no such response was recorded. In addition, higher antigen-specific antibody production after in vivo intra-peritoneal injection was observed with the 40 nm spherical AuNPs as compared to the antigen-coated particles of different designs or antigen alone The overall conclusion of the study was that AuNPs can act as a vaccine adjuvant in a size- and shape-dependent manner (Niikura et al. 2013).

8 Lipid-Based Nanocarriers(s) for Cancer Immunotherapy

Liposomes are lipid-based unilamellar/multilamellar, spherical vesicles which deliver encapsulated or entrapped bioactive(s) such as antigen(s), proteins, and DNA to lymphatic organs/APCs and exhibited great potential to revolutionize cancer immunotherapy. Owing to their biomimetic property, liposomes are allowed for efficient internalization by APCs and therein efficiently deliver, loaded immune bioactive(s) as a consequence generate the robust immune response as compared to soluble antigen(s). Antigen(s) internalization *by* APCs occurs through various pathways, the uptake of nanocarriers enhances and simultaneously the immune response is also augmented in a proportional manner. Immune bioactive(s) loaded nanocarriers can provoke both cytotoxic as well as humoral (antibody-based) responses against tumor. Since, the ultimate goal of liposomal vaccines is usually to target dendritic cells, therefore they are surface engineered and functionalized enabling them to activate and/or deliver the antigens to these cells. Liposomes carrying immunogenic peptides are capable of fusing with the membranes of dendritic cells or being pinocytosed. Subsequently, the peptides/proteins can be processed *via* (a) cytoplasmic MHC class I pathway, or (b) the endosome/ lysosome

MHC class II pathway. However, the major disadvantage of the liposomal delivery system is stability issues and a short systemic half-life of liposome, as they are readily intercepted and cleared by the reticulo-endothelial system. This is a definite drawback if one wants to target the tumor directly with the liposomes. In the recent years, numerous approaches have been attempted to develop novel lipid based systems as an effective adjuvant. The systems demonstrated great therapeutic potential in preclinical studies (Poggi and Zocchi 2006; Krishnamachari et al. 2011; Karande et al. 2010; Van Broekhoven et al. 2004).

- The "long circulating liposomes" or "Stealth liposomes" or "PEGylated liposome" could effectively to evade this drawback. These are the surface modified liposomes that have been sterically stabilized using long chain hydrophilic polymer(s), i.e., PEG. They are able to avoid their uptake by, the mononuclear phagocytes of the RES. This steric stabilization of lipids has been shown to increase circulation time of liposomes and improved CD8+ T lymphocyte response to antigens (Krishnamachari et al. 2011; Chik et al. 2002; Ignatius et al. 2000; Frank 1993).
- Targeted liposomes or ligands appended liposomes have been designed to achieve cell specific delivery. Targeting moieties include carbohydrates, glycoproteins, growth factors, peptides mAb or fragments or receptor ligands. The first humanized mAb approved by the FDA as monotherapy for metastatic breast cancer was anti-HER2 trastuzumab (Herceptin®, Genentech Inc., and Vacaville, CA, USA). Targeted liposomes offer numerous advantages such as improving the degree of uptake through receptor-mediated endocytosis. The reduction in nonspecific uptake by DCs and better TLR ligands stability within the nanocarriers resulted in a heightened response with the lower doses of adjuvant resulting in an improved safety profile (Medina et al. 2004; Cruz et al. 2010). Similarly, another investigation suggested that liposomes based DCs targeting is an outstanding platform to induce a robust antitumor immune response (Van Broekhoven et al. 2004). Preclinical investigations conducted on liposome DNA complexes have been described to be an effective strategy to elicit antitumor immunity (U'ren et al. 2006). Phase-I clinical trial of a liposomal cancer vaccine has already been reported for breast, ovarian, and prostate cancer. It has been proved that the peptide-based vaccine is safe and fairly immunogenic intended to elicit multifunctional T cell immune responses (Berinstein et al. 2012; Chan et al. 2014). Hobo et al., developed the activated DCs-based vaccine wherein DCs were activated using lipid nanoparticles, with improved immunogenic potential by combining PD-1 ligand siRNAs and target antigen mRNA delivery. They observed efficient and specific silencing of programmed death ligand 1 and 2 (PD-L1 and PD-L2) on DCs using respective siRNAs-LNPs, without affecting the phenotype or migratory capacity of the DCs. Furthermore, they concluded that these PD-L silenced DC loaded with MiHA mRNA have superior stimulatory potential and could effectively boost ex vivo antigen specific CD8 T cell immune responses in transplanted cancer patients (Hobo et al. 2013). In another approach, fusogenic liposomes (FLs) carrying tumor cell lysate (TCL) contain in accessory proteins

from *sendai* virus (for retaining membrane fusion ability) have been developed. This approach demonstrated the usefulness of FLs as TCL-delivery carriers for ex vivo DCs activation and their subsequent use an in vivo for direct immunization in the murine B16BL6 melanoma model. The prepared FLs can encapsulate various antigen candidates, such as crude tumor lysate or tumor extract, purified whole or partially processed TAA, and TAA-coding DNA or RNA. The fabricated liposomes showed cross-presentation phenomena and improved delivery of loaded TAAs to APCs. The liposome mediated ex vivo activated DCs-based immunization strategy; however, gave better results than direct immunization in murine B16/BL6 melanoma model (Yoshikawa et al. 2006). In a similar study, Yuba et al. developed pH-sensitive delivery system by derivatizing naturally occurring polysaccharide dextran with methylated glutaraldehyde residues. They observed that these ovalbumin (as model antigen) MGlu-Dex-modified liposomes were taken up by DCs and delivered their contents efficiently into the cytosol. These MGlu-Dex-modified liposomes were found to be useful as an antigen delivery system for the induction of antigen-specific immunity, which showed a marked therapeutic effect on tumor-bearing mice (Yuba et al. 2014).

9 Conclusion

Although immense progress has been made in chemotherapy, radiotherapy, and surgery for the effective treatment of cancer yet the battle against this disease seems to need more successes through the combination of different therapeutic modalities. Oncologists are continuously exploring new treatment regimens for successful management of this disease. Advancement in the field of nanomedicine, nanobiotechnology, and emerging collaborations between nanomaterial scientists and immunologists have paved a way for immune cell mediated therapy named as "Immunotherapy". Immunotherapy refers to therapeutic approaches to treat cancer by using patient's own immune system. Nanocarriers, for instance nanoparticles and liposomes, put forward an attractive approach for delivery of cytokines/antigens/ligands/antibody either alone or along with the immune components (cells) in a programmed manner to elicit prolonged effect or and memory immune response. The majority of the treatment modalities have demonstrated promising preclinical results in terms of prophylactic and/or therapeutic effects. Some have progressed to phase-I, II, and III clinical trials advocating the safety and potential therapeutic index of these nanocarrier systems; still it is difficult at this early stage to prognosticate as to which systems could be most progressive as an established vehicles for cancer immunotherapy. The optimization of the corresponding nanostructures is definitely required prior to the clinical adaptation of the effective approaches. Nevertheless, apart from the pragmatic curative benefits, other decisive factors comprises of biocompatibility, the ease and cost of manufacture and storage, along with amenability of materials in regard to design features.

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Nano-therapeutic Approaches for Targeting Cancer Stem Cells

Mintu Pal and Sabyasachi Maiti

Abstract

Emerging evidences suggest that a small population of highly tumorigenic cancer stem-like cells (CSC) or tumor-initiating cells (TICs) is responsible for sustaining multiple types of tumor. Like normal stem cells, CSCs can self-renew and differentiate to other tumor cell types and give rise to non-tumorigenic daughter cells that constitute the tumor bulk. These cells are highly resistant to chemo- and radiotherapies causing drug resistance, tumor recurrence, and the formation of distant metastases. CSCs often overexpress drug efflux transporters, and consequently, CSCs can escape conventional chemotherapies. Therefore, CSCs offer an attractive target for therapeutic intervention. Nanocarrier-based therapeutics is being targeted to CSCs for elimination and prevention of recurrence and metastasis of tumors in addition to achieving prolonged blood circulation times, stability, and bioavailability over current therapies. In this chapter, we focus on the problems in delivering drugs to the CSCs, and current status of CSCs therapy including inhibition of drug efflux transporters, targeting tumor microenvironment and nanometric drug delivery approaches to prevent tumor recurrence.

Keywords

Cancer stem-like cells (CSCs) · Nano-therapeutics · Tumor microenvironment · Drug efflux transporters

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1 Introduction

The self-renewal and differentiation are the most important properties of normal stem cells (NSCs). Evidence suggests that the growth and propagation of tumors depends on the presence of a small subset of cells known as cancer stem cells (CSCs). CSCs or tumor-initiating cells possess peculiar molecular aspects that resemble NSCs (O'Flaherty et al. 2012). This tumor subpopulation can also self-renew, differentiate and proliferate (Zhou et al. 2009). Although they have been found dormant in cell cycle like NSCs, they are highly influenced by signals from their microenvironment (Scatena et al. 2011). In terms of their abundance, CSCs may range from a small subpopulation to entire cells of a tumor. CSCs have been identified in various tumors including brain, prostate, colon, liver, breast, and others (Ischenko et al. 2008).

In general, CSCs are considered as the driving force for tumor progression, therapeutic resistance, metastasis, and recurrence (Medema 2013). The somatic stem cells (SSCs) and CSCs retain stem cell properties by sharing common signaling pathways. The wingless and integration site growth factor (Wnt)/β-catenin, notch and sonic hedgehog (Shh) signaling pathways are related to self-renewal properties. At present, the hormone replacement, tumor ablation, radiotherapy, and chemotherapy are the first line of treatments for cancer patients.

Conventional therapy shows high response rate at initial phases of treatment; however, the progression of cancer occurs due to intrinsic or acquired resistance and therefore, the disease relapsed (Dean et al. 2005). Moreover, the genetic and/or epigenetic changes in cancer cells may also be responsible for cancer recurrence due to their uncontrolled proliferation, invasion, and metastasis (Johnstone et al. 2002). CSCs are thought to have an impact on drug resistance and recurrence after traditional therapy and thus on tumorigenesis (Liu et al. 2013a). High expression of membrane glycoproteins as drug efflux transporter, resistance to drugs, deactivation of apoptotic signaling pathways, greater efficiency to repair DNA, and reprogrammable metabolic processes are the possible reasons behind the failure of conventional therapy (Vinogradov and Wei 2012).

To achieve better efficacy of cancer therapy, some therapeutic strategies against CSCs must be sought. CSCs surface markers, ATP-binding cassette (ABC) transporter, self-renewal and survival signaling pathways, and tumor microenvironment are the current strategic points in order to achieve better anti-CSCs therapeutics (Beck and Blanpain 2013). Despite the screening of a number of anti-CSCs therapeutic agents, the lack of target specificity, low water solubility, short circulation time, unfavorable bio-distribution, and low therapeutic index are their major limitations for clinical applications (Chen 2010). Nanoscale drug delivery systems such as liposomes, polymeric micelles, carbon nanotubes, and metal nanoparticles (NPs) have shown a great promise in overcoming these limitations (Davis et al. 2008). The nanosystems can accommodate multiple drug molecules and improve pharmacokinetic and pharmacodynamic properties, with consequent reduction of detrimental effects to normal cells (Sun et al. 2014a). Novel multifunctional

nanoparticles are under preclinical evaluation for developing CSC-specific nanomedicines.

For eradication of CSCs in treating malignant tumors, the identification of CSCs-specific therapeutic agents and selection of delivery strategies is of utmost importance (Chen et al. 2013). In this chapter, we briefly discuss current therapeutic strategies against CSCs, followed by latest developments in anti-CSC nano-therapeutic approaches. Finally, the future scope in developing clinically effective anti-CSC nanomedicines has been prescribed.

2 Hurdles to the Delivery of Drugs to CSCs

In continuation of our foregoing discussion, CSCs-rich tumors are resistant to conventional therapies and can proliferate and/or disseminate to distant lesions. Despite continuous improvements, traditional therapy resistance and tumor recurrence still occur in most patients.

Conventional therapeutic strategies are often based on targeting the tumor population but are not CSCs-specific. As a consequence, the chemo-resistance and multiple biological mechanisms support CSCs to remain unresponsive to the chemotherapeutic drugs and render the shielded cancer cells almost untreatable.

Colak et al. (2014) reported that the differentiated colon cancer cells that constitute most of the tumor mass underwent chemotherapy-induced apoptosis but resistance persisted in poorly differentiated CSCs in colon cancers. The quiescent state of colon CSCs protects them against conventional treatment, which mostly target active proliferating cells and save the colon CSCs, to repopulate overall growth of tumor (Di Franco et al. 2014). O'Hare et al. (2006) found that CSCs isolated from liver, lung, pancreatic, breast cancers, and others were resistant to chemotherapeutic drugs such as gemcitabine, cisplatin, 5-fluorouracil, and imatinib. Bao et al. (2006) identified more CD133+ (CSCs) cells in irradiated mice with gliomas than those with non-irradiated tumors. Diehn et al. (2009) reported twofold more CSCs of breast, head, and neck cancer in irradiated mice. The reports suggested that CSCs were resistant to radiotherapy and chemotherapy in various cancers. Thus, it becomes indispensable to search for novel CSCs targeting strategies in order to overcome drug resistance toward chemo-radiation therapies.

The low selectivity of current chemotherapeutic drugs is associated with side effects and lack of efficacy in many patients. Recent efforts therefore focused on the development of targeted approaches (e.g., nanoparticles, antibody–drug conjugates) to deliver drugs selectively to cancerous cells, thus enhancing drug efficacy and reducing nonspecific toxicity (Hubbell and Chilkoti 2012). However, the targeted anticancer therapeutic systems, which typically utilize monovalent molecular recognition between targeting molecules (e.g., antibody and aptamers) and receptors on cancer cells, still suffer from poor targeting and cellular internalization efficiency, selectivity, and overall killing efficacy (Huang et al. 2009; Keefe et al. 2010; Ray and White 2010).

In addition to CSC's self-renewal and proliferative abilities in acquiring resistance, the cancer cell microenvironment plays also crucial role in tumor metastasis and responds accordingly to the therapeutic molecules or approaches (Hanahan and Weinberg 2011). Tumor microenvironment is rich in cancerous and non-cancerous cells comprising endothelial cells, pericytes, vessels, fibroblasts, myofibroblasts, inflammatory cells, immune cells, dendritic cells, etc. The microenvironment assists in maintaining self-renewal, resistant properties of CSCs through the formation of hypoxic niches (Mannino and Chalmers 2011). The abundance of CSCs near vessels and the endothelial cells of microenvironment with other components help themselves to maintain their inherent and chemo-/radio-resistant properties, respectively (Borovski et al. 2011). Because low blood vessel formation decreases the CSC population in tumors and tumor growth, the intervention of CSC vascular niches is a promising therapeutic strategy. Importantly, CSCs reside in hypoxic environment within tumor cells that impedes effective enrichment of conventional therapeutics due to insufficient vascularization (Mohyeldin et al. 2010).

The fundamental understanding of the molecular signaling pathways involved in chemo-resistance mechanisms may help in identification of drug targets in resistant tumors and empower the chemotherapeutic drugs to achieve success in cancer therapy. CSC-targeted therapeutic approaches may improve the patient survival rate by prohibiting the frequency of tumor relapse. To triumph over cancer, the therapeutic strategies must have the ability to target proliferating cancer cells as well as tumorigenic CSCs (Shukla and Meeran 2014).

In addition to therapeutic resistance conferred by a small fraction of CSCs, the blood–brain barrier (BBB) restricts the permeation of drugs and cause poor prognosis of glioblastoma multiforme (GBM) brain tumor. An alkylating agent—temozolomide (TMZ)—and radiation, in combination were little effective in GBM patients in that they extended median survival time only by 2.5 months relative to radiation therapy alone (Kim et al. 2015a). Despite overwhelming primary responses, most of the patients later developed recurrences wherein TMZ became ineffective (Kim et al. 2015b). Considering the importance of CSCs in initiation and recurrence of tumors, current focus has been given to the targeting of CSCs for specifically delivering the therapeutics. Nanoparticles can selectively deliver payloads to relevant target cells of our body, and therefore, the development of NPs for CSCs-specific anticancer therapies is of substantial interest. However, in case of brain tumors, the enhanced permeability and retention (EPR) effect is very unlikely to achieve sufficient drug concentrations throughout the dense brain matrix under nanoparticle delivery via passive approaches (Sehedic et al. 2015). The chemotherapeutic drugs are accumulated in passive manner in the BBB area, disrupted by tumor sites in GBM (Bhowmik et al. 2015). However, the level of disruption depends on tumor sites and considered insignificant for certain tumors. Because BBB remains intact near growing edge of infiltrative tumor (Wen and Kesari 2008), it becomes difficult to efficiently target tumor cells by passive targeting mechanism (Juillerat-Jeanneret 2008). It is therefore an urgent need of finding ways to transport therapeutic molecules across the BBB. Nanomedicines that rely on receptor-mediated transcytosis might help to achieve effective

concentration of drugs in the affected distinct area of the tumors (Kim et al. 2014a). The specific antibodies or ligands for transferrin receptors (TfR) may facilitate transcytosis of nanoparticles via binding to the endothelial cells (ECs) receptors (Hendricks et al. 2015). They conjugated anti-TfR antibody to the NPs as an active targeting moiety. The nanoparticles bound to TfR on cerebral ECs, and actively traversed BBB via receptor-mediated transcytosis and efficiently delivered the drugs to intracranial tumors.

An understanding of CSCs provides an overview of therapeutic implications; nevertheless, challenges still persist in improving delivery of anti-CSC agents to the tumors, due to lower extent of water solubility, stability, circulation, cellular uptake, and unacceptable cytotoxicity.

3 Current Status of CSCs Therapy

In recent years, a considerable progress toward development of novel nanoscale strategies for cancer therapy including nanoliposomes, polymeric nanoparticles, and inorganic nanocomposites/particles has been noticed because they can carry a reasonable amount of drugs, and display greater absorption and pharmacodynamic activity.

The anticancer drugs are being continually developed. Some have undergone clinical trials and got approval for clinical application (Jain and Stylianopoulos 2010). The delivery of drug into target cancer cells in sufficient amount through reliable and safe methods is the key behind successful development of nanomedicines. However, a series of biological barriers significantly affect in vivo delivery of drugs to the tumor site (Alonso 2004). Following systemic administration, NPs undergoes extravasation, diffusion through blood vessels and extracellular matrix, respectively. Then, they can penetrate cell membranes of tumors before releasing the drug into cytoplasm (Kim et al. 2009). To overcome such barriers, NPs are delivered either by passive or active targeting approach.

Due to rapid and imperfect angiogenesis, the blood vessels in tumors form leaky endothelium and allow the entry of 400-nm-size macromolecules (Yuan et al. 1995). After intravenous administration, the nanoparticles passively extravasate into tumor tissue through the leaky vasculature, accumulate in the tumor bed through dysfunctional lymphatic drainage, and release therapeutic agents into the vicinity of tumor cells. This enhanced permeability and retention (EPR) effect constitutes the key mechanism for solid tumor targeting (Matsumura and Maeda 1986). The nanomedicines approved for clinical use in solid tumors rely on the EPR effect (Peer et al. 2007). As the EPR effect depends entirely on drug diffusion, the drugs must possess significant lipophilic property.

Ligands such as antibodies, peptides, or aptamers are attached to the surface of NPs that can bind specifically to targeted antigens or receptors and offers greater therapeutic effect by accelerating cellular uptake of the bioactive molecules via receptor-mediated endocytosis (Jain 1994). The NPs decorated with ligand

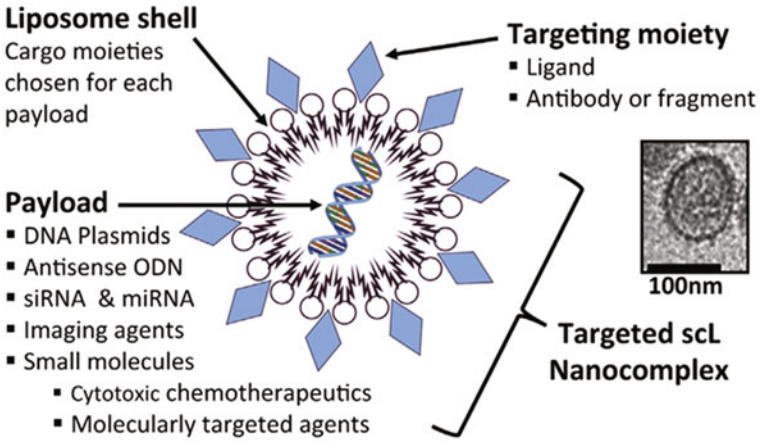

Fig. 1 Schematic representation of targeted TfR-binding NPs (scL nanocomplex). scL is composed of a targeting and cargo moiety. Various payloads can be packaged inside a cargo moiety made of cationic liposome shell. TfRscFv was incorporated on the surface of scL as a cancer cell and CSC-targeting moiety to form nano-sized complexes. A representative electron microscopic image of scL complex is shown. The scale bar represents 100 nm. [Reprinted from Biochemical and Biophysical Research Communications, 468, Kim, S.-S., Harford, J.B., Pirollo, K.F., Chang, E.H., Effective treatment of glioblastoma requires crossing the blood–brain barrier and targeting tumors including cancer stem cells: The promise of nanomedicine, 485–489, Copyright (2015), with permission from Elsevier]

molecules increase drug concentration in tumor tissues and CSCs due to high affinity toward the receptors overexpressed on the CSCs surface (Xia 2014). The nanoparticles loaded with drugs and engineered with the targeting moiety are depicted in Fig. 1. Identification of CSCs markers has now enabled us to design suitable methods for the delivery of anticancer drugs to CSCs in various cancers. Only limited studies are focused on surface antigen-specific targeting of CSCs (Naujokat 2014; Lang et al. 2015).

CSCs share various signaling characteristics with normal stem cells, but they also have unique, disease-specific pathways for exploitation as therapeutic targets (Hu and Fu 2012). Molecular differences between CSCs and their tissue-specific stem cell counterparts must be explained for the design of specific drug molecules and delivery strategies for CSCs. A successful therapy must kill all proliferating tumor cells; induce well differentiation of CSCs; or eliminate these cells. In this regard, novel therapeutic strategies for targeting CSCs have been investigated and some of them are discussed herein.

3.1 Targeting of Specific Cell Surface Molecules

CSCs can be abolished by targeting specific cell surface molecules overexpressed on cancer stem or progenitor cells. The development of specific antibodies or

immunotoxins against cell surface antigens could eradicate CSCs selectively. Gemtuzumab ozogamicin, an anti-CD133 monoclonal antibody when conjugated with calicheamicin (cytotoxic antibiotic), induced remissions in relapsed CD133+ acute myeloid leukemia (AML) (Tsimberidou et al. 2006). Swaminathan and coworkers (2013) incorporated paclitaxel into poly (lactic-co-glycolic acid) nanoparticles and conjugated anti-CD133 mAbs (CD133NPs). CD133NPs notably reduced the mammosphere and colony-forming ability of Caco-2 cells, which usually express abundant CD133 compared to only paclitaxel treated cells. In MDA-MB-231 xenograft, the NPs caused marked lowering of the CSCs and improvement of therapeutic effectiveness relative to non-targeted NPs.

CD44, a transmembrane glycoprotein, is a promising target because it is over-expressed on AML stem cells relative to normal stem cells. Salinomycin (SAL) has poor water solubility and exhibit toxicity, which prohibits its therapeutic application in killing CSCs. Yao and groups (2014) studied chitosan-enveloped single-wall carbon nanotubes—hyaluronic acid conjugates of SAL. This complex caused selective eradication of CSCs (CD44+ cells) from gastric cancer cells by inhibiting the capacity to self-renew CD44+ cells. Jin et al. (2006) found that the administration of H90 anti-CD44 antibody into non-obese diabetic/severe combined immunodeficiency (NOD/SCID) mice transplanted with human AML markedly reduced leukemic repopulation. Another cell surface target on AML stem cells is CD123, the receptor for IL-3 (Jordan et al. 2000). A diphtheria toxin–IL-3 fusion protein has shown selective toxicity toward acute AML in vitro. An efficient AML engraftment in NOD–SCID mice indicated its effect on leukemic stem cells. In conjunction with cytarabine—an anticancer agent—the complex effectively reduced the leukemic burden of NOD–SCID mice engrafted with human AML (Hogge et al. 2004). Haraguchi et al. (2010) demonstrated that anti-CD13 antibody treatment suppressed self-renewal and tumor-forming abilities of dormant CSCs in liver cancer. In addition, therapy against CD133 also represents a promising strategy for treatment of lung cancer (Bertolini et al. 2009), liver cancer (Rountree et al. 2009), and glioblastoma (Brescia et al. 2013).

3.2 Targeting of the Tumor Microenvironment of CSCs

CSCs are believed to exist in the microenvironment consisting of mesenchymal, vascular, and inflammatory cells (Plaks et al. 2015). Besides cellular components, extracellular matrix (ECM) and secreted molecules support tumor development and progression. These supportive niches provide assistance to maintain the properties of CSCs, protect them from apoptosis, and facilitate in spreading cancer to the distant organs (Ye et al. 2014). Apart from direct intervention with CSCs niches, the targeting of niche factors has been proved to be a powerful modality for the treatment and prevention of tumor progression.

Bevacizumab inhibits the progression of tumor by normalization of tumor vessels and disruption of CSC vascular niche (Narita 2013). The signaling pathway mediated by CXCR4/CXCL12 changes the tumor microenvironment after

chemotherapy or radiotherapy (Kioi et al. 2010). This in turn may cause tumor recurrence. Plerixafor, a CXCR4 antagonist prevents restoration of cancer vasculature, and thereby cancer recurrence. In mouse skin cancer, CSCs produced VEGF and stimulated the expression of neuropilin-1, co-receptor of VEGF receptor 2 and induced self-renewal of CSCs (Beck et al. 2011). VEGF in CSCs also induced angiogenesis and expanded the vascular niche for proliferation and maintenance of CSCs. Therefore, inhibitors of VEGF signaling pathway could suppress the progression of tumor by interrupting CSC renewal.

Cell types in CSC niche diverge from fibroblasts to endothelial cells, immune cells, and so on. However, the original features of these non-carcinoma cells are changed in normal tissues or organs and the acquired phenotype protects CSCs from traditional anticancer chemo- and radiotherapies. Hence, the underlying cellular mechanisms, implicated in maintaining immature phenotype of CSCs and in drug resistance, must be understood to kill CSCs (Kise et al. 2015). Targeting tumor hypoxia could be another option for manipulating the niche of quiescent, drug-resistant cells.

The inoculation of CSC-like cells with endothelial cells (ECs) into immuno-deficient mice enhanced tumor growth compared to only CSC-like cells, suggesting that ECs are the essential niche components that support the growth of glioblastoma. The release of nitric oxide (NO) from ECs acted as a stemness factor (Calabrese et al. 2007). The treatment of mice-bearing orthotopic U87 glioma cell xenografts with anti-VEGF monoclonal antibody, bevacizumab, extensively reduced the microvasculature density and thereby tumor progression and metastasis. These findings revealed that bevacizumab might disrupt microenvironment niche of the brain CSCs, and thereby impaired the self-renewal capacity (Calabrese et al. 2007).

The development of immunotherapies can selectively eliminate CSCs from cancer cells. Vik-Mo and groups (2013) highlighted vaccination strategy against autologous CSCs with dendritic cells in glioblastoma cancer patients. They observed threefold higher cancer-free survival of vaccinated patients. Therefore, the development of immunotherapy based on targeting of CSC-specific tumor antigens might be of great importance for clinical improvements in cancer treatment. A study by Motz et al. (2014) demonstrated that the inhibitors of VEGF and prostaglandin E2 could suppress Fas ligand expression in tumor ECs, resulting in infiltration of CD8 T cells. Another study indicated that the inhibition of VEGF signaling directly suppressed PD1 expression in T cells. Therefore, PD-L1 expression on cancer cells prohibited induction of T cell-mediated immunotolerance and resulted in suppression of the tumor growth (Voron et al. 2015).

Heterogeneous signals from fibroblasts, myofibroblasts, mesenchymal cells, and endothelial cells of tumor microenvironments cultivate CSCs (Hanahan and Coussens 2012). The therapeutic interventions of these signaling systems thus can eradicate CSCs in the tumor mass. Ma and coworkers (2013) found that the inhibition of stroma-derived signals by prostaglandin E2 receptor antagonist (RQ-15986) ensured complete fortification from the immunosuppressive effects of the breast cancer cell microenvironment.

3.3 Targeting ATP-Binding Cassette (ABC) Transporters

Even if CSCs are targeted by specific drugs, not all CSCs can be killed without hampering the function of drug efflux pumps. ABC transporters are membrane proteins that utilize adenosine triphosphate (ATP) to translocate various substrates across the cellular membranes. The expression of ABC transporter provides another way of drug resistance to human cancer cells due to efflux characteristics, which causes lowering of intracellular drug levels (Fletcher et al. 2010). Out of 49 ABC transporters, only ABCB1 (P-glycoprotein), ABCC1, and ABCG2 (breast cancer resistant protein, BCRP) have been extensively examined in multi-drug resistance (MDR) of cancers (Holohan et al. 2013). Overexpression of ABCB1 is associated with chemo-resistance in cancers like leukemia, liver, and colon (Holohan et al. 2013). ABCG2 causes chemo-resistance in CSCs in ovarian, breast, hepatocellular carcinoma, and glioblastoma. The breast CSCs overexpress caveolin-1, and β-catenin/ABCG2 signals mediate chemo-resistance (Wang et al. 2014a). Zhang et al. (2013a) described ABCG2 as a marker for CSC in hepatocellular carcinoma (HCC) because overexpression of ABCG2 enhanced malignant behavior of doxorubicin-resistant HCC cells.

The nanomedicines could overcome multi-drug resistance, an intractable obstacle for targeting CSCs (Markman et al. 2013). Due to chemical conjugation of drugs with NPs or encapsulation into NPs, the ABC drug efflux pumps could not physically recognize these drugs as substrates. Cell-penetrating peptides—or ligand-modified NPs—significantly increase intracellular concentration of chemotherapeutic agents due to the bypassing of efflux mechanism via endocytosis or macropinocytosis (Livney and Assaraf 2013) and consequently enhance their cytotoxic effects.

Since ABC transporters regulate the efflux and influx of drugs, they have the capability of affecting chemo-resistant properties to CSCs through direct efflux of drugs and cancer-causing substances in the tumor microenvironment. ABCB1 inhibitors—verapamil and cyclosporine—are toxic at the doses responsible for inhibition of P-gp function under clinical trials. The effect of ABC transporter inhibitor, tariquidar and anticancer agent, docetaxel, has also been tested in ovarian, cervical, and lung cancers (Patil et al. 2009; Blanpain et al. 2011; Minderman et al. 2004). However, low inhibitory effect and toxic potentials toward healthy cells prohibited their clinical development. CSCs overexpress transporter proteins that inhibit entry of most of the drugs and keep themselves nonresponsive.

Inhibition of ABC transporters could retard drug resistance and consequently the cancer cells respond better to chemotherapy. The inhibition of ABCG2 transporter by fumitremorgin C (Rabindran et al. 2000) and tryprostatin A (Woehlecke et al. 2003) could sensitize and henceforth kill CSCs. Drug resistance of CSCs may arise due to overexpression of other efflux transporters. A wider substrate specificity of ABCB1 is currently believed to be a barrier for effective therapy of leukemias and solid tumors (Nobili et al. 2006). Frank and colleagues (2003) found that ABCB5 could provide chemotherapeutic resistance to human malignant melanomas. They used monoclonal antibody (mAb) against ABCB5 for sensitization of melanoma

cells to doxorubicin, and thus, highlighted the importance of efflux pumps in drug resistance (Frank et al. 2005).

Instead of modulation of single mechanism, broad-spectrum inhibition is indeed a need in preventing drug resistance and CSCs eradication. Nanocarriers could bypass drug resistance due to ABC transporters. It has been demonstrated that doxorubicin-tethered gold NPs efficiently delivered doxorubicin to breast CSCs via poly (ethylene glycol) (PEG) spacer and an acid labile hydrazone bond. Gold NPs reduced stem cell-like behavior of CSCs by overcoming their MDR, and inhibited tumor growth during or after treatment (Sun et al. 2014b).

3.4 Poly-aptamer-Drug Approach

In nature, biological systems often use multivalent, cooperative interactions where multiple ligands on one biological entity simultaneously bind to receptors on another to achieve high binding affinity and selectivity (Mammen et al. 1998). Inspired by nature, engineered multivalency has become an emerging and powerful strategy of improving targeting efficacy and selectivity in drug delivery. The cooperative, multivalent binding between targeting molecules immobilized on a polymer scaffold or nanoparticles and receptors at target sites can improve not only the affinity but also the specificity of molecular interactions involved in drug delivery (Kolonko and Kiessling 2008). Intriguingly, multivalent ligand-receptor binding at the cell membrane can promote cellular internalization, likely through energy-dependent endocytic pathways. However, current methods to prepare multivalent-targeted drug delivery systems are complex.

Recently, Zhao and colleagues exploited a simple, powerful isothermal enzymatic reaction called rolling circle amplification (RCA) to synthesize multivalent scaffolds (Zhao et al. 2008, 2012). In RCA, DNA polymerase extends DNA from a primer by replicating a circular DNA template many folds to yield a single-stranded DNA product (Fig. 2a) (Hamblin et al. 2012). RCA products contain repetitive sequence units that are complementary to the circular DNA template and therefore can be easily modified. This versatility allows them to incorporate aptamers (single-stranded nucleic acid sequences that specifically bind to non-nucleic acid targets) into the RCA product that selectively binds to cancer but not normal cells. Further multivalent aptamers produced by RCA on a microfluidic-device could selectively capture flowing cancer cells with significantly higher efficiency than monovalent aptamers or antibodies due to their high binding avidity and unique structural and mechanical properties (Zhao et al. 2012). They reasoned that the aptamer-containing DNA molecules could act as a simple molecular scaffold to construct multivalent, targeted drug delivery systems. The multivalent aptamer composition may induce cooperative binding that increases the strength and frequency of interactions with target cells. Moreover, the DNA scaffold can be tailor-designed to incorporate a spacer domain between aptamers. These spacers can hybridize with complementary strands to form duplex, drug loading domains (Bagalkot et al. 2006; Zhu et al. 2013) on which DNA-intercalating

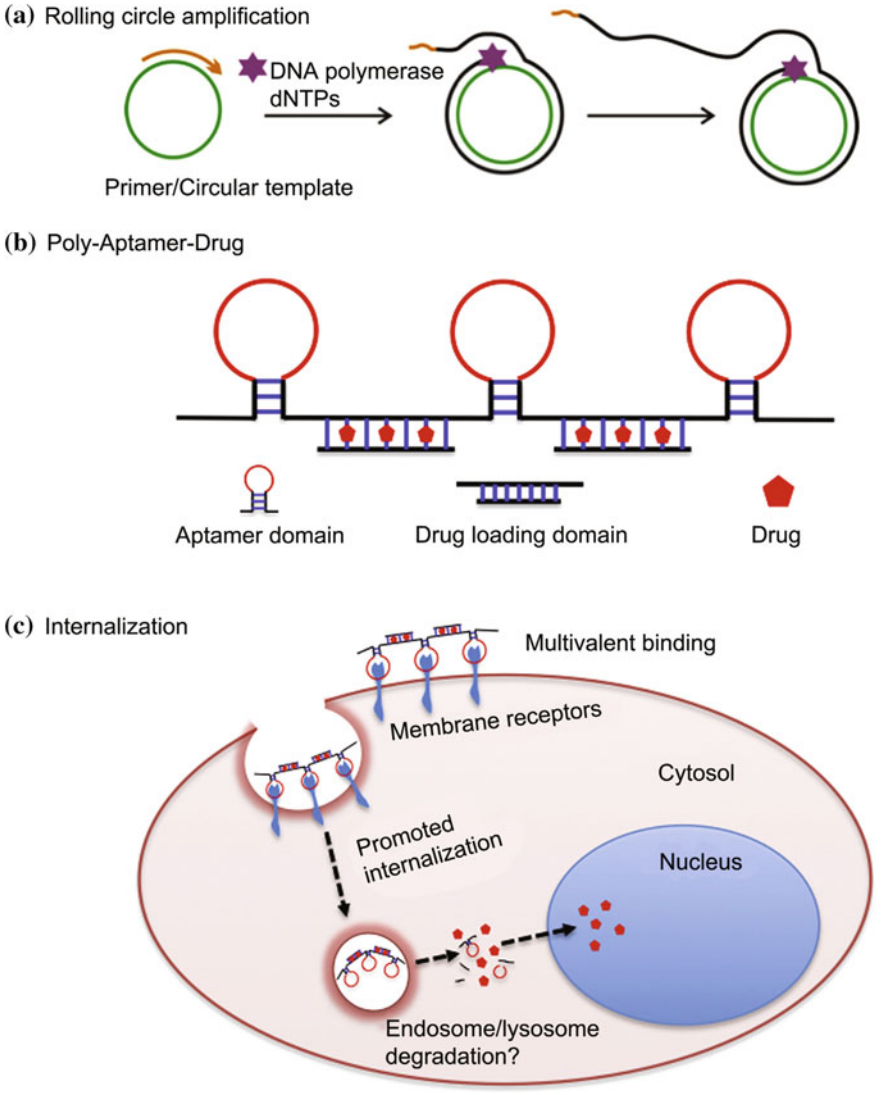

Fig. 2 Concept of using Poly-Aptamer-Drug to target and kill cancer cells: **a** Generation of long single-stranded DNA using rolling circle amplification (RCA). A short DNA primer is annealed to a circular DNA template. Amplification initiates upon addition of Phi29 DNA polymerase (purple star) in the presence of deoxyribonucleotide triphosphates (dNTP) mix; **b** Poly-Aptamer-Drug composition: Poly-Aptamer (*red loops*) complex with a short complementary sequence (shown as duplex) and drug; **c** Poly-Aptamer-Drug specifically binds to target receptors on cancer cells followed by enhanced cellular internalization due to multivalency effects. Poly-aptamer-drug might be degraded by intracellular nucleases to facilitate drug release. [Reprinted from Biomaterials, 34, Zhang, Z., Ali, M.M., Eckert, M.A., Kang, D.-K., Chen Y.Y., Sender, L.S., Fruman, D.A., Zhao, W., A polyvalent aptamer system for targeted drug delivery, 9728–9735, Copyright (2013), with permission from Elsevier]

chemotherapeutic agents (e.g., doxorubicin) can be readily incorporated via physical association without the need for chemical modification of the drug or the scaffold (i.e., poly-aptamer-drug) (Fig. 2b). They hypothesized that this poly-aptamer-drug system will exhibit high cancer cell targeting efficiency and enhanced cellular internalization due to multivalent effects (Fig. 2c). Zhang et al. (2013b) demonstrated that leukemia cell-binding aptamer–doxorubicin system was more effective than its monovalent counter part in targeting and killing leukemia cells due to about 40-fold higher binding affinity and cell internalization via multivalent effects. They suggested that poly-aptamer-doxorubicin system could effectively target and treat cancers while minimizing the side effects associated with chemotherapy treatment.

3.5 Nanometric Drug Delivery Platforms

Like conventional agents, CSC-specific chemotherapeutics do possess some undesirable physicochemical and biological properties. Here is an overview of various drug delivery devices under experimental stages for the application of this kind of agents (Fig. 3).

Despite excellent anticancer properties, the use of curcumin is limited due to lack of considerable hydrophilicity, stability, and favorable pharmacokinetics. Wang et al. (2011) reported that stearic acid–*graft*–chitosan micelles were efficiently internalized and exhibited higher cytotoxicity of oxaliplatin against colorectal cancer cells. However, the micellar particles reversed the chemo-resistance of CD133+/CD24+ CSC subpopulations in vitro and in vivo. Later they incorporated curcumin into chitosan micelles and also found that curcumin was largely accumulated in cancer cells, and significantly inhibited the subpopulation of $CD44^{+}CD24^{-}$ putative colorectal CSCs (Wang et al. 2012a).

Nanocarriers can improve stability and bioabsorption of the macromolecular drugs having anticancer activity. MicroRNAs (miRNAs) are a diverse family of small RNA molecules that behaves as a post-transcriptional regulator in different cellular functions. The regulatory role of miRNA in CSCs and the use of miRNAs as prognostic biomarkers for tumor progression and anticancer agents are recently reported (Ma et al. 2010; Liu et al. 2011). Its powerful ability of RNA interference (RNAi) could suppress the expression of target gene, needed for the treatment for various cancers (Pai et al. 2006). Hu et al. (2008) noted that the suppression of siRNA-mediated Octamer 4 (Oct-4) gene caused apoptosis of CSCs in lung and breast cancers. However, the therapeutic benefits of miRNAs and siRNAs are questionable due to nonspecific cellular uptake and systemic toxicity (Muthiah et al. 2013).

Therefore, it is an urgent need to design delivery systems that can restrict degradation of nucleic acid drugs and enhance drug accumulation in intended tissues, thereby improving overall efficacy of cancer therapeutics. Yang et al. (2012) described that cationic polyurethane–polyethylenimine (PU–PEI) nanoparticles could deliver plasmid DNA encoding a tumor-suppressive microRNA145 (miR145). They reported radio-sensitivity and chemo-sensitivity of CSC-derived brain tumors and longer animal survival after local intracranial injection of these

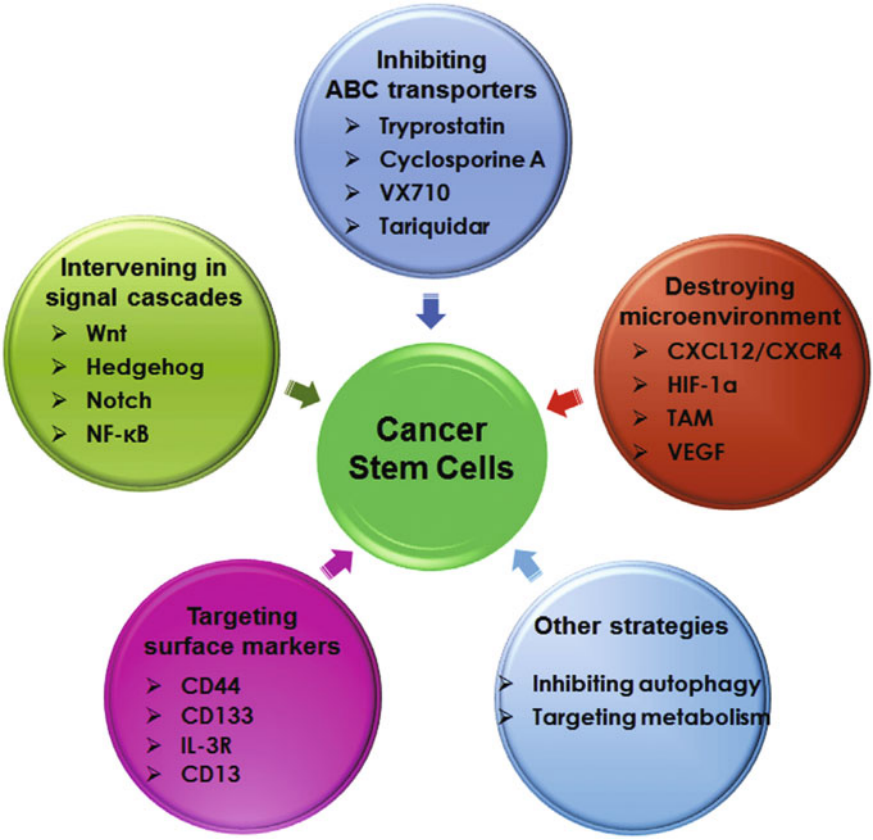

Fig. 3 Possible therapeutic strategies that can eliminate CSCs. Four main kinds of therapy aimed at eradicating CSCs have been developed in recent years, including selectively inhibiting ATP-driven efflux transporters, intervening in crucial signaling pathways, destroying the tumor microenvironment, which nurtures or nurses CSCs, and targeting surface markers of CSCs. Other strategies, such as inhibiting the autophagy of CSCs and targeting metabolism, have also been investigated in recent years. [Reprinted from Biomaterials, 74, Shen, S., Xia, J.-X., Wang, J., Nanomedicine-mediated cancer stem cell therapy, 1–18, Copyright (2016), with permission from Elsevier]

NPs. The delivery of miR-145 NPs into glioblastoma CD133+ cells significantly inhibited the tumorigenicity and facilitated their differentiation into CD133− non-CSCs. Although NPs lacked targeting ligands, CSC-like properties were reduced because miR145 down-regulated Oct4 and Sox2 genes expression for stemness. Nonetheless, the lack of active ligands for CSCs and intracranial administration may be the problems behind translation of this delivery system into the clinic. Shi et al. (2013) described solid lipid NPs for delivery of miR-34a that could resist both CSC differentiation and metastasis by direct repression of the CSC marker CD44 in lung cancer cells for eradication of CSCs.

Incorporation of siRNA into nanoparticles represents one strategy of overcoming their therapeutic limitations. The siRNA-based nanomedicine for targeting GLUT3 has been reported for simultaneous inhibition of self-renewal property of glioma stem cells and glioma bulk cells in glucose-restricted tumor microenvironment. NP-mediated GLUT3 knockdown significantly inhibited tumor growth in a U87MG xenograft model (Xu et al. 2015). Alternately, the nanocarrier-based siRNA delivery approach can be used to silence genes encoding drug efflux transporters, thereby sensitizing CSCs to chemotherapeutic drugs. To improve stability and amount of siRNA in human colon CD133+ CSCs, Liu and coworkers tested PEI–lipid NPs for the delivery of MDR1-silencing siRNA. A higher chemo-sensitivity to paclitaxel was observed (Liu et al. 2009). Nevertheless, the depletion of CSCs may be insufficient in complete tumor regression if the residual differentiated tumor cells are still likely to switch to CSCs and sustain the growth of a tumor mass (Bu and Cao 2012). The strategies that can tackle both non-CSCs and CSCs may hold a great promise in imparting therapeutic activity.

Zhang et al. (2012) developed octreotide (Oct)-decorated PEG-*b*-poly (caprolactone) (PCL) micelles for the delivery of paclitaxel (PTX) and SAL in combination. The combination therapy responded strongly due to the activity of PTX against bulk, breast cancer cells and that of SAL against CSCs. Zhou and coauthors (2013) tested HPMA copolymer–cyclopamine conjugate (P-CYP) and HPMA copolymer–docetaxel conjugate (P-DTX) in combination for exhibiting preferential toxicity of P-CYP to CSCs and that of P-DTX in debulking tumor mass. Overall, the combined drug inhibited tumor growth by killing both CSCs and non-CSC fractions. Ke and colleagues (2014) utilized a mixture of acid-functionalized poly(carbonate) (PAC) and PEG diblock copolymer (PEG–PAC) and urea-functionalized poly(carbonate) (PUC) and PEG diblock copolymer (PEG–PUC) to capture doxorubicin and thioridazine (THZ) for selective killing of CSCs. A strong inhibitory effect on CSCs and antitumor efficacy in BT-474 xenografts was noted following co-delivery of doxorubicin and THZ.

Nanocarriers can accommodate reasonable amount of multiple therapeutic molecules in their structures. Sun et al. (2015) encapsulated all-trans retinoic acid (ATRA), and doxorubicin in NPs for breast cancer therapy. The systemic administration enriched tumor tissues and CSCs with drugs and prodigiously enhanced the tumor suppression and reduced CSCs synergistically. This type of delivery systems was also investigated for co-delivery of nucleic acid drugs and chemical agents for improving their pharmacokinetics and bio-distribution in tumor tissues. Liu and coworkers (2013b) co-delivered miR-200c (CSCs inhibitor), and docetaxel (DOC)-loaded gelatinase-responsive NPs and evaluated their synergetic inhibitory effect on CSCs and non-CSCs. They noticed that miR-200c/DOC NPs significantly reduced cell proliferation and the expression of E-cadherin and CD44. Notably, the systemic delivery of miR-200c/DOC NPs led to greater in vivo drug accumulation, retention, and sustained antitumor activity into xenograft gastric cancer mice.

In addition to low molecular weight drug molecules and nucleic acid-based drugs, some anticancer mAbs demonstrated anti-CSCs potential and induced tumor regression in clinical trials (Vinogradov and Wei 2012). The role of antibodies in

antibody–drug conjugates and antibody-modified NPs is to guide the therapeutic molecules to CSCs. Since CSCs can express specific surface biomarkers, the use of antibodies against these biomarkers for target-specific drug delivery is justified.

Hyaluronic acid (HA) is an extracellular glycosaminoglycan matrix component and this has been accounted as a ligand for binding to CD44 that is overexpressed in most of the CSCs during carcinogenesis (Wei et al. 2013). Such binding affinity of HA provided an opportunity for developing CSC-targeted therapies. Ganesh et al. (2013) synthesized a series of CD44-targeted HA-based nanostructures for siRNA delivery. The delivery efficiency and gene silencing activity of siRNA-loaded HA nanosystems in drug-resistant CD44 receptor-overexpressing tumor models was appreciable. Recently, a nanodoxorubicin system has been developed by Rao et al. (2015). They encapsulated DOX in Pluronic F127 NPs after surface functionalization with chitosan that structurally resembles HA. The cytotoxicity of DOX was found to increase significantly compared to free DOX for eliminating CD44+ CSCs residing in 3D mammary tumor spheroids. They further revealed that the nanosystems could trim down the size of tumors in an orthotopic xenograft tumors without any evidence of systemic toxicity. Wang and coworkers (2012b) developed anti-CD44 antibody-decorated liposomal NPs for HCC therapy. They demonstrated that doxorubicin-encapsulated and mAb-directed liposomes could selectively target CD44+ HCCs and alleviate the side effects of conventional chemotherapy.

Transferrin receptor (TfR) is overexpressed on both CSCs and non-CSCs. TfR-targeted nanocomplex could efficiently deliver payloads in both populations and eliminate them simultaneously. The systemic administration of TfR-targeted nanocomplex carrying WTp53 gene inhibited tumor growth and induced cell death in subcutaneous colorectal cancer xenografts (Kim et al. 2014b). Some workers examined antitumor potential of cetuximab-conjugated iron oxide NPs in intracranial rodent GBM models. Cetuximab had the capability of binding extracellular domain of EGFR and EGFRvIII expressed on CSCs and non-CSCs of GBM, respectively. Kaluzova et al. (2015) observed a significant suppression in tumor growth as well as increased animal survival rate after treatment. Following binding of ligands to TfR, the complex is internalized via receptor-mediated endocytosis.

TfR represents a vital target for GBM cancer therapy. In vitro treatment of CSC-enriched GBM tumors with Tf-conjugated and miR1-loaded lipopolyplex NPs (Tf-NPs) significantly suppressed cell migration and EGFR expressions (Wang et al. 2014b). However, they did not assess in vivo targeting ability in GBM CSCs after systemic administration of Tf-NP-miR1 lipoplexes. Using an anti-TfR antibody as a targeting ligand, Kim et al. (2014b) examined targeting propensity of the NPs to the tumor cells and CSCs. The studies demonstrated that the NPs could cross BBB via transcytosis and efficiently deliver its load into CSCs and non-CSCs in GBM. Li et al. (2012) demonstrated that Tf conjugation to poly (amidoamine) NPs increased the accumulation of tamoxifen in glioma cells. Therefore, continuous efforts must be put in developing nanomedicines for effective anti-CSCs therapies.

4 Conclusion

Considering the troubles in eradicating CSCs from bulk tumor cells, a number of strategies have been adopted to resolve the issues. However, many issues remain unresolved for clinical developments of anti-CSC nanomedicines. Besides exploring mechanisms behind CSC-related drug resistance, and screening of CSC-targeting agents, special focus must be given to the fabrication of safe, effective delivery systems using nano-technological, and immunotherapeutic approaches. The innovative and effective strategies must ensure safety, better cellular internalization, and deeper tumor extravasation. Despite significant advances in this area, a multidisciplinary approach is an urgent need to achieve clinical success of nanomedicines.

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Dendrimers as Nanostructured Therapeutic Carriers

Sabyasachi Maiti and Sougata Jana

Abstract

The unique features of dendrimers, such as their high degree of branching, multivalency, globular architecture, and well-defined molecular weight, make them promising carriers for the delivery of therapeutics. Dendrimer nanostructures represent outstanding nanocarriers in medicine. Dendrimeric structures are of particular interest in the field of drug delivery due to their peculiar structural properties including controllable internal cavities bearing specific species for the encapsulation of guest drugs and external periphery with 3D multiple functional moieties for solubilization, conjugation of bioactive compounds and targeting molecules, and recognition purposes. In addition, the low polydispersity of dendrimers provides reproducible pharmacokinetic behavior. Polyamidoamine (PAMAM) dendrimers, polypropyleneimine dendrimers, and peptide-based dendrimers such as those based on polylysine have been tested as promising nanostructured carriers for the delivery small drug molecules and macromolecules. Despite their broad applicability, it is generally necessary to modify the surface amine groups of these dendrimers with neutral or anionic moieties to avoid the toxicity associated with their polycationic surfaces. In recent years, much effort has been devoted to the preparation of dendrimers that are designed to be highly biocompatible, biodegradable, and water-soluble. In the past decade, research has been increased on the design and synthesis of biocompatible dendrimers and their application in the field of drug and macromolecules delivery. Recent progress in ocular, oral, brain, and tumor drug targeting application using dendrimers is discussed herein.

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1 Introduction

The term dendrimer is derived from a Greek word dendron that means "tree," which is logical in view of their typical structure with a number of branching units and "meros" meaning "part" in Greek. It was first discovered in the early 1980s by Donald Tomalia and co-workers (Tomalia et al. 1985).

Dendrimers are well-defined branched structure with three-dimensional globular shape, monodispersed, nanosized macromolecules (1–100 nm). Three distinct domains of dendrimers are as follows. A central core of dendrimer consists of an atom or a molecule having at least two identical chemical functions. The branches, emanating from the core, constituted of repeat units having at least one branch junction, whose repetition is organized in a geometrical progression that results in a series of radially concentric layers called "generations." Generation is common for all dendrimer designs and the hyperbranching when going from the center of the dendrimer toward the periphery, resulting in homostructural layers between the focal points (branching points). The number of focal points when going from the core toward the dendrimer surface is the generation number. A dendrimer having five focal points when going from the center to the periphery is denoted as the 5th generation dendrimer and abbreviated as G5-dendrimer. The core of the dendrimer is sometimes denoted generation "zero" (G0). The core structure thus presents no focal points, as hydrogen substituents are not considered focal points. Thus, in PPI dendrimers, 1,4-diaminobutane represents the G0 core structure and in PAMAM Starburst dendrimers ammonia represents the G0 core structure. Intermediates during the dendrimer synthesis are sometimes denoted half-generations. Dendrimers of lower generations (0, 1, and 2) have highly asymmetric shape and possess more open structures as compared to higher generation dendrimers. As the chains growing from the core, molecule become longer and more branched (four and higher generations) dendrimers adopt a globular structure (Caminati et al. 1990).

The size of dendrimer increases as the generation number increases and becomes tightly packed. Divergent and convergent methods are most frequently used for dendrimer synthesis. In the **divergent methods**, dendrimer grows outwards from a multifunctional core molecule. The core molecule reacts with monomer molecules containing one reactive and two dormant groups giving the first generation dendrimer. Then the new periphery of the molecule is activated for reactions with more monomers. The process is repeated for several generations and a dendrimer is built layer after layer. The divergent approach is successful for the production of large

quantities of dendrimers. In the **convergent approach**, the dendrimer is constructed stepwise, starting from the end groups and progressing inwards. When the growing branched polymeric arms, called dendrons, are large enough, they are attached to a multifunctional core molecule. The convergent approach does not allow the formation of high generations because steric problems occur in the reactions of the dendrons and the core molecule (Klajnert and Bryszewska 2001).

Many terminal functional groups are generally located at the surface of dendritic architecture. These surface groups can be tailored to provide a template for drug delivery and are vital in determining the properties of dendritic macromolecules (Tomalia et al. 1990; Frechet 1994).

Dendrimers have some unique properties because of their globular shape and the presence of internal cavities. The most important one is the possibility to encapsulate guest molecules in the macromolecule interior. Toxicity considerations for the dendrimers are crucial because of the growing interest in using them in biomedical applications.

Toxicity of dendrimers is ascribed mainly to the end group present on its periphery. Generally, amine-terminated PAMAM and PPI dendrimers display concentration-dependent toxicity and hemolysis (Roberts et al. 1996; Malik et al. 2000; Jevprasesphant et al. 2003), whereas neutral or anionic groups terminated dendrimers have shown comparatively less toxicity and hemolysis (Fuchs et al. 2004; Gillies et al. 2005). Fortunately, the toxicity of cationic dendrimers can be overcome by partial or complete modification of their periphery with negatively charged or neutral groups (Chen et al. 2004). Although both PAMAM and PPI dendrimers have terminal amino groups, yet they display different pattern of toxicity. In case of cationic PAMAM dendrimers, toxicity increases with each generation but unpredictably cationic PPI dendrimers do not follow this pattern of toxicity (Malik et al. 2000). The cytotoxicity behavior of cationic dendrimers is widely explained by the favored interactions between negatively charged cell membranes and the positively charged dendrimers surface, enabling these dendrimers to adhere to and damage the cell membrane, causing cell lysis. The masking of cationic end groups or conversion of end groups of dendrimers to neutral or anionic groups have resulted in dendrimers with decreased toxicity or even non-toxic dendrimers as was observed in case of neutral dendrimers like polyester, polyether, and surface engineered dendrimers, for example, glycosylated, PEGylated dendrimers, etc. (Ihre et al. 2002; Kesharwani et al. 2011).

Furthermore, the cytotoxicity was found to be generation dependent, with higher generation dendrimers being the most toxic (Jevprasesphant et al. 2003). The degree of substitution as well as the type of amine functionality is important, with primary amines being more toxic than secondary or tertiary amines (Fischer et al. 2003).

Regardless of their toxicity, dendrimers have been considered as “smart” carrier owing to their ability as intracellular drug delivery vehicle, to cross biological barriers, to circulate in the body during time needed to exert a clinical effect, and to target-specific structures.

The end groups of dendrimers may possess positive, negative, or neutral charges, which are vital in the exploration of dendrimers as drug delivery vehicles. This polyvalency can be exploited to play an important role in the application of dendrimers as gene carrier because cationic dendrimers like poly-l-lysine, PPI, PAMAM, etc., can form complexes with negatively charged DNA. In addition, the positive charges of dendrimers facilitate its interaction with negatively charged biological membranes leading to applicability of dendrimers for intracellular drug delivery. The polyvalency of dendrimers also leads to the toxicities (Jain et al. 2010), and thus it has important implication on the properties of dendrimers and provides a potential arena for scientists working in the field of dendrimers-mediated drug delivery (Agashe et al. 2006).

Many commercial small molecule drugs with anticancer, anti-inflammatory, and antimicrobial activity have been successfully associated with dendrimers such as poly(amidoamine) (PAMAM), poly(propylene imine) (PPI or DAB), and poly (etherhydroxylamine) (PEHAM) dendrimers, either via physical interactions or through chemical bonding ("prodrug approach"). Targeted delivery is possible via targeting ligands conjugated to the dendrimer surface or via the enhanced permeability and retention (EPR) effect (Svenson 2009).

About 40% drugs are rejected by the pharmaceutical industry at the development stages due to poor bioavailability as a consequence of low water solubility. Hence, the core shell nanostructures of dendrimers could be suitable for drug solubilization applications (Gupta et al. 2006). Further, they are being considered as additives in several routes of administration, including intravenous, oral, transdermal, and ocular (Cheng et al. 2008a).

The applications of dendrimers have been reviewed exhaustively by many scientists (Svenson and Tomalia 2005; Gillies and Fréchet 2005; Li et al. 2007; Cheng et al. 2008b; Svenson 2009; Menjoge et al. 2010). In this chapter, the drug delivery applications of dendrimers are described emphasizing the latest developments in ocular, brain, oral, and tumor-targeting therapeutics.

2 Ocular Therapeutics

Indeed, eyes have a quasi-impermeable corneal surface epithelium, which necessitates a long residence time to increase the efficiency and the bioavailability of the instilled drug, to deliver it in the inner eye structure. Furthermore, lachrymal drainage poses problems to obtain sufficiently high therapeutic drug concentration inside the eye, notably when treating disorders such as diabetic retinopathy or glaucoma, to name as a few (Sigurdsson et al. 2007). The most common method for improving the bioavailability of a drug consists in increasing the viscosity by adding water-soluble polymers to enhance the bioadhesion of the solutions instilled (Sahoo et al. 2008). However, such conventional formulations may induce a temporarily disturbed vision, particularly for people suffering of "dry eye" disorder (Stern et al. 1998). Thus, penetrating the ocular surface still presents a challenge for

chemotherapy, and it appears tempting to use dendrimers instead of polymers in the formulation. The dendrimers have multiple extremities, which may increase the bioadhesion, but they have also very distinct properties compared to polymers, in particular a very low intrinsic viscosity (Uppuluri et al. 1998; Merino et al. 2001), and a perfectly defined structure. Vandamme anf Brobeck (2005) reported the use of dendrimers (PAMAM) for the in vivo ocular delivery of pilocarpine nitrate. They determined the influence of a controlled incremental increase in size, molecular weight and number of amine, carboxylate, and hydroxyl surface groups in several series of poly (amidoamine) (PAMAM) dendrimers for controlled ocular drug delivery. New Zealand albino rabbit was used for qualitative and quantitative assessment of ocular tolerance and retention time after a single application of 25 μl of dendrimer solution containing pilocarpine nitrate to the eye. Residence time was longer for the solutions containing dendrimers with carboxylic and hydroxyl surface groups. No prolongation of remanence time was observed when dendrimer concentration (0.25–2%) increased. The remanence time of PAMAM dendrimer solutions on the cornea showed size and molecular weight dependency. The novel macromolecular carriers with prolonged drug residence time were suitable for the ophthalmic route.

Spataro et al. (2010) attempted to use dendrimers for the ocular delivery of a drug to treat glaucoma and ocular hypertension, which are among the most frequent and severe ocular diseases, susceptible to degenerate to blindness. These diseases require a very constraining life treatment, with instillations all the 3 or 4 h. Increase of the residence time of the drug could decrease the number of daily takings.

They tested the well-known drug carteolol, a β-blocker and an ocular antihypertensive agent. The structure of the dendrimers was engineered in order to ensure an electrostatic association with the amino group of carteolol and limit the use of benzalkonium chloride as preservative. They thought that a quaternary ammonium group as core of dendrimers could replace benzalkonium chloride and carboxylic acid terminal groups could help to interact with the amino function of carteolol to afford catanionic (saline) species. They selected tris (2-chloroethyl) amine hydrochloride as analog of benzalkonium chloride for use as core of the dendrimer. Neutral carteolol was obtained by reacting the hydrochloride derivative (the commercially available form of the drug) with K_2CO_3. A known volume of water was added to dendrimer 12-G0 and carteolol. The resulting mixture was stirred for 24 h to afford a clear solution, which was then lyophilized. The resulting powder was washed with ether to eliminate the slight excess of drug. Dendrimer 13-G0 was isolated as a white powder. The structures of dendrimer 12-G0 and 13-G0 are depicted in Fig. 1.

The smallest saline species was soluble in water, but the largest ones were only poorly soluble. All the compounds (from generation 0 to generation 2) in solution in milliQ water were instilled in the eyes of rabbits. No irritation was observed, whatever the catanionic dendrimer was used and even after several hours. The quantity of carteolol penetrated inside the aqueous humor showed practically no difference between carteolol alone and carteolol entrapped with the generation zero. Due to the very low solubility of the second generation, the quantity of carteolol

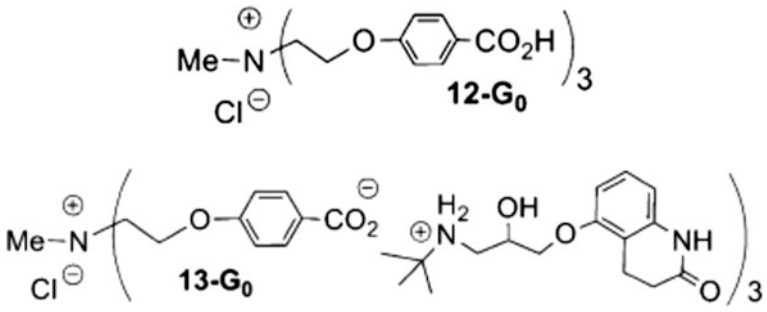

Fig. 1 The structures of dendrimer 12-G0 and 13-G0 (Spataro et al. 2010)

instilled was low, but the quantity of carteolol that penetrated inside the eyes was 2.5 times larger than expected, when compared with carteolol alone. Thus, even if the solubility is a real problem, these pharmacodynamic observations highlighted the biocompatibility and usefulness of this approach for drug delivery.

More than 90% of the marketed ophthalmic formulations are in the form of eye drops, and most of them target the "anterior segment eye diseases (Lang 1995). The ophthalmic diseases like glaucoma, diabetic retinopathy (DR), age-related macular degeneration, and various forms of retinitis pigmentosa are damaging the back of the eye, which may result in impaired vision and even blindness (Ranta et al. 2010). Unfortunately, treatment of "posterior segment diseases" is still an unsolved issue. Delivery of drugs to the posterior segment is more challenging than to the anterior segment, because of the ocular barriers, acellular nature of the vitreous body and the longer diffusion distance (Eljarrat-Binstock et al. 2010). Thus, posterior segment of the eye has become an important target for drug delivery in the therapeutic range, while reducing the side effects (Del Amo and Urtti 2008). Pharmacological medication is essential for DR treatment besides controlling the diabetes-related physiological factors. Dexamethasone (DEX) is a commonly used corticosteroid drug for the treatment of many of the retinal diseases including DR. Current treatment of DR requires intravitreal injection or intravitreal implants that are invasive and risky methods. Even the subconjunctival application is a promising delivery route for retinal drug delivery, low trans-scleral transmission of many drugs is a limitation for reaching the efficient drug concentration at the target area.

Dendrimer nanostructured polymers have been an important research field in ocular drug delivery. Most of the ocular diseases would benefit from long-lasting drug delivery of dendrimers and dendrimer-based drug delivery systems. It was already reported that dendrimers present practical solutions to drug delivery issues by enhancing solubility and biodistribution as well as ocular permeability (Kaminskas et al. 2011). Poly(amidoamine) (PAMAM) dendrimers, especially those with –OH and –COOH terminal groups are noncytotoxic and are cleared intact through the urine at lower generations (Jain et al. 2010). Ongoing studies in developing improved ocular dendrimeric systems may not only serve to enhance the drug delivery to the ocular surface, but may also provide effective delivery of

therapeutic agents to intraocular tissues, such as the retina or choroid, using non-invasive delivery methods (Yavuz et al. 2013).

Yavuz et al. (2015) evaluated the effect of various PAMAM formulations on DEX delivery to the back of the eye, especially to vitreous and retina via topical and subconjunctival (less invasive than intravitreal route) applications. On the basis of the literature information (Bourges et al. 2003; Amrite et al. 2005), nanoparticular systems bigger than 100 nm are expected to stay longer in the ocular injection site. Thus, longer retention time is predicted for dendrimers with higher generations based on their bigger particle sizes. Results indicated that DEX complexation increased particle size 3–4 times and sizes change in a range approximately between 125 and 250 nm with the exception of PAMAM G4–DEX complex that was measured as 423 nm.

The evaluation of the cytotoxicity is an important parameter in ocular drug delivery because dendrimer cytotoxicity might restrict their biopharmaceutical application. Methyl-thiazol-tetrazolium (MTT) assay on human corneal epithelium (ARPE 19) cell line results showed that DEX itself has a slight cytotoxicity and the blank dendrimers has no effect on cell viability. The presence of DEX affected cell viability, regardless of the dendrimer type. The cationic dendrimers (with amine end groups) PAMAM G3 and PAMAM G4 did not induce more cytotoxicity than dendrimers with peripheral carboxyl or hydroxyl functional groups, which was surprising, because cationic macromolecules including cationic dendrimers were reported to induce toxicity due to the chemical nature of polycations.

DEX–PAMAM complex formulations were designed to enhance solubility of DEX and improve its ocular permeation across rabbit cornea or sclera, choroid, and retinal pigment epithelium (SCRPE) following topical or subconjunctival application. Following topical application, drug has to cross through corneal barrier in order to reach the back of the eye. On contrary, following subconjunctival application, SCRPE were the barriers that drug needs to pass through to reach retina. DEX transport across SCRPE could be higher than corneal transport because cornea has more intact tight junctions when compared with SCRPE. It was reported that RPE should be considered as a major barrier for the transport of hydrophilic substances, but for hydrophobic materials, the choroid-RPE and sclera were approximately equivalent barriers (Pitkanen et al. 2005).

All anionic dendrimer complexes showed higher drug transport level than DEX solution and the cationic dendrimers. Dendrimers with –COOH ending groups showed slightly higher transport profile than dendrimers with –OH ending groups. Furthermore, G3 dendrimers had higher transport than G4 dendrimers across both cornea and SCRPE. SCRPE transport was found higher than corneal transport. Thus, the surface charge is a significant factor for transport across cornea and SCRPE. Overall, ex vivo transport studies indicated that both corneal and SCRPE transport of DEX could be enhanced with PAMAM dendrimer complexation. Transport is highly affected by charge and surface group of the dendrimers as well as the generation. DEX–PAMAM complex formulations enhance DEX delivery to retina following both topical and subconjunctival application. Especially, DEX–PAMAM G3.5 and G4.5 complex formulations having –COOH ending might be

suggested as strong candidates for topical DEX delivery to the back of the eye, as they have provided higher DEX tissue levels. DEX suspension stays at the injection site and the tissue distribution was poor.

Yao et al. (2010) evaluated puerarin–PAMAM dendrimer complexes as an ocular drug delivery system. Puerarin released more slowly from puerarin-dendrimer complexes than free puerarin in deionized water and phosphate buffer solution (pH 6.8). The in vitro release rate of puerarin complexed with full generation dendrimers was lower than that with half-generation dendrimers. Furthermore, puerarin–dendrimer complexes produced longer ocular residence times in rabbits compared with puerarin eye drops. No damages to the epithelium or endothelium were observed after the PAMAM dendrimer administration in this corneal permeation study in rabbits. Puerarin-dendrimer complexes had potential as an ocular drug delivery system to improve the efficacy of drug treatment.

Iezzi et al. (2012) previously reported that intravitreal administration of dendrimer fluocinolone acetonide (D-FA) conjugates selectively co-localized in activated microglial cells and provided sustained neuroprotection for a period of 30 days at a 40-fold lower dose compared to non-erodible controlled release implant for FA, in a rat RCS model of retinitis pigmentosa.

Triamcinolone acetonide (TA) is an FDA-approved glucocorticosteroid administered intravitreally for the treatment of diabetic macular edema, macular edema associated with retinal vein occlusion (Zhang et al. 2014), proliferative diabetic retinopathy (Zhang et al. 2008), etc. It has also been used for post-operative retinal surgery related inflammation (Matsuda et al. 2005). However, the hydrophobicity, lack of solubility, and the side effects limit its effectiveness in the treatment of retinal diseases. Increasing the solubility of TA can lead to an increase in bioavailability, permeation through ocular tissue, and intracellular transport. If better drug solubility is achieved, it may result in prolonged efficacy at significantly lower concentration thereby reducing the chances of drug toxicity (Durairaj et al. 2010; Holden et al. 2012). Following, intravitreal administration, TA forms epiretinal crystals in the vitreous humor due to its lack of solubility, prolonging the therapeutic effect of TA but also cause side effects (Chung et al. 2007). Drug aggregation and precipitation can lead to visual obscurity, unequal distribution, mechanical damage, and local toxicity to the retinal tissue (Narayanan et al. 2006). To address insolubility and sedimentation issues, TA formulations with benzyl alcohol or benzalkonium chlorides as vehicle preservatives were commercialized but resulted in vehicle-mediated toxicity and sterile-endophthalmitis (Kleinman et al. 2010; Chang et al. 2011).

PAMAM dendrimer encapsulation of anti-glaucoma drugs resulted in better efficacy and also increased the drug uptake in corneal cell layers (Holden et al. 2012).

Kambhampati et al. (2015) explored a PAMAM G4 dendrimer–TA conjugate (D-TA) as a potential strategy to improve intracellular delivery and efficacy of TA to target cells. The structure of D-TA and amine-functionalized ethylenediamine core generation four PAMAM dendrimers (G4-OH) are displayed in Fig. 2.

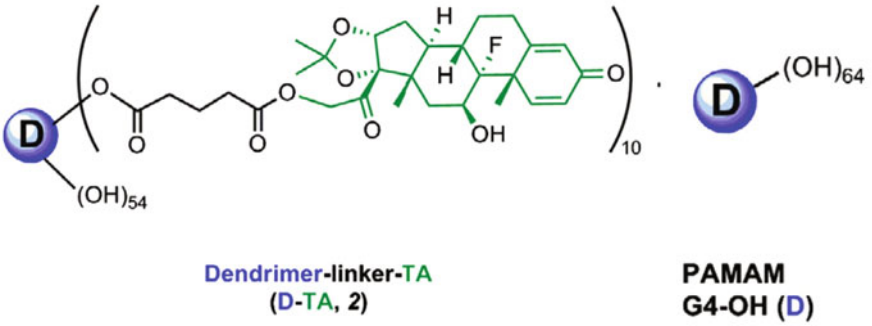

Fig. 2 Chemical structures of dendrimer-triamcinolone acetonide conjugate (*left*) and hydroxyl- and amine-functionalized ethylenediamine core generation four PAMAM dendrimers (G4-OH) (*right*) (Kambhampati et al. 2015)

Compared to free TA, D-TA demonstrated a significantly improved toxicity profile in two important target cells (microglial and human retinal pigment epithelium (RPE)). The D-TA was ~100-fold more effective than free TA in its anti-inflammatory activity (measured in microglia), and in suppressing VEGF production (in hypoxic RPE cells). Thus, dendrimer-based delivery may improve the efficacy of TA toward both its key targets of inflammation and VEGF production, with significant clinical implications.

3 Brain-Targeted Therapeutics

Gene therapy has emerged as a promising strategy to treat cerebral diseases such as glioma, Alzheimer's and Parkinson's diseases, which affect a large percentage of the world's population and hardly respond to intravenously administered, small molecule treatment (Schlachetzki et al. 2004; Gabathuler 2010). Although the genetic basis for many of these diseases is known, the possibility of using genes as medicines is currently limited by the lack of safe and efficacious delivery systems which are able to cross the blood–brain barrier (BBB) and deliver DNA to the brain after intravenous administration. The permeability properties of BBB prevent the delivery of more than 98% of drugs, including nucleic acids, to the brain (Pardridge 2001, 2007). In addition, locally administered treatments fail to achieve a widespread gene expression in the target cells throughout the entire brain, which is necessary for successful treatment of most cerebral pathologies (Begley 2004). However, the BBB does possess specific receptor-mediated transport mechanisms that can potentially be exploited as a means to target drugs and genes to the brain. The transferrin receptor (TfR) is of particular interest because it is overexpressed on the brain capillary endothelial cells (Jefferies et al. 1984). The antibodies that bind to the TfR have been shown to selectively target the brain microvascular

endothelium due to the high levels of TfR expressed by these cells (Bickel et al. 1993; Friden 1994; Moos and Morgan 2001). Several strategies have been explored to formulate TfR-targeted delivery systems able to transport nucleic acids to the brain following intravenous administration (Dufès et al. 2013). Numerous non-viral gene delivery systems are currently under development, due to their low immunogenicity, stability, unrestricted plasmid size, and versatility in types of modifications (Dufès et al. 2005; Li and Huang 2007). Among these delivery systems, generation 3-diaminobutyric polypropylenimine dendrimer (DAB) appears to be particularly promising. Koppu et al. (2010) recently prepared a transferrin (Tf)-bearing generation 3-diaminobutyric polypropylenimine dendrimer (DAB-Tf), able to increase the cellular uptake and gene expression of DNA by cancer cells overexpressing transferrin receptors compared to non-targeted delivery systems, in vitro and in vivo. Importantly, the treatment was well tolerated by the animals, with no apparent signs of toxicity.

Transferrin (Tf) was conjugated to generation of 3-diaminobutyric polypropylenimine dendrimer (DAB) using dimethylsuberimidate (DMSI) as a cross-linking agent as reported previously (Koppu et al. 2010; Lemarié et al. 2012). Briefly, DAB was added to transferrin and dimethylsuberimidate (1:2) in triethanolamine HCl buffer (pH 7.4). The reaction took place for 2 h at 25 °C while stirring. The conjugate was purified by size exclusion chromatography using a Sephadex G75 column and freeze-dried.

Later, they investigated the potential of DAB-Tf gene delivery system in improving the delivery of plasmid DNA to the brain (Somani et al. 2014). In vitro, the transferrin conjugation to the dendrimer increased the DNA uptake by bEnd.3 murine brain endothelioma cells overexpressing transferrin receptors, by about 1.4-fold and 2.3-fold compared to that observed with the non-targeted dendriplex and naked DNA. In vivo, the intravenous injection of transferrin-bearing dendriplex produced more than double gene expression in the brain compared to the unmodified dendriplex and decreased nonspecific gene expression in the lung. Gene expression was at least threefold higher in brain than that in any tested peripheral organs and was at its highest 24 h following the injection of the treatments. Thus, transferrin-bearing polypropylenimine dendrimer showed high promise as gene delivery system to the brain.

HIV-associated dementia (HAD) was strongly determined by active HIV-1 replication in the CNS and a neuropathological inflammatory response known as HIV-1 encephalitis (HIVE) (Budka 1991; del Palacio et al. 2012). The treatment of CNS disorders by systemic administration or local delivery of drugs is currently inefficient in many cases due to restrictions of molecular size or hydrophilicity imparted by BBB (Banks et al. 2006). Finding ways to get therapeutic drugs to the CNS effectively, safely, and conveniently is becoming more important than ever. Drug delivery vehicles that can target the inflammatory cells in different areas of the brain may provide new alternatives for sustained therapy.

Serramía et al. (2015) studied the viability of dendrimers and dendriplexes in human primary astrocytes, as well as their uptake by astrocytes. Briefly, carbosilane dendrimer 2G-$(SNMe_3I)_{12}$ was obtained by addition to vinyl terminated derivatives

of excess 2-dimethylaminoethanethiol hydrochloride $HS(CH_2)2NMe_2{\cdot}HCl$, then base and finally excess of methyl iodide. The 2G-$(SNMe_3I)_{12}$ obtained was purified by ultrafiltration (Fuentes-Paniagua et al. 2014).

Dendriplexes were prepared by mixing equal volumes of dendrimer and siRNA dissolved in sterile water at concentrations depending on the charge ratios and molar concentrations desired. The 2G-$(SNMe_3I)_{11}$-FITC dendrimer transported siRNA efficiently into the brain, crossing the BBB without histological and morphological changes in different brain regions. The results provided the basis for the development of novel nanoformulations with conjugated siRNA, which could not only effectively cross the BBB, but also facilitate significant uptake of the nanoplex by HIV-1 infected cells in the CNS, thereby permitting increased bioavailability of the anti-retrovirals and significant reduction of viral infection in the brain. They speculated that carbosilane dendrimers could be very useful as an effective therapeutic strategy in the treatment of neuro-AIDS and other neurological disorders.

Targeted dendrimer drug delivery systems using various targeting ligands have been developed. Lactoferrin, a newly explored brain targeting ligand, was coupled to the dendrimer for targeted delivery of DNAs and the lactoferrin-coupled dendrimer was reported to have high BBB-crossing efficiency (Huang et al. 2009). Doxorubicin was conjugated to peptide (RGD)-coupled PEGylated PAMAM dendrimer via a degradable disulfide spacer for controlled release in the treatment of glioma tumors (Kaneshiro et al. 2009).

Further, the PEPE dendrimers could cross the BBB in vitro in significant amounts without disrupting the tight junctions (Dhanikula et al. 2006). It has been described that an intact BBB hinders the dendrimer entry into the brain parenchyma (Iezzi et al. 2012), indicating their negligible brain uptake in healthy or tumor-bearing animals with an intact BBB (Li et al. 2012). Previously, they studied transcytosis of dendriplexes (carbosilane dendrimers/siRNA) through the BBB in an in vitro model (Jimenez et al. 2010). The main objective was to determine on the capacity of dendriplex to cross the BBB in an in vivo model, and in this context, the results demonstrated that the dendriplexes crossed the BBB in a BALB/c mouse model, without histological and morphological changes in different brain regions. Moreover, 2G carbosilane dendrimers were highly efficient at transfecting the non-CD4 + human primary astrocytes. A reduction of around 30–50% of viral infectivity with a concentration of siRNA of 100 nM was noted. Although we did not find any variation of viral production in NHA cultures isolated from human fetal brain cortex tissue at this concentration, it should be noted that it is possible that at higher concentrations of siRNA we could achieve robust inhibition of HIV-1 viral replication as observed in astrocytoma cells (Jimenez et al. 2010).

Glioblastoma multiforme (GBM) is the most aggressive central nervous system (CNS) tumor because of its fast development, poor prognosis, difficult control and terrible mortality. Poor penetration and retention in the glioblastoma parenchyma were crucial challenges in GBM nanomedicine therapy. Nanoparticle diameter can significantly influence the delivery efficiency in tumor tissue. Decreasing nanoparticle size can improve the nanoparticle penetration in tumor tissue but

decrease the nanoparticles retention effect. Therefore, small nanoparticles with high retention effect in tumor are urgently needed for effective GBM drug delivery.

Little work has been done on small nanoparticles to improve drug delivery to GBM tissue by increasing enhanced permeability and retention (EPR) effect and tissue penetration. EPR effect was a vital factor for nanomedicine therapy of tumor (Danhier et al. 2010), and the nanoparticle permeability and retention in brain tumor was relative weak than peripheral tumors owing to special glioma microenvironment (Sarin et al. 2009). Hobbs et al. (1998) reported that the microvascular pore size of the U87 glioma was 7–100 nm, significantly smaller than that of noncerebral tumors (380–780 nm). Given that, proper small particle size is the basic precondition of good nanoparticle permeation in glioma tissue. Furthermore, it has been reported that for poorly permeable tumors only small size nanoparticles (not more than 30 nm) can reach deeper section and achieve a broader tumor tissue distribution (Cabral et al. 2011) and more intracellular accumulation than conventional size ($\sim$100 nm) nanoparticles (Huang et al. 2012). As the non-edge region of brain tumor has a higher pressure (Boucher et al. 1990), and small nanoparticles are easier to flush back into the blood, resulting in a decreased retention in brain tumor tissue. Therefore, small nanoparticles with high retention effect in tumor are still urgently needed for effective GBM drug delivery.

Zhao et al. (2015) developed a small nanoparticle drug delivery system by conjugating fibrin-binding peptide CREKA to PAMAM dendrimer, where PEGylated PAMAM was used as drug carrier due to its small size and good penetration in tumor and CREKA was used to target the abundant fibrin in GBM for enhanced retention in tumor. In vitro binding ability tests demonstrated that CREKA could significantly enhance nanoparticle binding with fibrin. CREKA-modified PAMAM achieved higher accumulation and deeper penetration in GBM tissue than unmodified one and thus constituted a promising strategy for brain tumor therapy.

4 Tumor-Targeted Therapeutics

In the gene delivery field, the study of small interfering RNA (siRNA) has increased due to their potential as a therapeutic macromolecule for the treatment of cancer therapy. siRNA can inhibit production of oncogenic regulators associated with tumor growth, transformation, and metastasis by a sequence-specific gene silencing effect (Miele et al. 2012; Burnett and Rossi 2012). The introduced siRNA interacts with the RNA-induced silencing complex (RICS) in the cell cytoplasm and promotes cleavage of messenger RNA (mRNA) which consists of complementary sequences (Bernstein et al. 2001). Therefore, siRNA has a very powerful mechanism of silencing specific genes that has been reported both in vitro and in vivo with various cell types and diseases including solid tumors, respiratory syncytial virus infection, inherited skin disorder, and age-related macular degeneration (McManus and Sharp 2002; Davis et al. 2010; Kaiser et al. 2010). However, siRNA still has

limited to clinical applications due to the lack of efficiency and the low stability in the human body. Therefore, siRNA has not been approved by the Food and Drug Administration (FDA or USFDA).

To overcome the drawbacks of siRNA, various non-viral vectors, such as polycationic polymer, polyanionic polymer, liposomes, and micelles have been studied. These non-viral vectors have shown lower transfection efficiency than viral vectors but have several advantages including lower cytotoxicity, non-immunogenicity, stability, and large-scale production. Hence, the studies using non-viral vector in the field of gene therapy has increased. Nanosized polyplexes for easy accumulation at the tumor site through EPR effect (Liu 2010; Park et al. 2013); cell penetrating peptide (CPP) modified non-viral vectors (Kim et al. 2009, 2011); amine-rich non-viral vectors to enhance the gene condensing ability and easy escape from endosome to cytoplasm (Zintchenko et al. 2008); bio-reducible non-viral vectors for easy release of genetic materials from polyplex in reductive environments (Breunig et al. 2008; Nam et al. 2012) and ligand-conjugated non-viral vectors for target-specific delivery (Yhee et al. 2013) have been tested to enhance transfection efficiency.

Previously, dendrimer type bio-reducible polymer (PAM-ABP) was synthesized using arginine grafted bio-reducible poly(cystaminebisacrylamide-diaminohexane) (ABP) and polyamidoamine (PAMAM) and evaluated for application as a pDNA delivery carrier (Nam et al. 2012; Won et al. 2013). The polyplex with genetic material at a low complex ratio (weight ratio or N/P ratio) increased its cellular uptake into the cells and easy release of genetic material.

Nam et al. (2015) synthesized dendrimer type bio-reducible polymer (PAM-ABP) using arginine grafted bio-reducible poly(cystaminebisacrylamide-diaminohexane) (ABP) and polyamidoamine (PAMAM) to deliver anti-VEGF siRNA into cancer cell lines including human hepatocarcinoma (Huh-7), human lung adenocarcinoma (A549), and human fibrosarcoma (HT1080) cells and access their potential as a siRNA delivery carrier for cancer therapy. PAM-ABP and siRNA formed polyplexes with average diameter of 116 nm. The siRNA in the PAM-ABP/siRNA polyplex was released by degradation of the disulfide bond of PMA-ABP in presence of 5 mM DTT and heparin. VEGF gene silencing efficiency of PAM-ABP/siRNA polyplexes was shown to be more effective than PEI/siRNA polyplexes in three cell lines with the following order HT1080 > A549 > Huh-7.

Polycationic dendrimers have been extensively studied for gene delivery because they aid in efficient internalization of DNA following endocytosis and membrane destabilization, and facilitate escape of gene/dendrimer polyplexes from endosomes and lysosomes. Covalent coupling of targeting ligands to the dendrimer is a viable approach to develop efficient targeted therapeutic modalities for drug delivery. Epidermal growth factor receptor (EGFR) overexpression occurs in multiple human solid tumors, including cancers of the head and neck, lung, breast, colon, and brain (Mendelsohn 2001). EGF12 and anti-EGFR antibody such as Cetuximab (Wu et al. 2006) have been used as targeting ligand to selectively enhance cellular uptake of drug-carrying vehicles by human carcinomas.

Yuan et al. (2010) designed EGF-containing polyamidoamine (PAMAM) Generation 4 dendrimer and found that EGF-conjugated dendrimers did not stimulate growth of EGFR-expressing cells. G4.0-triglycine (GGG)-EGF nanoparticles localized within cells that expressed the EGFR in a receptor-dependent manner, whereas uptake into cells lacking the receptor was low. They used vimentin shRNA (shVIM) plasmid and yellow fluorescent protein (YFP) siRNA to test the delivery and transfection efficiency of the constructed targeted vector. Significant knockdown of expression was observed, indicating that this vector was useful for introduction of nucleic acids or drugs into cells by a receptor-targeted mechanism.

CpG-oligodeoxynucleotides (CpG-ODNs) can stimulate the immune system via interaction with Toll-like receptor 9 (TLR9) (Weiner 2009). These unmethylated CpG-ODN motifs are characteristic for bacterial and viral DNA. In humans, TLR9 is expressed in numerous cells of the immune system and also in cancer cells, including breast, brain, gastric, lung, and prostate. TLR9, specifically recognizing unmethylated CpG oligonucleotides in vertebrates, is localized at endoplasmic reticulum. Then, it is translocated to the endosomal/lysosomal compartment for ligand recognition (Heeg et al. 2008). After binding to ligand, TLR9 and its associated adaptors, such as MyD88 and TRIF, recruit intracellular signaling mediators that activate transcription factors, such as nuclear factor kB (NF-kB) (Kawai and Akira 2007). Activation of TLR9 leads to triggering apoptosis in various types of malignant cells including breast cancer cells. Therefore, TLR9 agonist could be considered as candidates for cancer treatment. However, successful transfer of the CpG-ODN to the tumor site is dependent on the development of an efficient delivery vector to overcome various hurdles, such as rapid degradation by serum nucleases and poor diffusion across the cell membrane. Due to their growing clinical significance, different formulations and delivery systems have been designed to improve the stability and efficiency of cellular uptake of CpG-ODNs (Pan et al. 2007; Zhang et al. 2013).

Magnetic nanoparticles (MNPs) based on iron oxide as targeted drug delivery system have received specific attention due to their simplicity, ease of synthesis, and ability to tailor their properties for special biological purposes. These nanoparticles carrying the anticancer agent can be targeted to the tumor site, and accumulated in cancer cells by the help of an implanted permanent magnet or an externally applied field. However, the use of magnetic nanoparticles needs lot of surface modification so as to protect them from reticuloendothelial system and to increase their stability in vivo. Organic ligands such as poly(amidoamine) (PAMAM) dendrimer, polyethylene glycol, dextran, and aminosilanes are commonly used to stabilize these nanoparticles. PAMAM dendrimers with abundant terminal groups on the surface can form stable complexes with plasmid DNA and oligonucleotides.

Pourianazar and Gunduz (2016) fully utilized the advantages of MNPs and PAMAM dendrimers to solve the problems associated with CpG-oligodeoxynucleotides (CpG-ODNs) delivery to tumor cells, such as poor diffusion across cell membrane and rapid degradation by exonuclease or

endonuclease, by fabricating nanoscale delivery system composed of MNPs covered with different generations of PAMAM dendrimers.

CpG-ODNs activate Toll-like receptor 9 (TLR9), which can generate a signal cascade for cell death. They utilized three-layer magnetic nanoparticles composed of a Fe_3O_4 magnetic core, an aminosilane (APTS) interlayer and a cationic poly (amidoamine) (PAMAM) dendrimer. The dendrimer-coated magnetic nanoparticles (40 ± 10 nm) having high positive charges on their surface attached to CpG-ODN molecules via electrostatic means and induced cell death in MDA-MB231 and SKBR3 (human breast cancer cell lines) tumor cells and could be considered a suitable targeted delivery system for CpG-ODN.

Considerable interest has been raised to capitalize on dendrimer nanocarriers for the delivery of the emerging RNA interference (RNAi)-based nucleic acid drugs. RNAi is an endogenous process during which long double-stranded RNA molecules are processed by the endoribonuclease Dicer to yield small double-stranded RNA fragments of 19–23 nucleotides called small interfering RNAs (siRNAs). These siRNAs are then incorporated into the RNA-Induced Silencing Complex (RISC), with the antisense strand to bind in a fully complementary manner to mRNA, resulting in mRNA cleavage in sequence-specific manner and thereby gene silencing and turning-off of protein synthesis (Hannon 2002). Recently, Dicer has been shown to be a component of the RISC and involved in the entry of the siRNA duplex into RISC (Pham et al. 2004). Dicer-substrate siRNAs (dsiRNAs) can be designed to generate higher and more durable RNAi potency (Kim et al. 2005). Indeed, siRNA molecules are intrinsically unstable against various enzymes (Soutschek et al. 2004) and also highly negative charged, thus requiring delivery systems able to protect them and transport them cross the cell membranes.

To address this challenge, structurally flexible triethanolamine (TEA) core poly (amidoamine) (PAMAM) dendrimers have been developed as nanovectors for RNA delivery (Zhou et al. 2011; Liu et al. 2012, 2014). Several siRNA molecules have been established to knock down Heat shock protein 27 (Hsp27), leading to effective anticancer activity (Rocchi et al. 2006). Liu et al. (2014) disclosed for the first time, the dendrimer-based targeted delivery of Hsp27 Dicer-substrate siRNA (dsiRNA) in prostate cancer models to silence the target gene and successfully accomplished the desired and improved anticancer activity. They used a structurally flexible triethanolamine-core poly(amidoamine) dendrimer of generation 5 as the nanocarrier, which gave rise to a much greater RNAi response than that produced with conventional siRNA. Further decoration of the dsiRNA/dendrimer complexes with a dual targeting peptide simultaneously promoted cancer cell targeting through interacting with integrins and cell penetration via the interaction with neuropilin-1 receptors, which led to improved gene silencing and anticancer activity. Altogether, they opened a new avenue for therapeutic implementation of RNAi using dendrimer nanovector-based targeted delivery.

The properties of dendrimers have attracted great interest in exploring their potential applications in the field of tumor-targeted drug delivery, whereby drug compounds could either be incorporated into the dendrimer core or covalently conjugated to the dendrimer surface. Two common virtues of these nano-DDS are

Table 1 The drug delivery attributes of various dendrimer-based tumor-targeted systems

Dendrimers/nanosystems	Anticancer drug (cell line)	Study objectives	Outcomes	References
Chitosan (CS)-poly (amidoamine) (PAMAM)	Methotrexate (human lung adenocarcinoma epithelial cell line, A549 cells)	To examine methotrexate delivery efficiency of CS-PAMAM on A549 cells	Low cytotoxicity on cells, pH-dependent release of methotrexate from CS-PAMAM, significant improvement in the cytotoxicity of free methotrexate on A549 cells	Leng et al. (2013)
1,2-dipalmitoyl-sn-glycero-3-phosphocholine and G4 PAMAM	Paclitaxel (IGROV-1 ovarian cancer cell line)	To develop lipid-dendrimer hybrid (LDH) nanosystem for the hydrophobic anticancer drug, and evaluate its anti-cancer activity in ovarian cancer models	78% encapsulation efficiency of paclitaxel in the 37.6 ± 6.1 nm size LDH system, 37-fold improvement in the potency of paclitaxel compared to free drug, synergistic action of paclitaxel and PAMAM G4.0 in killing ovarian cancer cells. Despite the use of a tenfold lower paclitaxel dose, the survival of IGROV-1 ovarian tumor-bearing animals significantly prolonged	Liu et al. (2015)
G3 PAMAM dendrimer surface modified with lauryl chains and conjugated with paclitaxel via glutaric anhydride linker	Paclitaxel (human colon adenocarcinoma cell line (Caco-2) and primary cultured porcine brain endothelial cells	To study the ability of G3 PAMAM dendrimer in enhancing permeability of paclitaxel and overcoming cellular barriers	Conjugation of lauryl chains and paclitaxel on dendrimer significantly increased cytotoxicity against both cell types, conjugate had approximately 12-fold greater permeability across both cell monolayers compared to paclitaxel alone	Teow et al. (2013)
Gelatin NP core surface decorated with doxorubicin (DOX) and arginine-glycine-aspartic acid (RGD) peptide conjugated dendritic poly-L-lysine (DGL)	Doxorubicin (DOX) (Mouse mammary breast tumor cell line (4T1) and human umbilical vein endothelial cells (HUVEC)	To develop multistage drug delivery system which could intelligently shrink its size from large size to small size in the presence of matrix metalloproteinase-2 (MMP-2) which were highly expressed in tumor tissues	Size of multistage NP effectively shrank in the presence of MMP-2. RGD–DOX–DGL–GNP could penetrate deep into tumor spheroids, Higher tumor retention and deeper penetration than both DOX–DGL and DOX–GNP. The multistage system could benefit from its large size for better retention effect in tumor and then shrunk to small size to contribute to better penetration efficiency	Hu et al. (2015)
PEG 1100 attached to surface amino groups and doxorubicin linked through 4-(hydrazinosulfonyl) benzoic acid linker via surface α-amino groups of G5 polylysine dendrimer	DOX (Walker 256 carcinoma cells)	To characterize pharmacokinetics of doxorubicin after administration of t PEGylated dendrimer (D-DOX) and compare the same with DOX-loaded within PEGylated liposome (L-DOX) and DOX solution	Enhanced accumulation of dendrimer- and liposome-associated DOX in tumors compared to DOX alone, all three formulations reduced tumor growth to a similar extent, Dendrimer-doxorubicin displayed similar antitumor efficacy to PEGylated liposomal doxorubicin, but with lower systemic toxicity	Kaminskas et al. (2012)

(continued)

Table 1 (continued)

Dendrimers/nanosystems	Anticancer drug (cell line)	Study objectives	Outcomes	References
Herceptin-diglycolamic acid (DGA) functionalized G4 PAMAM	Cisplatin (Human ovarian cancer cell lines PA-1 and SKOV-3)	To explore herceptin as ligand to specifically target HER-2 expressing tumors and maximize intracellular concentration of chemotherapeutics	Enhanced anticancer activity with reduced systemic side effects when targeted with herceptin, Lower IC50 value, improved S-phase arrest, and enhanced apoptosis due to increased cellular uptake and accumulation than untargeted DGA-G4-cisplatin and free cisplatin, Herceptin-DGA-G4-cisplatin was more effective in inducing tumor regression as compared to free cisplatin, Overall improvement of chemotherapeutical efficacy with a pH sensitive DGA functionalized PAMAM dendrimer with the aid of herceptin	Kesavan et al. (2015)
OH-terminated G4 PAMAM and folic acid on graphene oxide (GO)	Doxorubicin (HeLa human cervical cancer cells)	To achieve effective drug loading ability and, consequently efficient drug delivery	Small hybrid carriers possessed 1.3 times lower loading capacity for doxorubicin (DOX) than the large hybrid carriers (1500 nm GO). Small hybrids led to higher cell viability, and efficient cellular internalization (97% at 62.5 mg/ml). Small hybrids consisting of 100 nm GO carrier successfully loaded and delivered DOX into HeLa cells. Dendrimer had a role of stable suspension of the hybrids in water, and folic acid acted as an effective anchoring of the hybrids on HeLa cells	Siriviriyanun et al. (2015)
PEGylated G4 polylysine	Doxorubicin (breast carcinoma MAT 13762 IIIB cells)	To explore whether PEGylated polylysine conjugated with DOX viaan acid labile 4-(hydrazine sulphonyl) benzoic acid linker provides therapeutic benefit against lung-resident cancers compared to inhaled or IV administration of drug solution	After intratracheal instillation to rats, approximately 60% of the dendrimer was rapidly removed from the lungs (within 24 h) via mucociliary clearance and absorption into the blood. This was followed by a slower clearance phase that reflected both absorption from the lungs and biodegradation of the dendrimer. After 7 days, approximately 15% of the dose remained in the lungs. PEGylated dendrimers have potential as inhalable drug delivery systems to promote the prolonged exposure of lung-resident cancers to chemotherapeutic drugs and improve anti-cancer activity	Kaminskas et al. (2014)

their ability to reduce the unwanted side effects of the drug with concomitant improvement in the pharmacological activities, as well as their ability to improve delivery through targeting the drug to disease sites such as tumor tissue by EPR effect. The outcomes of various dendrimer-based tumor-targeted nanoscale delivery systems for small drug molecules are summarized in Table 1.

5 Oral Therapeutics

Oral drug delivery is among the most preferred routes of administration owing to its noninvasive nature, cost-effectiveness and patient compliance. Oral delivery of polymer therapeutics is hindered by several barriers in the gastrointestinal tract (GIT). These include their large size and hydrophilicity, which limit transepithelial transport. Furthermore, variations in pH along with high enzymatic activity in the GIT can adversely affect the stability of polymer therapeutics leading to premature release of the drug or degradation of the constructs (Ponchel and Irache 1998). By investigation and optimization of their stability in the GIT, and absorption across the gut, polymer therapeutics can be developed for oral administration. Owing to their compact structure and high surface charge density, dendritic polymers can be tailor made to permeate across the epithelial barrier of the gut. Polymers with dendritic architecture such as poly(lysine), and poly(amido amine) (PAMAM) have been observed to transport across the epithelial barrier of the intestine, thus showing promise in oral delivery. PAMAM dendrimers have been the most widely studied dendritic polymers for oral delivery purposes. They have the advantage of having a very open internal structure and cavities for accommodating guest therapeutic molecules that need to be solubilized or targeted for drug delivery.

The surface of dendrimers can be functionalized with drugs, targeting moieties, and biologically active components. Drugs or genes can be either encapsulated within the dendrimers through non-covalent strategies such as hydrophobic, ionic, and hydrogen bond interactions or conjugated to the peripheral groups of dendrimers via covalent methods. PAMAM and PPI dendrimers are the extensively used ones among the numerous dendrimers used for drug delivery. A bountiful reports are available which evidence for the drug-carrying potential ofdendrimers through properties like increased solubility of the hydrophobic drugs, sustained drug release behavior and increased efficiency of the drugs (Patri et al. 2005; Gupta et al. 2006; Cheng et al. 2008a, b). Dendrimer-based drug delivery systems provide an attractive platform for loading and release of conventional drug molecules, which improve the pharmacodynamic and pharmacokinetic behaviors of several families of drugs (Svenson 2009). These in turn reveal the promising future of dendrimer-based drug delivery systems.

Dendrimers such as PAMAM and PPI possess cationic primary amine groups at the surface. Even though they showed excellent drug delivery efficacy, their cytotoxicity is still a burning issue that limits the clinical applications of most these dendrimer-based drug formulations (Duncan and Izzo 2005; Jain et al. 2010). They

exhibited high cytotoxicity on numerous cell lines and serious hemolytic activity on red blood cells, which is dependent on dendrimer generation, surface functionality/charge and concentration (Jeyprasesphant et al. 2003). Cationic groups on dendrimer surface are the major determinant factor in generating cytotoxicity and hemolytic activity. These cationic charges interact with the phosphates on the cell membrane, which leads to disturbance of lipid bilayers resulting in leakage of intracellular components (Smith et al. 2010). In addition, the surface cationic dendrimers are rapidly cleared from the blood circulation systems, which result in limited therapeutic efficacy and bioavailability of the administered drug–dendrimer formulation. In order to overcome this problem, the cationic charges on dendrimer surface can be neutralized.

Nimesulide (NMD) is a non-steroidal anti-inflammatory drug (NSAID) with analgesic and antipyretic properties. It is mainly prescribed for the treatment of acute pain, the symptomatic treatment of osteoarthritis (Davis and Brogden 1994). However, its poor solubility in aqueous medium (0.01 mg/ml) (Piel et al. 1997) gives rise to difficulties in the design of pharmaceutical formulations and leads to variable oral bioavailability. Further, poorly water-soluble drugs often require high doses in order to reach therapeutic plasma concentrations after oral administration. Hence, to increase the therapeutic efficiency of poorly soluble drugs, its solubility in aqueous medium has to be increased. Murugan et al. (2014) prepared a quaternized G3 PPI dendrimer (QPPI) and utilized as carrier for nimesulide. QPPI (G3) was prepared by reacting surface amine groups of PPI (G3) dendrimer with glycidyltrimethyl ammonium chloride. The aqueous solubility of drug dramatically increased up to 72-folds in the presence of G3 QPPI and also sustained the drug release. After 5 h, 90.32% of drug was released from G3 PPI formulations whereas 35.69% of drug was released from G3 QPPI formulation. These results suggested that the G3 QPPI dendrimer significantly contributed to the sustained release of drug than their parent dendrimer formulation in phosphate buffer solution (PBS). Cytotoxicity study on Vero and HBL-100 cell lines revealed that this dendrimer could increase the biocompatibility.

Another non-steroidal anti-inflammatory drug (NSAIDs), ketoprofen is effective in the long-term management of rheumatoid arthritis, ankylosing spondylistis, osteoarthritis and acute out, as well as mild to moderate pain and dysmenorrhea. ketoprofen has been administered orally, three or four doses per day (Cho and Choi 1998), its significant adverse effects limit the use of ketoprofen, which include gastrointestinal side effects, renal side effects. Previously, PAMAM dendrimers was attempted as a potential drug carrier to improve the solubility of the drug in water (Cheng and Xu 2005). As poor solubility is generally related to a low bioavailability, this presents a major challenge during drug formulation.

Na et al. (2006) used G5 PAMAM dendrimer to investigate the potential of PAMAM dendrimers as a carrier of hydrophobic drugs as exemplified by ketoprofen. PAMAM dendrimers possess empty internal cavities, where drug molecules can easily be encapsulated in the dendrimer interior. The special structure and high density of amino groups in PAMAM may be expected to have potential applications in enhancing the solubility of the low aqueous solubility drugs and as delivery

systems for bioactive materials. The release of ketoprofen from the drug–dendrimer complex was appreciably slower compared to pure ketoprofen. Anti-nociceptive studies using the acetic acid-induced writhing model in mice showed a prolonged pharmacodynamic behavior for the ketoprofen–G5PAMAM dendrimer complex. Maximum plasma ketoprofen concentration in ketoprofen–PAMAM dendrimer complex treated mice was attained after 1 h compared with 0.5 h for the pure ketoprofen treated ones. Higher plasma ketoprofen concentrations were observed in mice after oral treatment with ketoprofen–dendrimer complex than those treated with pure ketoprofen from 1 to 4 h during the experiment period, indicating a sustained release.

Site-specific delivery of indomethacin to the target site remains the best choice to overcome the side effects such as gastric intestinal hemorrhage, renal dysfunction. Chandrasekar et al. (2007) synthesized folate–dendrimer conjugates as suitable vehicle for site-specific delivery of anti-arthritic drug (indomethacin) to inflammatory regions. Folic acid was coupled to the surface amino groups of G4-PAMAM dendrimer (G4D) and loaded with indomethacin. The drug content and percent encapsulation efficiency increased with increasing folate content for the dendrimer conjugates. The conjugation of folate moiety resulted in 1.17–3.43 times increased indomethacin encapsulation when compared with G4D. The in vitro release rate in PBS (pH 7.4) was decreased for the folate conjugates when compared with unconjugated dendrimer (DNI). The increased folate coverage (21.38 molecules corresponding to 33.41% surface coverage) provided more shielding effect to the entrapped drug from outer aqueous environment leading to the release of only 86.27% drug in 24 h. The decrease in release rate correlated well with the increase in folate content of the conjugates. Thus, the increasing folate conjugation with G4D not only increased the encapsulation efficiency, but also serves as a controlled delivery system. Pharmacokinetic and tissue distribution studies in arthritic rats had shown preferential higher accumulation of indomethacin at the inflamed paw by the folate conjugates when compared with dendrimer at equivalent dose.

Propranolol–PAMAM dendrimer conjugate was investigated for transport across Caco-2 cell monolayers and it was observed that the conjugate could reduce the effect of P-glycoprotein on intestinal absorption of propranolol. Hence, dendrimers could bypass P-glycoprotein efflux transporter and facilitate oral administration of drugs (D'Emanuele et al. 2004). Najlah et al. (2007) investigated the oral delivery potential of prodrug of naproxen based on PAMAM dendrimers. Conjugates with lactate ester linker were more stable in plasma than diethylene glycol linker-based conjugate. The conjugate based on lactate ester linker could serve as promising candidate for controlled release.

6 Conclusion

Dendrimers offers an electrifying opportunity for scientists to fabricate macromolecular structures for drug delivery. The dendrimers are highly monodisperse macromolecular spherical structures with good control over final size. Further, the dendrimer surface can be modified with ligands for targeting therapeutics via various routes. The tailor-made surface of dendrimers provides opportunities for designing and tuning properties that are not possible with other types of nanocarriers. It was noted that dendrimers have significant potential as a versatile delivery system for small drug molecules, siRNA, gene, oligonucleotides, etc. The dendrimers can highly enhance bioavailability and therapeutic efficacies compared to plain drug and decrease the side effects of drugs. Still dendrimer applications to drug delivery are in infancy and scientists are exploring different aspects of dendrimers as drug delivery vehicle. Due to their tunable physicochemical properties, biocompatible dendrimers could represent an outstanding choice as nanocarriers for a large variety of drugs. However, the toxicity of the dendrimer nanosystems will have to be fully explored in human beings before clinical applications. Overall, dendrimer constructs appears to be highly promising as targeted drug delivery carriers and have a bright future as a new generation of drug delivery systems.

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Chitosan-Based Nanoparticulate Systems: Implication Towards Therapeutics Application

Anurag Dobhal, Prachi Bangde, Anomitra Dey, Prajakta Dandekar and Ratnesh Jain

Abstract

Recently polymeric nanoparticulate systems have gained a great impetus in the biopharmaceutical research, primarily due to their ability to enhance the pharmacokinetics and pharmacodynamics of various drugs and bioactives. Some of these polymeric systems render an improved biodegradability and biocompatible properties. There is a wide array of natural and synthetic polymers that have been utilized to develop various nanocarriers for delivery of drugs, genes, and other bioactive compounds. Polymeric nanoparticles offer moderately high encapsulation efficiency, along with enhancement of stealth properties in the circulatory system when suitably surface modified, which further ensures a sustained release of the encapsulated agent. Moreover, the polymeric nanoparticles have a mesh-like framework that can accommodate bioactive materials along with a drug moiety forming a dual delivery system, which can be further coated with another polymer giving rise to core–shell nano-conjugate system to offer better targeting of drugs/genes and bioactives. Chitosan and their chitosan derivatives have found applications in various delivery systems. Chitosan is one of the abundant biopolymers existing in nature, which has made it a polymer of choice in various drug delivery applications. This chapter focuses on the practical considerations for development of chitosan nanoparticles, along with a detailed account of various genes and bioactives that may be encapsulated or loaded on to them. Further, emphasis will be given on the physicochemical behavior of such systems and their subsequent cellular responses, besides their

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biodistribution and toxicity profiles. The discussion will also highlight the implication of polymeric nanoparticles in various therapies and limitations pertaining to them. The manuscript will also address the commercial status of polymeric nanoparticles.

Keywords
Chitosan · Nanoparticles · Drug delivery · Gene delivery and protein delivery

1 Introduction

Almost two decades polymeric nanoparticles have captivated significant interest as new delivery vectors for small molecules, DNA/genes, proteins, with adequacy to overcome poor drug solubility and cell permeability. The controlled, safe, and reproducible delivery of bioactive molecules is a critical aspect for its tissue engineering applications. In this context nanotechnology-based delivery systems enable enhanced administration of selected drugs in order to meet temporal and unique needs of target sites ex vivo, as well as in vivo and in vitro (Bachar et al. 2011; Raouane et al. 2011). Chitosan is one such biodegradable biopolymer having exceptional properties for biomedical and especially for drug delivery applications. Chitosan is one of the most widely explored biomaterials worldwide due to its abundant availability in nature, only second to cellulose (Dutta et al. 2004; Nasti et al. 2009). In recent times, this polymer has received increasing attention in medical and pharmaceutical fields (Shahbazi et al. 2008). It is a polysaccharide having a structure very similar to cellulose, obtained by deacetylation of chitin and is composed of 2-amino-2-deoxy-h-d-glucan combined with hemiacetal glycosidic linkages (Berscht et al. 1994; Dodane and Vilivalam 1998; Felt et al. 1998; Kumar 2000). Chitin is the principle component of the marine crustacean exoskeletons and is also present in some insects and cell walls of yeast and fungi (Mao et al. 2001; Nasti et al. 2009; Özbaş-Turan et al. 2002). Crustaceans contribute to a significant population of coastal organisms and their byproducts (exoskeleton) add to the marine pollution in coastal areas (Dutta et al. 2004). As chitosan is a deacetylated product of chitin, it is available as a renewable source as it is the main waste product from the marine food processing industry (Leong and Candau 1982; Maitra 1984). Large-scale production of chitosan is conducted by deacetylation of chitin (*N*-acetyl-D-glucosamine), which results in a copolymer of D-glucosamine (GlcN) and *N*-acetyl-D-glucosamine units linked together by β 1,4-glycosidic linkages. The ratio of the individual components of chitosan and hence its molecular weight and other physicochemical properties depend on the degree of *N*-deacetylation of chitin. However, completely deacetylated polymer has not yet been possible due to the complex nature of its parent polymer (Dykxhoorn et al. 2003; Luisi et al. 1988). Currently newer technologies based on enzymes and green processes have enabled

commercial synthesis of chitosan possessing a degree of deacetylation as high as 95%, as claimed by various manufacturers (Ubaidulla et al. 2009).

Chitosan is well-accepted polymer among pharmaceutical scientists due to its inherent property of being a cationic polymer that enhances its electrostatic interactions with cells. Furthermore, the polymer exhibits nontoxicity, biocompatibility, and biodegradability (Aiedeh and Taha 2001; Liu and De Yao 2002; Richardson et al. 1999; Win et al. 2003). These properties make it appropriate for various biomedical applications like drug and gene delivery and also in tissue engineering for fabricating scaffolds for cell proliferation and wound healing (Dutta et al. 2004). The potential of chitosan in drug delivery has stimulated extensive research in nanoparticulate systems of chitosan. The fabrication of chitosan-based nanoparticles requires various physicochemical considerations of the polymer. Here, the degree of deacetylation and the molecular weight of the polymer play a vital role in the formation of stable polymeric nanoparticulate systems (Jiang et al. 2007). Physiological properties of chitosan like degree of deacetylation and molecular weight can be controlled, which makes chitosan a suitable biomaterial for nano/microparticle synthesis (Kas 1997; Yao et al. 1995). Other advantages of chitosan for nanoparticle formation include the following: its ability to facilitate controlled release of active agents, ability to absorb or encapsulate wide range of bioactive molecules, solubility profile (it is soluble in mild acidic environment) that precludes the use of hazardous organic solvents while production of nanoparticles, presence of free amine groups that are readily accessible for cross-linking, cationic nature that allows for ionic cross-linking with multivalent anions, and mucoadhesive behavior, which adds to residual time at the site of absorption (Bernkop-Schnürch and Dünnhaupt 2012). Along with all these advantages, other properties like increased water solubility (derivatization by alkyl or carboxymethyl groups), increased chelating properties, enhanced bioavailability, antibacterial, and anti-fungal properties, and induction of polyampholytic properties can be improved by chemical modifications of its functional groups (–OH, $–NH_2$) (Alves and Mano 2008; Jayakumar et al. 2010; Lu et al. 2007; Muzzarelli et al. 1982).

This chapter focuses on recent expansion of chitosan-based systems for DNA, siRNA antigens, and therapeutic proteins delivery via different administration routes. Formulation aspects of chitosan delivery systems like methods of preparation of nanoparticles will be discussed; in addition to its application as a coating material for other polymeric nanoparticles and its modifications to improve the physiological properties of nanoparticles will also be discussed.

2 Properties of Chitosan the Polymer

2.1 Structure

Chitosan is a semi-crystalline copolymer of *N*-acetyl glucosamine and glucosamine. It is *N*-deacetylated product of chitin with degree deacetylation being 50% and above (Mao et al. 2001; Rudzinski and Aminabhavi 2010). The degree of

deacetylation (DA) is a vital parameter that contributes to its intrinsic properties. The structure of chitosan possesses hydroxyl and amines groups which facilitate easy modification of the polymer.

2.2 Source and Molecular Weight of Chitosan

Predominantly chitosan is obtained from the waste produced by the industrial processing of marine organism. The main donors of chitosan are shrimps, squids, crab, lobsters, etc. where the production of the polymer runs into million tons per year on an average (Thanou et al. 2002; Zhang et al. 2007c). However, chitosan produced from these sources is highly contaminated with proteins from the exoskeleton that protects the delicate organisms and is therefore closely associated with their flesh. The exoskeletons also contain a high amount of calcium that provides them with strength and rigidity. These calcium compounds also form one of the major contaminants of the polymer. Furthermore, the chitosan produced from crustaceans possesses a very high molecular weight and degree of deacetylation. Therefore, it requires extensive purification, including demineralization, deproteination, and deacetylation steps. Chitosan of this origin also possesses certain drawbacks in terms of its solubility and viscosity (Thanou et al. 2002). Thus, fungal sources are being extensively explored for producing chitosan. The fungal chitosan has many advantages such as a moderate to low molecular weight and a low degree of deacetylation. Moreover, production of fungal chitosan is possible by fermentation under a controlled environment which enables manipulation of polymer properties along with the yield (Wong et al. 2006). Another study described chitosan as the "material of choice" in the development of particulate systems due to its versatile molecular weight and degree of deacetylation (Noureddini et al. 2005; Ubaidulla et al. 2009).

2.3 Degree of Deacetylation of Chitosan

DD is a quantitative character for chitosan that determines its suitability for nanoparticulate systems. The DD is a measure of the presence of amino groups in the chitosan polymer. These amino groups provide a site of protonation that in turn makes the chitosan soluble in aqueous acidic condition. Thus the DD is relative to the degree of protonation of the chitosan polymer. Therefore, while working with chitosan polymer, calculation of precise value of DD is essential. There are various methods reported for calculating this parameter, but invariantly ^{1}H liquid state NMR is the most accurate of all (Mao et al. 2001). IR spectroscopy has also been used to determine the DD of chitosan (Mansouri et al. 2006; Pan et al. 2014; Tan and Zhang 2005). However, these calculations give an approximation of the DD value. Potentiometric titration, elemental analysis, enzymatic reaction, solid-state ^{13}C, solid-state NMR, and ^{15}N solid-state NMR are the few other methods reported for

determining DD of chitosan (Jiang et al. 2006; Liu et al. 2003; Mathew et al. 2010; Sanjai et al. 2014).

2.4 Solubility of Chitosan

The semi-crystalline nature of chitosan results in extensive hydrogen bonding within the polymer, due to which chitosan is not soluble in organic solvents but is readily soluble in dilute acid (Yoksan and Akashi 2009; Yoo et al. 2005). Numerous investigations have been dedicated toward solubility of chitosan, wherein it was observed that chitosans are soluble in the acids which possess a very low pKa value, which are mostly weak acids and their dilute solutions (Rudzinski and Aminabhavi 2010). Acetic acid is a commonly used solvent for dissolving chitosan, where the strength of the acid used can be as high as 2% (Satoh et al. 2006). Other solvents used are formic acid, lactic acid, hydrochloric acid, and nitric acid, albeit only in a few and scattered investigations (Yoo et al. 2005). The solubility of chitosan in these acids with low pKa is due to the fact that these acids partially dissociate in the solution, thus resulting in protonation of chitosan. Also at basic and neutral pH, the free amino groups form intra- and inter-hydrogen bonding. Under acidic condition they undergo protonation (Liu et al. 2003) due to which the individual chitosan chains repel each other resulting in dissociation of hydrogen bonds. This facilitates the solubility of the polymer. Besides, DD also plays a crucial role in the distribution of the acetyl group within the polymer, wherein random arrangement of acetyl groups enhances the solubility of chitosan (Hu et al. 2006).

3 Chitosan: Ideal Choice for Particulate System

The chitosan polymers possess primary amine groups, due to which the polymer possesses a cationic nature and exhibits mucoadhesive properties. This renders it useful in pharmaceutical applications, especially for delivering drugs, genes, and bioactives (Chae et al. 2005; Kim et al. 2001; Lee et al. 2007; Liu et al. 2001). Chitosan has the ability to bind to the mucosal membrane and facilitates the opening of the gap and tight junction which helps the polymeric vehicle to enter the retico-endothelial system. Further being biocompatible it possesses antibacterial properties having the ability of coagulation thus activating the immune system (Ubaidulla et al. 2009). Especially chitosan nanoparticles, being mucoadhesive, can rapidly open the tight junctions of epithelial cells (Liu et al. 2001). These properties render chitosan a suitable polymer for development of particulate systems. The degradability profile of the polymers is superior as chitosan breaks down into its monomer glucosamines, which are amino sugars that are easily assimilated into the body causing no side effects or bioaccumulation (Schmitz et al. 2007). Moreover, the LD_{50} of chitosan in mice is comparable to the LD_{50} values of salts and sugars

(Teijeiro-Osorio et al. 2009). Moreover, this polymer has been recommended as a functional food ingredient by Japan's Health Department in 1992 (Kim et al. 2003; Opanasopit et al. 2008; Rojanarata et al. 2008; Yoksan and Akashi 2009). Along with these desirable attributes, chitosan can be effortlessly sterilized by various methods without undergoing any degradation or loss of properties. The dexterity of this polymer lies in its linear polyamine chains that make it feasible to be cross-linked through ionic and electrostatic interactions. Owing to the presence of both amino and hydroxyl groups, chitosan can be easily modified by chemical functionalization with diverse functional groups (Yoksan and Akashi 2009).

3.1 Methods of Preparation of Chitosan Nano- and Microparticles

Different methods have been used to prepare chitosan-based particulate systems. The choice of the method depends upon factors such as requirement of particle size, thermal and chemical stability of the active agent to be encapsulated, reproducibility of release profiles, stability, and safety of the nanoparticles. Our subsequent discussion will focus on micro- and nanoparticulate systems of chitosan and its derivatives; we will restrict our discussions only on these aspects.

3.1.1 Emulsion Cross-Linking for Preparation of Chitosan Nanoparticles

This method utilizes the reactive functional amine group of chitosan to cross-link with reactive functional group of the cross-linking agent. The method comprises four steps. Initially, an aqueous solution of chitosan is emulsified in oil phase to form water-in-oil (w/o) emulsion (Kataoka et al. 2000; Ohya et al. 1994; Yokoyama et al. 1998). The aqueous droplets formed in the emulsion are stabilized by adding optimum amount of suitable stabilizer (Kawashima et al. 1986). Once the emulsion is stabilized, the aqueous droplets are hardened by adding appropriate cross-linking agent like glutaraldehyde, genipin, etc. Particles thus formed are further filtered and repeatedly washed with *n*-hexane, followed by ethanol, to remove free cross-linking agent and stabilizer. Finally, the particles are dried before further use (Fig. 1) (Akbuğa and Durmaz 1994; Banerjee et al. 2002; Bodnar et al. 2005; Karnchanajindanun et al. 2011).

In this method the final size of particles depends upon three factors, viz., (1) Size of aqueous droplets formed, thus on the method used to add the aqueous phase to the oil phase; (2) The amount and type of cross-linking agent used, higher the ratio of cross-linking agent to chitosan used, larger the size of the particles that is obtained; and (3) Speed and time of homogenization used during formulating the emulsion and addition of the cross-linking agent (Bodnar et al. 2006; Kumbar et al. 2002; Oliveira et al. 2005; Patil and Bhoskar 2014).

Ohya et al. (1994) reported for the first time the preparation of chitosan-gel nanospheres (average diameter 250 nm) containing 5-fluorouracil (5-FU) or immobilized 5-FU derivatives (aminopentyl-carbamoyl-5-FU or aminopentyl-estermethylene-5FU)

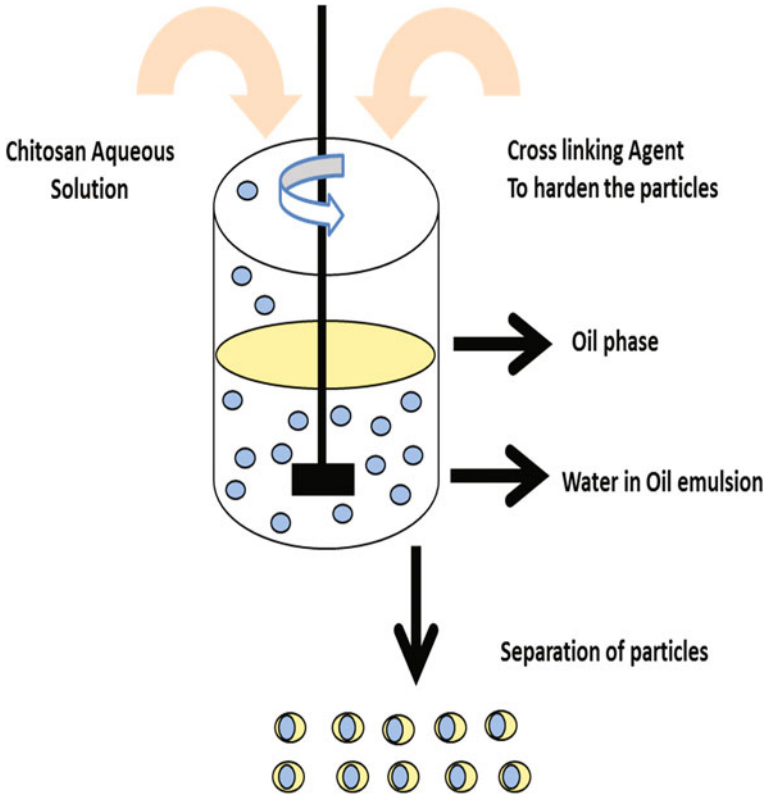

Fig. 1 Preparation of chitosan particles by emulsion cross-linking method

using w/o emulsion method, using glutaraldehyde for cross-linking the amino groups of chitosan (Ohya et al. 1994). Emulsion cross-linking method has few drawbacks as the overall process is quite tedious and involves the use of harsh chemicals in the form of cross-linkers, like sulfuric acid, which may adversely react with the polymer or active agent loaded in it. Complete removal of the cross-linking agent is also not possible in some cases (Hosseinzadeh et al. 2012; Kumbar et al. 2002).

Kumbar et al. used emulsion cross-linking method to synthesize chitosan microparticles encapsulated with diclofenac sodium, using glutaraldehyde, sulfuric acid, and heat treatment for cross-linking. The size of the microparticles ranged between 40 and 230 μm (Kumbar et al. 2002). Particles obtained by heat treatment for 6 h exhibited the lowest particle size and highest drug release profile. The results indicated that sulfuric acid-based cross-linking resulted in highest particle size, while glutaraldehyde resulted in lowest drug release. The same researchers also worked with chitosan derivatives and used polyacrylamide-g-chitosan to synthesize nifedipine-loaded microparticles, using different concentrations of glutaraldehyde as a cross-linking agent (Kumbar and Aminabhavi 2003). The microparticles showed rough surface, with a mean particle size of 450 μm.

Thanoo et al. prepared chitosan microspheres by emulsion technique, wherein chitosan solution in paraffin oil was cross-linked using glutaraldehyde and dioctyl sulfosuccinate was used as the stabilizing agent. Addition of stabilizing agent during particle formation produced microspheres with spherical geometry and smooth surface (Thanoo et al. 1992). Jameela et al. used a mixture of mineral oil/petroleum ether in the ratio of 60/40 (v/v) as the oil phase to prepare chitosan microspheres, using glutaraldehyde as the cross-linking agent and Tween-80 as the emulsifier. Smaller microspheres with narrow distributions were produced when chitosan/solvent ratio and drug/chitosan ratio were lower (Jameela et al. 1998).

3.1.2 Thermal Cross-Linking of Chitosan Particles

Orienti et al. demonstrated a novel technique to formulate chitosan microparticles, using citric acid as the cross-linking agent. In this method citric acid was added to aqueous acetic acid solution of chitosan. The chitosan cross-linked solution was cooled to 0 °C and then added to corn oil also maintained at 0 °C, under continuous stirring. This emulsion was further added to corn oil solution maintained at 120 °C and cross-linking was performed under vigorous stirring (1000 RPM) for 40 min (Orienti et al. 1996).

3.1.3 Coacervation/Precipitation for Preparation of Chitosan Nanoparticles

This method utilizes physicochemical properties of chitosan, i.e., its insolubility at alkaline pH, to precipitate/coacervate the polymer in alkaline solution. Herein, particles are produced by adding chitosan solution into alkaline solutions like sodium hydroxide, NaOH–methanol, or ethanediamine, using a compressed air nozzle, syringe pump, or manually, to form coacervate droplets (Nishimura et al. 1986). Further, the particles are separated by filtration/centrifugation, followed by successive washing with hot and cold water. Variations in compressed air pressure or spray–nozzle diameter or flow rate of syringe pump can control the size of the particles and further use a cross-linking agent to harden the particles can control the drug release. Separation and purification of particles are done by filtration/centrifugation, followed by successive washing with hot and cold water.

Recombinant human interleukin-2 (rIL-2)-loaded chitosan microparticles have been prepared by adding the rIL-2 with sodium sulfate solution in acidic chitosan solution under slow stirring. Due to the addition of the sodium sulfate solution, chitosan in the solution gets precipitated into particles with the protein (rIL-2) incorporated into them (Özbaş-Turan et al. 2002).

Another study demonstrates the preparation of chitosan–DNA complexes by coacervation technique. The study shows that at pH of 6, when the amino-to-phosphate group ratio was 3:8 and the chitosan concentration was 100 μg/mL, the particles obtained had size range of 100–250 nm and the zeta potential ranged from 112 to 118 mV. These particles were found to protect the DNA molecules from nuclease degradation, indicating coacervation as one of the prominent techniques for preparation of DNA-loaded particles (Mao et al. 2001).

3.1.4 Spray Drying

Spray drying is a one-step method to synthesize chitosan nanoparticles (Grenha et al. 2007b). The principle of the method is based on drying of atomized droplets in a stream of hot air. Chitosan is first added in aqueous acetic acid solution, then drug is dissolved or dispersed in the solution, and thereafter a suitable cross-linking agent is added. This solution or dispersion is then atomized in a stream of hot air. Atomization leads to the formation of small droplets, from which solvent evaporates instantaneously leading to the formation of free flowing particles (He et al. 1999) (Fig. 2). The particle size in this method depends on the size of nozzle, spray flow rate, atomization pressure, inlet air temperature, and extent of cross-linking (He et al. 1999).

Ganza-Gonzalez et al. have demonstrated that spray drying technique is fast, simple and reliable for formulating chitosan microspheres incorporated with metoclopramide hydrochloride, using different amounts of formaldehyde as

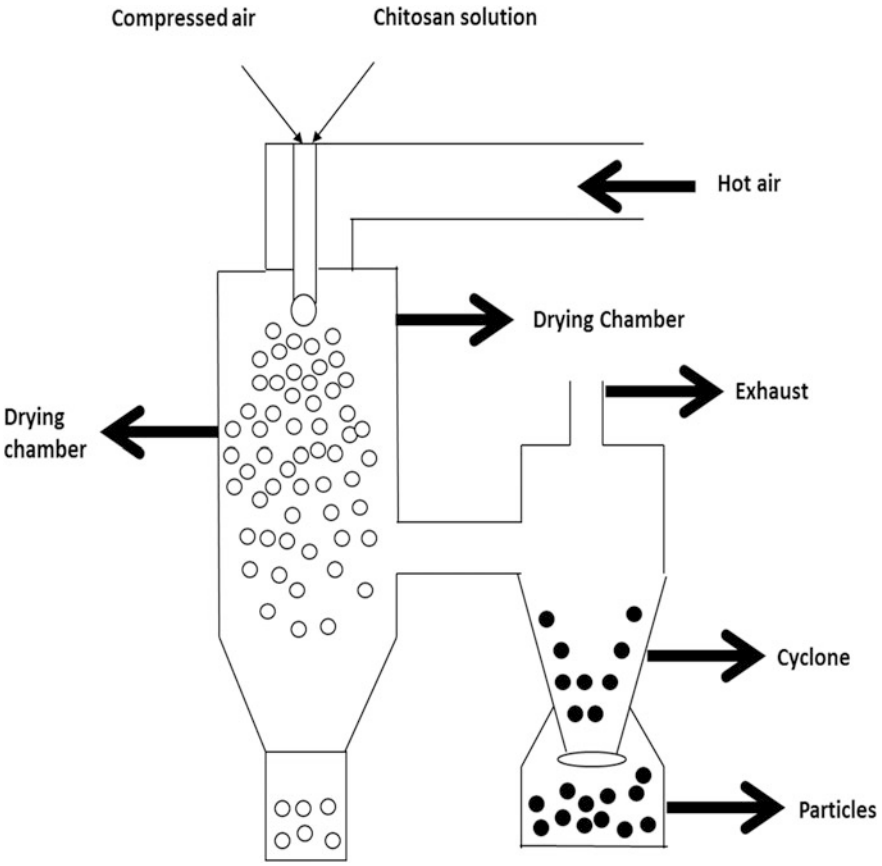

Fig. 2 Preparation of chitosan particles by spray drying technique

cross-linking agent (Ganza-Gonzalez et al. 1999). He et al. prepared both cross-linked and non-cross-linked chitosan microparticles, using spray drying, for the delivery of cimetidine, famotidine, and nizatidine. Particle size of uncross-linked particles was lying between 4 and 5 μm and that of cross-linked particles ranged from 2 to 10 μm (He et al. 1999). Conti et al. synthesized cetylpyridinium-incorporated chitosan nanoparticles, having smooth surface by this technique (Conti et al. 1998). In another studies (Shi and Tan 2002), vitamin D2 (ergocalciferol), ampicillin (Giunchedi et al. 1998), sodium diclofenac (Lorenzo-Lamosa et al. 1998), and betamethasone disodium phosphate (Huang et al. 2002)—loaded chitosan particles were prepared using spray drying technique. All the studies show the ability of the spray drying technique to produce smooth spherical microparticles (2–15 μm) having narrow size distribution.

3.1.5 Emulsion-Droplet Coalescence Method

This method was developed by Tokumitsu et al. (1999). In this method, precipitation is induced by allowing chitosan droplets to combine with NaOH droplets (Fig. 3). Gadolinium-bound chitosan nanoparticles were prepared with this technique, which involved both emulsion cross-linking and precipitation. A stable emulsion containing aqueous solution of chitosan and drug was produced in liquid paraffin oil. Simultaneously, a stable emulsion containing chitosan in aqueous solution of NaOH was produced in the same way. When both these emulsions were mixed under high-speed stirring, droplets of each emulsion collided at random, coalesced, and finally precipitated as small size particles. Nanoparticles were obtained within the emulsion droplets (Polk et al. 1994; Tokumitsu et al. 1999).

3.1.6 Ionic Gelation

This method is based on the conjugation of differentially charged macromolecules to synthesize chitosan particles (Liu et al. 1997; Polk et al. 1994). Tripolyphosphate (TPP) is generally used for cross-linking as it is a multivalent molecule that is nontoxic and is able to form gelates through ionic interaction with positively charged amino groups of chitosan (Kawashima et al. 1985). This method was first

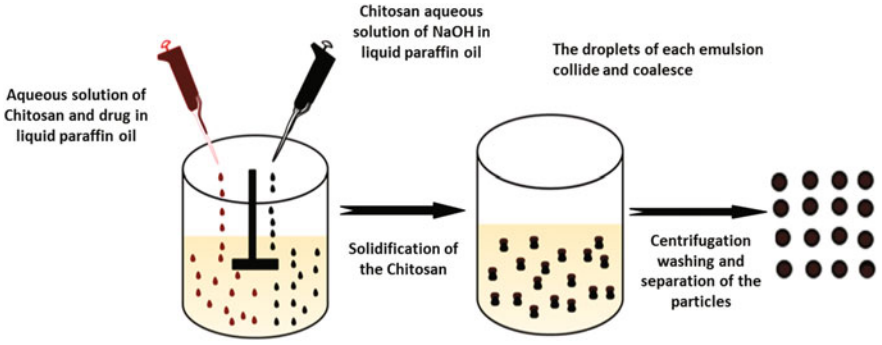

Fig. 3 Preparation of chitosan particles by droplet coalescence method

reported by Bodmeier et al. to synthesize TPP–chitosan complex by dropping chitosan droplets into TPP solution (Bodmeier et al. 1989). In this method TPP solution in water is added dropwise to the acidic solution of chitosan or vice versa, depending upon the requirement of particle size. Due to the complexation between oppositely charged species, chitosan undergoes ionic gelation and precipitates to form spherical particles (Aydin and Akbuğa 1996; Calvo et al. 1997a, b; Sezer and Akbuğa 1995; Shiraishi et al. 1993; Shu and Zhu 2000) (Fig. 4).

Fernandez-Urrusuno et al. reported the formulation of insulin-bound nanoparticles (300–400 nm) by dropwise addition of TPP–insulin solution to chitosan solution under continuous stirring (Fernandez-Urrusuno et al. 1999). Xu and Du demonstrated synthesis of chitosan particles of having diameter in the range of 20–200 nm (Xu and Du 2003). FTIR studies of the particles confirmed that tripolyphosphoric groups of TPP are associated with ammonium groups of chitosan. Ko et al. prepared felodipine-loaded chitosan particles by ionic gelation method, having particle size range between 500 and 710 μm (Ko et al. 2002).

3.1.7 Reverse Micellar Method

Reverse micelles are thermodynamically stable liquid mixtures of water, oil, and surfactant. Macroscopically, they are homogeneous and isotropic mixtures, structured on a microscopic scale into aqueous and oil micro-domains separated by surfactant-rich films (Chattopadhyay et al. 2002). Size of reverse micelles generally lies between 1 and 10 nm and these are highly mono-disperse systems; the method is thus the most suitable to formulate polymeric nanoparticles with narrow size distribution (Leong and Candau 1982; Maitra 1984). To form chitosan particles first

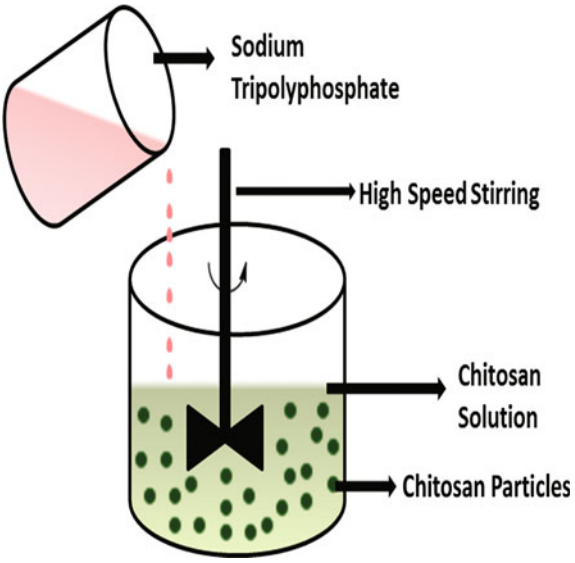

Fig. 4 Preparation of chitosan particles by ionic gelation method

surfactant is added in organic phase to form reverse micelles and then aqueous phase containing chitosan and drug is dissolved or dispersed in it under continuous stirrings. The amount and concentration of aqueous phase is coordinated in such a way as to keep the entire mixture in an optically transparent micro-emulsion phase. Finally, a cross-linking agent is added to the obtained transparent solution under constant stirring. The size of particles obtained by this method is dependent on the amount of aqueous phase and cross-linking agent; the higher the aqueous phase and cross-linking agent, the larger the particles (Ubaidulla et al. 2009). Later organic phase is evaporated to achieve dry mass of particles, which is further resuspended in water and centrifuged and washed to remove excess surfactant and cross-linking agent. The final resuspension is freeze dried before further use (Fig. 5). Mitra et al. demonstrated the preparation of doxorubicin–dextran encapsulated nanoparticles (diameter: 100 ± 10 nm) by reverse micellar method (Mitra et al. 2001). Tang et al. 2007 demonstrated this method for proteinase immobilization on nanoparticles having a diameter of 45 ± 5 nm (Tang et al. 2007).

Preparation of ultrafine chitosan nanoparticles with narrow size distribution could be achieved by using reverse micellar method (Leong and Candau 1982). The aqueous core of the reverse micellar droplets is used as nanoreactors to prepare such particles (Maitra 1984). Since micellar droplets are in Brownian motion, they undergo continuous coalescence followed by re-separation on a timescale that varies between millisecond and microsecond. The size, polydispersity, and thermodynamic stability of these droplets are maintained in the system by a rapid dynamic equilibrium (Luisi et al. 1988).

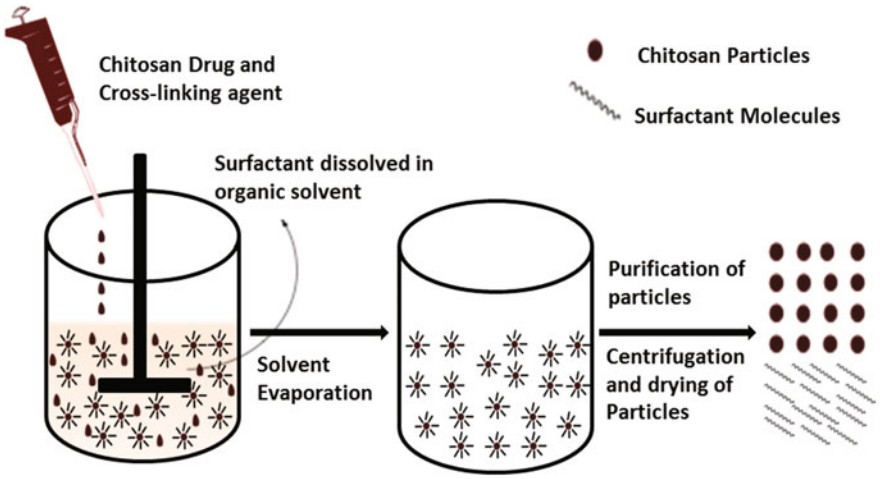

Fig. 5 Preparation of chitosan particles by reverse micellar method

3.1.8 Sieving Method

This method was developed by Agnihotri and Aminabhavi to produce clozapine-loaded microparticles. At first thick jelly mass of chitosan is prepared by dissolving suitable amount of chitosan in acetic acid solution, followed by addition of glutaraldehyde as cross-linking agent. In this method, chitosan concentration has to be optimized such that it does not form a sticky cross-linked mass. This mass is then passed through a sieve having suitable mesh size to obtain microparticles. The particles are further washed with NaOH solution to remove excess glutaraldehyde. The dried particles of clozapine synthesized by this method possessed entrapment efficiency up to 98.9% and diameter in the range of 543–698 μm (Agnihotri and Aminabhavi 2004).

3.2 Derivatives of Chitosan Used for Particulate Systems

Although native chitosan alone has exhibited advantages over existing polymers, these properties can be further enhanced by functionalization to afford targeting ability to chitosan particles. The applications of chitosan can be expanded through its chemical modification with varying functional groups to result in different chitosan "personalities" (Table 1). The presence of –OH and $–NH_2$ groups in chitosan can be easily targeted to functionalize the polymer chemical means or graft copolymerization. Thus resulting polymer possess properties such as increasing chelating or complexation properties or bacteriostatic effect or enhancing adsorption properties (Chen et al. 2013). Many chitosan derivatized particulate systems are described in the literature which are known specifically to increase the efficiency of drug carriers; a generalized particulate system with different properties is depicted in Fig. 6. Chitosan can be modified in such a way in order to increase its water solubility, mucoadhesive properties, permeation effect, transfection efficiency, less cell toxicity, and facilitating targeted delivery with diagnosis.

3.2.1 Improved Water Solubility Due to *N*-Alkylation/Improved Cationic Properties

Chitosan is known to be soluble only below pH 6.4, due to protonation of amine groups present on the D-glucosamine moieties. Thus numerous literature reports suggest superior ability of quaternized chitosan to be able to conserve its positive charge at a higher pH, thus significantly increasing its aqueous solubility. This observation is due to the fact that amine group of chitosan acquires a permanent positive charge, thereby decreasing its intra- and inter-hydrogen bonding, which exposes its functional groups to aqueous environment to improve its solubility at higher pH. The increased solubility is due to the presence of permanent positive charge, which breaks the crystallinity of chitosan and hence exposes chitosan chains to the aqueous environment. This property affects solubility of chitosan in

Table 1 Examples of chitosan derivatives mediated by DNA/siRNA delivery

Derivative	Type of modification	Reason for modification	References
Arginine–chitosan	Hydrophilic modification	Improve water solubility of chitosan and enhance its gene transfection efficiency	Liu et al. (2003)
Polyethyleneglycol (Chan et al.)–Chitosan		Improve stability of chitosan, reduce the opsonisation, enhances the plasma circulation time and prolong the gene transfer	Jiang et al. (2006), Zhang et al. (2007c)
Quaternized chitosan (Trimethyl chitosan (TMC))		Makes the chitosan over a wide pH range, increases its transfection efficiency	Thanou et al. (2002)
Polyethyleneimine (PEI)–Chitosan		Enhance transfection efficiency with low cytotoxicity owing to induction of proton sponge effect by PEI	Thanou et al. (2002), Wong et al. (2006)
Chitosan-g-L-phenylalanine		A higher degree of swelling and faster degradability	Yoksan and Akashi (2009)
Glycol chitosan		Facilitate endocytic uptake of the complex and effectively deliver DNA to the cells in the presence of serum, enhancing transfection efficiencies in vitro as well as in vivo	Yoo et al. (2005)
6-amino-6-deoxy-chitosan		Soluble in water at neutral pH, the transfection efficiency was superior to chitosan	Satoh et al. (2006)
5b-cholanic acid-modified glycol chitosan (CGC)	Hydrophobic modification	Decreased particle size and improved stability against serum facilitate endocytic uptake	Yoo et al. (2005)
Stearic acid–chitosan		Increase of escape of complexes from endosome	Hu et al. (2006)
Alkylated chitosan		Easier unpacking of DNA from carriers, enhance perturbation of the model membrane system and increase the entry into cells	Liu et al. (2003)
Deoxycholic acid-modified chitosan		Increase of cell membrane–carrier interactions and/or destabilization of cell membrane	Chae et al. (2005), Kim et al. (2001)

(continued)

Table 1 (continued)

Derivative	Type of modification	Reason for modification	References
Thiolate chitosan		Possess enhanced mucoadhesiveness and cell penetration properties	(Lee et al. 2007)
N-dodecylated chitosan (NDC)		Enhanced the thermal stability of DNA	Liu et al. (2001)
Chitosan–thiobutylamidine		Introduce the property of extracellular stability and intracellular pDNA release by forming reversible disulfide bonds	Schmitz et al. (2007)
Hybrid chitosan/CD		Promote cellular uptake, and decrease the cytotoxicity of the systems	Teijeiro-Osorio et al. (2009)
Urocanic acid-modified chitosan		Enhance endosomal rupture through a proton sponge mechanism	Kim et al. (2003)
Quaternized *N*-(4-*N*,*N*-dimethylaminobenzyl) chitosan		To provide the hydrophobic moiety for the improved hydrophobic interaction with pDNA, and quaternized to render chitosan soluble	Rojanarata et al. (2008)
N-(4-pyridinylmethyl) chitosans		Produce hydrophobicity for improved hydrophobic interaction with pDNA	Opanasopit et al. (2008)
Galactosylated chitosan	Ligand modification	To induce the receptor-mediated endocytosis to bind asialoglycoproteins (ASGP) for hepatocyte targeting An efficient transfection by bearing a galactose group for liver specificity and by grafting dextran for stability in water	Park et al. (2000)
Galactosylated chitosan-graft-poly (ethylene glycol)		For hepatocyte targeting with improved solubility	Park et al. (2001)
Galactosylated chitosan (Sun et al.)-graft–poly (vinyl pyrrolidone)		An efficient transfection by bearing a galactose group for liver specificity and by grafting PVP for stability in water	Park et al. (2003)
KNOB protein (C-terminal globular domain of the fiber protein)		Enhance the transfection efficiency through the specific receptor-mediated endocytosis mechanism	Mao et al. (2001)

(continued)

Table 1 (continued)

Derivative	Type of modification	Reason for modification	References
Folate–chitosan		A ligand for targeting cell membrane and allows nanoparticles endocytosis via the folate receptor (FR) for higher transfection yields	Chan et al. (2007), Mansouri et al. (2006)
Trimethyl chitosan-g-poly (*N*-isopropyl-acrylamide)	Thermosensitive modification	A thermoresponsive copolymer having zeta potential and DNA affinity adjustable by controlling the solution temperature below or above the LCST (lower critical solution temperature)	Mao et al. (2007)
N-isopropylacrylamide/vinyl laurate (NIPAAm/VL) copolymer with chitosan (PNVLCS)		The transfection efficiency of PNVLCS/DNA complexes was improved by dissociation of the gene from the carrier by temporarily reducing the culture temperature to 20 °C	Sun et al. (2005)

physiological solution. The positive groups of chitosan interact with cells, when administered in humans. In addition, this cationic nature of quaternized NPs has added advantage to encapsulate anionic therapeutic molecules and provide protection against enzymatic attacks. The cationic nature of quaternized chitosan also improves its mucoadhesive property.

Trimethyl chitosan (TMC) a methylated derivative has been studied as a vehicle for nasal delivery of proteins. TMC NPs of size range 360–480 nm were prepared and loaded with model protein albumin. TMC NPs exhibited excellent loading efficiency of 79.8% due to their cationic nature which enabled loading of negatively charged protein on their surface (Amidi et al. 2006). Further another study states the use of TMC NPs loaded with monovalent influenza A subunit H3N2 of particles of size of 800 nm and positive surface charge were studied for treating influenza by nasal delivery loading efficiency as 78% (Amidi et al. 2007b). Different types of alkyl group attached to amine group are also known to affect the efficiency of NPs. Few reports have suggested that smaller alkyl groups showed more effective mucoadhesion than the bulky substituents present on amine group of chitosan. Various quaternized chitosan derivatives, such as dimethylethyl chitosan (DMEC), diethylmethyl chitosan (DEMC) (Sadeghi et al. 2008a) and triethyl chitosan (TEC), have been studied to evaluate the effect of length of alkyl groups attached to amine

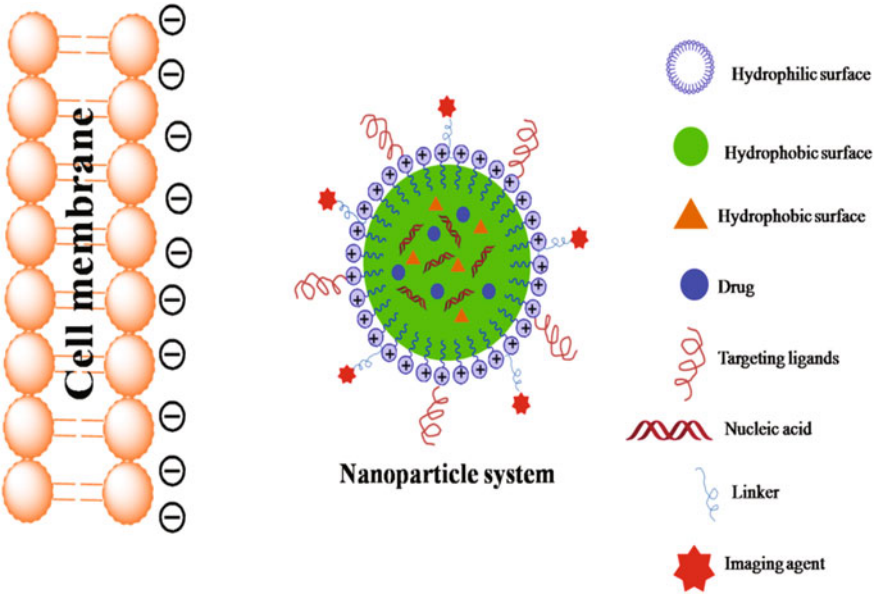

Fig. 6 Generalised chitosan-based polymeric system with different functions

groups (Sadeghi et al. 2008b). Here, the availability of positive charge was observed to decrease when the alkyl chain length increased; hence the trend of effective behavior of quaternized NPs observed was TMC >DMEC >DEMC> TEC= >CS (Sadeghi et al. 2008a).

Another examples of water-soluble chitosan are carboxymethyl chitosan, *O*-carboxymethyl chitosan (O-CMC), and *N*,*O*-carboxymethyl chitosan (N,O-CMC), which have been studied for cytotoxicity. NPs formulated from these polymers were in the range of 80–100 nm. Almost complete viability was observed in MCF-7 cell line (Anitha et al. 2009). Another study demonstrated by Shi et al. showed that increase in molecular weight and degree of substitution of carboxymethyl chitosan enhanced its drug encapsulation properties, as long as polyionic chain can easily fold up to encapsulate ionic drug compared to short polymer chain (Shi et al. 2006). Apart from attachment methyl groups, different lengths of alkyl groups can be attached to the chitosan polymer yielding modified polymers like triethyl, dimethylethyl chitosan, etc. These polymers are quaternized chitosan possessing a net positive charge polymer chain having size in the range of 170–270 nm. These quaternized polymers have displayed greater transport of drug through colon membrane and higher absorption efficiency (Bayat et al. 2008). Another polymer derivative chitosan succinate shows hydrophilic characters which have been successfully demonstrated to deliver drugs like insulin, diclofenac sodium, etc. in basic environment of intestine (Aiedeh et al. 2005; Ubaidulla et al. 2009). The possible hydrophilicity of chitosan particles at higher pH is attributed to the generation of deprotonated carboxylic moieties yielding carboxylate anions (Aiedeh and Taha

2001). Phosphorylated chitosan is yet another derivative of chitosan which have potential importance for targeted drug delivery in gastrointestinal fluid owing to its higher water-soluble properties. Phosphorylated chitosan nanocarriers were tested for in vitro release of model drug ibuprofen at various pH media. The release profile indicated increase in drug release with increase in pH; the behavior is attributed to the ionization of phosphorous groups of chitosan along with repulsive force acting between phosphate groups in phosphorylated chitosan and negatively ionized carboxyl groups of ibuprofen (Win et al. 2003).

3.2.2 Amphiphilic Chitosan Derivatives

Chitosan, being a biocompatible cationic polymer, is known to deliver anionic molecules. In order to widen scope of its applications, hydrophobic chitosan exhibiting amphiphilic nature has been synthesized (Aranaz et al. 2010). Molecular design of chitosan was modified by the introduction of hydrophobic groups like succinyl, oleoyl chloride, deoxycholic acid, linoleic acid, stearic acid, etc., all of which imparted amphiphilic characteristics to chitosan (Aranaz et al. 2010; Yang et al. 2014). It has been reported that chemical modification with hydrophobic groups can help in formation of micellar structures, with hydrophobic inner core and the hydrophilic outer shell in aqueous media. The hydrophobic inner core is surrounded by a hydrophilic outer shell that provides a stabilizing interface between the micelle core and the aqueous environment (Thurmond et al. 1996). The micellar formation ability of amphiphilic chitosan is a spontaneous process which is easy and cost-effective method to synthesize nanosized particles with functional geometries and properties (Yang et al. 2014).

Thus this nature of self-forming amphiphilic chitosan NPs can be explored to effectively load hydrophobic moieties with higher drug payload than unmodified chitosan nanoparticles, in addition to improved mucoadhesive properties. The combination of hydrophobic and hydrophilic character on chitosan induces the formation of nanosized particles above critical micellar concentration (CMC). This observation was reported in *N*-phosphorylcholine chitosan which displayed low toxicity against NIH/3T3 cells, indicating its safety for biomedical applications (Zeng et al. 2012). Another safe amphiphilic derivative, namely *N*-octyl-*O*-glycol chitosan (OGC), was studied for delivery of water-insoluble anticancer drug paclitaxel (Huo et al. 2010). Derivative of *N*-octyl-*N*-trimethyl chitosan (OTMCS) demonstrated encapsulation and controlled release of hydrophobic drug 10-hydroxycamptothecin (10-HCPT) (Zhang et al. 2007a). Amphiphilic NPs *N*-mPEG-*N*-octyl-*O*-sulfate chitosan (mPEGOSC) was studied for delivery of paclitaxel drug (Yao et al. 2007).

A pH-dependent insulin release was observed for PEG-grafted chitosan nanoparticles (Mao et al. 2005a). Similar effect was observed for pH-responsive dextran sulfate–chitosan derivative loaded with insulin (Sarmento et al. 2006). It is important to note that addition of molecules on hydroxyl groups has been found to maintain the cationic nature of amino units of chitosan for various functions like mucoadhesion, permeation, and transfection. Such derivatives can be synthesized by protection group strategies to preserve amine groups of chitosan as reported by Li

et al. They have synthesized amphiphilic chitosan by reaction of poly (L-lactic acid) (PLLA) on hydroxyl groups on phthaloyl chitosan (PHCS) and later deprotection of *N*-phthaloyl groups was done using hydrazine to yield back active amine groups (Li et al. 2008). This derivative has been studied to encapsulate anti-HIV drug lamivudine which showed pH-dependent controlled release of drug (Dev et al. 2010).

3.2.3 Improved Mucoadhesive and Permeation Properties

Epithelial lining of mucosal tissue is protected by gel layer called as mucus, which is primarily composed of mucin. The glycoprotein mucin possesses functional groups that serve as sites of interaction for mucoadhesion. These sites include carboxylate group (of sialic acid) and sulfate group (of *N*-acetyl glucosamine-6-sulfate) which is responsible for imparting negative charge and hence results in electrostatic interaction with positively charged moieties promoting mucoadhesion. Although chitosan nanoparticles (CS-NPs) have been reported to exhibit enhanced adhesion and permeation (Pan et al. 2002; Zhang et al. 2004), these mucoadhesive interactions are weak and short lasting. However, the interactions can be fortified by the presence of permanent charge or other groups like thiol. Thiolated chitosans display higher interaction due to the formation of inter- and intramolecular disulfide bonds resulting from oxidation of thiol groups at physiological pH. Numerous thiolated chitosan derivatives have been reported to have enhanced properties including CS–thioglycolic acid (CS–TGA) (Kast and Bernkop-Schnürch 2001), CS–cysteine (CS–Cys) (Wang et al. 2009), CS–glutathione, and CS–thioethylamidine (CS–TEA) (Kafedjiiski et al. 2005), chitosan-2-iminothiolane (Andreas et al. 2003), etc. The enhanced mucoadhesion of thiolated chitosans may be attributed to the formation of disulfide bonds between thiol groups of chitosan and the cysteine present in glycoprotein of mucus layer, which results in stronger interaction than the usual ionic bonding between unmodified chitosan and anionic mucosal moieties (Di Colo et al. 2008).

Further to enhance mucoadhesive nature of chitosan, quaternized chitosan can be modified with additional moieties thus to imparting extra functionalities to the polymer. One such example is denoted by enhanced adhesion, and permeation effects of TMC were combined in a conjugate of TMC with cysteine (TMC-Cys). This complex displayed enhanced effects over TMC NPs owing to disulfide bond formation between TMC-Cys and mucin, which was confirmed through DSC studies (Anitha et al. 2011; Yin et al. 2009). The presence of thiol groups on the nanoparticle surface at high concentration demonstrated enhanced mucoadhesion of nanoparticles by forming covalent bonds with the cysteine residues of mucus (Bravo-Osuna et al. 2007).

3.2.4 Increased Transfection Efficiency

Delivery of naked gene possesses serious limitations to be used as therapeutics agent. Injection of exogenous nucleic acid undergoes rapid degradation by attack nucleases present in plasma, in the absence of any shield around naked gene (Mansouri et al. 2004). Therefore, to successfully cross multiple blockages exogenous nucleic acid must be protected from the external factors. Further, gene

being electronegative in nature tends to inhibit itself from entering most negatively charged cell membranes (Liu and De Yao 2002). At the cellular level, the nano-carriers are intended to deliver the nucleic payload to the cytoplasmic compartment of the cell through endocytosis of the aggregated nucleic-loaded nanocomplex outside cell. This is subsequently transferred across the membrane into the cytosol, where endosome swells due to swelling of DNA-loaded complex resulting in rupture of endosome. Finally, the nanoconstructs must liberate nucleic material in order to integrate with the host cell DNA (Richardson et al. 1999; Rudzinski and Aminabhavi 2010).

Nucleic acids, being negatively charged, do not easily traverse across the negatively charged cell membranes. In this regards, cationic biopolymers like chitosan have demonstrated superior ability to transfect nucleic acids owing to their inherent surface properties as well as their versatility to be modified suitably. Polycationic chitosan has been used as a nonviral delivery carrier to effectively interact with polyanionic DNA resulting in spontaneous formation of complexes that protect the DNA from nuclease degradation. At the cellular level, these nanocarriers are intended to deliver the nucleic acid payload to the cytoplasmic compartment of the cell through pinocytic capture of the nucleic acid-loaded noncomplexes. These are then transferred across the cell membrane into the cytosol. Finally, the nanoconstructs release the loaded DNA to integrate with the host cell DNA (Richardson et al. 1999). Richardson et al. investigated the effect of molecular weight of chitosan on their ability for intracellular DNA delivery. The researchers observed that the toxicity of polymers increased with increasing molecular weight; thus low molecular weight chitosan was found to be nontoxic against L132 human embryonic lung cells. The researchers also demonstrated that significant decrease in degradation by DNase II. The possible reason proposed for this decrease DNase II degradation was due to interaction-induced changes in DNA tertiary structure causing steric hindrance which protects the DNA from the enzymatic degradation (Richardson et al. 1999).

Transfection efficiency of chitosan-based NPs is found to be low. Therefore, studies emerged which intended to design copolymer system to enhance transfection efficiency. Chitosan–graft–polyethylenimine (C–g–PEI) demonstrated higher transfection efficiency than PEI alone in HeLa, 293T, and HepG2 cell lines (Jiang et al. 2007). In view of the limited transfection capability of naked chitosan, work has also focused on modifying the chitosan structure to improve transfection efficiency. To achieve higher transfection efficiency use of ligands like monoclonal antibodies, peptides, and sugars conjugated with NPs results in targeted delivery to specific cell surface receptors (Rudzinski and Aminabhavi 2010). One such approach demonstrates the use of transferrin and KNOB protein in conjugation with DNA-loaded chitosan NPs. This NP system displayed improved transfection efficiency fourfold increase in HEK293 and HeLa cells when in conjugation with transferrin, while KNOB-conjugated NPs improved gene expression level in HeLa cells by 130-fold (Mao et al. 2001). Another appealing ligand folic acid was investigated for targeted gene delivery through chitosan NPs (Mansouri et al. 2006).

3.2.5 Biomedical Imaging

Bioimaging enables a noninvasive assessment of biochemical processes inside living cells. This technology has immense potential to increase our understanding of cellular functioning defects and understand drug delivery mechanism. Therefore, biopolymers like chitosan have garnered attention for bioimaging technology owing to its biocompatibility and less toxicity toward mammalian cells. When chitosan conjugated with different purpose moieties results in new specific functional complexes, which possess specific functional, physiochemical, and targeted delivery properties, the conventional optical imaging agents like nuclear agents, organic fluorophores, microbubbles, gadolinium (Gd) and superparamagnetic iron oxide, etc. possess many limitations. The major drawbacks are rapid degradation of agents, sensitive to local environment (e.g., pH, ions, temperature, etc.) of cells, less contrast due to auto-fluorescence from tissues. To exploit the properties of hydrophilic chitosan polymer as bioimaging agents, chitosans have been successfully derivatized by the incorporation of imaging agents such as iron, Gd be used for bioimaging.

Photoluminescence property of chitosan polymer due to its functional groups was studied by Pan et al. (2014). The study was conducted on the basis that amine group of chitosan polymer is converted to carbamato anion (fluorophore) by reaction of amine groups with CO_2 which exhibited photoluminescence property. The formation of carbamato species was confirmed by UV studies which reflected maximum emission from excitation at 336 nm. The CO_2-treated chitosan was successfully demonstrated as an imaging probe for MCF-7 cell at two excitation wavelengths of 405 and 473 nm, resulted in blue emission and bright green emission under confocal microscopy (Pan et al. 2014).

Quantum dots (QDs) are highly luminescent semiconductor, which exhibits a wide range of size-tunable, different color dots, which can be activated using a single laser source. To increase QDs applicability in in vivo cell imaging, chitosan nanostructure were prepared to encapsulate QDs and paramagnetic gadolinium diethylene triamine pentaacetate (Gd-DTPA). These characters produced multifunctional biomarkers (fluorescent and paramagnetic) which can function both as fluorescent marker cell studies as well as contrast agents for magnetic resonance imaging (MRI). The prepared nanobeads displayed an intense narrow emission peak at 567 nm, suggesting its application in biomedical field (Tan and Zhang 2005).

Superparamagnetic iron oxide coated with chitosan (SPIONPs–CS) nanoparticles were prepared in order to overcome drawbacks possessed by pure iron oxide NPs which tends to aggregation in contact with water or tissue fluid, hence limits its applicability magnetic-based isolation and detection strategies. This complex was demonstrated for potential MR contrast agents in tissue environments in the human body (Sanjai et al. 2014).

Further, to combine properties of fluorescence with targeted DNA, delivery was achieved with lanthanide-Fe_3O_4-doped chitosan nanospheres (Fe_3O_4-LDCNs), which successfully resulted in gene delivery and transfer efficiency assessment simultaneously (Wang et al. 2012). Another, nanosystems with similar function are using chemically modified chitosan derivative as base polymer was studied.

Carboxymethyl chitosan (CMCS) conjugated with folic acid doped with zinc sulfide (ZnS:Mn) quantum dot (FA-CMCS-ZnS:Mn) NPs was prepared. The NPs were used for targeted drug delivery of anticancer drug 5-Fluorouracil against breast cancer MCF-7 cells. Due to quantum dots the NPs showed bright luminescence image of the drug carrier in cancer cells (Mathew et al. 2010).

4 Chitosan-Based Particles for Delivery of Biomolecules

4.1 DNA

In recent decades, gene delivery-related research has rapidly grown due to its potential as a future therapeutic strategy to treat various inheritable or acquired genetic diseases by replacing defective genes, inserting new functional genes which are missing from the body or silencing any unwanted gene expression. Due to the very sensitive nature of the nucleic acid chains toward environmental factors (Kim et al. 2007), development of efficient and safe carriers for genes and siRNA is an essential prerequisite for successful gene therapy (Rolland 2005). At present, majority of the gene delivery strategies use either viral or nonviral vectors. Although viral vectors are very efficient for in vivo gene transfection and immunization, they possess numerous drawbacks including complicated development techniques, immunogenicity, inflammatory potential, and insertional mutagenesis (Glover et al. 2005; Lim et al. 2006). Nonviral vectors have attracted increasing attention due to many advantages such as ease of synthesis, low immune response, and potential benefits in terms of safety and efficacy (Remaut et al. 2007). Nonviral vectors include liposomes, complexes of negatively charged plasmids with cationic polymers, and polymeric/lipidic nanoparticles (Ishida et al. 2005). Here, chitosan with its cationic amine has been seen as an attractive option for efficient DNA packaging. Furthermore, the polymer has well-defined physiochemical properties and high molecular diversity, both of which render this material as an ideal carrier for gene and siRNA delivery (Li and Huang 2000) (Fig. 7).

Chitosan has positive charge on its backbone and forms polyelectrolyte complexes with negatively charged nucleotides via electrostatic interactions. (Lee et al. 2005; Shu and Zhu 2002). When the pH values of the medium are slightly acidic, interaction between negatively charged DNA/siRNA and positively charged chitosan backbone leads to spontaneous formation of nanosized complexes (polyplexes) in aqueous medium (Lee et al. 2005). However, gel electrophoresis studies have shown that when the external conditions are neutral or alkaline, chitosan has only a slight positive charge and secondary nonelectrostatic interactions, such as hydrogen bonding and hydrophobic interactions may govern the binding between chitosan and DNA (Messai et al. 2005).

At sufficient nitrogen to phosphate ratio [ratio between chitosan nitrogen (N) per DNA phosphate (P)] (N:P) chitosan can efficiently condense the/siRNA into nanocomplexes suitable for efficient cellular uptake, simultaneously preventing

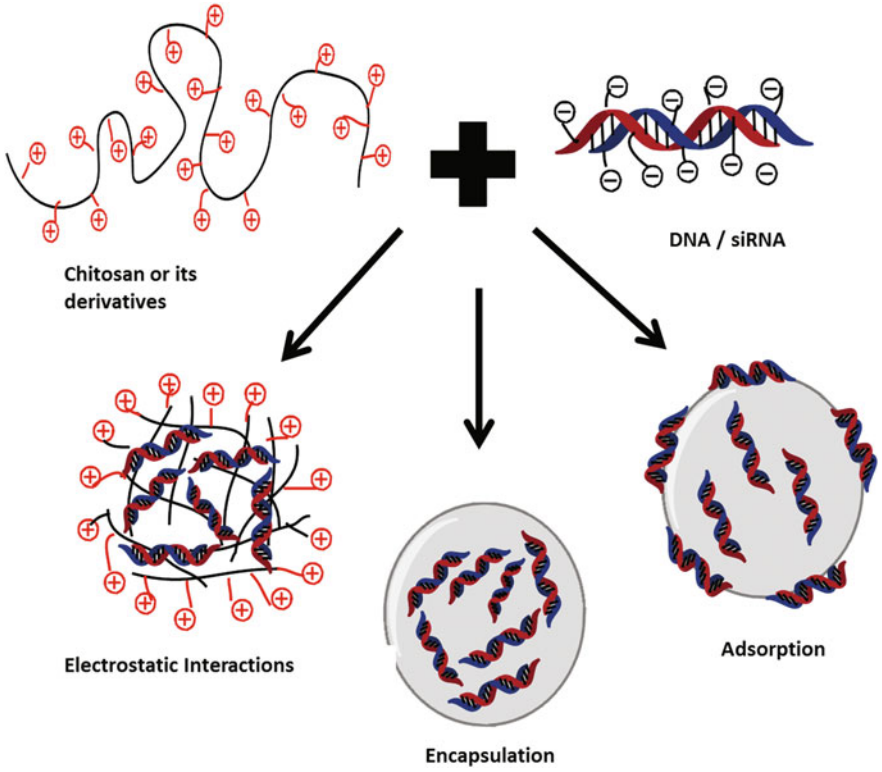

Fig. 7 Mechanism of DNA/siRNA complexes with chitosan particles

them from nuclease-mediated degradation by offering steric protection. However, it should be noted that very strong electrostatic interactions between chitosan and DNA can hamper the release of nucleic acid at the site of action. Therefore, formulation parameters need to be adjusted to achieve optimum electrostatic interaction between chitosan and DNA (Huang et al. 2005).

4.1.1 Factors Affecting DNA Delivery via Chitosan Particulate Systems

Effect of Molecular Weight of Chitosan on Chitosan–DNA Complexes

Studies show that there is decrease in the size of complex as the molecular weight of chitosan decreases (MacLaughlin et al. 1998). Huang et al. (2005) demonstrated that particle size decreased from 181 to 155 nm when Mw was decreased from 213 to 48 kDa. However, further reduction in the molecular weight reversed the trend, as particle sizes of 269 and 289 nm were observed when chitosans of 17 and 10 kDa were used. It has been found that different cell lines have different transfections and uptake behaviors for different molecular weights of chitosan (Ishii et al. 2001; Weecharangsan et al. 2008). Ishii et al. demonstrated the correlation between

gene expression and cellular uptake by using chitosan of different molecular weights (40, 84, 100 kDa) complexed with pGL3-Luc plasmid in SOJ cells (Ishii et al. 2001). Chitosan of molecular weights 20, 40, and 300 kDa has effective transfection in CHO-K1 cell lines (Weecharangsan et al. 2008). In A549 cells, B16 melanoma cells, and HeLa cells, 15 and 25 kDa chitosan promoted luciferase activity. However, when 100 kDa chitosan is used the transfection efficiency is less in all these cell lines (Sato et al. 2001).

Studies explain that appropriate balance needs to be maintained between protection and release of DNA for biological functions, as high molecular weight chitosan is superior compared to low molecular weight chitosan to protect or enhance the stability of DNA, whereas low molecular weight chitosan mediates higher and faster cellular transfection (Köping-Höggård et al. 2001, 2004; MacLaughlin et al. 1998).

Degree of Chitosan Deacetylation

Koping-Hoggard et al. have proved that DD of chitosan must exceed 65% to form stable complexes with pDNA (Köpping-Höggård et al. 2001). Studies show that DD affects DNA binding and gene transfection efficiency in vitro and in vivo. A decrease in DD was reported to decrease the overall in vitro gene expression levels of luciferase transgene in HEK293, HeLa, and SW756 due to instability of particles in serum proteins. However, this instability and increased chitosan degradation resulted in higher luciferase transgenic expression levels in tibialis muscle muscles of female Balb/c mice (Kiang et al. 2004). It was further reported that maximum expression levels were obtained by lowering molecular weight (MW) and increasing DD or either increasing molecular weight and lowering DD. In the study wide range of chitosan (varying in respect to MW and DD) were tested in human embryonic kidney, HEK 293 cell lines for gene transfer. Results indicate a predominant role of particle stability, through cooperative electrostatic binding, in determining efficiency of the chitosan–DNA complex (Lavertu et al. 2006).

N/P Ratio

The N/P ratio is defined as the ratio between chitosan nitrogen (N) per DNA phosphate (P). The surface charge of chitosan/ DNA polyplexes has have been observed to depend on the molar stoichiometry of DNA to chitosan (N/P ratio), which finally influences the transfection ability of the particles (Huang et al. 2005; Nafee et al. 2007). It was recently observed that cationic particles can interact with anionic microtubles and molecular motor proteins and move to nuclear membrane via cytoskeletal network, promoting cytoplasmic trafficking (Jeong et al. 2007). Moreover, increase in N:P implies an increase in chitosan concentration in polyplexes, which leads to higher osmotic pressure in the endosomes and thus the efficiency of plasmid release may increase (Köpping-Höggård et al. 2001). On the contrary polyplexes with neutral surface charge tends to aggregate in cytoplasm due to lack of inter-particulate repulsive forces (De Smedt et al. 2000). Studies state the N:P should be optimized for individual chitosan or its derivative since too low N:P can lead to physically unstable complexes and poor transfection, while too high N:P

results in stable complexes having poor transfection ability (Köping-Höggård et al. 2001, 2003; Sato et al. 2001).

In an another study it was reported that transfection efficiency of chitosan/DNA complexes in SOJ cells increased at charge ratios of 3 and 5 but decreased when the ratio was further increased. The results were explained as, to get the maximum transfection there must be optimum N/P ratio (3–5), whereas increasing the ratio further (7–12) leads to aggregation of the chitosan–DNA complexes resulting in decrease of their transfection (Ishii et al. 2001). Sato et al. demonstrated that pGL3/chitosan complexes at the N:P ratio of 1:5 resulted in highest luciferase activity in human lung carcinoma A549 cells (Sato et al. 2001). Different studies have shown that morphology of chitosan/DNA complexes is also dependent on N:P ratio. DNA–chitosan complexes of 150–600 nm size were found to have spherical, annular, globular, or toroidal shapes, depending upon charge ratios (Erbacher et al. 1998; Köping-Höggård et al. 2003; Liu et al. 2005). It has also been observed that high molecular weight chitosan requires low N:P ratio to form a complex and gives superior transfection efficiency whereas low molecular weight chitosan requires a high N:P ratio to completely form the complex (Lavertu et al. 2006; Romøren et al. 2003; Weecharangsan et al. 2008).

Effect of Chitosan Salt Form

Unlike chitosan, its salts such as chloride and lactate salts are soluble in water and have enhanced transfection capability. Weecharangsan et al. demonstrated the transfection efficiency of DNA–chitosan hydrochloride, DNA–chitosan lactate, DNA–chitosan acetate, DNA–chitosan aspartate, and DNA–chitosan glutamate. In vitro results in CHO-K1 cell lines indicated that all the salt forms had superior transfection efficiency as compared to native chitosan (Weecharangsan et al. 2008).

Nucleic Acid Concentration

Studies have shown that increase in plasmid concentration leads to increased diameter of chitosan–DNA complexes. A greater increase in size was observed when particles were formulated with high molecular weight chitosan (102 kDa), which implied that the diameter of complexes can be controlled by using appropriate concentration of plasmid and chitosan of suitable molecular weight (MacLaughlin et al. 1998). Romøren et al. demonstrated the effect of plasmid concentration on epithelioma papulosum cyprini (EPC) cells. Expression was higher at 2.5 μg/well compared to 0.5 μg/well, but further increase in concentration to 5 μg/well resulted in saturation in the level of expression (Romøren et al. 2003). In another study with primary chondrocytes, the transfection efficiency was observed to linearly increase with increase in plasmid concentration from 0 to 8 μg/well. Further increase in plasmid concentration to 16 and 32 μg/well resulted in drastic decrease in transfection efficiency, which was attributed to aggregation of nanoparticles containing higher concentration of plasmid amount resulting in reduced cellular uptake (Zhao et al. 2006b).

pH of the Culture Medium

A change in pH of culture media affects the transfection efficiency of the DNA–chitosan complex as the charge density of chitosan is dependent on environmental pH. At a pH of 5.5–5.7, 90% of the amino groups of chitosan are protonated, which decreases as the pH moves toward neutral values (Mao et al. 2001). Studies have proved that as pH decreases, the DNA binding capacity of chitosan increases as more protonated amine groups are available to bind to negatively charged DNA, thereby resulting in smaller sized particles (Köping-Höggård et al. 2003). Surface charge analysis of these complexes showed that the zeta potential values decrease as the pH is adjusted to neutral and electrostatically neutral particles are achieved at around 7–7.4 pH (Mao et al. 2001). At pH of 6.9, DNA/chitosan complexes demonstrate a positive charge and can bind to negatively charged cellular membranes. Thus a slightly lower than neutral pH is superior to pH 7.6 for enhanced transfection in A549 cells (Sato et al. 2001). Another study reported that at a pH of 6.5, transfection efficiency in HEK 293 cells was higher as compared to that at a pH of 7.1 (Lavertu et al. 2006). In yet another study in primary chondrocytes it was found that highest transfection efficiency was obtained at pH 6.8 and 7.0, whereas the transfection efficiency decreased when the pH was further increased to 7.4 (Ishii et al. 2001). In contrast, at more acidic pH values transfection efficiency was found to decrease due to very strong electrostatic interactions between DNA and chitosan which caused difficulty in dissociation of DNA from the complexes (Liu et al. 2005). Two reasons were attributed for poor transfection around neutral pH; first to the possibility of release of free plasmid from the complex and second to the probability of polyplex aggregation at neutral pH values.

Presence of Serum

Many studies have stated that the presence of serum enhances the gene expression via chitosan–DNA complexes at optimum molecular weight and N:P ratio (Ishii et al. 2001; Sato et al. 2001; Zhao et al. 2006b). In Hela cells, chitosan/DNA complexes demonstrated efficient transfection which was independent of the presence of 10% (v/v) serum in the medium. In contrast, PEI/DNA complexes show reduced transfection in the presence of the same concentration of serum (Erbacher et al. 1998). Another study showed that in the presence of serum, stability of DNA/chitosan complexes was dependent on the N:P ratio and the molecular weight of chitosan. Here, the complexes formulated with 7 kDa chitosan were unstable and dissociated when challenged with 10% (V/V) serum. Thereafter, a series of complexes made at the N/P ratio of 1:2, with molecular weight of chitosan ranging between 32 and 102 kDa, were challenged with serum. It was found that complexes prepared with 32 kDa chitosan dissociated as compared to all the other complexes. Aggregated and unstable formulations were observed when same series of experiments were conducted by changing the N:P to 1:6 (MacLaughlin et al. 1998). It was found that 10–20% v/v of serum increased the transfection efficiency of pGL3/chitosan in A549 cells. At 20% (v/v) of serum, the transfection efficiency increased by 2–3 folds. The results were explained by the fact that the cell functions

are increased in the presence of high serum concentration. Finally, the addition of 30–50% FBS was found to induce cell damage and was hence concluded as being unsuitable during investigations (Sato et al. 2001).

Effect of Additives

Experiments were done to formulate complexes of DNA/chitosan (10 kDa) and DNA/chitosan (10 kDa)/alginate (12–80 kDa) and their transfection efficiencies were studied in 293T cells. DNA/chitosan/alginate particles were smaller and stable compared to DNA/chitosan particles and exhibited higher transfection capability (Douglas et al. 2006). It was thus concluded that inclusion of anionic biopolymers with low molecular weight chitosan could thus be used as a strategy to improve their stability and transfection ability. These results were corroborated by the association of hyaluronic acid with low molecular weight chitosan to produce stable and uniform nanoparticles having increased transfection efficiency of pEGFP in 293T cells. Thus, it was also concluded that when low molecular weight chitosan was used to prepare DNA/chitosan/polyanion complexes, and the resulting transfection was more prominent (Duceppe and Tabrizian 2009).

Stability Against Polyanions

Extracellular matrix of cells consists of various proteoglycans, which are proteins covalently cross-linked with carboxylic or sulfated glycosaminoglycans (GAGs). Heparin sulfate, chondroitin sulfate, and hyaluronic acid are examples of GAGs which have been found to interact with polyplexes having positive surface charge. This in turn is known to affect their integrity, mobility in extracellular matrix, and accessibility to target cells (Pitkänen et al. 2003; Ruponen et al. 1999, 2003). MacLaughlin et al. demonstrated that plasmid (containing luciferase)/chitosan complexes (102 kDa; N:P = 1:2) when formulated with SDS and heparin (sodium salt) had improved in vivo applications (MacLaughlin et al. 1998).

Chitosan as an Additive

Chitosan has been used as coating material for nanoparticles synthesized with negatively charge polymers like PLGA to render them a cationic nature and hence improve their DNA loading. Chitosan-coated PLGA particles are known to possess high cationicity and are hence stabilized by interparticle repulsion and exhibit higher cell uptake (Nie et al. 2008). Chitosan-coated PLGA nanoparticles have been reported as a suitable delivery system antisense for oligonucleotides in lung cancer cells (Nafee et al. 2007). Chitosan coating hinders the initial burst release and promotes sustained release of nucleotides (Tahara et al. 2008). Another study demonstrated that chitosan is competent of enhancing the emulsion as a gene delivery vehicle. Chitosan enables the precondensation of plasmid DNA and increases transfection efficiency of the emulsion of in vitro in hepatoma cells (HepG2). In vivo studies have reported that residence time of chitosan-enhanced emulsions complexes in organs of mice was longer compared to DNA emulsions (nDNA/E) complexes (Lee et al. 2005). Moreover, when liposomes are coated with

chitosan, expression levels of luciferase were significantly reduced due to augmented instability of chitosan-loaded liposomes (Colonna et al. 2008).

4.2 siRNA

In contrast to gene therapy via DNA transfection, gene silencing [RNA interference (RNAi)] via siRNA transfection has attained significant attention in recent time (Morris et al. 2004). RNA interference is a biological mechanism found in most of the eukaryotic cells, in which siRNA molecules inhibit gene expression by destroying protein-specific mRNA molecules. Detailed mechanism has been depicted in Fig. 8 (Dykxhoorn et al. 2003; Elbashir et al. 2001).

RNAi shows numerous advantages over other therapeutic methods as very specific, safe effects were often observed during chemotherapy and the induction of interferon response of antisense therapy (Zhang et al. 2007b). Compared to other antisense strategies like antisense DNA oligonucleotides and ribozymes, RNAi is more competent. Importantly its higher competence of RNAi means that the effector molecules may function at much lower concentrations than antisense oligos or ribozymes.

However, the delivery of siRNA is faced with severe challenges like its rapid degradation in plasma and cellular cytoplasm, failure of permeation of naked siRNA through positively charged cell membrane, venerability of enzyme degradation, size of the siRNA, etc. (Sioud 2005; Zhang et al. 2007b). Thus, successful delivery of siRNA is essentially dependent on formulation of a delivery vehicle that can be administered efficiently, conveniently, and consistently and can overcome the aforementioned delivery challenges (Kumar and Clarke 2007). Katas and Alpar were the first to demonstrate chitosan as siRNA delivery vector in vitro (Katas and Alpar 2006). As with DNA delivery, chitosan particles meant for siRNA delivery also require optimization with respect to certain parameters which have been elaborated subsequently. The siRNA structure, which is significantly different from that of pDNA, is more prone to enzymatic degradation that further poses additional delivery challenges.

4.2.1 Factor Affecting siRNA Delivery via Chitosan Particulate Systems

Chitosan Molecular Weight and Concentration

Chitosan molecular weight greatly influences the physiological properties like size, surface charge, and surface morphology of the siRNA-loaded particles/complexes and thus affects its gene silencing capability. Stability of complexes is essential to assure the integrity and protection of siRNA and also its dissociation from the complex at the target site. This has been reported to be achieved by choosing optimum amount of chitosan of appropriate molecular weight.

Katas et al. demonstrated that the size of siRNA/chitosan particles is dependent on the molecular weight of the polymer. The authors prepared complexes using 110 and

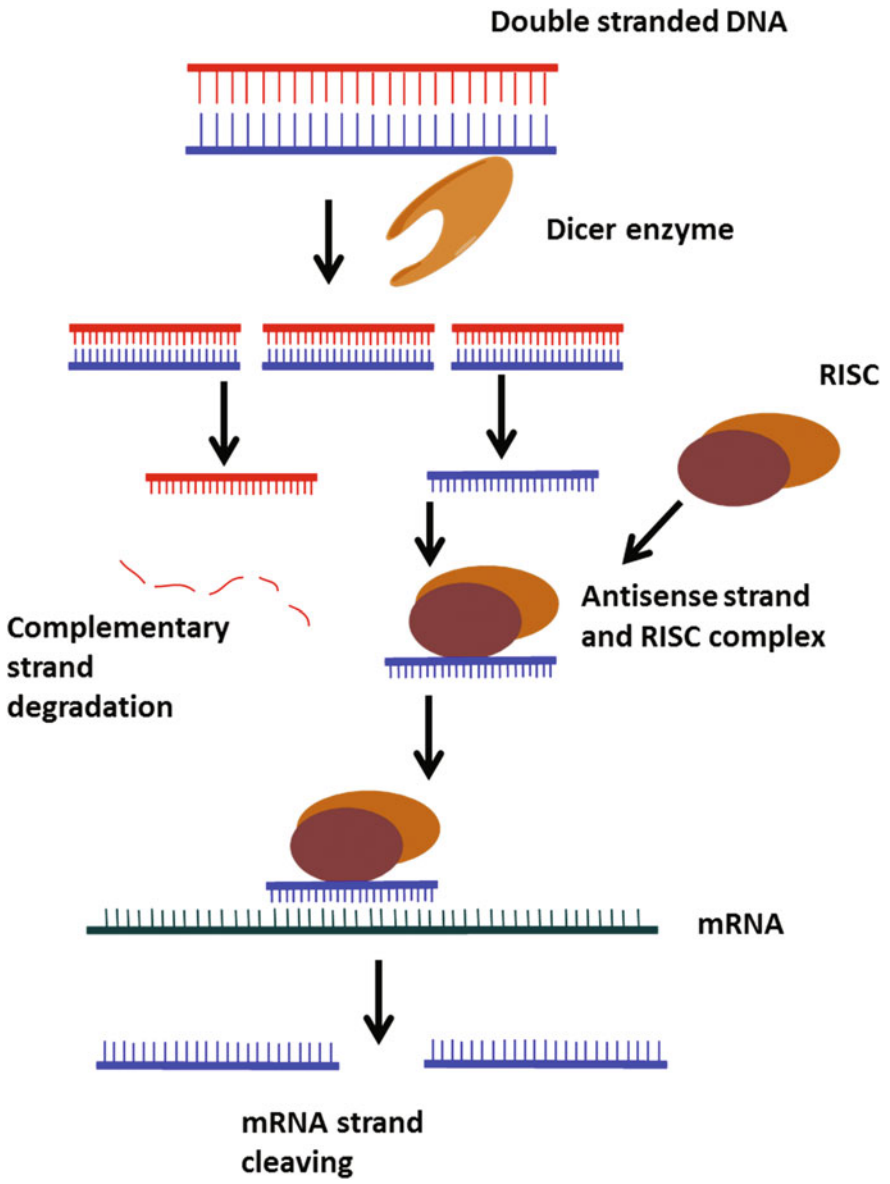

Fig. 8 Mechanism of siRNA-mediated gene silencing

270 kDa chitosan and demonstrated the production of larger particles (450–500 nm) by using the higher Mw chitosan compared to lower Mw chitosan (140–300 nm) (Katas and Alpar 2006). Liu et al. explored chitosan having molecular weights of 64.8, 170, and 10 kDa, to form siRNA/chitosan particles and studied them for in vitro siRNA delivery in H1299 human lung carcinoma cells. Results showed that stable

complexes and significant knockdown were achieved with 64.8 and 170 kDa chitosan particles, whereas 10 kDa chitosan was unable to compact siRNA to form stable particles and resulted in negligible gene knockdown (Liu et al. 2007). Results concluded that high molecular weight chitosan is the primary requirement for siRNA-mediated gene knockdown. Another study revealed that mean particle size and the surface charge (zeta potential) of the chitosan/siRNA particles prepared by simple complexation of both, chitosan hydrochloride and glutamate, increased with increase in chitosan concentration from 25 to 300 μg/mL. The increase in size with increase in concentration was also observed when the siRNA–chitosan complexes were prepared by ionic gelation method using TPP as a cross-linker. Further in vitro studies were done in CHO K1 and HEK 293 cell lines and it was concluded that chitosan–TPP nanoparticles with entrapped siNA are better vectors compared to chitosan–siRNA complexes possibly due to their high binding capacity and loading efficiency.

Degree of Chitosan Deacetylation (Noureddini et al.)

Positive charge on the chitosan is directly proportional to the DD. It has been reported that chitosan with higher DD exhibited higher siRNA–EGFP binding efficiency. Particles formed with high DD chitosan (64.8–170 kDa) demonstrated higher knockdown efficiencies compared to low DD chitosan (10 kDa), in H1299 human carcinoma cells (EGFP expressing). Non-transfected cells were used as negative control and commercial TransIT-TKO was used as a positive control (Liu et al. 2007).

N/P Ratio

Liu et al. demonstrated that size of the chitosan (Mw 114 kDa)/siRNA nanoparticles is dependent on N:P ratio. Results showed the production of 181.6 nm particles at 71 N:P and 223.6 nm at N:P of 6. N:P has been also stated to influence gene knockdown in H1299 human lung carcinoma cells. EGFP knockdown was higher with nanoparticles formulated at N:P ratio of 50, compared to those formulated at the N:P ratio of 10. Highest knockdown (80%) was observed at the N:P ratio of 150. The study concluded that increase in N:P resulted in stability of the particles, enabling them to be more potent for gene knockdown (Liu et al. 2007).

Chitosan Salt Form

Studies have reported that chitosan glutamate (470, 160 kDa) having higher molecular weight than chitosan hydrochloride (270, 110 kDa) resulted in smaller siRNA (siRNA targeting against pGL3 luciferase gene) loaded nanoparticles. However, lower sized complexes were obtained when lower Mw chitosan was used in case of both chitosan glutamate and chitosan hydrochloride. This was explained due to the decreased viscosity of the lower concentration or molecular weight of chitosan which resulted in better solubility and generated its molecular character as a polyelectrolyte material that allows more efficient interaction between negatively charged siRNA and the cationic chitosan. Moreover, loading efficiency was also higher in chitosan glutamate particles (83 ± 0.9% for 470 kDa and 90 ± 0.3% for 160 kDa) compared to chitosan hydrochloride particles (72 ± 1.1% for 270 kDa

and 59 ± 0.8% for 110 kDa). In addition, highest gene silencing was achieved using chitosan glutamate (470 kDa)/siRNA complexes in CHO-K1 cells. It was concluded that chitosan glutamate was more efficient to wrap the siRNA molecules and hence protecting it against extracellular degradation thus increasing its transfection efficiency (Katas and Alpar 2006).

pH of the System

Electrostatic interactions between siRNA and chitosan (12 kDa, 77% DD) have been observed to have pH-dependent. It has been reported that the strength of these interactions decreases with an increase in the pH of the environment from 4.1 to 9.5 [(phosphate buffered saline) PBS solution]. Around neutral pH, negligible electrostatic forces are observed in chitosan/siRNA complexes (Xu et al. 2007). However, it has also been shown that particle size of chitosan/siRNA complex remained the same when prepared in acetate buffer of pH 4.5 (0.1 M) compared to when prepared in distilled water (pH 6.5), for all the tested chitosans. The results were explained by the possibility of reduced electrostatic interactions between chitosan and siRNA at neutral pH resulting in instability of the chitosan–siRNA complexes. However, chitosan–siRNA complexes remain stable at pH of 4.5 due to sufficient electrostatic interactions between chitosan and siRNA (Katas and Alpar 2006).

Presence of Serum

Studies have demonstrated that up to 10% (V/V) serum is suitable for maintaining stability of chitosan–siRNA complexes and chitosan–TPP-siRNA nanoparticles in cell culture medium (Katas and Alpar 2006). It was reported that when free and chitosan complexed siRNA were incubated in 5% FBS at 37 °C, free siRNA was intact only up to 30 min and completely degraded after 48 h. Whereas siRNA loaded on chitosan nanoparticles degraded after 72 h, thus exhibiting higher stability. When experimental conditions were altered by changing serum concentration to 50% (V/V), naked siRNA degraded as soon as it was mixed with the medium while siRNA loaded on chitosan nanoparticles started degrading only after 7 h and completely degraded after 48 h of incubation (Katas and Alpar 2006).

Methods of siRNA Association

Three different methods namely simple complexation, ionic gelation (siRNA entrapment), and adsorption of siRNA on preformed chitosan nanoparticles have been explored. Interestingly, cell permeation of the particles in CHO K1 and HEK 293 cell lines was observed to be the highest when the particles were prepared by ionic gelation method. Gene silencing studies also showed that the same particles were more superior in gene knockdown than the particles prepared by alternative methods. It was explained that when particles were formed by ionic gelation, they have high binding capacity and loading efficiency due to a better cross-linked platform of chitosan and TPP (cross-linker) to entrap the siRNA molecules (Katas and Alpar 2006).

Chitosan as the Coating Material

Chitosan has been extensively used for coating negatively charged nanoparticles for enhancing the siRNA loading and delivery. Pille et al. reported the formulation of anti-RhoA siRNA-loaded, chitosan-coated poly-isohexylcyanoacrylate nanoparticles. On intravenous administration in nude mice with xenografted aggressive breast cancers, the particles were found to hinder the tumor growth by 90% compared to untreated controls (Pillé et al. 2006). Another study reported the preparation of antisense oligonucleotides (20-*O* methyl RNA (OMR))—loaded chitosan-coated PLGA nanoparticles and OMR-loaded-PLGA nanoparticles. Very slight difference in size was observed between OMR-PLGA and OMR-PLGA–chitosan nanoparticles. Excellent permeation of particles (OMR-PLGA–Chitosan) and significant gene silencing was observed in A549 cells after 6 h of incubation compared to OMR-PLGA particles (Nafee et al. 2007).

4.3 Peptides/Proteins via Chitosan Particulate Systems

Most of the proteins are delivered via parenteral routes to overcome the problems associated with low bioavailability and poor immunogenicity of the proteins delivered via alternative routes (Giudice and Campbell 2006). However, the parenteral route faces challenges of high cost and reduced patient compliance. Furthermore, inherent physical, chemical, proteolytic instability, and large size are some of the major factors responsible for poor penetration of therapeutic proteins across mucosal surfaces. The nasal and respiratory passages are covered by mucosal layer, which is constantly removed by mucociliary clearance and hence causes additional hindrance for mucosal delivery of proteins (Patton 1996; Patton et al. 2004; Soane et al. 2001). These delivery challenges can be overcome by formulating proteins using external excipients that can protect them against degradation, prevent their rapid elimination, and help in their targeted administration inside the body. As described earlier, chitosan exhibits mucoadhesive and/or absorption-enhancing properties and is also capable of opening the tight junctions between epithelial cells. These properties stimulate absorption of proteins or antigens on chitosan-based particles (Amidi et al. 2006; Calvo et al. 1997a; Fernandez-Urrusuno et al. 1999; Kotzé et al. 1997; Mao et al. 2005a, 2006; Thanou et al. 2000, 2001; Van Der Lubben et al. 2001a, b; Vila et al. 2004). Due to its mucoadhesive and absorption promoting properties, chitosan enhances intracellular uptake of proteins and their transport across epithelial barriers. In addition, particulate carrier systems promote interaction of proteins with epithelial cell membranes and mucus, intensify the residence time of proteins at the site of administration, protect the labile proteins from enzymatic degradation, and stimulate the intake of free proteins via the paracellular pathway as well as transcytosis of the encapsulated proteins (Artursson et al. 1994; Borchard et al. 1996; Giudice and Campbell 2006; Illum et al. 1994; Lee et al. 2000; Lehr et al. 1992; Van der Merwe

et al. 2004). In this section, we have discussed chitosan-based particulate systems that have been explored for protein delivery via non-parenteral (nasal and pulmonary) and parenteral routes.

Chitosan-Coated Particles for Delivery of Peptides/Proteins

Scientists have employed layer-by-layer self-assembly technique, wherein alginate and chitosan were alternately adsorbed onto the surface of cationic liposomes prepared by dissolving 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), cholesterol, and a cationic surfactant; dimethyldioctadecyl-ammonium bromide (DDAB) in a chloroform–methanol mixture (4:1, v/v). DDAB was used in a 4% molar concentration to tailor the surface charge of the liposomes. Further they loaded BSA on these particles, with >80% loading efficiency. The particles were found to release the protein in a sustained manner over a period of one month (Haidar et al. 2008). In another study, melamine formaldehyde (Polk et al.)—loaded microparticles were prepared. These particles had self-assembled multilayer chitosan/alginate coating on them. After deposition of multilayers, the microparticle template was removed by dissolving the core at low pH. Further insulin was loaded into the hollow chitosan/alginate microparticles. In order to slow down the release kinetics, cross-linked particles were prepared by adding a calcium chloride ($CaCl_2$) solution to the insulin-loaded particles. The chitosan/alginate particles showed a sustained release of insulin of 80% in 15 h, which was decreased to 50% for the cross-linked particles (Ye et al. 2006). In another study, investigators prepared salmon calcitonin-loaded chitosan-coated nano-emulsions and lipid nanoparticles having size of about 300–500 nm and a positive surface charge. Improved mucosal absorption of salmon calcitonin was observed in in vivo experiments conducted in rats (Garcia-Fuentes et al. 2005; Prego et al. 2005).

Chitosan-coated gold nanoparticles were studied for mucosal protein delivery (Bhumkar et al. 2007). Chitosan was chosen due to its reducing properties, which helped in the synthesis of gold nanoparticles, in addition to its mucoadhesive and permeation augmenting properties. Insulin was adsorbed through electrostatic interactions onto the surface of these nanoparticles. The efficiency of adsorption was 50% and the particles were stable for 6 months. Further, pharmacodynamics effect of these particles was investigated by their intranasal administration in diabetic rats wherein chitosan-coated particles were found to be more effective in reducing blood glucose level as compared to insulin-loaded gold nanoparticles prepared by using sodium borohydride as the reducing agent. The results were explained by the facts that chitosan provides numerous electrostatic binding sites with insulin compared to that of gold nanoparticles (Bhumkar et al. 2007).

Pegylated Chitosan Particles for Delivery of Peptides/Proteins

Studies have reported grafting of Poly (ethylene glycol) on chitosan copolymers to boost its solubility and improve its biocompatibility (Zhang et al. 2008a, b). Zhang et al. reported the preparation of pegylated chitosan nanoparticles by reacting peg-aldehyde group with amine group of chitosan. Further, the investigators formulated insulin-loaded PEG–chitosan nanoparticles by ionic gelation method using

TPP. On studying the effect of molecular weight of chitosan and PEG on insulin release, they found that the higher the molecular weight of chitosan, the higher and faster was the release behavior as the higher molecular weight chitosan had longer chain segments, which influenced the diffusion of insulin as well as the rate of degradation of the nanoparticles. Further it was demonstrated that raising the molecular weight of PEG graft reduced the release rate. PEG–chitosan–insulin nanoparticles showed increased insulin release compared to chitosan–insulin nanoparticles as chitosan–insulin has high positive charges, forming a tight network and effectively retarding insulin release. This was accompanied with higher plasma insulin concentration in female New Zealand rabbits (2–3 kg, 3 months old) and accelerated reduction in blood glucose (Zhang et al. 2008a, b).

TMC Particulate Systems for Delivery of Peptides/Proteins

Extensive research has been done in mucosal delivery of peptides/proteins prepared with soluble TMC; however, few studies report the delivery of proteins administered in the form of particulate (nanoparticles or microparticles) systems (Fernandez-Urrusuno et al. 1999; Kotzé et al. 1997; Kumar 2000). Amidi et al. reported preparation of ovalbumin-loaded TMC nanoparticles for the nasal delivery, by ionic cross-linking of TMC with TPP. Western blot analysis shows the preserved integrity of the proteins in this system. Loading efficiency was up to 50% (w/w) and release studies show that 30% of the loaded proteins were released in a burst manner whereas the remaining protein was intact with TMC nanoparticles for at least 3 h in PBS (pH 7.4) at 37 °C. Calu-3 cells were taken as model for respiratory epithelial cells and no toxic effects of albumin–TMC nanoparticles was found, though partially reversible cilio-inhibiting effect on the ciliary beat frequency of chicken trachea was observed. FITC-labeled albumin-loaded TMC nanoparticles were easily taken up by the nasal epithelial cells and NALT (Nasal-associated lymphoid tissue) of rats (Amidi et al. 2008b).

In another study, PEG (molecular weights 5 and 550 kDa) was grafted on TMC of molecular weights ranging from 5 to 400 kDa to improve the biocompatibility of TMC. Results indicated reduced in vitro cytotoxicity, particularly of low molecular weight TMCs. Upon insulin loading, cytotoxicity of the particles was further reduced (Mao et al. 2005b). In another study, polyelectrolyte complexes (PEC) consisting of chitosan or TMC and insulin were prepared and characterized. The stability of nanocomplexes was dependent on the molecular weight of chitosan/TMC and stable particles were obtained when the molecular weight of the polymers was above 25 kDa. Particles prepared with lower molecular weights of TMC showed severe aggregation. Pegylated-TMC protected insulin from chemical degradation even at 50 °C. All complexes could be lyophilized without affecting the particle size and stability of insulin. These PECs were aimed for nasal protein delivery. In an alternative study, polyelectrolyte nanocomplexes (PECs) consisting of chitosan or TMC and insulin were formulated (Thanou et al. 2000). Scientists reported that stable particles were obtained when molecular weight of polymers was above 25 kDa while particles formed with lower molecular weight TMC exhibited severe aggregation (Mao et al. 2006).

Chen et al. studied the effect of TMC with different degrees of quaternization (DQ) on nanoparticle characteristics and protein loading, as well as the release of two model proteins with different isoelectric points (pI), bovine serum albumin (pI 4.8), and bovine hemoglobin (pI 6.8). For hemoglobin, the loading efficiency of nanoparticles was low (30%) which was thought to be due to weak negative charge of the protein at neutral pH. However, for BSA the loading efficiency was 95%. For TMC nanoparticles with lower DQ, slower release kinetics was observed due to lower charge density and lower hydrophilicity. Also as these particles have significant swelling properties the diffusion of the protein is further reduced from the nanoparticle network. Alginate is incorporated on the surface of these particles to lessen the burst effect (Chen et al. 2007).

Sandri et al. prepared nanoparticles loaded with fluorescein isocyanate dextran using TMC with different DQs and chitosan. These nanoparticles which were formulated for mucosal peptide delivery were investigated in vitro in Caco-2 cell model and ex vivo in rat jejunum model. Results demonstrated that except for nanoparticles formulated using TMC with high DQ (90%), all other nanoparticles systems interacted with the Caco-2 cells reducing the transepithelial electric resistance (TEER) and increasing Lucifer Yellow (LY) Papp (paracellular pathway marker). It was concluded that TMC nanoparticles with intermediate DQ (35%) are superior carriers for mucosal peptide/protein delivery compared to chitosan nanoparticles having lesser transfection efficiency for the protein (Sandri et al. 2007).

Zheng et al. prepared folate-decorated TMC nanoparticles by conjugating TMC with folate. These particles were designed to bind to folate receptors of cells for enhancing intracellular delivery of macromolecules. Further FITC-BSA-loaded folate–TMC nanoparticles were prepared by ionic cross-linking using alginate. Results of in vitro studies showed up to fourfold higher uptake in SKOV3 cells (folate receptor expressing cells) compared to nonfunctionalized TMC nanoparticles. The folate receptor-mediated uptake could be inhibited by the presence of folate in the culture medium demonstrating that the uptake of the targeted particles was indeed via the folate receptor (Zheng et al. 2009).

In another study self-assembled TMC (DQ 40%)-poly(γ-glutamic acid) (PGA) nanoparticles were prepared for oral delivery of insulin. These particles demonstrated loading efficiency of as high as 74% and showed a sustained release of insulin for 12 days, at physiological pH. As compared to insulin–chitosan nanoparticles, insulin–TMC-γ-PGA nanoparticles showed improved colloidal stability at a broad pH range. Further, in vitro *studies were carried out* using Caco-2 cells, ZO-1 tight junction's proteins were stained, and confocal microscopy was carried out. Results confirmed that insulin-loaded TMC-γ-PGA nanoparticles opened the tight junctions in cells to facilitate the uptake of insulin along the paracellular pathway (Mi et al. 2008).

Effect of Different Formulation Factors on Chitosan Particle Size, Polydispersity, and Protein Loading Efficiency

Koppolu et al. demonstrated the effect of different formulation parameters such as such as chitosan concentration, precipitant salt type and concentration, molecular

weight, rate of precipitant addition, protein size and sonication power, on particle size, polydispersity, and protein loading efficiency of chitosan nanoparticles (Prasanth Koppolu et al. 2014). Results show that chitosan particles were successfully prepared and loaded with FITC-BSA (Bovine serum albumin), BSA, ovalbumin, FITC-insulin, and FITC-concanavalin (ConA). It was found that lower molecular weight and concentration of chitosan resulted in smaller particles. Hoffmeister series salts containing strongly hydrated anions resulted in particles with low PDI. Increase in sonication power reduced the particle size and resulted in a more homogenous distribution. Protein loading ranged from 14.3 to 99.2%, and was inversely proportional to the hydration capability of precipitant salts, molecular weight of protein, and directly proportional to concentration and molecular weight of chitosan. Larger particles showed increased release rates of proteins while high molecular weight proteins were released at slower rates.

5 Effect of Route of Administration

5.1 Effect of Route of Administration on DNA Delivery

Appropriate route of administration should be selected during gene therapy to improve the bioavailability of nucleic acids. Due to physiological characteristics of DNA, it is commonly delivered via parenteral route. However, the mucoadhesive properties of the chitosan make it an attractive material to be explored for mucosal gene delivery via oral, intranasal and intra-tracheal routes (Dang and Leong 2006; Kim et al. 2007). In a study dealing with chitosan nanoparticles loaded with dominant peanut allergen gene (pCMVrah2), the nanoparticles were orally administered in mice. The experiments resulted in transduced gene expression in the intestinal epithelium of the animals and resulted in production of secretory IgA and serum IgG2a. Mice immunized with nanoparticles showed significant lowering of allergen-induced anaphylaxis, compared to non-immunized mice (treated with naked DNA) (Roy et al. 1999). Further Guliyeva et al. demonstrated immune responses against peanut allergen Arah-2 in swiss albino mice by oral administration of the chitosan/DNA microparticles (Guliyeva et al. 2006). In another study, the major capsid protein (MCP) gene of lymphocystis disease virus (LCDV) was encapsulated in chitosan microspheres with encapsulation efficiency of 94.5%. Significant transfection of the gene was observed in Japanese flounder, after oral administration (Tian et al. 2008).

All these studies show that the transfection efficiencies via the oral route are comparable to that of parenteral route demonstrating the competence of the oral route administration of DNA–chitosan complexes.

A few studies have also explored the intranasal route for delivering chitosan/DNA complexes. Kumar et al. efficiently prepared chitosan nanoparticles containing a cocktail of plasmid DNAs encoding all RSV antigens. On nasal administration of the nanoparticles in mice, the animals demonstrated no change in airway reactivity to

methacholine and no apparent pulmonary inflammation. Results also showed a convincing induction of RSV-specific IgG antibodies, nasal IgA antibodies, cytotoxic T lymphocytes, and Interferon–γ production in the lung and splenocytes compared to controls (Kumar et al. 2002). In another study chitosan nanoparticles loaded with plasmid DNA (CTL epitope from the M2 protein of RSV) were formulated. After intranasal administration, the particles resulted in significant lowering in the virus load as compared to the control group (Iqbal et al. 2003). Studies pertaining to pulmonary administration of DNA/chitosan complexes have also been explored. In one such study, DNA incorporated into chitosan nanoparticles was found to promote increased levels of IFN-γ secretion in HLA-A2 mice compared to the native plasmid solution administered via intramuscular route (Bivas-Benita et al. 2004). Another study showed that chitosan/DNA complexes in powder form possessed higher transfection potency than solutions containing the same amount of DNA, when administered via pulmonary route (Okamoto et al. 2005).

5.2 Effect of Route of Administration on siRNA Delivery

Intranasal administration of siNS1-loaded chitosan nanoparticles in RSV (respiratory syncytial virus)—infected mice models showed decreased virus titers in lungs, decreased inflammation, and airway reactivity compared to controls (IFN-deficient Vero cells, transfected with siNS1) (Zhang et al. 2005). In a similar study, EGFP knockdown was observed in mice models where EGFP siRNA–chitosan nanoparticles were delivered via intranasal administration. It was observed that there is 47% knockdown in mice in which chitosan-loaded siRNA was given compared to control (only siRNA was given) (Howard et al. 2006).

Another study demonstrated knockdown of TNF-α gene in systemic macrophages when the siRNA [TNF-alpha Dicer-substrate siRNA (DsiRNA)]—loaded nanoparticles were administered via intraperitoneal route. Knockdown was followed by reduced systemic and local inflammation (Howard et al. 2009). Another study compared biodistribution of chitosan/siRNA nanoplexes after their administration via intravenous and intraperitoneal routes. Compared to intravenous route, particle accumulation was seen in the peritoneal cavity via intraperitoneal route. Further, it was concluded that retention of chitosan formulation within a serum-free environment permitted nanoparticles to enter native peritoneal macrophages in a form that could successfully deliver antitumor necrosis factor-α siRNA into macrophages to provide targeted anti-inflammatory therapy (Gao et al. 2009).

Gao et al. were the first to demonstrate that chitosan could change the biodistribution pattern of siRNA. This conclusion was based on observation that high accumulation of siRNA-loaded chitosan nanoparticles was observed in the kidney after intravenous injection in mice. After 30 min of administration, 10-fold and 100-fold increases in accumulation were seen in the liver as compared to lungs and spleen, respectively. The level of intact siRNA in liver was maintained even after 24 h implying that chitosan/siRNA complexes preferentially accumulate in the kidneys while circumventing the rapid glomerular filtration observed for naked

siRNA, showing the potential for development of clinically relevant RNAi therapeutics for renal diseases with targeting effect (Gao et al. 2009).

5.3 Effect of Route of Administration on Peptides/Proteins Delivery

Recently, several studies have demonstrated the use of chitosan particles or polyelectrolyte complexes for nasal delivery of therapeutic proteins (Fernandez-Urrusuno et al. 1999; Teijeiro-Osorio et al. 2008; Wang et al. 2009; Zhang et al. 2008b). Studies reveal that insulin-loaded chitosan nanoparticles intensify the nasal absorption of insulin as compared to chitosan solution (Fernandez-Urrusuno et al. 1999; Zhang et al. 2008b). According to Wang et al., insulin-loaded thiolated chitosan nanoparticles show improved adsorption of insulin on nasal mucosa as compared to non-thiolated chitosan due to higher mucoadhesive properties of the former. Moreover, in vivo studies with thiolated chitosan nanoparticles revealed decreased glucose levels in blood, similar to levels when insulin is injected subcutaneously (Wang et al. 2009). In another study polysaccharide nanoparticles of chitosan and cyclodextrin were analyzed for nasal delivery of insulin in rabbits and in Calu-3 cell lines. In vivo studies showed more than 35% reduction in plasma glucose levels, whereas results of in vitro studies showed that chitosan–cyclodextrin nanoparticles caused a reversible reduction in the transepithelial resistance of the cell monolayer, thus increasing the membrane permeability for the insulin delivery and thus induces reduction of plasma glucose level (Teijeiro-Osorio et al. 2008).

Grenha et al. formulated insulin-loaded chitosan/TPP microparticles by spray drying technique, which possessed appropriate aerodynamic properties for pulmonary delivery (Grenha et al. 2005). These particles demonstrated good protein loading capacity (65–80%), providing the release of 75–80% insulin within 15 min. Further, many research groups demonstrated formulations of protein-loaded chitosan nanoparticles suitable for pulmonary delivery by spray drying technique (Grenha et al. 2007a, 2008). These particles were studied for their biocompatibility and penetration efficiency in A549 and Calu-3 cells as model cells for alveolar and respiratory epithelial pathways. Results indicated no opening of tight junctions and very low toxicity in both the cell lines (Lim et al. 2001; Witschi and Mrsny 1999). Further, confocal microscopy results indicated very low internalization of the particles, which attributed to the low concentration of chitosan employed in the preparation of nanoparticles (Grenha et al. 2007a). Lim et al. prepared nanoparticles using chitosan glutamate, which showed better internalization as compared to chitosan nanoparticles (Lim et al. 2001). Yang et al. studied the effect of chitosan on physiological stability of calcitonin by formulating inhalable salmon calcitonin-loaded chitosan nanoparticles by spray drying technique. The dissolution rate of the protein decreased when formulated with chitosan, which might be due to an irreversible complex formation between the (aggregated) protein and chitosan during the drying process (Yang et al. 2007). However, chitosan-coated PLGA nanoparticles, which were aerosolized with a nebulizer, show improved absorption

of calcitonin after pulmonary administration. Elimination of chitosan-coated nanoparticles was retarded, as compared to uncoated particles, due to mucoadhesive properties of chitosan and the pharmacological action of calcitonin with coated particles was more sustained compared to uncoated particles (Yamamoto et al. 2005). In another study, effects of chitosan oligomers (dimer, tetramer, hexamer, and water-soluble chitosan) on pulmonary absorption of interferon alpha (IFN) were examined by in vivo pulmonary absorption experiments in rats. In all the experiments, a significant increase in serum IFN concentrations was observed after intra-tracheal administration, but pulmonary absorption of IFN was seen with 0.5% w/v chitosan hexamer. In addition, it was concluded that water-insoluble chitosan used in the experiments (22–96 kDa) were not effective in enhancing the pulmonary absorption of IFN (Yamada et al. 2005).

Amidi et al. utilized supercritical fluid technology to produce insulin-loaded-TMC microparticles (6–10 μm), suitable for pulmonary administration (Amidi et al. 2008b). HPLC, GPC, and CD analyses confirmed no degradation and no changes in secondary structure of the insulin. After one-year storage at 4 °C, the particle characteristics were maintained and the insulin structure was largely preserved, thus indicating the process to be suitable for preparation of insulin-loaded particles. On further investigations, potential of TMC–insulin powder formulations was evaluated in diabetic rats via pulmonary delivery. Results showed enhanced systemic absorption of insulin, with bioavailability of about 95% relative to subcutaneously administered insulin (Amidi et al. 2008a).

Kim et al. modified glycol chitosan with 5-β-cholanic acid (HCG) groups and used it to synthesize nanoparticles by solvent evaporation method. The HCG group helps in holding the particles together, whereas glycol groups stabilized the particles in aqueous environment. Further, the researchers encapsulated anti-angiogenic RGD peptides in the particles, with 85% loading efficiency. Sustained release of the encapsulated peptide was observed for a period of one week. In vivo studies in mouse models, using intra-tumoral administration, demonstrated significant decrease in tumor growth as compared to intravenously administered RGD peptide (Kim et al. 2008).

Chitosan (DD 80%) particles having mean diameter of 2.5 μm augmented the cytolytic activity of peritoneal macrophages in promoting the production of colony-stimulating factor (CSF) in vitro and in vivo by macrophage, spleen cells, and bone marrow cells. These particles were also effective in producing IL-1 by both resident and thioglycolate-induced peritoneal macrophages. Chitin particles of 2.5 μm were unable to activate peritoneal macrophages in vivo and the production of IL-1 in vitro. However, they increased the production of CSF in serum in vivo. Shibata et al. demonstrated that chitin and chitosan particles required to be phagocytised by alveolar microphages in order to enhance the expression of interferon gamma (INF-γ). Van der Lubben et al. demonstrated that diphtheria toxoid (DT)-loaded chitosan microparticles could possess 100% loading efficiencies. Local immune responses of these particles were investigated after oral and nasal delivery in mice models and enhanced neutralizing antibody levels and secretory IgA antibody levels were observed by both the routes (Van Der Lubben

et al. 2001b). Further TMC having degree of quaternization 20% was explored to formulate DT-loaded microparticles and their immunization was studied following intranasal administration. Results concluded that DT-loaded TMC microparticles facilitate the formation of both IgG and IgA analogous to that of soluble TMC co-administered with the DT antigen. Moreover, influenza hemagglutinin-loaded TMC (DQ 20%) nanoparticles induced superior immune response compared to intranasal administration of soluble TMC and influenza antigen (Amidi et al. 2007b).

Another study demonstrated the competence of diphtheria toxoid-loaded TMC (DQ 60%) microparticles for mucosal vaccination. When these particles were administered via pulmonary route in guinea pigs, the animals showed enhanced systemic and local immune responses compared to subcutaneously administer alum-adsorbed DT vaccine (Amidi et al. 2007a). Sayin et al. prepared tetanus toxoid (TT)-loaded anionic mono-*N*-carboxymethyl chitosan (MCC) and cationic TMC (DQ 57%) nanoparticles and administered in mice models. Both particles were taken up by murine macrophages, irrespective of their surface charge. Higher serum antibodies were developed in the mice models which received TT–chitosan or TT–TMC nanoparticles via intranasal route, compared to those who received TT–MCC particles intranasally. Serum antibody levels in control mice which were vaccinated subcutaneously with free TT showed comparable results to mice vaccinated with TT–chitosan and TT–TMC nanoparticles by intranasal administration. Further it was concluded that antigen formulated with soluble TMC and MCC shows comparable results to their nanoparticle formulations (Sayın et al. 2008).

6 Chitosan-Based Formulations for Vaccine Delivery

Chitosan-based particulate systems have been broadly studied for parenteral and mucosal delivery of antigens (Amidi et al. 2007b; Boonyo et al. 2007; Davis 2002; Illum et al. 2001; Köpping-Höggård et al. 2001; Nagamoto et al. 2004; Sato et al. 2001). In these studies various antigens showing both systemic and local immune responses were delivered with chitosan in the forms of micro- or nanoparticles or via particles coated with chitosan. In a phase-I clinical study, intranasal immunization with influenza vaccine formulated with soluble chitosan glutamate showed positive effects of the polymer on the immune responses raised in the vaccinees (Read et al. 2005).

Chitosan-Based Micro- and Nanoparticles for Parenteral and Mucosal Vaccination

Chitosan nanoparticles can protect antigens from degradation and increase the uptake of the particles by antigen-presenting cells (APCs), macrophages and M-cells at mucosal sites and other sites of administration. Chitosan (DD 80%) particles having mean diameter of 2.5 μm augmented the cytolytic activity of peritoneal macrophages in promoting the production of colony-stimulating factor

(CSF) in vitro and in vivo by macrophage, spleen cells, and bone marrow cells. These particles were also effective in producing IL-1 by both resident and thioglycolate-induced peritoneal macrophages. Chitin particles of 2.5 μm were unable to activate peritoneal macrophages in vivo and the production of IL-1 in vitro. However, they increased the production of CSF in serum in vivo. Shibata et al. demonstrated that chitin and chitosan particles required to be phagocytised by alveolar microphages in order to enhance the expression of interferon gamma (INF-γ). Van der Lubben et al. demonstrated that diphtheria toxoid (DT)-loaded chitosan microparticles could possess 100% loading efficiencies. Local immune responses of these particles were investigated after oral and nasal delivery in mice models and enhanced neutralizing antibody levels and secretory IgA antibody levels were observed by both the routes. Further TMC having degree of quaternization 20% was explored to formulate DT-loaded microparticles and their immunization was studied following intranasal administration. Results concluded that DT-loaded TMC microparticles facilitate the formation of both IgG and IgA analogous to that of soluble TMC co-administered with the DT antigen. Moreover, influenza hemagglutinin-loaded TMC (DQ 20%) nanoparticles induced superior immune response compared to intranasal administration of soluble TMC and Influenza antigen (Amidi et al. 2007b).

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Another work reported the formulation of polyelectrolyte complexes (PECs) involving two polysaccharides viz chitosan and dextran. The colloids were either negatively or positively charged depending on the concentration of dextran or chitosan used. Further, interaction of model protein p24 (the capsid protein of HIV-1 virus) was studied with these colloids and it was found that negatively charged colloids had superior binding capacities, rapid kinetics, and improved stability of the adsorbed p24. Results of in vivo subcutaneous administration of antigen with both cationic and anionic antigen-loaded colloids were similar to those found after administration of Freund adjuvanted vaccine (Drogoz et al. 2008).

Zhu et al. demonstrated that chitosan microparticles loaded with tuberculosis subunit antigen “Ag85B-MPT64 (190–198)-Mtb8.4 (AMM)” possessed loading

efficiency of >99%. The particles released the antigen within 16 days in PBS. Subcutaneous administration in mice splenocytes immunized with AMM-loaded chitosan microparticles produced higher levels of IFN-gamma, IgG (H+L), IgG1 and IgG2a compared to free antigens in PBS. Subcutaneous administration in mice splenocytes immunized with AMM-loaded chitosan microspheres produced higher levels of IFN-gamma compared to administration of AMM in PBS upon stimulation with Ag85B and synthetic peptide MPT64 (190–198). The levels of Ag85B-specific IgG (H+L), IgG1 and IgG2a in sera of mice immunized with AMM-loaded chitosan microspheres were also higher than those with AMM in PBS. These results indicate that chitosan microspheres when used as a carrier for fusion protein AMM could elicit strong humoral- and cell-mediated immune responses (Zhu et al. 2007).

Chitosan-Coated Particles for Parenteral and Mucosal Vaccination

Nagamoto et al. prepared chitosan particles (CP) of varying sizes and chitosan-coated emulsions (CCE) by ethanol injection method (Maitani et al. 2001). Further ovalbumin (OVA) and cholera toxin (CT) were adsorbed on the particles, with adsorption efficiency of around 96%. Further these conjugates were analyzed for their immunization in rats by intranasal and intraperitoneal administration. The results show that IgG elicited by intranasal administration of CP was comparable to that produced by intraperitoneal administration. IgA elicited by 0.4 and 1-μm-sized CP was higher as compared to control (OVA and CT). IgA and IgG elicited by intranasal administration of 2 μm size CCE were notably higher compared to control (OVA and CT) (Nagamoto et al. 2004). Another study concluded that chitosan-coated diphtheria toxoid–liposomes exhibited reduced burst release compared to uncoated DT liposomes. Further these particles were administered subcutaneously in mice and DT-loaded liposomes coated with chitosan had improved Th1 and Th2 immune responses compared to uncoated DT liposomes. It was concluded that along with improving mucoadhesivity and immunogenicity of vaccines, chitosan also helped in enhancing the encapsulation efficiency of antigens and regulated release of the antigens (Marón et al. 2007).

Jaganathan and Vyas demonstrated the preparation of chitosan-coated PLGA microparticles loaded with hepatitis B surface protein (HBsAg). When administered by intranasal route, these particles induced similar systematic but more pronounced local and cell-mediated immune responses against HBsAg compared to subcutaneously administered alum-adsorbed HBsAg (Jaganathan and Vyas 2006; Zhao et al. 2006a).

Fischer et al. demonstrated the preparation of chitosan-coated PLGA microparticles and explored them as a tool for ex vivo antigen loading of antigen-presenting cells such as dendritic cells (DCs) to be used as cellular vaccines. Results indicated that both coated and uncoated particles had equal internalization by monocyte-derived DCs (MoDCs) but did not induce their maturation in terms of surface expression of molecules for antigen presentation and T-cell activation (Fischer et al. 2007).

Another study involved the preparation of chitosan-coated (cationic) and uncoated poly-ε-caprolactone (PCL) (anionic) micro- and nanoparticles by double emulsion solvent evaporation technique. These particles were further loaded with streptococcus equi-antigens. The loading capability of both cationic and anionic particles was comparable and on subcutaneous administration strong mix of Th1 and Th2 responses was observed in both cases.

Further it was observed that chitosan-coated PCL particles elicited higher IgG1 and IgG2a antibody titer, higher levels INF-γ and IL-2, and Th1-dependant cytokines whereas PCL particles elicited higher levels of the cytokine IL-6 (Florindo et al. 2008).

7 Biodistribution of Chitosan Particulate System

Biodistribution aspect of chitosan polymer derivatives and its particulate systems is less explored area to give a clear insight of chitosan used as drug carrier systems. It is reported that biodistribution of chitosan systems is affected by all aspects of particulate system from charge and size of the particulate system along with molecular weight and type of chemical modification. Once chitosan particulate system dissociates into free polymer and active drug molecule, chitosan polymer is eliminated from the body either by renal clearance or by biodegradation. At the same time, it is important to enhance prolonged circulation of nanoparticles in body which is largely governed by factors like size, which should be small enough to pass through capillaries of size 7 μm and avoid organ uptake and its interaction with biomolecules within body.

Few studies report biodistribution of technetium-labeled chitosan particulate system in organs after 30 min post-intravenous injection. The chitosan nanoparticulate system of size 100 nm was found highest in liver (24%) followed by spleen (15%), blood (13%), lungs (9%), and kidney and stomach (8%), while 5% in bone and heart. The high activity of radiolabeled chitosan in liver, spleen, blood, and lungs can be accounted for the circulating blood passing through the organs. The study also highlights that chitosan particle system of size 100 nm shows prolonged circulation in blood post 2 h injection of radiolabeled chitosan, which indicates minimum uptake by the cells of reticuloendothelial system (RES). It is postulated that particles escapes RES results due to the presence of same charge on proteins (opsonins) and on the surface of the nanoparticles thus decreasing adsorption (Banerjee et al. 2002). Another study also demonstrates effect type of reducing agent on accumulation of the techniticum-labeled chitosan particulate systems in various organs. The study highlights the use of stannous chloride and sodium borohydride as a reducing agent for preparation radiolabeled chitosan hydrogel nanoparticles. The activity of nanoparticles was found to be highest in RES organs (liver, lungs, and spleen) when treated with stannous chloride, while the lower accumulation was observed in RES organs upon treatment with sodium borohydride as a reducing agent. The possible reason for such results is accounted to the

generation of colloidal tin oxides impurities during the preparation of nanoparticles which remain adhered to chitosan nanoparticle surface results into different biodistribution profiles when injected in body. The chitosan nanoparticle prepared using sodium borohydride does not result into impurities hindering radioactivity of chitosan particulate system, hence give a different biodistribution profile showing increased accumulation in blood, but also displaying high activity in liver (Banerjee et al. 2005).

Glycosylated chitosan a hydrophobic nanoparticulate system of size in the range of 250–300 nm was studied for its distribution in different organs post-intravenous injection. High activity of florescent-labeled nanoparticle was observed in kidney indicating rapid excretion of particles along with high activity in tumor cells. It is postulated that as molecular weight increases from 20 to 250 kDa increase in activity of chitosan nanoparticle is observed in tumor cells (Park et al. 2007). Yet another study confirmed docetaxel-loaded glycolated chitosan nanoparticle showing high activity in tumor cells and prolonged circulation of nanoparticles up to 72 h. Further, it was shown nanoparticle size plays an important role for prolonged circulation in body. The particles of sizes in the range of 150–300 nm were found to be rapidly opsonized by mononuclear phagocytic system (MPS) cells present in liver and spleen, whereas particles of size 200–400 nm were not opsonized by MPS. The possible reason for such difference could be attributed to deformities of nanoparticles undergone once particles interact with blood molecules (Hwang et al. 2008).

8 In Vitro and In Vivo Toxicity

In vivo studies were carried out in test animals, which did show any acute toxicity effect when tested as injectable, eye, and skin test (Rao and Sharma 1997). Few studies have been conducted to evaluate toxicity of chitosan as dietary supplement when administered in human. It was found that a maximum dose of 6.75 g was reported as safe for human consumption as cholesterol treatment (Tapola et al. 2008).

Also when chitosan is used in pharmaceutical formulations, suitable molecular weight should be used which can undergo renal clearance, if not the polymer used should undergo biodegradation by the action of enzymes like lysozymes to yield fragments suitable for renal clearance. It is observed that chitosan-based NPs of molecular weight ranging from 10 to 213 kDa showed cytotoxicity when concentrations increased beyond 0.741 mg/ml was neatly demonstrated on A549 Cells. The study showed that when degree of acetylation increases IC_{50} value increases 1.7-fold. The possible reason for high toxicity with higher degree of deacetylation is attributed to the fact of availability of maximum number of positive amine groups and extended 3-D conformation increases interaction with negative groups of cell membrane surface (Huang et al. 2004).

But unclear cytotoxic profiles of chitosan and chitosan-based NPs are reported. The major factors responsible for varied cytotoxicity profile depend on concentration, polymer molecular weight and on type of chitosan salt used

(Carreño-Gómez and Duncan 1997). It is shown that chitosan hydrochloride showed maximum toxicity compared to other polycationic polymer poly-L-lysine (Kean et al. 2005). Toxicity is found to be dependent on degree of deacetylation and molecular weight (Kean and Thanou 2010). Low molecular chitosan and its derivative TMC were found to be less toxic than high molecular chitosan and its corresponding TMC when tested on MCF-7 cell lines. While similar value of IC50 on was found to be COS-7 for TMC low molecular weight chitosan and high molecular weight chitosan with least degree of trimethylation. As degree of trimethylation increased, cytotoxicity increased linearly. This study also indicates that cytotoxicity varies from cell type to cell type (Kean et al. 2005). Cytotoxicity of chitosan-based nanoparticles can be advantageous when targeted to tumor cells. A study has shown that chitosan has limited cytotoxic effect on normal human cells L-02 when compared to toxic effect on BEL7402, BGC823, and Colo320 tumor cells (Qi et al. 2005). Cytotoxicity is said to increase with net positive charge present on the NP. Hence, a pronounced cytotoxic effect of chitosan can be observed when chitosan is chelated with metal ions like copper (Cu^{2+}). Another aspect to consider for cytotoxic effect of NPs is particle size. It was demonstrated that as particle size decreases cytotoxicity activity increases, due to higher accumulation at tumor cells (Qi et al. 2005).

9 Stability of Chitosan

Currently, chitosan-based products are marketed for applications like hemostatic dressings, preparations for wound healing, and nutraceutical. Despite the great applicability of chitosan, it has found limited market status to be used for biopharmaceutical applications. The major factor responsible is due to poor stability over a period of time, which is mainly affected by strong hygroscopic nature of chitosan and varies depending on various sources from which chitosan is extracted in terms of purity, degree of deacetylation, and molecular weight (Szymańska and Winnicka 2015). In order to reduce physiochemical degradation upon storage of chitosan few scientists have suggested its storage at low temperature (5 °C) under controlled humidity and in addition with stabilizing agents. Stabilizing agents like polyols (glycerol, mannitol, sorbitol, polyethylene glycol, etc.) enhance shelf life of chitosan polymer considerably by providing a protective environment for chitosan's reactive functional groups. Hence, inherent factors like molecular weight, degree of deacetylation and moisture content along with external factors like storage conditions, form of chitosan, thermal processing, and sterilization are crucial factors responsible for the stability of chitosan-based formulations (Szymańska and Winnicka 2015).

Stability of chitosan NPs suspension is also well-affected on charge present on the surface. The higher the positive charge on surface of NPs, the more the repulsion, hence overcome the natural tendency to form aggregates (Qi et al. 2005). When NPs are administered in human body, it comes in contact with enzymes

which degrade polymer. Hence, to maintain stability of chitosan NPs with the human body, degree of deacetylation should be maintained maximum.

10 Conclusion

This chapter highlights the emerging applications of nanotechnology in the delivery of therapeutic molecules delivery, with a special focus on chitosan biopolymer. The interesting structural characteristics of chitosan have been explored by various scientists, which helped us to understand chitosan behavior at different conditions. The presence of different functional groups on chitosan polymer has been extensively explored for manipulating the polymer behavior. Further, chitosan is only cationic polymer found in nature, which is biodegradable and nontoxic in human body. Therefore, chitosan polymer has been explored for nasal, oral, ocular, and colonic drug delivery of both polar and nonpolar drugs. However, due to its high crystallinity, it applications in pharmaceutical field are limited. Chemical modification of chitosan provides derivatives with enhanced solubility and varying hydrophobic and hydrophilic properties. This chapter gives a detailed insight on factors affecting chitosan-mediated delivery of molecules like protein, vaccines, nucleic acids, and polysaccharide moieties. This chapter has briefly mentioned various methods to synthesize chitosan particulate systems and derivatives with specific functional properties. Later, various chitosan derivatives have combinational properties of both targeted delivery drug and simultaneously imagining which have opened new avenues for multipurpose applications. The major properties of chitosan which determine its applicability are its molecular weight and degree of deacetylation. In addition to these properties, investigations suggest that chitosan-based drug delivery systems can be tuned effectively to target diseased cells, without harming normal cells. But it is observed that chemically modified, chitosan-based nanovehicles do not follow a uniform toxicity profile as it may change its structural properties completely, which remain a major point of concern.

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Carboxymethyl Polysaccharide-Based Multiunit Hydrogel Systems for Drug Delivery

Sabyasachi Maiti and Sougata Jana

Abstract

In recent years, natural polymers are being modified chemically to search for new materials for drug delivery applications. Natural polymers possess functional groups like hydroxyl, carboxyl, and amide that make them amenable for various modifications. Chemical modification is desirable to confer smartness to the polymers achieving pH-sensitivity, thermo-responsiveness of the delivery devices for controlled drug release. At the same time, some undesirable physicochemical properties of native polymers are elimination. The design of synthetic polymer-based particulate systems mostly involves organic solvents. However, their use in the design of drug delivery system is questionable in terms of long-term viability due to flammability, health hazards, and stringent governmental regulation. Henceforth, the scientists are involved in preparing drug-loaded particles avoiding the use of organic solvents. The gelling ability of the some native biopolymers as well as modified biopolymers with metallic salts have been utilized to fabricate particulate systems in aqueous environment. Carboxymethylation is an important reaction in offering gelling ability to the native polymers. Furthermore, the graft copolymers, hydrophobic conjugates, and interpenetrating networks have been tested in preparing drug delivery particles. This chapter discusses the carboxymethylation techniques, synthesis of particulate systems, and recent developments in drug delivery applications of some natural polymers including xanthan gum, guar gum, locust bean gum, pullulan, and curdlan.

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1 Introduction

The organic solvent evaporation technique has been extensively employed in the fabrication of synthetic polymer-based particles for drug delivery applications. However, traces of organic solvents in the particulate dosage forms may cause toxicity upon chronic use. Further, the flammability, environmental toxicity, and regulatory issues limit their use in dosage form design (Harris and Ghebre-Sellassie 1989; Halder et al. 2005). Currently, there is a trend to restrict the use of organic solvents in pharmaceutical formulations as low as possible. Due to nontoxic and biodegradable properties, natural polymers are considered more advantageous than synthetic polymers for the design of pharmaceutical dosage forms (Bhardwaj et al. 2000). In last few decades, polysaccharide-based hydrogel particles have been developed through ionic gelation technique and tested for their potential use as drug carriers. The drug can be encapsulated in the polymer particles in an environment-friendly environment by this technique (Patil et al. 2010). Compared to single-unit dosage form, multiunit systems offer a number of potential benefits on chronic dosing. The small size and distribution of the particles helps to avoid delay in gastric emptying and variation in transit rates through the gastrointestinal (GI) tract. Therefore, the drug is released uniformly and consequently, the gastric mucosa is less exposed to a high drug concentration at a time, and mucosal irritation and damage are prevented (Davis et al. 1984; Follonier and Doelkar 1992). In general, the synthesis of polysaccharide microparticles involves metal ion cross-linking with the existing carboxyl functional groups or newly introduced carboxymethyl groups on the polysaccharide backbone (Thimma and Tammishetti 2001; Das and Ng 2010a). The chemical cross-linking of hydroxyl group of the polysaccharide with glutaraldehyde or polyethyleneimine has also been investigated (Setty et al. 2005; Das and Ng 2010b). The dual cross-linking (ionic and chemical) strategies have also been adopted (Gotoh et al. 2004). It is worthy to note that the preparation of hydrogel beads by gelation technique results in low encapsulation efficiency of water-soluble drugs. However, the entrapment efficiency of the hydrogel particles can be improved with the use of drug-resin complex, instead of using free drug.

To tune physicochemical properties, the polysaccharides are graft-copolymerized (Kumar et al. 2009; Malik and Ahuja 2011), oxidized (Tang et al. 2012), thiolated (Kaur et al. 2012), and carboxymethylated (Kumar and Ahuja 2012). Among various approaches, the carboxymethylation is usually preferred due to easy synthesis procedure and involvement of low cost reagents. The aqueous solubility and gelling property of carboxymethyl polysaccharides have been investigated earlier.

Besides the ability to retard drug release, carboxymethylation can modify the physicochemical properties of the polysaccharides like xanthan gum, guar gum, locust bean gum, pullulan, and curdlan. Xanthan gum is an exopolysaccharide produced by the bacterium *Xanthomonas campestris*. It is composed of two glucose and mannose units and one glucuronic acid unit with variable *O*-acetyl and pyruvate content (Becker et al. 1998; García-Ochoa et al. 2000). Nonionic guar gum is isolated from the endosperm of *cyamopsis tetragonoloba* bean. It has a linear backbone of β-1,4 linked D-mannopyranosyl units with α-1,6 linked D-galactopyranosyl residues as pendent chains (Hoffman and Svensson, 1978; McCleary et al. 1985). The mannose to galactose ratio typically ranges between 1.6 and 1.8 (Cheng et al. 2002). Locust bean polysaccharide (LBP) is extracted from the seeds of *Ceratonia siliqua* tree. It consists of α-1,4-linked β-D-mannopyranose backbone with branch points from their 6-positions linked to α-D-galactose (1,6-linked α-D-galactopyranose) (Dea and Morrison 1975). Pullulan is produced by *Aureobasidium pullulans* (Shingel 2004). Its linear chains are formed from α-1,4-linked glucose units, which are included in α-1,6-linked maltotriose units. Curdlan, is a naturally occurring insoluble polysaccharide composed of 1, 3-β-linked D-glucose units produced by a strain of *Alcaligenes faecalis* (Harada et al. 1993).

The hydrogel network of carboxymethyl polysaccharides can swell in biological fluids and release the drug for prolong period in simulated gastrointestinal fluids. Earlier studies demonstrated that the particulate dosage forms based on hydrophilic polysaccharides could control oral release of various bioactive molecules. Carboxymethyl derivative of hydrophilic biopolymers like guar gum, locust bean gum, pullulan, xanthan gum, and to a lesser extent curdlan gum have been found to form hydrogel beads in presence of di- and trivalent metal ions. Sometimes, single biopolymer cannot impart desirable physicochemical and extended drug release properties to the hydrogel particles. Recently, the formation of an interpenetrating network (IPN) represents an interesting strategy to develop particulate drug delivery systems. In IPN composite network structures, at least one polymer network is cross-linked independently in presence of the other. An IPN combination of guar gum (Soppimath et al. 2000), and xanthan gum (Ray et al. 2010b) with poly (vinyl alcohol) has been reported for a variety of drugs. However, the design of IPN using two carbohydrate polymers containing carboxyl groups is least studied in the field of drug delivery. The key features of one component are conferred in the IPN structures without hampering critical attributes of the other. It is possible that the IPNs may exhibit entirely new properties, not pertaining to either of the polymer networks (Myung et al. 2008). The mechanical strength can be improved after the formation of IPNs using homopolymer networks (Zhang and Peppas 2000). Moreover, interpenetration of the networks creates void spaces that can accommodate drug molecules (Kulkarni et al. 2001). The electrostatic repulsive or attractive forces generated by ionizable groups on the polymer chain determine the degree of swelling of the hydrogels in simulated GI fluids. Depending on pH, ionic strength, and type of counter ions in the medium, the hydrogels either swells or

shrinks. In acidic medium, the weakly acidic carboxylic groups are not ionized and therefore shrunk; however, the hydrogel swells in basic pH due to electrostatic repulsive forces created by ionized carboxylic functional groups. Henceforth, the drug release is expected to be minimal at low pH of stomach, and relatively high at the pH of intestinal tract. This smart behavior of hydrogels is important in controlling the drug release rate at variable pH environments of GI tract. Thus, the ionic nature of the hydrogels is crucial in drug delivery application. For example, anionic hydrogels can be used for site-specific delivery of therapeutic proteins to the large intestine for prolonged biological activity (Satish et al. 2006), while cationic hydrogels can be useful for stomach-specific drug delivery for the treatment of *Helicobacter pylori* infections (Patel and Amiji 1996). Therefore, the formation of IPN hydrogel appears to be a better approach in controlling drug delivery (Changez et al. 2003). In such systems, the extent of cross-linking dictates the extent of drug release (Kulkarni et al. 2001; Rokhade et al. 2006).

Nanosized delivery systems (10–1000 nm) are more advantageous than microparticles in that they demonstrate higher intracellular uptake and easily penetrate blood–brain barrier. In addition, they can target the desired organs or tissues following systemic administration without blockade of fine capillaries. These properties are beneficial in targeting nanoparticles to cancer cells through enhanced permeability and retention (EPR) effect (Maeda et al. 2000). The nanoparticles can respond to pH or temperature and accordingly release the bioactive molecules and improve the bioavailability of drugs with significant reduction of side effects.

The grafting of synthetic polymers onto polysaccharides offers another possibility to tailor the properties of polysaccharide-based drug delivery particles. In a graft copolymer, the synthetic polymer or one/more species of monomers is covalently attached to the main polysaccharide backbone as pendant chains (Zohuriaan-Mehr 2005). During graft copolymerization, free radical sites are generated on the polymer backbone, and the monomers are then added up through the chain propagation process.

The purpose of this chapter is to provide detailed information on some carboxymethyl polysaccharide-based particles reported so far for controlled drug delivery applications. The carboxymethylation procedure and the fabrication of particulate systems have also been discussed.

2 Carboxymethylation of Polysaccharides

2.1 Dry Method

In this method, known samples of galactomannan and finely powdered sodium bicarbonate ($NaHCO_3$) are mixed, and the surface is wetted with trace amount of ethanol. Monochloroacetic acid (MCA) is then added to the mixture (Parvathy et al. 2005). The reaction can be carried out at ambient or elevated temperature for about 2 h. The reagents are intermittently mixed, neutralized with the use of dilute acetic

acid, and the salts formed are then removed by repeated ethanol washing and solvent exchange drying. By varying the ratio of catalyst and the reagent, CM derivatives of differing degree of substitution (DS) can be obtained. The surface wetting step can be omitted during carboxymethylation.

The solution viscosity and degree of carboxymethyl substitution are found to decrease at higher base concentration (Parvathy et al. 2005). The viscosity of CM guar is reduced as the temperature and catalyst concentration is increased. A maximum DS value of 0.675 is obtained at 80 °C for guar gum and then decreased. However, in case of tara gum, the DS value (0.098) increased to 0.231 at 98 °C. MCA concentration has no significant effect on the DS, but retains viscosity considerably. No appreciable improvement is noticed in DS or solution viscosity at higher reaction time (>2 h).

2.2 Wet Method

This method of carboxymethylation is illustrated citing locust bean gum (LBG) as an example (Setty 2005). LBG (2%) is dispersed in cold water containing 3% sodium hydroxide. An aqueous solution of monochloroacetic acid (1.5%) is slowly added and the temperature of mixture is maintained at 15–18 °C. The temperature is gradually raised to 60–65 °C and stirred for 1 h. The derivatized product is washed with 80% methanol and the suspension is adjusted to pH7.0. Finally, the suspension is filtered and the residues are dried.

Maiti et al. (2010) slightly modified the procedure. They washed LBG with methanol to remove the organic impurities and dried the same before starting the carboxymethylation reaction. Because LBG is not hydrated at low temperature, they heated the washed and dried LBG powder in presence of water at 80 °C for 15 min and cooled. Then, a solution of NaOH in ice-cold water was added and the rest of the procedure remained same.

However, the etherification reaction may cause nonspecific degradation at elevated temperature by β-elimination and/or peeling reaction at the reducing sugar unit at alkaline pH (Whistler and BeMiller 1958). Thus, the molecular weight and solution viscosity of derivatized material are reduced. For example, guar gum undergoes degradation to saccharinic acids in alkaline solution (Whistler and BeMiller 1958).

3 Preparation of Hydrogel Particles

3.1 Nanoprecipitation and Dialysis Cross-Linking

This process was followed by Soumya et al. (2010) for the preparation of guar gum (GG) nanoparticles. GG (1%) was hydrolyzed in presence of mannanase (0.1216 units/mg) at pH5.2 and incubated at 30 °C for 24 h. The hydrolyzed

suspension of GG was sonicated for 10 min and then filtered. The nonsolvent was added to an aqueous mixture of depolymerized GG solution, surfactant, and cross-linker to cause nanoprecipitation of hydrolyzed GG. For dialysis cross-linking, a mixture of GG hydrolysate and Triton X-100 in dimethylsulphoxide (DMSO) was dialyzed against 0.1% tripolyphosphate (TPP) or borate solution for 24 h. A sequentially mixing of hydrolyzed gum, lipase PS, Triton X-100, polyethyleneimine, isopropyl alcohol, TPP or boric acid, and glutaraldehyde was required for the enzyme functionalization of the nanoparticles. The vortexing and sonication of the solution at room temperature allowed the formation of uniform nanoparticles.

3.2 Jeffamine/ POX Cross-Linking

Before the addition of cross-linker, carboxymethyl galactomanan is oxidized according to the procedure described by Bruneel and Schacht (1993). For example, 4% carboxymethyl pullulan (CMP) was oxidized in presence of 0.4% potassium iodate (KIO_4) for 24 h under dark at room temperature. The oxidation reaction was quenched with the addition of trace amount of ethylene glycol. The oxidized product was purified by dialysis and lyophilized. Then, Jeffamine was reacted with oxidized CMP solution (pH 10) for 24 h at room temperature. Excess sodium borohydride was used to reduce imine bonds to amine groups (Mocanu et al. 2014). CMP in hydrogen form was swollen in DMSO and dispersed in paraffin oil containing Span 85. After 30 min, *N, N*-dicyclohexyl carbodiimide (DCCI) was added and the reaction was continued for 2 h. Then, Jeffamine ED-2003 and dimethylaminopyridine were reacted for 48 h at room temperature. After isolation by filtration, the hydrogel microparticles were washed with acetone to remove the unreacted Jeffamine and dicyclohexylurea byproducts. The dried hydrogel microparticles ranged between 20 and 100 μm (Mocanu et al. 2012). Similarly, poloxamer (POX) was substituted by Mocanu et al. (2011). The possible chemical structures of cross-linked structures were depicted in Figs. 1 and 2.

3.3 Hydrophobization of Carboxymethyl Derivative

The carboxymethyl polysaccharides can be cross-linked with either trimethaphosphate (TMP) or epichlorohydrine in organic suspension media (Mocanu et al. 2002). To an organic suspension medium, 25% alkaline CMP solution was dispersed and epichlorohydrine or TMP was added as 50% solution. After a reaction period of 24 h at 50 °C, the microspheres were filtered, washed, and dehydrated from ethanol. The cross-linked dry CMP microspheres were hydrophobized by reacting with palmitoyl chloride in DMF-pyridine system for 5 h. Finally, the microspheres were dried from methanol (Mocanu et al. 2004).

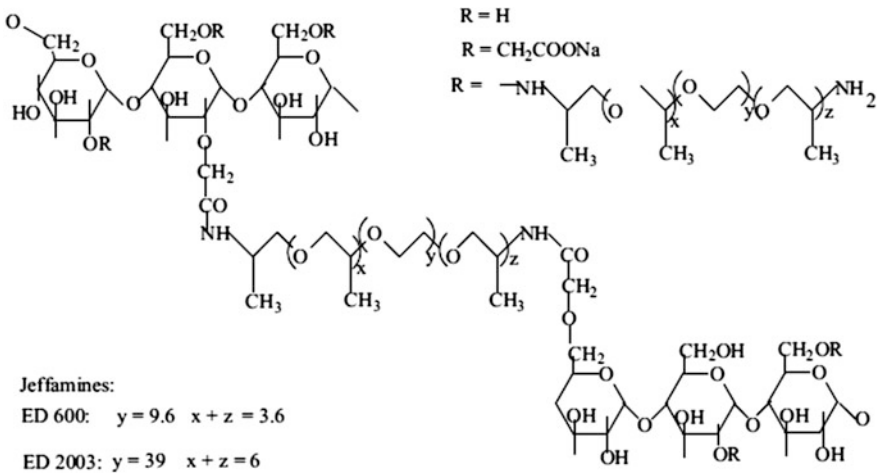

Fig. 1 Possible structure of Jeffamine cross-linked carboxymethyl pullulan hydrogel microparticles (Mocanu et al. 2012)

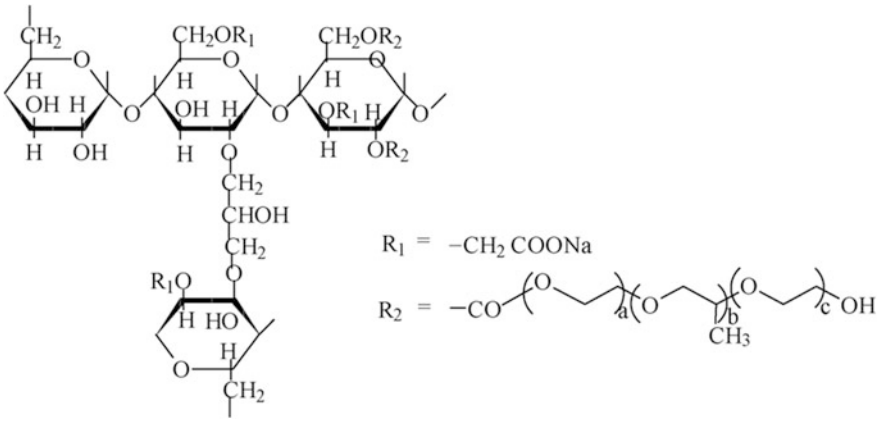

Fig. 2 Chemical structure of POX-substituted carboxymethyl pullulan microparticles and their synthesized functional derivatives (Mocanu et al. 2011)

3.4 Ionotropic Gelation Method

In this method, either a single carboxymethyl polymer or a blend of carboxyl group containing polymers (IPN) is used to load drug in aqueous environment. The polymer–drug dispersion is added drop wise through flat-tipped hypodermic needle into aqueous metallic salt solution such as $CaCl_2$, $AlCl_3$, or $BaCl_2$. Following incubation, the beads were filtered, washed with water and dried. This method was

mostly followed by researchers to design hydrogel beads. Carboxymethyl LBG–sodium carboxymethyl cellulose blend microbeads have been prepared by Bhattacharya et al. (2012).

3.5 Emulsion Cross-Linking

The water-in-oil (w/o) emulsion cross-linking method has been described earlier (Ray et al. 2010a; Banerjee et al. 2012). They reported the preparation of polysaccharide-poly (vinyl alcohol) (PVA) IPN hydrogel microspheres. First, PVA is dissolved in water at 80 °C. After that, carboxymethyl polysaccharide was dispersed in PVA solution. Then, ethanol solution of drug was added to the polymer mixture in order to obtain homogeneous drug dispersion. The drug dispersion was then added slowly to liquid paraffin containing Span 80 under mechanical agitation to produce w/o emulsion. The microspheres were cross-linked by glutaraldehyde (GA), washed to remove excess paraffin oils, GA, and Spans. The microspheres were finally dried at 40 °C.

3.6 Surfactant-Facilitated Reticulation

This method was followed by Maiti et al. (2014) for the synthesis of carboxymethyl locust bean gum (LBG) nanoparticles. First, the drug was dissolved in 1% (w/v) carboxymethyl polymer solution in water. After the addition of 2% Span 80, 5 ml 2.5% (w/v) $AlCl_3$ solution was slowly dropped into this solution and homogenized at 5000 rpm for 15 min. The nanoparticles were recovered by centrifugation at 8000 rpm. The supernatant liquid was decanted from the tube and the precipitate at the bottom of the tube is washed with a small volume of chloroform (1 × 10 ml) to remove residual surfactant and air-dried.

3.7 Simultaneous or Sequential Cross-Linking

These methods were adopted by Mitra et al. (2015) for loading of drug–resin complex into carboxymethyl xanthan hydrogel beads. In simultaneous method, the resinate-loaded aqueous gum solution droplets were cross-linked in a mixture of aluminum chloride ($AlCl_3$) and GA solution. In sequential method, the polymer droplets were ionically cross-linked, and then the dried hydrogel beads were further cross-linked with GA solution. After a definite cross-linking period, the hydrogel beads were repeatedly washed with water and dried.

4 Drug Delivery Applications

4.1 Hydrogel Particles of Single CM-Biopolymer

4.1.1 Xanthan Gum

Carboxymethyl xanthan gum microparticles (MPs) were synthesized by Maiti et al. (2007) by Al^{3+} ion induced gelation method for oral delivery of proteins. As high as 82% bovine serum albumin (BSA) was entrapped into the MPs. Higher swelling of the MPs in acidic medium caused the release of a higher amount of protein than that in alkaline medium. The pH of gum solution influenced the swelling behavior and protein release characteristics to a considerable extent. A maximum degree of swelling was observed for the MPs when prepared with a gum solution of pH 8.0. Since the solubility of BSA is lowest at isoelectric pH 4.7 and bears positive charge at pH < 4.7; the anionic polymer ionically interacted with the protein molecules at pH 4.0 and 5.0 and caused phase separation. Hence, the gum solution was set at pH 6.0 and 7.0 for effective BSA loading. In another study, the gelled microparticles were further coated with 0.5% carboxymethyl xanthan or alginate solution (Maiti et al. 2009). The strength of $AlCl_3$ affected BSA entrapment efficiency of the uncoated MPs. Higher strength of ionic cross-linker (>1%) decreased the entrapment efficiency from 86–61%. BSA entrapment efficiency (78–79%) dropped for the coated MPs than uncoated ones. Almost 50% BSA was released from the uncoated microparticles in NaCl-HCl buffer solution (pH 1.2) in 2 h. On contrary, the coated MPs prevented a substantial amount of entrapped protein from release in acidic medium. Further, the alginate- and xanthan-coated particles prolonged the duration of BSA release in alkaline medium (pH 7.4) up to 10 and 12 h, respectively. They further ensured that the integrity of BSA was not affected after incorporation into the carboxymethyl xanthan particles. Ray et al. (2008) developed a multiunit sustained release dosage form wherein diltiazem–resin complex was incorporated into Al^{3+} ions cross-linked carboxymethyl xanthan beads. Gelation period, $AlCl_3$ strength, and gum concentration influenced the drug entrapment efficiency. Higher gelation period and cross-linker concentration reduced the percentage of drug entrapment in the MPs. However, the effect reversed at higher gum concentration. The drug displacement from the resinate by the Al^{+3} ions was found responsible for variable drug entrapment efficiency of the MPs. The drug release was dependent on the pH of dissolution media. The particles released 75–82% drug in 2 h in simulated gastric fluid and 75–98% content in 5 h in simulated intestinal fluid. Recently, Mitra et al. (2015) slightly modified the synthesis process in developing diltiazem–resin complex loaded carboxymethyl xanthan particles to achieve higher drug entrapment efficiency and extended release profile. The sequential (SEQ) cross-linking produced smaller beads with desirable drug entrapment efficiency and release characteristics compared to ionic cross-linking and simultaneous methods. Increasing the concentration of covalent cross-linker and cross-linking time gradually decreased the drug release rate. Thus, the

sequential method was better than other methods in producing hydrogel particles for controlled drug delivery applications.

4.1.2 Guar Gum

Reddy and Tammishetti (2002) reported that Ca^{+2}- and Ba^{+2}-carboxymethyl guar gum (CMGG) beads could encapsulate sensitive drugs like proteins. Ba^{+2} cross-linked CMGG beads were much more efficient in retarding drug release in NaCl-HCl buffer (pH 1.2). The beads released almost the entire encapsulated bovine serum albumin in TRIS-HCl buffer (pH 7.4). They indicated that Ba^{+2} cross-linked CMGG beads were suitable for gastrointestinal drug delivery. They further emphasized that trivalent $A1^{+3}$ and Fe^{+3} ions were superior to divalent metals for microencapsulation of acid-sensitive drugs (Reddy and Tammishetti 2002). The maximum retention of bovine serum albumin was obtained for trivalent ions at concentration much lower than divalent metal ions. Relative to divalent ions, trivalent metal cross-linked microbeads liberated the protein over a prolonged period in enzyme-free simulated intestinal fluids. Thimma and Tammishetti (2003) studied complex coacervation of gelatin with sodium CMGG as a function of pH. An effective coacervation was realized over the pH range of 2.5–4.0. They reported that CMGG/gelatin system successfully encapsulated clove oil and sulphamethoxazole. Sullad et al. (2011) loaded abacavir sulfate into GG and CMGG microspheres by w/o emulsion method. The drug release profiles in simulated conditions of stomach (pH 1.2) and intestine (pH 7.4) notably differed. Both GG and CMGG matrices were able to retard the drug release up to 28 h, but the rapid release of drug from GG matrices was reduced after carboxymethylation. Soumya et al. (2010) prepared guar gum particles by nanoprecipitation and dialysis methods. The smallest nanoparticles (NPs) were obtained with the use of isopropyl alcohol as nonsolvent. The dielectric constant of nonsolvent decided the stability of the NPs. A dielectric constant of 18.3 was optimum for the production of nanosized particles (<100 nm). The phosphodiester bonds were formed between two polysaccharide chains as a consequence of the cross-linking reaction between TPP and hydroxyl functional groups of the sugar units. On contrary, boric acid specifically cross-linked cis-diol groups of the mannose sugar units. The stable, smallest size ($\sim$32 nm), and narrow size distribution (polydispersity index 0.491) was evident with the use of boric acid cross-linker. TPP cross-linking resulted in particles of $\sim$50 nm size with a polydispersity index of greater than 0.8. Upon lipase conjugation, the particle size reduced to 25 nm with a more narrow size distribution (polydispersity index 0.129). The surfactants-Aerosol OT (AOT) and Span 60 did not produce stable NPs. A triphasic release pattern was reported for the NPs. An initial faster release up to 24 h was followed by a very slow process reaching a plateau level beyond 75 h. The delivery efficacy of NPs was further evaluated by the release of crystal violet. The dye was completely depleted in $\sim$24 h and therefore, the guar gum particles showed their potential for biosensing and drug delivery applications.

4.1.3 Locust Bean Gum (LBG)

The gelling ability of carboxymethyl LBG toward aluminum ions was explored for the development of hydrogel particles to entrap an antidiabetic drug by ionic gelation process (Maiti et al. 2010). No appreciable changes were noticed in the drug entrapment efficiency of the particles as a function of gelling ion concentration. Lower degree of swelling corroborated with the slower drug release profiles in KCl-HCl buffer (pH 1.2). However, the swelling and drug release tendency was opposite in phosphate buffer (pH 7.4). Regardless of the pH of dissolution fluids, the particles prepared at higher aluminum concentration, released the drug gradually up to 10 h from without being disintegrated. In vivo hypoglycemic action of the glipizide-loaded particles was encouraging. These quality attributes supported the potential application of carboxymethyl LBG beads for controlled oral drug delivery application. Maiti et al. (2014) prepared nanoscale etherified locust bean polysaccharide (ELBP) particles (43–197 nm) containing lamivudine by surfactant assisted homogenization-reticulation technique. The variation in $AlCl_3$ strength and drug: polymer mass ratio affected the properties of the nanoreticulations. Only 44% drug entrapment efficiency was achieved. In simulated intestinal fluid (pH 7.4), the drug release inversely correlated to the strength of $AlCl_3$; however followed a proportional relationship with the drug: polymer ratio. Thermal analysis and X-ray studies confirmed amorphous nature of drug following nanoreticulations.

4.1.4 Pullulan

Mocanu et al. (2004) synthesized and characterized palmitoyl carboxymethyl pullulan microspheres. The hydroxyl groups of the polysaccharide were esterified with palmitoyl groups. The interaction of carboxymethyl pullulan with TPP generated 0.05–0.20-mm-size microspheres. Higher carboxymethyl substitution assisted in greater adsorption of lysozyme. Cross-linked pullulan without negative charges could physically entrap a least amount of lysozyme. They realized that both anionic and hydrophobic groups could improve the adsorption properties of bioactive molecules on the same support. The release of lysozyme was dependent on anionic and hydrophobic functionalities. They further studied the interaction between 3-(glycid oxypropyl) trimethoxysilane)-cross-linked carboxymethyl pullulan microparticles and biologically active molecules, such as enzymes (lysozyme) and drugs (propranolol, quinidine) (Mocanu et al. 2008). The ability to retain bioactive compounds and in vitro release behavior supported potential applications of the microparticles in the field of controlled drug delivery and enzyme immobilization. Despite high hydrophilicity, the CMP microparticles retained small amount of tetanus anatoxin (AT) inside the polysaccharide network than poloxamer (POX) derivatives (Mocanu et al. 2011). Compared to the electrostatic interaction, the hydrophobic interactions occurred preponderantly in the retention of antigen. Despite a remarkable difference in degree of swelling of hydrogels, the release rate did not differ considerably at different pH values. More substitution of hydrophobic POX units prohibited the AT release kinetics of the hydrogels. POX-substitution on the hydrogel assisted in controlling AT release in acidic and alkaline media. The release of protein from CMP was almost independent on the temperature. The slow

and fast release rate respectively in acidic and alkaline medium suggested oral controlled release application of the hydrogels. Mocanu et al. (2014) prepared thermo-associative amphiphilic nanoparticles of 159–230 nm size by cross-linking oxidized carboxymethyl pullulan with difunctional Jeffamines: ED-600 and ED-2003. More pronounced thermosensitive properties were observed for ED-2003 cross-linked nanoparticles. Jeffamines are polyoxyalkyleneamines (polyethylene oxide [PEO], polypropylene oxide [PPO]) with thermo-associative properties (Belbekhouche et al. 2011, 2013). The amphiphilic polysaccharide nanoparticles were loaded with diclofenac. Less cross-linked NPs released the drug at a rate faster than highly cross-linked NPs at double molar ratio of Jeff/glucopyranose. An initial rapid release of about 18–32% drug was associated with easy desorption of drug from the surface of NPs. Thereafter, the drug release occurred in sustained fashion. The NPs also exhibited thermosensitive properties and showed promise as smart drug delivery systems.

4.2 Interpenetrating Particles of Dual Biopolymers

4.2.1 Xanthan Gum IPNs

The use of nonsteroidal anti-inflammatory drugs (NSAIDs) for the treatment of rheumatoid arthritis and osteoarthritis is usually associated with side effects such as gastric and duodenal ulceration, perforation, and gastrointestinal bleeding. The development of controlled delivery system could reduce the dose-related side effects of this kind of drugs. Ray et al. (2010a) evaluated the ulcerogenic and anti-inflammatory effects of ibuprofen (IBP)-loaded IPN hydrogel beads in male albino Wistar rats. The beads were prepared by cross-linking CMXG and ALG with aluminum chloride cross-linker. The ulcerogenicity decreased significantly after encapsulation of IBP into the beads. The anti-inflammatory activity of IPN beads lasted for a longer duration. Kundu et al. (2012) showed that aceclofenac-loaded hydrogel beads could prevent the release of a substantial amount of drug in stomach and complete the depletion of the same in small intestine in slow and sustained manner. IPN beads of carboxymethyl xanthan gum (CMXG) and carboxymethyl cellulose were prepared by the same procedure. The drug delivery properties of the IPN beads were influenced by the mass ratio of polymers, concentration of total polymer, drug, and $AlCl_3$. The beads liberated only 14% drug in acid solution, however provided controlled release in phosphate buffer solution (pH 6.8). IPN hydrogel beads of pectin and CMXG were prepared by Al^{+3} induced gelation and covalent cross-linking method for sustained delivery of diltiazem hydrochloride (Giri et al. 2013). The carboxyl groups of polysaccharides underwent ionization and an osmotic pressure was built up inside the hydrogel matrices resulting in faster swelling and drug release at higher pH.

4.2.2 Guar Gum and Other Polysaccharides

Bajpai and Sharma (2006) reported that barium ions cross-linked sodium alginate/CMGG bipolymeric beads swelled in simulated gastric fluid (SGF) in 3 h.

Following transfer of the hydrogel beads into simulated intestinal fluid (SIF), the swelling degree was almost doubled. The total release of Vitamin B12 in SGF was nearly 20% in 3 h and about 70% in SIF in next 7 h. The vitamin entrapment reached to ~50% for the beads cross-linked with 5–6% (w/v) $BaCl_2$. Phadke et al. (2014) blended CMGG with gelatin to obtain semi-IPN microspheres by w/o emulsion method. They found that 56–74% theophylline was encapsulated in the CMGG/gelatin interpenetrating network, which gradually emptied their content over 26 h.

4.2.3 Locust Bean Gum and Others

Kaity and Ghosh (2013) utilized carboxymethyl LBG and poly (vinyl alcohol) to design IPN microspheres for controlled release of buflomedil hydrochloride. Acute oral toxicity and biodegradability data suggested that carboxymethyl LBG was safe for the fabrication of oral drug delivery system. The microspheres could retard the drug release up to 12 h in vitro. IPN microparticles of carboxymethyl LBG and sodium carboxymethyl cellulose (SCMC) containing diclofenac sodium were prepared by Al^{+3} mediated gelation process (Bhattacharya et al. 2012). The drug was stable without any evidence of chemical interaction but transformed into amorphous state after encapsulation. The mean drug entrapment efficiency was 98% at 1:2 ratio of modified LBG: SCMC for 30 min gelation period; but the same was about 97% after 1 h of gelation. It decreased to 85% with the increase in $AlCl_3$ strength from 2.0 to 4.0%. Higher metal ion strength reduced drug release rate in simulated gastric and intestinal fluids. The drug release profiles were depicted in Fig. 3.

The amount of drug release was considerably less in acidic medium than in alkaline media. The drug release extended up to 8 h in phosphate buffer solution. On many instances, homopolymer networks alone failed to restrict the drug delivery rate. Dey et al. (2013a) tested IPN microspheres of alginate (ALG) and carboxymethyl LBG for glipizide release. IPN particles entrapped 94% drug in their structures and suppressed the drug release for 8 h. Al^{+3}-ALG particles released

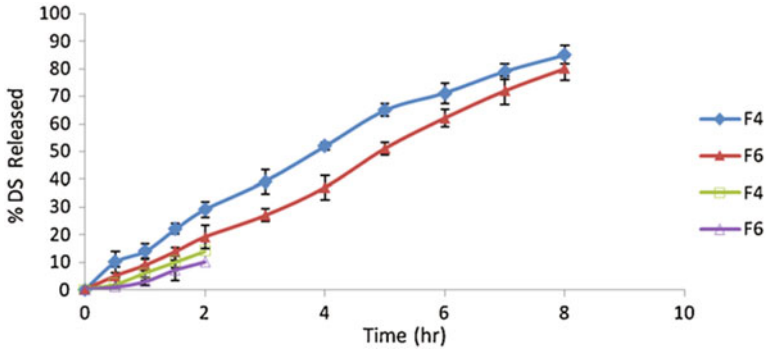

Fig. 3 Drug release profiles in acidic solution (*open symbols*) and phosphate buffer solution (*closed symbols*) from IPN microbeads prepared as a function of $AlCl_3$ at fixed ratio of LBG–cellulose (1:2) and gelation time (1 h). Key: $AlCl_3$ 2% (w/v) (F4) and 4% (w/v) (F6) (Bhattacharya et al. 2012)

almost entire content in 3.5 h. Like the IPNs, Al^{+3}-carboxymethyl LBG particles also discharged their load slowly and uniformly up to 8 h. The drug release rate was always faster in phosphate buffer (pH 7.4) than in acidic solution. The beads swelled depending upon the pH and released the drug accordingly. IPNs had greater mechanical strength (63.79 MPa) than Al^{3+}-carboxymethyl LBG network. The study revealed that both Al^{3+}-CMLBG and IPNs could control diabetes over longer periods. In another study, they reticulated ALG and carboxymethyl LBG in presence of $AlCl_3$. IPN hydrogels encapsulated about 93–98% glipizide (Dey et al. 2013b). The pH-dependent swelling of IPN regulated the drug release rate over 8 h. IPNs exhibited significant control over blood glucose levels on male Wistar rats over 10 h. Dey et al. (2015) studied the effect of hydrogelation period on drug entrapment efficiency and glipizide release from carboxymethyl LBG and ALG IPN beads. The longer gelation period caused 8% loss of the drug and affected their drug release performance of IPN beads. The IPNs cured for 0.5 h exhibited slower drug release kinetics in HCl (pH 1.2) and phosphate buffer (pH 7.4) solution than those incubated for longer periods. The beads had the capability of releasing the drug for a minimum period of 8 h in accordance with the pH-dependent swelling propensity of the IPNs. Henceforth, the gelation period must be optimized in order to have a control over the delivery of drug. They suggested low gelation period for the successful entrapment and release of the drug.

4.2.4 Pullulan and Others

CMP and poly (N-isopropylacrylamide) (PNIPAAm) IPNs responded to changes in temperature and pH reversibly (Asmarandei et al. 2013). At low pH and <VPTT (volume phase transition temperature), the IPNs swelled to a lesser extent than pure PNIPAAm because the protonated carboxyl groups formed hydrogen bonds with other carboxyl or PNIPAAm amide groups. Conversely, the swelling ratio of IPNs was higher in phosphate buffer solution (pH 7.4). The carboxylic groups of CMP were ionized, the hydrogen bonds dissociated, and the hydrogel network expanded due to electrostatic repulsions. The release of diphenhydramine (DPH) was higher at >pH10 buffer solution at 37 °C. In pseudo-physiological fluids, the hydrogel released DPH more quickly at 20 °C than that observed at 37 °C.

Cevher et al. (2015a) tested potential of chitosan/pullulan composite nanoparticles for the nasal delivery of diphtheria toxoid (DT). The antigen-loaded composites were prepared by polyion complexation of *N*-trimethyl chitosan shloride (TMC), chitosan chloride (C-Cl), and chitosan glutamate (CG) with carboxymethyl pullulan (CMP) having particle size of 239–405 nm. Their immunological effects were studied after intranasal administration to mice. Composite nanoparticles induced higher levels of IgG responses than particles formed with chitosan derivative and antigen. Nasally administered TMC–pullulan composites showed higher DT serum IgG titre when compared with the other composites. TMC/pullulan composite nanoparticles induced highest cytokine levels compared to those of chitosan salts. These findings demonstrated that TMC-CMP-DT composite nanoparticles were promising delivery system for nasal vaccination. They further demonstrated that a mass ratio of 2:1 for TMC-CMP combination produced

stable nanocarriers (Cevher et al. 2015b). For CCl-CMP and CG-CMP formulations, a mass ratio of 3:1 was needed. Loading efficiency was >90% for all nanoparticles (207–603 nm). SDS-PAGE integrity of the model antigen was also demonstrated. MTT studies showed that nanocomposites were less toxic to Calu-3 cells than the particles of cationic polymers alone. FITC-labeled BSA-loaded nanoparticles were efficiently taken up by J774A.1 macrophages as was confirmed by confocal microscopy, thus highlighting the potential of these nanocarriers for nasal vaccination.

4.3 Grafted CM-Polysaccharide Hydrogel Particles

4.3.1 Xanthan Gum-Based Grafting

Badwaik et al. (2016) described the synthesis of graft copolymer of carboxymethyl xanthan gum using acrylamide monomers. They reported only thermal stability of the copolymers. However, they did not exploit the potential of this copolymer as a carrier for drugs. Some reports on native xanthan gum-based copolymer are available. Kulkarni and Sa (2008a) prepared smart IPN hydrogels of polyacrylamide-grafted-xanthan (PAAm-g-XG) and SCMC for oral delivery of ketoprofen. The hydrogel beads were sensitive to pH in terms of swelling and drug release characteristics. In another report, they indicated that pH-sensitive hydrolyzed PAAm-g-XG copolymer beads resisted the exposure of a significant amount of drug to gastric mucosa. Stomach histopathology of albino rats revealed that the drug-loaded beads could diminish gastric side effects such as ulceration, hemorrhage, and erosion of gastric mucosa (Kulkarni and Sa 2008b). Maiti and Mukherjee (2014) reported that hexadecyl xanthan copolymer self-associated into nanoscale core–shell structures, which accommodated entire glibenclamide into their cores and slowly discharged the content in phosphate buffer solution (pH 6.8). They incorporated drug-loaded copolymer particles into carboxymethyl xanthan hydrogels for further lowering of drug release rate in simulated gastrointestinal fluids. The drug was released in accordance with the swelling degree of the hydrogel particles. The formulations showed immense potential in regulating blood glucose level in animal model for a longer period. Thus, the entrapment of drug-loaded copolymer micelles into hydrogel particles presented a different strategy for the management of diabetes.

4.3.2 Guar Gum Grafting

Adhikary et al. (2011) grafted polyacrylamide onto carboxymethyl guar gum (CMGG) backbone using ceric-ion induced solution polymerization techniques and examined the flocculation efficiency. The graft copolymer exhibited better flocculation performance than its non-grafted counterparts. Pala et al. (2011) used redox systems and microwave energy for the synthesis polyacrylamide-grafted CMGG copolymer. They investigated flocculation characteristics of modified and non-modified polysaccharides in kaolin suspension. Microwave-assisted CMGG copolymer demonstrated best flocculation characteristics. Gupta et al. (2015) synthesized new thermo-associating nanopolymer particles. The copolymer consisted

of amino-terminated poly (ethylene oxide-co-propylene oxide) (PEPO) and CMGG. EDC/NHS-mediated coupling reaction between NH_2 groups of PEPO and COOH groups of CMGG yield the copolymer. The pyrene intensity ratio (I_1/I_3) decreased at 40 °C indicating the formation of hydrophobic microdomains in CMGG-g-PEPO copolymer. They speculated that PEPO-rich phase was formed above transition temperature of the grafted chains, and pyrene was solubilized in polymer-rich hydrophobic microenvironment. Based on sharpness and amplitude of the transition, the hydrophobic domain was found weaker in case of graft copolymer than PEPO homopolymer. They further prepared silver nanoparticles (AgNPs) using this thermo-associating CMGG-g-PEPO copolymer (Gupta et al. 2014). The polymer acted as reducing agent and stabilizer. They evaluated the potential of silver nanoparticles in monitoring the release of an anticancer drug doxorubicin hydrochloride (DOX). DOX binding onto copolymer and NPs indicated the involvement of charge-transfer mechanism between DOX and polymer that united both the entities.

4.3.3 LBG Grafting

To the best of our knowledge, carboxymethyl LBG-based graft copolymer nanoparticles are not reported so far. However, native LBG has been used for graft copolymerization. Giri and coworkers (2015) applied microwave energy and potassium persulphate initiator for the synthesis of graft copolymer of locust bean gum (LBG) and acrylamide. The data indicated that the graft copolymer had better flocculation properties than native polymer. The graft copolymer did not manifest any toxic symptoms following administration of single oral dose and all mice survived beyond an observation period of 14 days. The same group prepared pH-sensitive hydrogel beads of hydrolyzed polyacrylamide grafted LBG and carboxymethyl cellulose (CMC) by using ionic and covalent cross-linkers for oral delivery of diclofenac sodium (Giri 2015). The developed hydrogel beads survived the harsh acidity of stomach and preferably released the drug in intestine. The hydrogel beads liberated diclofenac sodium in solution of pH 1.2 at a rate slower than that noted in buffer solution of pH6.8. They reported that higher $AlCl_3$ concentration decreased drug release rate from the hydrogel beads. The hydrogel beads sustained the drug release profiles in consistent with swelling degree.

4.3.4 Pulluan Grafting

Grafting copolymerization is considered as a convenient way of combining the properties of natural and synthetic polymers. Research reports are available in the scientific databases on grafting of synthetic polymers onto natural polysaccharide pullulan (Jiao et al. 2004; Wu et al. 2009). Most of the research articles focused on the construction of pH-sensitive NPs, self-assembled particles and nanobioconjugates for the delivery of anticancer drug. Bataille et al. (1997) synthesized amphiphilic carboxymethylpullulans derivatives by graft copolymerization with alkyl chains. Hydrophobically modified carboxymethylpullulans (HMCMPs) spontaneously developed inter- or intramolecular interactions in aqueous solution, depending on the graft chain length and grafting efficiency (Duval-Terrié et al.

2003). These interactions created lipophilic microdomains that could hold hydrophobic drugs. Na and Bae (2002) investigated the potential of hydrogel NPs of pullulan acetate (PA) and sulfonamide conjugates in targeting tumors. The nanoconjugates were sensitive to tumor extracellular pH. They synthesized pH-responsive polymer by conjugating sulfadimethoxine (SDM), to succinylated pullulan acetate. Adriamycin (ADR) was tested for loading into and release from the nanoparticles at various pH. The mean diameter of nanoparticles was less than 70 nm with unimodal size distribution. CAC was 3.16 μg/ml at pH 9.0. The nanoparticles were stable at pH 7.4, but aggregated below pH 7.0. The release of ADR from the PA/SDM NPs was pH-dependent around physiological pH and significantly enhanced at pH <6.8. Due to pH-responsiveness, PA/SDM nanoparticles could aggregate, thereby enhancing drug release rate at tumor pH.

Poly (L-lactide) (PLLA) with terminal amino groups (PLLA-NH_2) was grafted onto hydrophilic carboxymethyl pullulan (Ouchi et al. 2004). The copolymer was synthesized via coupling reaction between PLLA-NH_2 and CM-pullulan using 2-ethoxy-1-(ethoxycarbonyl)-1,2-dihydroquinoline as condensing reagent. The copolymer having >78% sugar unit formed nano-aggregates in water. The nanoparticles offer a number of advantages such as large surface area, easy surface modification, long blood circulation avoiding reticuloendothelial system (RES), specific interaction with cell surface receptors, etc. The tumor-specific targeting of nanoparticles allows selective killing of tumor cells without causing any harm to healthy cells or tissues (Jeong et al. 2012; Zwicke et al. 2012; Studenovsky et al. 2012). As the nanoparticles themselves do not possess tumor-specific targeting attributes, the tumor-specific ligands can be attached to the surface of nanoparticles to bestow active drug targeting properties. For example, the folate receptor is overexpressed in virtually all types of tumor cells, however hardly found in healthy cells and tissues (Zhang et al. 2012; Yue et al. 2012). Therefore, folic acid-conjugated nanoparticles could specifically target anticancer drug via folate receptors (Gao et al. 2012; Zhao et al. 2012). Lee and coworkers (2015) conjugated folic acid to pullulan-*g*-poly (lactic-co-glycolic acid) (PLGA) copolymers for targeting DOX to tumor cells. Self-assembled copolymer nanoparticles (<200 nm) were used to target folate receptor overexpressing KB human carcinoma cells. Fluorescence microscopy revealed that DOX-loaded nanoparticles induced strong red fluorescence in KB cells in absence of folic acid. However, the fluorescence intensity decreased after blockade of folate receptors. The antitumor activity of nanoparticles against KB cells was improved by blocking folate receptors. Thus, nanoparticles had the ability of active targeting anticancer agents to folate receptors of tumor cells.

4.3.5 Curdlan-Based Graft Copolymers

Na et al. (2000) substituted hydrophobic sulfonylurea (SU) moieties onto carboxymethyl curdlan to load all-*trans* retinoic acid (ATRA) in self-assembled nanostructures of the copolymer. The degree of substitution (DS) was considered as the number of SU groups per 100 anhydroglucose units of the polymer. Monomodal size distribution and a mean diameter of <300 nm were noticed. The critical

aggregation concentration (CAC) was 0.042, 0.031, and 0.019 mg/ml for the copolymer having DS values of 2.4, 5.6, and 7.2, respectively. ATRA loading efficiencies of curdlan-SU nanoparticles increased with the increase in SU substitution. The degree of substitution controlled ATRA release. Lactobionic acid (LBA) was conjugated to carboxymethyl curdlan for ensuing specific interaction with hepatic carcinoma cells (HepG2). LBA functionalized carboxymethyl curdlan-SU hydrogel NPs added extra benefit in treating liver cancer due to immunomodualting activities of CM-curdlan, the ligand–receptor specific interactions, and controlled release properties. Gao et al. (2008a, 2008b) investigated self-aggregates behavior of hydrophobically modified carboxymethyl curdlan for further use as drug carriers. Deoxycholic acid (DOCA) was covalently anchored onto CM-curdlan by ester formation. The degree of DOCA substitution was 2.1, 3.2, 4.1, or 6.3 DOCA groups per 100 anhydroglucose units. DOCA-modified-CM-curdlan (DCMC) nanoconjugates provided spherical, monodispersed particles of 192–347 nm size that decreased with increasing DS. The CAC of the DCMC was 0.014–0.052 mg/ml depending on the DOCA substitution. The same research group loaded epirubicin (EPB) in cholesterol-conjugated CM-curdlan nanoparticles (Li et al. 2010). EPB-loaded nanoparticles (DS 3.5) exhibited pH-sensitive release profiles. The cytotoxicity and cellular uptake were assessed in human cervical carcinoma (HeLa) cells. EPB-loaded NPs showed more cytotoxicity and widely distributed in the cells compared to free drug. The mean residence time (MRT), biological half-life, and extent of absorption were enhanced by fourfold, 4.31-fold, and 6.69-fold, respectively for the EPB-loaded nanoparticles compared to free EPB in rats after intravenous injection. The drug was significantly concentrated in liver relative to heart in comparison to the free EPB. Moreover, EPB-loaded nanoparticles demonstrated greater antitumor efficacy than the free drug. Overall, the nanoparticles showed promise as anticancer drug carriers. They further demonstrated that DCMC nanoparticles (327–511 nm) were highly cytotoxic than free drug, possibly due to greater intracellular uptake (Gao et al. 2010). The uptake of free EPB and EPB-loaded DCMC nanoparticles was also investigated in MCF-7 cell line. Confocal microscopy confirmed better uptake of EDNs in MCF-7 cells as shown in Fig. 4. MCF-7 cells incubated with EPB-loaded DCMC nanoparticles emitted more intense fluorescence than those incubated with free EPB.

Higher uptake of EPB was noticed in the tumor after entrapment of the drug in DCMC conjugates without any remarkable side effects. However, the uptake of EPB decreased in kidney and heart. Lehtovaara et al. (2012) attempted to synthesize core–shell nanoparticles using curdlan instead of carboxymethyl curdlan. Self-assembled curdlan-*graft*-poly (ethylene glycol) copolymer nanoparticles containing DOX (109.9 nm) controlled the DOX release over 24 h. The hemolysis assay confirmed the biocompatibility of curdlan-*g*-PEG.

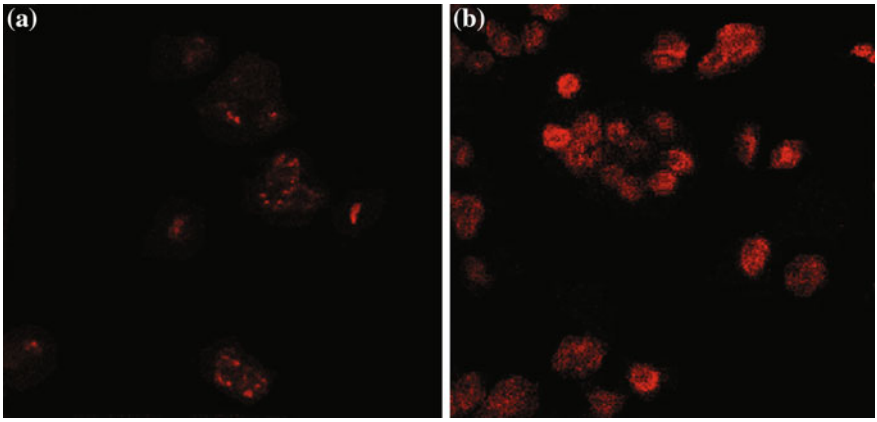

Fig. 4 Confocal microscopic images of MCF-7 incubated with free EPB **a** and EPB-loaded DCMC nanoparticles **b** with a drug loading content of 9.67% for 2 h (Gao et al. 2010)

5 Conclusion

There is a recent trend to modify chemical structures of natural polysaccharides for designing drug delivery systems. Out of various modification techniques, carboxymethylation constitutes an important approach for a number of polysaccharides to solve many inherent problems pertaining to the polysaccharides like excessive hydration, water solubility, high viscosity, etc., impart gelling characteristics, pH- and temperature-responsiveness on their particulate systems. Especially thermo-responsiveness and self-assembling behaviors are conferred after chemical conjugation with hydrophobic moieties or by grafting with other synthetic polymers. The swelling characteristics of the particulate systems have been controlled by cross-linking with other polymers or chemicals. The mechanical strength of the homopolymer particles has been improved by forming interpenetrating network with other polysaccharides or synthetic polymers. A significant advancement is noticed in this field regarding the characterization of the developed systems. One of the added advantages of the carboxymethylation is that the particulate systems can be fabricated in completely aqueous systems in presence of metal ion cross-linkers, thus eliminating the need of organic solvents. The particles could restrict the release of drug for an extended period of time which will not only reduce the chances of dose-related side effects of drugs but will also improve patient compliance by eliminating the need of frequent dosing during treatment. After a critical analysis of the open literatures, there is a limited report on the toxicity profiles of carboxymethyl polysaccharides. This must be taken into consideration in future studies. Overall, the results obtained with natural polymer-based particles are promising and encouraging. Hope in near future these tailor-made materials would find their way to clinical applications.

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Lipid Carriers: Role and Applications in Nano Drug Delivery

Naveen Chella and Nalini R. Shastri

Abstract

The ability of lipid carriers to enhance solubility, absorption and thereby the bioavailability of poorly soluble molecules by selective mechanism makes these systems a unique delivery option for certain classes of drugs. Different types of delivery systems that include liposome, solid lipid nanoparticles, nanostructured lipid carriers, lipid–polymer hybrid nanoparticles, lipoplexes, and phytosomes can be produced depending on the lipids/excipients used and the formulation technique employed. In this chapter, focus will be on different lipid-based carrier systems, their role in nano delivery and the advantages offered in improvement of solubility, absorption, and bioavailability with relevant case studies. Manufacturing methods of different carrier systems will be elaborated with a brief overview of scale up feasibility. Gene delivery with the use of charged lipids and delivery of herbal actives and neutraceuticals by phytosomes will be presented. Commercial products based on the lipid technology and recent patents in this area will be discussed.

Keywords

Lipid carriers · Drug delivery · Solid lipid nanoparticles · Liposomes · Phytosome · Gene delivery

Abbreviations

BCS	Biopharmaceutics classification system
GI	Gastrointestinal
GRAS	Generally regarded as safe

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HPH	High-pressure homogenization
LPHNs	Lipid polymer hybrid nanoparticles
LUV	Large unilamellar vesicles
MLV	Multilamellar vesicles
NLC	Nanostructured lipid carriers
PEG	Polyethylene glycol
PLs	Phospholipids
RES	Reticuloendothelial system
SCF	Supercritical fluid
SFEE	Supercritical fluid extraction of emulsions
SLNs	Solid lipid nanoparticles
SUV	Small unilamellar vesicles

1 Introduction

Poor bioavailability is one of the major reasons for the failure of most of new chemical entities to reach the clinical stage or market. According to the biopharmaceutics classification system (BCS) given by Amidon et al. (1995), BCS class II, III, and IV drugs face this problem due to either low aqueous solubility, poor permeability, or both. These problems combined with first pass metabolism and presence of efflux transporters on the membranes further contributes to their low oral bioavailability. Hence, development of suitable delivery system that can deliver the drug at target site in higher concentrations and in a stable form has become a challenging task to the pharmaceutical scientists.

Lipid-based drug delivery systems are developed based on the fact that ingested lipids (fats) enhance the absorption of lipophilic drugs. Lipid excipients are mainly comprised of monoglycerides, diglycerides, and triglycerides, and oils constituting various combinations of glycerides, phospholipids, and sphingolipids. Depending on the choice of excipients and formulation techniques, lipid-based delivery systems can range from simple solutions (liquid/solid solutions), emulsions, solid dispersions, and self-dispersing formulations such as self-emulsifying granules, powder to self-nano emulsifying systems, and colloidal carriers (vesicles and nanoparticles). Drug delivery using lipid-based carriers dates back to 1960s, where fat emulsions were administered through parenteral route for nutrition. The commercial success of lipid-based emulsions, Diazemuls (Diazepam) and Diprivan (Propofol) attracted many pharmaceutical industries to focus their development further on the lipid-based formulations (Wissing et al. 2004). The continuous efforts of pharmaceutical scientists led to the development of lipid-based carriers such as liposome, solid lipid nanoparticles, nanostructured lipid carriers, lipid polymer hybrid nanoparticles, and phytosomes. Lipid carriers are attractive systems for

delivery of drugs through different routes (oral, parenteral, topical, pulmonary, and ophthalmic) because of their size, biocompatibility, safety, ability to encapsulate variety of drugs, and flexibility for modulating the drug release with an added advantage of regulatory compliance of excipients used, scale up feasibility and commercial viability. Lipid carriers in addition may also exhibit enhanced bioavailability, improved therapeutic efficacy with reduced toxicity by altering the pharmacokinetics and bio distribution of drugs (Shrestha et al. 2014). The present chapter deals with only lipid-based carriers systems. For the convenience of readers, we have divided the lipid carriers into lipid nanoparticles and lipid vesicles. Based on the composition, lipid nanoparticles are further classified as solid lipid nanoparticles (SLNs), nanostructured lipid carriers (NLC), lipid polymer hybrid nanoparticles. Lipid vesicles are divided into liposomes, lipoplexes, and phytosome. The role of these lipid carriers in drug delivery will be discussed along with their preparation methods. General mechanism behind improved drug delivery by lipid-based drug delivery systems through various routes will also be dealt in the following sections.

2 Mechanism of Lipid Carriers in Improved Drug Delivery

Poor solubility and in adequate and poor permeability of drugs limits their deliverability not only by oral route, but also from parenteral, topical, and ophthalmic routes. Initial lipid-based formulations such as lipid emulsions and liposomes are intended for only parenteral administration, whereas the new generation carrier systems like stealth liposomes (PEGylated liosomes), solid lipid nanoparticles and nanostructured lipid carriers, and phytosomes find application in oral, topical, pulmonary, and ophthalmic delivery. The simple mechanism by which lipid-based carrier systems improves the delivery of BCS class II, III, and IV drugs is depicted in Fig. 1.

2.1 Oral Delivery

Low oral bioavailability is a result of poor aqueous solubility, poor intestinal permeability, first pass metabolism, and efflux process. Lipid carriers can overcome these obstacles by increased residence time, improved solubility and permeation, thereby enhancing drug absorption and lymphatic transportation. Adherence of small lipid particles (with greater surface area) to mucus membranes and the decreased movement of gastrointestinal (GI) tract due to presence of lipid, enhances the exposure of lipid particles to GI media thus resulting with increased residence time and thereby absorption. Bile acids and contents released into the intestine in response to stimulation by lipids in which the drug is dispersed, contribute to the

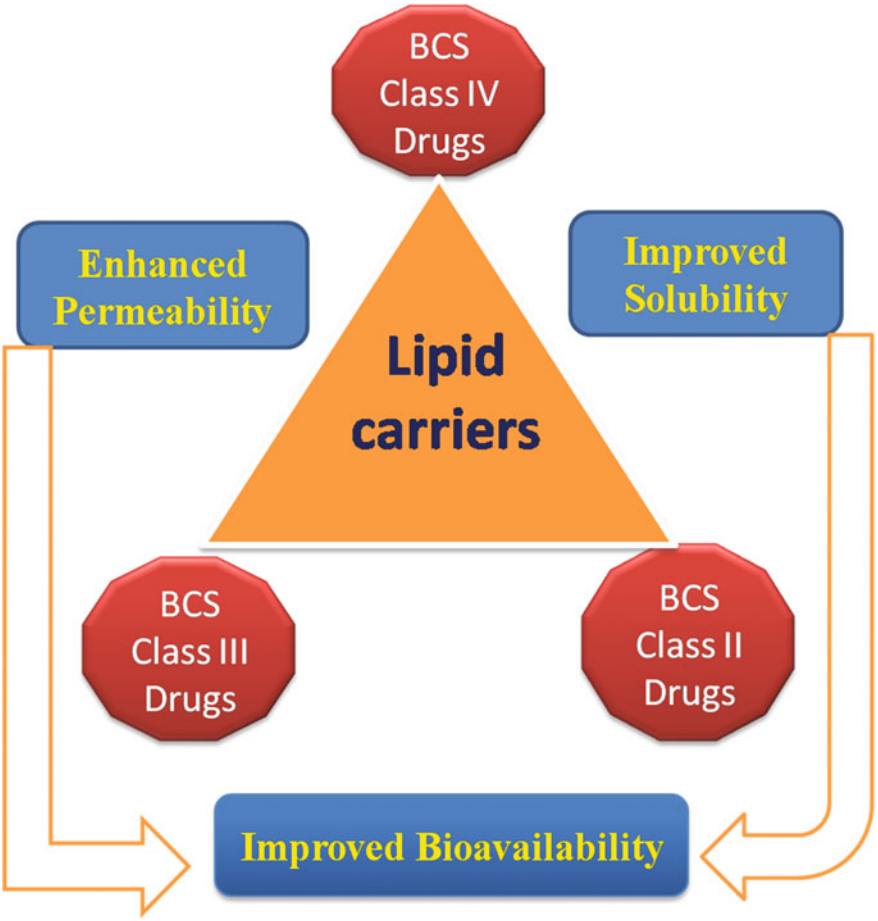

Fig. 1 Role of lipid carriers in improved delivery of different BCS class drugs

increased drug solubility of lipophilic compounds. Transport of lipid carriers into the enterocytes by endocytosis (clathrin mediated or caveolae-dependent) or particle uptake by M cells on the Peyer's patches results in enhanced drug absorption. Entry of carriers into lymphatic circulation due to smaller size prevents the exposure to liver and thereby metabolism. Simultaneous delivery of efflux inhibitors by co-encapsulation or use of excipients with Pg-P inhibition activity (either oils or surfactants) in the formulation, results in inhibition of efflux pump and increased plasma drug concentration. Surface modification of the particle with specific ligands can enhance mucosal contact, penetration, and uptake that further improve pharmacokinetics and bioavailability of the drug (Harde et al. 2011).

2.2 Topical Delivery

Drug delivery system used to deliver drugs through topical route should be non-toxic and non-irritant to the skin, especially on inflamed or damaged skin and should be able to modulate the release (skin penetration, prolonged delivery). The properties of lipid carriers such as small particle size, use of biocompatible lipids and film forming ability makes them more suitable for topical delivery over conventional formulations. Smaller size helps in adherence of particles to the skin, which provides sufficient interaction between particles, and skin to improve drug penetration through skin. Use of biocompatible lipids do not cause irritation or toxicity of dosage form after application and structural similarity of these lipids to the skin lipids further helps in penetration of drug molecules. Increased stability and prolonged release of drug due to encapsulation into lipid core helps in achieving higher concentration at the application site and improved penetration. Occlusion due to formation of lipid film on skin results in hydration of skin and increased penetration of loaded actives (Cannon 2014).

2.3 Pulmonary Delivery

Pulmonary delivery is preferred for local delivery in treating respiratory disorders or can be used as an alternate to oral route, to improve the bioavailability of drugs undergoing first pass metabolism. The large surface area, thin alveolar epithelium, easily permeable membrane, and the extensive vasculature that allows rapid absorption of drugs make pulmonary route an attractive route of administration for systemic delivery of drugs. Parameters that control distribution and penetration of drug in the respiratory tract are size, size distribution, and contact time with mucus membrane. The smaller size of lipid nanoparticles allows deeper penetration of drugs and increases the adherence to mucus membranes. Release of drug for prolonged periods allows increased drug exposure and improved drug delivery (Weber et al. 2014).

2.4 Ophthalmic Delivery

Poor permeability through cornea, removal of drug by nasolacrimal drainage, and short retention time are the major barriers that limit availability of drug in ophthalmic delivery. Lipid nanoparticles due to their large surface area provided by small size and mucoadhesion properties can overcome these barriers and show promising results in the delivery of drugs through ophthalmic route (Pignatello et al. 2015).

3 Lipid Nanoparticles

Lipid nanoparticles are colloidal dispersions composed of lipid matrix in which the drug is incorporated and stabilized by surfactants. General size for these particles is 150–300 nm. Depending on the application and administration route, particles smaller than 100 nm or larger than 300 nm (up to 1000 nm) can be produced (Müller et al. 2011). Lipid nanoparticles can be classified into two types based on the composition of lipid matrix. First-generation lipid nanoparticles are called solid lipid nanoparticles (SLNs) and the second-generation lipid nanoparticles are called nanostructured lipid carriers (NLCs).

3.1 Solid Lipid Nanoparticles

Solid lipid nanoparticles (SLNs) are aqueous colloidal dispersions in the size range of 10–1000 nm, composed of solid lipid matrix stabilized in an aqueous dispersion by surfactants or stabilizers (MuÈller et al. 2000). SLNs combine the advantages of lipid emulsions, liposomes, and polymeric carriers such as controlled release of incorporated drug, protection of drug from GI conditions, targeted release, and tolerability by minimizing the problems like stability, leakage of drug, and biocompatibility associated with the above mentioned carrier systems. Distribution of drugs in the solid lipid matrix prevents the mobility and thereby reduces the leakage of drugs from the particles, provides stability (physical, chemical, and mechanical) with controlled or sustained release of drug molecules if desired. The biocompatibility and biodegradability of the lipids, ability to encapsulate lipophilic and hydrophilic molecules, higher drug loading, enhanced solubility of lipophilic molecules, improved absorption and bioavailability, site-specific delivery of actives along with feasibility of scale up (large-scale manufacturing) makes them an attractive system for drug delivery (Manjunath et al. 2005).

SLNs are made up of solid lipids (solids at room temperature and body temperature), emulsifier, co-emulsifier and water. Depending on the application and need, other excipients like charge modifiers, cryoprotectants, and preservatives can be added. Solid lipids used in the preparation of SLN include fatty acids (stearic acid, palmitic acid, and behenic acid), triglycerides, and partial glycerides (tripalmitin, tristearin, and trimyristin) and waxes (cetyl palmitate, bees wax, solid paraffin). Emulsifiers include ionic (bile salts, sodium dodecylsulphate), nonionic (Tweens, poloxamer, polyvinyl alcohol, Brij, Solutol HS, Cremophore RH 40) and amphoteric (phosphatidyl choline from egg or soya). Butanol, sodium taurocholate, and sodium dodecyl sulfate can be added as co-emulsifiers along with lecithin to increase the stability of the particles and further decrease the size. Charge modifiers like stearylamine, cetyltrimethylammonium bromide, dipalmitoylphosphatidylcholine and dimyristoylphosphatidylglycerol can also be incorporated to improve the stability and site-specific delivery of drugs. Sugar-based cryoprotectants such as trehalose, mannose, and sucrose are added during lyophilization to prevent the

aggregation of redispersed particles (Manjunath et al. 2005; Mehnert and Mader 2001; Mukherjee et al. 2009).

3.1.1 Selection of Excipients

Specific guidelines or regulations have not yet been framed for the selection of excipients. In general, most of the lipids used in the preparation of SLNs are physiological lipids or generally regarded as safe (GRAS). However, one needs to consider the effect of excipients on key parameters like size, size distribution, entrapment efficiency, and drug loading in developing SLNs formulations. Physicochemical properties of the lipids like structure of the lipid, melting point, crystallinity, and purity of the lipid along with solubility or miscibility of drug in lipid melt will affect the size, size distribution, entrapment efficiency, and drug loading of SLNs. Higher melting lipids (MP > 70 °C) generally produces particles with larger size compared to lower melting lipids. For example, Ekambaram and Abdul (2011) reported, increase in particle size with high melting lipid (glyceryl monostearate) compared to lipid with a lower melting point (glyceryl monooleate). This may be due to increased viscosity and slower lipid crystallization of higher melting lipids from the hot homogenized condition. The highly ordered crystalline lipids like beeswax, cetylpalmitate, and tripalmitate generally gives lower entrapment efficiency due to expulsion of drug during recrystallization compared to lipids with less order of crystallinity such as and glyceryl behenate (Vyas et al. 2008).

Emulsifier or surfactants used in the formulation disperses the lipid in aqueous phase by reducing the interfacial tension and prevents aggregation leading to enhanced stability of the particles by coating on the surface. The emulsifiers should be nontoxic, give minimum size at lower concentrations, and prevent aggregation of particles during the storage and sterilization. The type and concentration of emulsifier used affects the charge and size of final formulation. The choice of the emulsifier depends on the charge, molecular weight, chemical structure, hydrophilic lipophilic balance (HLB) value, and route of administration. Surfactant used in parenteral formulations should be GRAS and should have regulatory acceptance. Lecithin, Tween 80, Span 85, Poloxamer 188, and sodium glycocholate are listed as GRAS (U.S. FDA 1972–1980) and no signs of toxicity have been reported at the concentration levels recommended. Nonionic surfactants are more preferred compared to ionic surfactants because of their low toxicity. Increase in concentration of emulsifiers decreases the particle size up to certain extent, further increase usually increases the particle size due to higher viscosity of the surfactants at higher concentrations (with poloxamer and poly vinyl alcohol) or due to formation of micelles (with lecithin) (Olbrich and Müller 1999; Salminen et al. 2014). Higher concentrations of surfactants may reduce the entrapment efficiency and result in higher amount of drug released in initial period (burst release). Generally, co-emulsifiers are included with a combination of surfactants or emulsifier to obtain SLNs with lower particle size and improved stability.

The distribution of drug in the lipid matrix can be explained by three models, namely solid solution model, drug-enriched shell model, and drug-enriched core model. The uniform dispersion of drug throughout the solid lipid matrix produces a

solid solution model (Müller et al. 2000). Cold homogenization technique yields this type of particles; where in formation of particle does not require surfactants or stabilizers. The dispersion of drug in the matrix by this method generally results in controlled release of drug from the system. Precipitation of drug before lipid recrystallization results in drug-enriched core model. This occurs when the amount of drug dissolved is closer to its saturation solubility. During cooling of the nanoemulsion, drug reaches supersaturation in the lipid melt and precipitates before lipids can recrystallize. Further cooling leads to the recrystallization of the lipid surrounding the drug as a membrane. Drug-enriched shell model explains the repartitioning of drug from water phase to lipid layer during cooling. Hot homogenization process leads to this type of SLNs. During homogenization at high temperatures, partitioning of drug takes place from lipid to water. As the temperature decreases during cooling process, the drug partitioning occurs from water to the lipid phase (Uner 2006).

3.1.2 Preparation Techniques

Preparation method has a significant effect on the size and performance of the final formulation. Different methods reported in the literature (Muéller et al. 2000; Manjunath et al. 2005; Mehnert and Mader 2001) for the production of SLNs are briefly described in the next section.

High-Pressure Homogenization

High-pressure homogenization (HPH) introduced in 1992 by Siekmann and Westesen (1992) and later developed by Müller and Lucks (1994). Easy operation and scale up feasibility of HPH process helps in large-scale production of SLNs. In the first step, lipid drug mix is obtained by dissolving or dispersing the drug in molten lipid. Depending upon the process conditions used subsequently, HPH is again classified into hot and cold homogenization.

(a) **Hot homogenization**

Hot homogenization is suitable for the formulation with high amount of lipids. Drug-loaded lipid melt is emulsified with hot aqueous surfactant solution maintained at 5–10 °C above the melting point of lipid, using a homogenizer. Homogenization is repeated in cycles till a desired particle size is obtained. SLNs are precipitated after cooling the emulsion to room temperature. This method offers effective lipid dispersion, reproducibility, handling of high-solid content and feasibility of scale up. However, the major limitation of this method is high temperature and application of high mechanical shear, which may lead to degradation of sensitive drugs.

(b) **Cold homogenization**

Cold homogenization is used to encapsulate thermolabile materials into SLNs. The molten lipid-containing drug is solidified in liquid nitrogen and subsequently milled

using a ball mill to obtain lipid micro particles (50–100 μm). These micro particles are dispersed in cold aqueous surfactant solution and the resulting suspension is homogenized at room temperature to obtain SLNs. Rapid cooling may enhance drug loading and reduce drug loss. Hydrophilic drugs can be easily encapsulated using this technology and the process can be easily scaled up. Polydispersity of the particles and expulsion of drug during storage are major drawbacks of this technology.

Solvent Emulsification-Evaporation Method

Preparation of SLNs by solvent emulsification method was described by Sjostrom and Bergenstahl (1992). The method involves preparation of emulsion with lipid phase (lipid and drug) dissolved in a water-immiscible organic solvent (e.g., dichloromethane, chloroform, and cyclohexane) and an aqueous surfactant solution by high shear homogenization. Ratio of organic to aqueous phase is the key parameter in producing a stable emulsion. Lipid nanoparticles are precipitated in aqueous phase after solvent removal by evaporation. Mixture of solvents can be used to dissolve the lipids and the drug. SLNs prepared by this method are of smaller size (30–100 nm) and can be advantageous for handling thermolabile materials. However, the presence of residual organic solvent in the formulation is the major limitation of this method.

Solvent Diffusion Method

Hu et al. (2002) introduced solvent diffusion method by simple modification of solvent evaporation method. Water miscible solvents like benzyl alcohol, ethyl acetate or butyl lactate is used to dissolve lipid phase instead of water-immiscible organic solvents. After dilution with water, the lipid precipitates as fine particles due to reduced solubility by diffusion of organic solvent in continuous phase. Saturation of organic solvent with water before dissolving lipid is necessary to ensure preliminary thermodynamic equilibrium between the two liquids. Optimum ratio of organic to the aqueous phase is 1:5 or 1:10. SLNs less than 100 nm can be successfully produced by this method. Presence of traces of organic solvent in the final formulation and capacity to incorporate low amount of lipid are the limitations of this method.

Solvent Injection Method

This method is a modification of solvent diffusion method by replacing water miscible solvent with semi polar, water miscible solvents like ethanol, acetone, isopropanol, and methanol. Lipid dissolved in the solvent, is rapidly injected into the aqueous phase containing surfactant(s). SLNs are produced by precipitation due to rapid distribution of organic solvent in continuous aqueous phase. Size of the particles is controlled by rate of diffusion of the organic solvent. Average particle size of SLNs obtained by this method is in the range of 10–200 nm.

Double Emulsion Method

In solvent evaporation method, hydrophilic molecules may diffuse into the aqueous phase during emulsion formation, resulting in lower entrapment efficiency. This can be prevented by first incorporating the hydrophilic drug in the aqueous phase, which is emulsified with an organic phase to obtain a primary emulsion. This primary emulsion is further diluted with an aqueous surfactant solution to obtain a W/O/W double emulsion as described by García-Fuentes et al. (2003). SLNs are precipitated on evaporation of the organic solvent. Encapsulation of the drug in the primary emulsion prevents the partitioning of the hydrophilic drug to the external water phase during solvent evaporation.

Micro Emulsion-Based Method

Gasco (1993) prepared SLNs by dilution of micro emulsions. Micro emulsion is prepared by adding an emulsifier, co-emulsifier(s), and water maintained at a temperature above the melting point of lipid, to a molten lipid containing the drug. The resulting transparent micro emulsion is added to a large quantity (1:50 ratio of emulsion to water) of cold water (2–3 °C) under stirring; to precipitate SLNs. Easy preparation and scale up feasibility are added advantages of this method. Key parameters that govern the quality of the final formulation are composition of emulsion, the temperature gradient, and the pH. Maintaining a higher temperature gradient is essential to cause rapid crystallization and prevent aggregation of particles.

High Shear Homogenization/Ultra-Sonication

The advantage of this method is that the equipment used is commonly available for lab scale. The solid lipid is heated 5–10 °C above its melting point, and homogenized in presence of aqueous surfactant solution previously heated to the same temperature as that of the lipid. The obtained pre-emulsion is sonicated by means of an ultrasonicater that leads to droplet breakage by acoustic cavitations. Subsequent cooling of the system results in the formation of nanoparticles. Organic solvents can be avoided and lower surfactant concentrations can be used while preparing SLNs by this method. High polydispersity, physical instability, poor encapsulation efficiency, and metal contamination are major limitations of this method.

Membrane Contactor Method

The lipid phase is pressed through the membrane pores at a temperature above the melting point of the lipid that allows formation of small droplets. These droplets are carried away by aqueous phase that flows tangential to the membrane surface. SLNs are formed on subsequent cooling of the preparation to room temperature. Particle size is affected by membrane pore size, lipid phase temperature, cross flow velocity, and the pressure used to pass the lipid through the membrane. Higher lipid content generally results in increased particle size and reduces the membrane performance (Charcosset et al. 2005).

Supercritical Fluid Method

Supercritical fluid (SCF) technology has gained interest in developing micro or nanoparticles in recent years due to solvent-free processing and its ability to formulate thermosensitive compounds. SCF is obtained above critical pressure and temperature of the compound. Carbon dioxide is the most widely used SCF due to its lower values of critical temperature, pressure, low cost, and nontoxic nature. Rapid expansion of supercritical solutions (RESS), gas antisolvent (GAS) process, particles from gas-saturated solutions/suspensions (PGSS), supercritical fluid extraction of emulsions (SFEE) are the four different methods reported in the literature for preparing micro or nanoparticles using SCF as solvent or anti-solvent (Luigi Battaglia et al. 2014). Generally, SFEE method is used to prepare SLNs in which, lipid, drug and surfactant are dissolved in organic solvent like chloroform to form an organic phase. An o/w emulsion is formed by dispersing this organic solution in aqueous phase containing a co-surfactant, using HPH. In an extraction column, o/w emulsion is introduced from top at a constant flow rate and the supercritical fluid is introduced counter-currently from bottom at a constant flow rate. Extraction of the organic solvent from emulsion by SCF results in precipitation of SLNs in the column (Chattopadhyay et al. 2007). Circumventing the use of organic solvent and existence of final formulation in dry condition are main advantages of this method. However, limited solubility of lipids and drugs in SCF is a major concern.

Hot Melt Extrusion Method

Hot melt extrusion is a new approach described Patil et al. (2015) to prepare SLNs. By combining hot melt extrusion with HPH, SLNs can be produced in large scale continuously. Lipid emulsion is obtained in hot melt extruder and size reduction is performed on high-pressure homogenizer. Lipid and drug is fed into the co-rotating twin-screw extruder operated at 5–10 °C above melting point of lipid and aqueous surfactant solution heated to the same temperature is injected into the feeder. The resulting hot emulsion was pumped directly into HPH for size reduction.

3.1.3 Application of SLNs in Drug Delivery

SLNs, due to their advantages compared to other carrier systems finds wide variety of applications in drug delivery through various routes of administration. Few applications are discussed in the following section.

SLNs for Improved Oral Drug Delivery

Oral route is the most preferred route of administration for drugs due to ease of administration, and patient convenience. However, many drugs exhibit low oral bioavailability due to poor solubility, poor permeability, degradation in GI tract, first pass metabolism, and efflux process. SLNs are proven to be a reliable system to improve the oral delivery by overcoming these obstacles. Kakkar et al. (2011) explored the role of SLNs in improving the bioavailability of poorly soluble compounds by using curcumin as a model drug. SLNs prepared by microemulsion technique showed a particle size of 134.6 nm, total drug content of 92.33% and

controlled delivery of curcumin for 7 days. In vivo studies performed with different doses in rats showed significant improvement in bioavailability at all doses indicating therapeutic usefulness of curcumin-loaded SLN in neurodegenerative and cancerous disorders. Few other drugs that reported improvement in bioavailability include vinpocetine (Luo et al. 2006), carvedilol (Venishetty et al. 2012), resveratrol (Neves et al. 2013), torcetrapib (Liu et al. 2015) and efavirenz (Makwana et al. 2015). Co-delivery of P-gp efflux inhibitor (verapamil) along with paclitaxel encapsulated in SLNs showed significantly higher cytotoxicity and improved cellular uptake of paclitaxel in MCF-7/ADR-resistant cells compared to PTX solution, due to downregulation of P-gp expression (Baek and Cho 2015). Improved intestinal permeability of sulpiride from SLNs in rat everted sac intestine model was reported by Ibrahim et al. (2014). SLN-loaded formulation showed marked enhancement in sulpiride permeability and the results obtained displayed good correlation with the in vitro release data. The enhanced permeability resulted in increased oral absorption of drug. In an another study, significantly higher concentration of lopinavir (4.9 folds) in lymph from SLNs was reported by Aji Alex et al. (2011) compared to pure drug administered in the form of suspension. This increased concentration in the lymph resulted in enhanced bioavailability and improved pharmacokinetics.

SLNs for Oral Delivery of Proteins and Peptides

Oral delivery of proteins and peptides suffers from low bioavailability as a result of poor permeability, and rapid clearance from circulation due to degradation by GI enzymes. SLNs by protecting from harsh GI environment, showed improvement in delivery of insulin (Sarmento et al. 2007) and an enhanced absorption of salmoncalcitonin (Martins et al. 2009). Surface modification of SLNs with a mucoadhesive polymer like chitosan has demonstrated enhanced oral absorption of insulin due to increase in contact time (Fonte et al. 2011).

SLNs in Topical Delivery

SLN are preferred in wound healing as they are well tolerated on inflamed or damaged skin due to biocompatibility and the nontoxic lipids used in the formulation. Improved topical delivery of drugs from SLNs has been reported due to epidermal targeting (Chen et al. 2006), controlled delivery of drugs (clotrimazole) for prolonged period at the site of application (Souto et al. 2004), and increased drug penetration (aceclofenac) (Raj et al. 2015).

SLNs in Pulmonary Delivery

SLNs can be administered as aqueous dispersion or converted to dry powder and used as dry powder inhalations. SLN showed promising results in the delivery of anticancer agents to treat lung cancer (Videira et al. 2012), proteins and peptides that are poorly bioavailable through oral route (Yang et al. 2012), and anti-tubercular agents (Gaspar et al. 2016).

SLNs in Ophthalmic Delivery

SLNs improve the ocular delivery of drugs by increasing residence time, improved interaction of carrier with ocular mucosa, enhanced penetration of drug and prolonged drug release. Studies have showed improvement in ocular delivery of drugs like gatifloxacin (Kalam et al. 2013), acyclovir (Seyfoddin and Al-Kassas 2013), methazolamide (Youshia et al. 2012), indomethacin (Hippalgaonkar et al. 2013) and ketoconazole (Kakkar et al. 2015) using SLNs as carriers.

SLNs in Parenteral Delivery

Parenteral delivery of SLNs provides targeted delivery, increased uptake by tissues, improved pharmacokinetics and prolonged drug release. Surface modification of SLNs with polyethyleneglycol (PEG) improves the circulation half-life by diminishing the identification and uptake by reticuloendothelial cells (Mehnert and Mader 2001). A study by Alyautdin et al. showed increased uptake of SLNs by brain, after parenteral administration, due to adsorption of a blood protein onto particle surface (Alyautdin et al. 1997).

SLNs for Targeted Delivery

SLNs can deliver the drugs at specific site by both passive and active targeting. The smaller size of the SLNs contributes to the passive targeting by enhanced permeation and retention effect, whereas the surface modification of SLNs can offer active targeting. Site-specific delivery of drugs by SLNs can enhance the therapeutic efficacy (for anticancer agents) and reduce the side effects (due to reduced exposure to other tissues/organs). Zhang et al. (2015) reported enhanced uptake of drug and greater tumor suppression from surface modified SLNs with epidermal growth factor compared to normal SLNs. Ana Rute et al. demonstrated targeting ability by surface modification of SLN, using apolipoprotein E, in brain targeting. Surface modified SLN showed increased permeability (1.5 folds) in transwell devices with hCMEC/D3 monolayers without any toxicity when compared to plain SLN without surface modification (Ana Rute et al. 2015).

3.2 Nanostructured Lipid Carriers

The major limitation of SLNs is inadequate solubility of drugs in solid lipids and the low drug entrapment efficiency due to expulsion of drug during recrystallization of lipid. To tackle these issues novel carrier system called nanostructured lipid carriers (NLC) were developed. NLCs are improved version of SLNs often called as second-generation SLNs. First reported by Müller et al. (2002), in NLCs, a portion of solid lipid is replaced with liquid lipids (oils). Incorporation of oil into solid lipid matrix enhances the solubility of drug in lipid and produces less ordered crystal structures with imperfections due to structural differences between solid and liquid lipid. This aids in accommodating more amount of drug thus improving the drug loading and encapsulation efficiency. Liquid lipid incorporation also causes melting point depression and prevents crystallization process, further improving the

encapsulation efficiency (Montenegro et al. 2016). In addition, the conversion of SLNs suspension into a dry powder becomes difficult due to low solid content and (1–30%) and high amount of water (Pardeike et al. 2009). The solid content in NLC is, however, more when compared to SLNs; NLCs can be produced with a high solid content up to 95% with higher drug loads. Removal of water content is cost effective and hence, NLCs can be easy converted into solid powders. Thus, NLCs combine all the advantages of SLNs, overcome the shortfalls of SLNs, and hence find application for delivering a wide range of therapeutics.

In general, incorporation of liquid lipids up to 35–40% w/w in NLC formulations have been reported in literature (Müller et al. 2002). Liquid lipid used should be nontoxic, GRAS and should have higher solubilization ability for the selected drug. Generally, vegetable oils like corn oil, castor oil, soybean oil, sun flower oil, medium chain triglycerides or oleic acid is preferred as liquid lipid (Tamjidi et al. 2013). The types of oil used play an important role on the properties of NLC. Yang et al. prepared NLCs using different liquid lipids with tristearin. Results indicated that the stability of NLCs was strongly affected by the type and amount of the oil used. Higher concentration of oils produced spherical particles with decreased crystallization, reduced melting temperatures and alteration in shape. The authors also found that NLCs required less concentration of surfactant for stabilization when compared to SLNs (Yang et al. 2014b). A comparison study performed on SLN, NLC and lipid nanoemulsion of quercetin by Aditya et al. (2014) revealed that, NLC produced smaller size particles with improved drug loading when compared to SLNs. Lipid nanoemulsion and NLC showed improved drug loading compared to SLNs. Decreased particle size was due to change in lipid composition (use of mono and di glycerides in NLC) and enhanced drug loading was due to formation of less ordered crystal lattice in both NLC and nanoemulsion.

3.2.1 Production Techniques

The technologies used to prepare SLNs can also be applied for the production of NLC. HPH is the most preferred method due to ease of operation and commercial viability for the production of NLCs. NLCs with high solid content (up to 80%) can be produced by multistep production technology (Radtke et al. 2005).

3.2.2 Classification of NLC

Depending on the method of manufacture and type of excipients used, three types of NLC can be produced. Imperfect type or type I, amorphous type or type II and multiple oil in fat water (O/F/W) type or type III (Uner 2005).

Type I or Imperfect Type NLC

Lipid matrix in type I NLC is composed of different types of liquid lipids with solid lipid. The mixing of different lipids with structural differences produces a highly disordered imperfect lipid matrix that can accommodate more amount of drug resulting in higher drug pay loads.

Type II or Amorphous Type NLC

In type II NLCs, amorphous particles are produced when medium chain triglycerides like Miglyol 8012 is used as a liquid lipid. The absence of crystallization prevents the drug expulsion caused by crystallization process during storage.

Type III or Multiple (O/F/W) Type NLC

High amounts of liquid lipid in lipid matrix produces type III NLCs. Use of liquid lipids in high concentrations causes phase separation during the cooling process that leads to formation of tiny oil droplets in the solid matrix. These oily droplets can accommodate large amount of drug due to high solubility of drug in the liquid lipid. Hence, more amount of drug can be dispersed in the lipid matrix with type III NLC (Radtke et al. 2005).

3.2.3 Applications of NLC in Drug Delivery

Parenteral administration of lipid carriers presently find applications in cancer chemotherapy, imaging studies, targeted delivery (brain, CNS), gene delivery, in treatment of cardio vascular diseases, parasitic diseases, and in conditions like rheumatoid arthritis (Joshi and Müller 2009). Studies showing improvement in delivery of variety of actives using NLCs in topical, oral delivery, ophthalmic, pulmonary, and parenteral routes are tabulated (Table 1).

3.3 Lipid Polymer Hybrid Nanoparticles

Lipid polymer hybrid nanoparticles (LPHNP) are submicron solid particles composed of polymer and lipid in which, drug is entrapped, adsorbed on the surface or covalently attached to the particles. These hybrid systems combine advantages of liposomes and polymeric nanoparticles. Resulting particles are biocompatible, non-toxic with more versatility in terms of drug loading and drug release. More choice in selecting the polymer opens the way for loading of different types of drugs (lipophilic, hydrophilic, salts, ionic).

The major components of hybrid particles are (i) polymer core, (ii) lipid outer shell, and (iii) a hydrophilic polymer stealth layer outside the lipid shell. The inner polymer shell acts as a carrier for hydrophobic drug molecules, provides mechanical strength to the particle and also acts as rate controlling membrane during drug release. Lipid shell acts as barrier for penetration of dissolution media into the core and for diffusion of drug from core thus controlling the drug release rate. The outer lipid layer conjugated with PEG provides long circulation half-life by increasing the in vivo stability and evading identification or recognition by reticuloendothelial system (RES). PEG further provides functional groups for addition of wide variety of ligands for targeting. LPHNP can be classified into five types based on arrangement of lipid and polymer layer (Mandal et al. 2013). The core-shell LPHNP is a major system used to deliver drugs, gene, protein, and vaccines. Based on this technology, Biovector Therapeutics developed a new delivery system called "Supra molecularbio-vector™" (SMBV™) in early 1990s.

Table 1 Applications of NLC in drug delivery through different routes

Drug	Route	Comment	Reference
Flurbiprofen	Topical	Improved C_{max} and enhanced drug accumulation in skin	Han et al. (2012a)
Dexamethasone acetate		Improved drug permeation rate by 7.3 times and skin deposition by 3.8 folds compared to drug ointment and solution	Tung et al. (2015)
Etoricoxib		Prolonged availability of drug in skin tissues and improved safety	Hafeez et al. (2015)
Miconazole nitrate		Improved drug delivery with wider zone of inhibition in antifungal studies	Singh et al. (2016)
Baicalin	Oral	Improved pharmacokinetics of drug from NLC compared to pure drug	Luan et al. (2015)
Oridonin		Significant improvement in absorption with a relative bioavailability of 171.01% in comparison to drug solution	Zhou et al. (2015)
Docetaxel		Improved mucoadhesion, drug absorption and accumulation in blood resulting in 12.3 folds larger area under the curve compared to docetaxel solution	Fang et al. (2015)
Mitoxantrone hydrochloride		Improved encapsulation efficiency, decreased efflux, enhanced oral bioavailability and cytotoxicity against resistant breast cancer cells	Ling et al. (2016)
Tobramycin	Pulmonary	Increased efficacy against *P. aeruginosa* in vitro, enhanced mucus penetration ability and large pulmonary distribution and retention in the in vivo studies	Moreno-Sastre et al. (2016)
Doxorubicin or paclitaxel and siRNA		Combined delivery of anticancer agent with siRNA, efficient suppression of tumor growth and prevention side effects on healthy organs	Taratula et al. (2013)
Montelukast		Improved efficacy with reduced toxicity	Patil-Gadhe et al. (2014)
Triamcinolone acetonide	Ocular	Sustained drug release, zero-order kinetics and strong tissue binding	Araújo et al. (2012)
Mangiferin		Sustained drug release with increased corneal permeation and increased ocular bioavailability	Liu et al. (2012)
Ofloxacin		Controlled drug release, improved pre-ocular residence time, and enhanced corneal bioavailability	Ustundag-Okur et al. (2014)
Tyrphostin AG-1478	Parenteral	Enhanced in vitro antitumor activity in human hepatocellular carcinoma HA22T/VGH cells	Bondì et al. (2014)
β-artemether		Superiority in terms of parasitaemia reduction and increased survival when compared to marketed formulation	Patil et al. (2012)

Table 2 List of polymers and lipids used in the preparation of hybrid particles

Polymers	Lipids
Polylactic-co-glycolic acid Polycaprolactone (PCL) Dextran Albumin	Lecithin, 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) 1,2-dipalmitoyl-3-trimethylammonium-propane (DPTAP) 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) 1,2-dioleoyl-glycero-3-phosphoethanolamine (DOPE)

SMBV is a nanoparticle formulation that contains a modified polysaccharide hydrogel core covered with phospholipids.

3.3.1 Preparation Methods

Different lipids and polymers used in the preparation of hybrid nanoparticles are given in Table 2. LPHNP can be prepared by two different approaches, two-step process and single-step process (Krishnamurthy et al. 2015). Other methods to prepare LPHNP are modified from the conventionally used solvent evaporation or nanoprecipitation methods to prepare nanoparticles. Type of lipid, lipid to polymer ratio, concentration of lipid, organic phase to aqueous phase ratio, viscosity of the polymer are the critical parameters that control size, size distribution, and zeta potential of the final formulation. In addition, drug properties like solubility of drug in given polymer, lipid and aqueous phase, charge of lipid and pH of the aqueous phase plays an important role in controlling entrapment efficiency and drug loading.

Single- and Two-Step Approach

In the two-step process, lipid vesicles and polymeric particles are prepared separately and fused together in certain molar ratios by various technologies like simple mixing, incubation, or use of electro static interactions to obtain hybrid nanoparticulate systems. Sonication or extrusion can be used for size reduction. The advantage of this technology is that two different actives can be loaded into the hybrid particles and the particle size and drug loading can be controlled easily. The major limitations of this process are leakage of hydrophilic drugs during incubation and longer processing time. Single-step process involves modification of solvent evaporation and nanoprecipitation methods to get hybrid particles in a single step.

Modified Methods

In a modified solvent extraction/evaporation method, polymer and drug are dissolved in water-immiscible organic solvent and lipid is dispersed in the aqueous phase with the aid of heat or sonication. The organic solution is added to aqueous phase-containing lipid under stirring. Finally, the solvent is removed to precipitate nanoparticles coated with lipid layer. A modified nanoprecipitation method involves dissolving polymer and drug in water miscible solvent and its drop wise addition to the aqueous phase containing lipid under stirring conditions. Diffusion of organic solvent into the water results in precipitation of particles in nano size range. Lipids self-assemble on the surface of polymer nanoparticles through hydrophobic interactions.

3.3.2 Role in Drug Delivery

The structural versatility of LPHNPs allows encapsulation of different kinds (hydrophobic, lipophilic, and hydrophilic) of drugs alone or in combination. Their surface can be modified with ligands for site-specific delivery and are promising systems to deliver chemotherapeutic agents or gene delivery.

Drug Delivery in Cancer

Delivery of anticancer agents using LPHNP showed improved cytotoxicity of doxorubicin in breast cancer cell lines (Wong et al. 2006b), enhanced uptake (Wong et al. 2006a), controlled drug delivery (Chan et al. 2009) and increased drug accumulation by overcoming multi drug resistance in breast cancer cell lines (Li et al. 2012). Anna et al. (Palange et al. 2014) reported significant reduction in number of adhering cells (approximately 70% reduction) and decrease in vascular adhesion (approx. 50%) with curcumin loaded LPHNPs.

Combination Therapy

Zhang et al. (2016) reported synergistic activity of doxorubicin and mitomycin C at tumor sites with combined delivery using hybrid nanoparticles. The authors also reported enhanced bio distribution, improved pharmacokinetics with increased local bioavailability and cytotoxicity of chemotherapeutic agents in murine breast tumor model. Higher levels of tumor cell apoptosis and reduced organ toxicity were reported with hybrid nanoparticles when compared to free drug mixtures.

Gene Delivery

LPHNPs play an important role in gene delivery. Prolonged release of siRNA over one month from LPHNPs showed greater silencing activity in luciferase-expressed HeLa cells and A549 lung carcinoma compared to lipofectamine 2000-siRNA complexes with significant improvement in tumor inhibition, both in vitro and in vivo (Shi et al. 2014).

Targeted Delivery

Agrawal et al. (2015) evaluated the efficiency of surface modified LPHNPs in targeting glioma cells bypassing blood–brain barrier. Hybrid particles were prepared by nanoprecipitation method using poly(lactic-co-glycolic acid), distearoylphosphatidylethanolamine-poly(ethylene glycol)-200 and lecithin. Folic acid was used as ligand for surface modification and the active therapeutic agent was peptide-conjugated paclitaxel. Cellular uptake studies indicated enhancement in uptake of folic acid coated hybrid nanoparticles. In vivo studies suggested significant improvement in survival time of mice administered with hybrid nanoparticles. Pharmacokinetic and distribution studies revealed significant concentration of drug in tumors thus preventing the exposure at nontarget sites and reducing the side effects. Zhang et al. (2008a, b) proved LPHNPs are better compared to poly (lactic-co-glycolic acid) nanoparticles in terms of drug loading, sustained drug release profile, stability in serum, cellular targeting ability.

4 Lipid Vesicles

4.1 Liposomes

Dr. Alec D. Bangham, a British hematologist observed the formation of bilayered vesicles by phospholipids, in his lab at the Babraham Institute, Cambridge in 1961 and published first article on the swollen phospholipids (Bangham et al. 1965). Since then, different reports were published (Bangham et al. 1967; Papahadjopoulos and Watkins 1967) on bilayered phospholipids initially named as bangosomes later changed to liposomes derived from two Greek words, "lipos" meaning fat and "soma" meaning body (Deamer 2010). The development of drug incorporation into liposomes concept, by Gregoriadis et al. led to the further growth of liposomes in drug delivery (Gregoriadis and Ryman 1971). First product to be introduced into the market was a cosmetic product (Capture) by the company Dior in 1986 (Müller et al. 2011). The first pharmaceutical product to enter the market was Alveofact® for acute respiratory distress syndrome through pulmonary route, first injectable liposomal formulation introduced was AmBisome® in 1991 (available in Europe), the first FDA approved liposome in the USA and first anticancer formulation was (Doxil®).

Liposomes are micro vesicles composed of one or more layers of amphiphilic lipid molecules with encapsulated aqueous phase or compartments. The presence of both lipid and aqueous core makes liposomes more useful in encapsulating both hydrophilic and hydrophobic drugs. Hydrophilic drugs are present in the aqueous core and lipophilic drugs are distributed in the lipid core. The other advantages of liposomes include, biocompatibility, increased stability and controlled release of drugs via encapsulation, tunability, or flexibility of the surface modification, and reduced toxicity of drugs due to site-specific delivery.

Phospholipids (PLs) (natural or synthetic origin) are the major constituent of liposome. However, cholesterol can be added to the phospholipids to increase the stability of liposome structures. PLs contain hydrophilic head group and hydrophobic tail linked to an alcohol. The variation in these head groups and length of hydrophobic chain results in different types of PLs. Detailed structure and properties of PLs are discussed elsewhere (Li et al. 2015). PLs exhibit emulsifying, surface-active wetting properties which makes PLs as suitable carriers for drug delivery (Yang et al. 2013). Soya phosphatidylcholine, egg phosphatidylcholine, and hydrogenated phosphatidylcholine, are commonly used PLs in different types of formulations.

4.1.1 Classification of Liposomes

Liposomes are classified based on their size, number of layers, method of preparation, and their application. Liposomes size can vary from 200 nm to 2.5 μm range. Depending on size, number of layers and method of preparation they are classified as small unilamellar vesicles (SUV), large unilamellar vesicles (LUV), and multilamellar vesicles (MLV), single or multilamellar vesicles made by

reverse-phase evaporation method (REV), stable plurilamellar vesicles (SPLV), frozen and thawed multilamellar vesicles (FTMLV), vesicles prepared by extrusion methods (VET), and dehydration-rehydration vesicles (DRV). Based on composition and application they are classified as conventional liposomes, stimuli-responsive liposomes (pH/thermo/heat sensitive), stealth liposomes, immuno liposomes, and cationic liposomes (Garg and Goya 2014). Stealth liposomes or PEGylated liposomes are surface-modified liposomes with hydrophilic polymers like polyethylene glycol to prevent the rapid clearance by mononuclear phagocyte system (MPS) due to opsonization process. This reduction in mononuclear phagocyte system (MPS) uptake and long circulatory half-life results in accumulation of drug in the tissues organs and thus can result in improved pharmacokinetics and reduced toxicity of drugs (Mahmood and Green 2005).

4.1.2 Liposome Preparation Methods

Liposomes are prepared by using three strategies such as mechanical methods, solvent removal methods and size transformation methods.

Mechanical Methods

Film Hydration Method

Liposomes are spontaneously produced upon hydration of thin lipid film as reported by Bangham et al. (1965). Lipid is dissolved in water-immiscible organic solvent (chloroform) and evaporated under reduced pressure using rotary evaporator. Thin lipid film is formed on flask surface during evaporation. Evaporation is continued for 2–3 h for complete removal of solvent. The film is then hydrated with aqueous phase, under stirring, to obtain vesicles. Hydration step should be carried above the phase transition temperature of lipid to obtain proper dispersion of lipid. Formation of thin and uniform film is necessary for efficient hydration. For encapsulation, hydrophilic drugs are dissolved in aqueous phase and lipophilic drugs are dissolved in organic solvent. Hydration time also plays a major role in the encapsulation of drugs into liposomes. MLV are produced using this method. SUV can be produced on ultra-sonication or high shear homogenization of the product.

Methods Based on Solvent Removal

Lipids dissolved in organic solvent and evaporated followed by hydration gives liposomes. However, solvent evaporation depends on the type of organic solvent. In case of water-immiscible solvents, emulsion is formed with aqueous phase and solvent evaporation takes place. Whereas, if water miscible solvent is used, then it diffuses into water and further dilution followed by evaporation results in liposomes.

Reverse-Phase Evaporation

Lipids are dissolved in organic solvents like, isopropyl ether or diethyl ether or a mixture of isopropyl ether and chloroform and emulsified with aqueous phase. Organic solvent is slowly evaporated under reduced pressure, which results in formation of viscous gel. Continuous evaporation process removes the traces of

solvent and disrupts the gel structure, leading to formation of liposomes. LUVs are produced using this method (Szoka and Papahadjopoulos 1980).

Ethanol Injection Method

This method was described by Batzri and Korn (1973). Lipids dissolved in ethanol are rapidly injected into an aqueous solution. Dilution of ethanol with aqueous phase precipitates liposomes. The obtained liposomal suspension is concentrated by ultrafiltration. Rapid evaporation of ethanol results in smaller particles whereas slow evaporation rates generally gives larger particles.

Ether Injection Method

Diethyl ether or ether–methanol mixture containing lipids is gradually injected to an aqueous solution at 55–65 °C. The removal of solvent leads to the formation of liposomes. The major limitation of this technique is that resultant product is heterogeneous (70–200 nm) and there exists a possibility of drug degradation due to exposure to organic solvents at high temperature (Akbarzadeh et al. 2013).

Size Transformation Methods

Freezing and Thawing

The lipid is dispersed in aqueous phase by sonication. The dispersion is frozen at −20 °C and kept at that temperature for 24 h. The frozen dispersion is then thawed at room temperature for 15 min and sonicated to obtain liposomes (Ohsawa et al. 1985).

French Press Extrusion

MLVs are converted to SUVs in French press cell under high shear. MLV are passed through a poly carbonate membrane (size < 0.2 μm) at 4 °C under high-pressure 20,000 psi. Generally, membranes with pore size greater than 0.2 μm yields polydisperse suspension of MLV liposomes. Hence, use of membranes having a pore size of ≤ 0.2 μm is recommended to get SUVs. Heterogeneous particles are produced after a single passage through membrane while repeated passages of MLV dispersion through the press results in SUVs of size range 30–50 nm (Szoka and Papahadjopoulos 1980). The system is attached to heating and cooling systems for temperature controlling. Liposomes produced by this method are more stable than the liposomes produced by sonication method. The major limitation of this method is that, only small volumes can be handled and cannot be used for large scale batches.

Dehydration/Rehydration Method

Kirby and Gregoriadis (1984) developed this method wherein, LUVs can be prepared from SUVs. SUVs dispersed in small amount of water are lyophilized and rehydration of the particles leads to formation of LUVs with improved encapsulation efficiency. This method is used to prepare liposomes in situ for parenteral delivery. Drugs can be added in the first step to SUVs or can be added during the final rehydration step. Limitation of this method involves use of cryoprotectants during lyophilization which may interfere with the formation of LUVs during rehydration.

Detergent Removal Method

Small unilamellar liposomes are produced from mixed micelles after dilution. Mixed micelles are obtained by dissolving lipid in presence of detergent like bile salts or alkyl glycosides. Detergents above their critical micellar concentration solubilize the lipid leading to formation of mixed micelles. Removal of detergent by dilution slowly precipitates the lipid in form of liposomes. Detergent can be removed by dilution, dialysis, or gel chromatography.

Recently, new methods such as bubble method (Talsma et al. 1994) and use of supercritical fluid (Zhao and Temelli 2015) was reported for the preparation of liposomes. These new methods reduce the use of organic solvents.

4.1.3 Drug Loading

Loading of active constituents into the liposomes is by either passive loading via incorporation of drug during the preparation of liposomes, or by active loading where drug is added after the liposomes are produced. For active or remote loading, liposomes are incubated in drug solution and diffusion of drug takes place due to proton gradient (Bally et al. 1988) or pH gradient (Haran et al. 1993). Active loading is advantageous in terms of achieving high encapsulation efficiency, and reduced leakage compared to passive loading methods. Parameters that influence loading are physicochemical properties of drug, pH of external media, gradient ions (ammonium and acetate), loading duration, and process temperature (Gubernator 2011).

4.1.4 Applications of Liposome in Drug Delivery

Since the invention of liposomes by Bangham, wide variety of application in drug delivery has been reported. Controlled delivery of drug from liposomes reduces the fluctuation in plasma concentration associated with multiple dosing and decreases the frequency of administration. Encapsulation of drugs into lipid layers protects the drug from gastric environment and increases the stability. Further, surface modulation to produce site-specific delivery reduces the toxic effects of drugs especially useful for anticancer agents. Liposomes can be used to improve the delivery and therapeutic efficacy of drugs with narrow therapeutic index drugs such as oxaliplatin (Yang et al. 2011), amphotericin B (Juliano et al. 1985), and glucocorticoids (van den Hoven et al. 2011).

In case of tumor, where, blood vessels are damaged, these circulating vesicles gain entry and accumulate at tumor sites. This type of phenomenon is called as enhanced permeation and retention (EPR) effect or passive targeting which improves the therapeutic activity of anticancer agents (Takeuchi et al. 2001; Solomon and Gabizon 2008). Enhanced bioavailability was observed when liposomes were used as carriers for drugs like fenofibrate (Chen et al. 2009), glucagon-like peptide-1 (Hanato et al. 2009), alendronate (Han et al. 2012b), and furosemide (Vural et al. 2011). Liposomes were also found beneficial in delivering proteins and peptides through oral route. Prolonged effect of insulin on blood glucose levels in rabbits was reported by Choudhari et al. (1994).

PEGylated doxorubicin product showed decrease in cardiotoxicity, myelosuppression, alopecia and nausea compared to conventional liposomal formulation in a study by Lyass et al. (2000). Additionally, PEGylated liposomes may increase the solubility and stability as reported by Yang et al. for paclitaxel (2007). Various surface modified liposomes such as antibody mediated (Ahmad et al. 1993), magnetic liposomes (Kubo et al. 2000), immunoliposomes (coated with monoclonal antibody) (Cheng and Allen 2008), transferrin coated (Daniels et al. 2012), folic acid (Yoo and Park 2004) containing anticancer agents showed improved activity in vitro and in vivo animal models.

The ability of liposomes to encapsulate both hydrophilic and hydrophobic drugs permits simultaneous delivery of two drugs and thus useful in combination therapy. The combination therapy may show synergistic effect resulting in increased activity of therapeutic drugs (Liu et al. 2014) at lower concentration than individual therapy and minimize the concentration related toxicity (Yang et al. 2014a). Co-delivery of drug along with efflux inhibitors via liposomes may increase the concentration of therapeutic molecules in plasma and enhance their activity (Tang et al. 2014). Liposomes also find application in improving the efficacy of drugs via topical route (Chen et al. 2014), ophthalmic route (Tsukamoto et al. 2013) and pulmonary route (Elhissi et al. 2014). Few marketed products are reported in Table 3.

Table 3 List of marketed liposomes

Brand name	Drug	Company
Doxil; Caelyx (PEGylated liposomes)	Doxorubicin HCl	Janssen Biotech; Centocor Ortho Biotech; Schering-Plough
Myocet		Elan Pharmaceuticals; Zeneus Pharma
Lipo-dox (PEGylated liposomes)		Sun Pharma Global FZE
DaunoXome	Daunorubicin	Gilead Sciences
DepotDur	Morphine sulfate	Pacira Pharmaceuticals (San Diego, CA, USA)
DepoCyt	Cytarabine	
AmBisome	Amphotericin B	Gilead (CA, USA); Astellas Pharma (US); Fujisawa Health care (Osaka, Japan)
Epaxal	Inactivated hepatitis A virus (strain RG-SB)	Crucell, The Netherlands
Inflexal V	Inactivated hemaglutinine of Influenza virus strains A and B	Crucell, The Netherlands
Visudyne	Verteporfin	QLT (Vancouver, Canada), Novartis (Basel)
Marqibo	Vincristine sulfate	CASI Pharmaceuticals; Spectrum Pharmaceuticals

4.2 Lipoplexes

Advancement in genetic engineering and understanding of disease pathology at molecular level has led to the development of nucleic acids as therapeutic molecules for treatment of cancers, vascular diseases, infectious diseases, arthritis, neurodegenerative disorders, and AIDS (Mhashilkar et al. 2001). Different molecules that show potential in therapeutics include plasmid DNA, antisense oligodeoxynucleotides (AS-ODN), small interfering RNA (siRNA), and micro RNA (miRNA). However, the therapeutic efficiency of these molecules depends on their successful delivery at the site of application in stable form. In order to improve nucleic acid delivery, both viral and nonviral vectors have been explored, with each system having its own advantages and disadvantages. Lipoplex is one such example in nonviral delivery vehicles focused in the present chapter.

Lipoplex is a self-assembling nanosystem (80–400 nm) produced by complexation of cationic liposome with DNA. These systems were introduced by Felgner et al. (1987) for gene delivery. Complex formation occurs spontaneously due to electrostatic interactions between cationic liposome and negative charge on phosphate backbone of DNA when two solutions are mixed together (Elouahabi and Ruysschaert 2005). Few examples of cationic lipids used in the preparation of lipoplexare 1,2-di-O-octadecenyl-3-trimethylammonium-propane (DOTMA), dioctadecylamido-glycylspermine (DOGS), DC-Cholesterol (3ß-[N-(N′,N′-dimethylaminoethane)-carbamoyl]cholesterol), 1,2-dioleoyl-3-trimethylammonium-propane(DOTAP) and 2,3-Dioleyloxy-N-[2(sperminecarboxamido)-ethyl]-N, N-dimethyl-1-propanaminium trifluoroacetate (DOSPA) (Martin et al. 2005). Liposome preparation method, mixing kinetics, temperature and concentration of liposome and DNA are the critical parameters that control the lipoplex formation (Zelphati et al. 1998). Complexation results in complete neutralization of charges on DNA, final complex may be positively charged to increase the interaction with negatively charged cell membrane and transfection efficiency. It is also reported that the charge on the complex can determine size of the resulting particles and increase the stability of DNA against cellular nucleases (Sakurai et al. 2000). Hence, ratio of lipid to DNA is adjusted to obtain a cationic complex with improved transfection efficiency. The structure of lipoplex depends on composition of lipid, ionic strength of buffer media, and lipid to plasmid ratio. Structural evaluation using Synchrotron X-ray scattering analysis reveals that cationic lipid–nucleic acid complexes spontaneously assemble into distinct liquid crystalline phases such as lamellar, inverse hexagonal, hexagonal, and gyroid cubic phases (Safinya et al. 2014).

4.2.1 Mechanism of Drug Delivery

Positively charged lipoplexes bind to negative charge (due to proteoglycans) on the cell membrane by electrostatic interactions. Lipoplex enters the cell by clathrin mediated endocytosis (Wasungu and Hoekstra 2006). In general, after endocytosis, particles fuse with lysosomes and release its components. In such cases, DNA will be degraded by cytoplasmic nucleases. However, for transfection to occur, DNA should reach nucleus without undergoing degradation by nucleases. Anionic lipids

located on the cytoplasmic face of the endosomal membrane diffuse into complex initiating destabilization of the endosome membrane. These anionic lipids form neutral ion pair by reacting with cationic lipids due to stronger affinity. This weakens the electrostatic interactions between DNA and cationic lipids and results in release of the DNA from the lipoplexes (Xu and Szoka 1996). The released DNA enters the nucleus by passive diffusion (small molecules) during cell division or by a facilitated diffusion through nuclear pore complex (large DNA molecules). Transfection efficiency depends on the lipid composition, presence excipients like helper lipid, fusogenic peptide, charge density and charge ratio (Ma et al. 2007).

Study by Flenger et al. revealed that formulations with dioleoylphosphatidylethanolamine(DOPE) showed greater transfection activity than formulations containing dioleolyphosphatidylcholine (DOPC) as helper lipid. The authors concluded that this enhancement was due to ability of DOPE to increase fusion of lipoplex with cell membrane compared to DOPC (Felgner et al. 1987). The major limitation of these systems is the toxicity associated with cationic lipids.

4.2.2 Applications of Lipoplexes in Drug Delivery

Lipoplexes find applications in successful delivery of nucleic acid based molecules that are difficult to deliver due to their size and negative charge. The successful delivery of genes in stable form at specific site (nucleus) results in increased transfection efficiency and improved therapeutic efficacy. Sonia Duarte et al. showed significant enhancement in the transfection efficiency with high in vitro antitumoral activity of HSV-tk suicide gene from folate associated lipoplexes compared to plainlipoplexes (Duarte et al. 2012). Guo X et al. prepared a novel ortho ester based pH sensitive liposomes and tested their ability to deliver plasmid DNA encoding a luciferase reporter gene. Gene expression activity was tested in CV-1 cells and CD-1 mice after intrathecal injection. DOC/DOPE/DNA lipoplexes showed 5–10 folds improvement in luciferase gene expression in both in vitro cell lines and in vivo animal models compared to acid stable DC-Chol/DOPE/DNA lipoplexes (Guo et al. 2014). Lipoplexes are also reported to show improved tumor accumulation and bio distribution of the actives (Gopal et al. 2006; Zhang et al. 2008a, b), gene delivery to liver (Mohr et al. 2001), efficacy in lung metastasis (Sakurai et al. 2003), neuronal siRNA delivery (Cardoso et al. 2008), gene delivery by EGF-lipoplexes (Buñuales et al. 2011), delivery of plasmid DNA (Kim et al. 2014). Few lipoplex based delivery systems under clinical trial are reported in Table 4.

4.3 Phytosomes

“Phytosome” is a patented technology that emerged in 1989 derived from the word ‘phyto’ meaning herb or plant and ‘some’ meaning cell like structure. Phytosomes are molecular complexes between natural herbal extracts and soy phospholipids that give micelles in aqueous media in nano size range (Kidd 2009). Phospholipids such as phosphatidyl choline (PC), phosphatidyl serine (PS), phosphatidyl ethanolamine (PE) and modified lipids like hydrogenated soy phosphatidylcholine (HSPC),

Table 4 List of lipoplexformulations in clinical trials

Lipoplex	Disease/condition	Phase level
siRNA-lipoplex (Atu027)	Solid tumors	Phase I
Tetravalent RNA-lipoplex	Advanced melanoma	Phase I
pbi-shRNA™ STMN1LP	Advanced cancer; metastatic cancer; solid tumors	Phase I
pGM169/GL67A	Cystic fibrosis	Phase II completed
pGT-1 gene lipid complex	Cystic fibrosis	Phase I completed

dipalmitoylphosphatidylcholine, and distearoyl phosphatidylcholine can be used as carrier molecules (Maiti et al. 2010). Phytosomes are produced in stiochometric ratio of 1:1, 1:2, and 1:3 of herbal extracts to phospholipids. Generally, herbal extracts contains flavanoids, polyphenols, terpenes that are water soluble or polar in nature. The polarity and large molecular size hinders the transport of these molecules across the biological membranes (intestine and skin) leading to poor bioavailability (Manach et al. 2004). As phosphatidyl choline is amphiphilic in nature (miscible in both water and oil) and is a major ingredient of lipid membranes, it easily permeates through the biological membranes resulting in higher concentration of the actives in circulation. A complex of herbal extract with phosphatidyl choline thus helps in enhancing the absorption and permeation of herbal actives across the intestinal membrane and skin (Mukherjee et al. 2015). Large surface area due to nano size, increased solubility, permeability and improved stability of herbal extracts incorporated in phytosomes further enhances the bioavailability. The advantage with phytosomes is the presence of soya lipids used in the preparation that are biocompatible, biodegradable, and with a well-proven safety profile. Phytosomes offer controlled or prolonged release of the actives with site-specific delivery for improved patient compliance and better therapeutic efficacy. Phytosomes can be used to deliver drugs through oral and topical routes.

The major difference between liposome and phytosomes is that, in liposomes the drug is in the core or central cavity without any chemical bonding. Whereas, in phytosomes the drug molecules are dispersed in phosphatidyl choline with formation of hydrogen bonds as evidenced by infrared spectroscopy and multi-nuclear spectroscopic studies (Bombardelli and Spelta 1991).

4.3.1 Method of Preparation

Drug (herbal extract) and phospholipid are dissolved in organic solvent or mixture of solvents in definite molar ratio (1:1 to 1:4) and the solution is refluxed. Different researchers have reported different temperature conditions (room temperature to 60 °C) for a period ranging from 3 to 24 h. Phytosomes are precipitated by addition of n-hexane to the solution or the solvent is evaporated either by spray drying or under vacuum or by lyophilization (Kidd 2009; Mukherjee et al. 2015; Panda and

Table 5 List of marketed phytosome products

Brand name	Composition	Biological activity
Casperome® (Boswellia Phytosome®)	Extract from Gum resin of *Boswelliaserrata*	Joint health
18β-Glycyrrhetinic acid phytosome®	18β-glycyrrhetinic acid	Soothing, lenitive
Bosexil®	*Boswelliaserrata*	Soothing, anti-photo aging
Ginkgoselect®	Standardized extract of *Ginkgo biloba* leaves	Cognition and circulation improver, antioxidant, vasokinetic
Ginseng phytosome	Extracts of *Panax ginseng*	Skin elasticity improver
Greenselect®	Extract from Green Tea leaves	Antioxidant activity, weight management
Hawthorn	Extract of *Crataegus* spp. Flowering top	Cardiovascular health, antioxidant
Leucoselect®	lower procyanidinoligimers (OPCs) from grape seed	Cardiovascular protector, UV protector, antioxidant
Meriva®	Curcumin	Joint health
Rexatrol®	Polygonum cuspidatum Sieb	Antioxidant, anti-aging
Virtiva®	*Ginkgo biloba* standardized extract	Cognitive enhancer
Siliphos®	Silybin	Anti-wrinkles, retinoic acid-like activity
Milk Thistle Phytosome®	Silymarin, dandelion powder, turmeric, artichoke powder	Against liver damage by hepatitis, cirrhosis

Naik 2008; Flaig et al. 2010). Few phytosome products with enhanced absorption and improved bioavailability of plant extracts are available in the market (Table 5).

4.3.2 Applications of Phytosomes

Enhanced Biological Activity of Phytoconstituents or Extracts

Many authors have reported improvement in the biological activity of phytoconstituents from phytosome complex compared to their original counter parts. Panda and Naik (2008) reported significant enhancement in the cardio protective effect of Ginkgo biloba from phytosome in isoproterenol-induced myocardial necrosis in rats. The authors concluded that the improved activity was due to improved absorption of active constituents from phytosomal formulation resulting in augmentation of the endogenous antioxidants and inhibition of lipid peroxidation of membrane. Mazumder et al. (2016) reported enhancement in wound healing activity with reduced toxicity of sinigrin from phytosome compared to pure drug. Ali et al. (2014) discussed the protective effect showed by silybin-phytosome-administered along curcumin and R-lipoic acid in liver damage. Antioxidant and antifibrotic activity of these phytoconstituents showed potential in protecting the liver from the biochemical abnormalities caused by ongoing hepatic damage.

Phytosome formulations are also reported for improved anti-inflammatory activity of glycyrrhetinic acid (Bombardelli et al. 1994), anti-aging properties of silymarin (Bombardelli et al. 1991), hepato-protective activity of milk thistle (Silybummarianum) (El-Gazayerly et al. 2014), antioxidant activity of naringenin (Maiti et al. 2006), improved oral absorption of boswellic acid (Hüsch et al. 2013), improved intestinal permeability of diosmin (Freag et al. 2013), improvedpharmacokinetics of silybin (Yanyu et al. 2006) and improved bioavailability curcumin (Zhang et al. 2013), oxymatrine (Yue et al. 2009) and ginkgo biloba extract (Chen et al. 2010).

4.3.3 Studies in Clinical Trials Using Phytosome Delivery

Recent studies indicates that silibinin phytosomes in high concentrations showed enhanced therapeutic effects in phase I (Flaig et al. 2010) and has now moved to phase II study in prostate cancer patients (ClinicalTrials.gov 2007). The effectiveness of combination of erlotinib and silybin phytosome in EGFR Mutant Lung Adenocarcinoma is under phase II (ClinicalTrials.gov 2014). A study on weight maintenance after weight loss in obese women was completed recently (September, 2015) using Greenselect Phytosome (ClinicalTrials.gov 2015).

5 Characterization of Lipid Carriers

The particle size plays a crucial role in determining properties of lipid carriers in vitro and its biological fate in vivo. Hence, precise characterization is necessary to develop a carrier system with required properties for desired application. Particle size and size distribution are determined by dynamic light scattering technique using zeta sizer. Particle shape and morphology is characterized by electron microscopy like scanning electron microscopy, transmission electron microscopy, and atomic force microscopy. Surface charge plays an important role in stability of colloidal particles that is measured in terms of zeta potential using zeta sizer. Crystallinity and polymorphism are identified by differential scanning calorimetry, and X-ray diffraction analysis. Presence of other colloidal structure and interactions between drug and carrier are detected by spectroscopic techniques such as nuclear magnetic resonance and electron spin resonance spectroscopy (Mehnert and Mader 2001).

6 Storage Stability of Lipid Carriers

Increased particle size, change in crystal morphology, gelation phenomenon, drug expulsion or leakage, and presence of other colloidal structures like micelles are the major problems of lipid carriers during storage. Increased particle size may occur due to crystal growth by Ostwald ripening. Drug expulsion may be due to transformation of less stable form (alpha form of lipid produced during cooling) of lipid

to more stable form with highly ordered crystal structures. Presence of aqueous phase in the formulations further enhances the crystallization process. Conversion of aqueous dispersion into dry powder is a promising way to improve the stability of lipid carriers. Lyophilization is found to be a suitable technique for conversion of lipid suspension to dry powder. Lipid suspension is frozen and water is removed by sublimation under vacuum. Cryoprotectants (sucrose, trehalose, polyvinylpyrrolidine, sorbitol, and mannose) added to the lipid suspension before lyophilization prevents the agglomeration of particles after reconstitution (Manjunath et al. 2005).

7 Conclusion

Lipid-based carrier systems offer promising nano drug delivery systems to deliver poorly soluble drugs thereby improving their therapeutic efficacy and reducing their side effects. Biocompatibility and GRAS status of the excipients (lipids), ability to deliver the drugs through various routes, ability to encapsulate both hydrophilic and hydrophobic drugs, site-specific delivering capability, market potential and commercially feasible production techniques makes them an attractive drug delivery system.

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Nanocrystals for Delivery of Therapeutic Agents

Rajesh Thipparaboina, Rahul B. Chavan and Nalini R. Shastri

Abstract

Clinical application of many emerging new chemical entities remains a herculean task due to poor aqueous solubility and bioavailability problems. Nanoscale orchestrations of solid state of such NCEs render faster dissolution rate, increased saturation solubility and enhanced bioavailability. Nanocrystals are crystalline particulate systems with dimensions less than 1000 nm. Unique surface properties, high loading capabilities, marked enhancements in bioavailability, lower fast/fed state variability, low incidence of side effects, delivery through various routes like enteral, parenteral, pulmonary, dermal etc., scope for active and passive targeting and wide range of technologies available for commercial applications offers potential platform for exploration of drug delivery using nanocrystals. It is predicted that nanocrystals would account for about 60% of all nanotechnology-based products with a market capture of 82 billion USD by 2021. Recent surge in marketed products and greater market capture amongst all nanoparticulate systems emphasizes the need for further development of nanocrystals. Exploring the potential of synchronized release with targeting could help in effective treatment of infectious diseases, pain-related disorders, and also aid in cancer chemotherapy. This chapter aims at providing a brief overview of formulation, preparation methodologies, stabilization techniques, characterization, evaluation, applications, biopharmaceutical aspects, safety and efficacy, and regulatory perspectives related to nanocrystals.

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DSC · Crystallinity · Polymer · Nanotoxicological classification · Crystallization · Precipitation

Abbreviations
AUC Area under the curve
PDI Polydispersity index

1 Introduction

High throughput screening has revolutionized drug discovery and development programmes, but has increased the risk of development of poorly soluble compounds, as high throughput screening hits are likely to have high molecular weight and LogP. Poorly soluble compounds lead to problems in in vitro and in vivo assays during preliminary screening and also pose a major financial risk in the drug development process (Di et al. 2012). Poor solubility of an estimated 75% drug development candidates is a major concern in drug discovery and development despite increasing costs of development (Di et al. 2009). Devising strategies to develop formulations for such BCS class II and IV (poorly water soluble) drugs has always been a major obstacle for formulation scientists (Gao et al. 2008). Poor solubility has been tailored using various approaches like crystal engineering (Blagden et al. 2007), amorphization (Van den Mooter 2012), micronization (Loh et al. 2015), prodrug synthesis (Stella and Nti-Addae 2007), cyclodextrin complexation (Jambhekar and Breen 2015), use of cosolvents, use of lipid vehicles and polymeric carriers (Mehnert and Mader 2001) etc. since long, with specific applications and occasionally with longstanding setbacks.

Various nanotechnology-based strategies like nanoemulsions, nanocrystals, polymeric micelles, lipid nanoparticles, dendrimers, and carbon nanotubes are being used to tackle poor solubility and bioavailability issues of BCS class II and IV drugs (Chen et al. 2011; Pathak and Raghuvanshi 2015). Nanocrystals constitute a unique group of all the nanotechnology-based products with majority of them designed for oral drug delivery. Nanocrystals are crystalline systems in the size range of 1–1000 nm with or without stabilizers. They act as a connecting link between crystalline form and amorphous form of a drug. Drug nanocrystals are comprised of 100% drug and do not contain any carrier/matrix materials like polymers or lipids. This differentiates nanocrystals from other nanoparticles. In the past few decades, extensive research is being carried out to develop new manufacturing technologies for nanocrystals, evaluate physicochemical properties of nanocrystals, understand and elucidate their stability and safety concerns. Benefits offered by nanocrystals in pharmaceutical field mainly include improved saturation solubility, enhanced dissolution velocity, improved bioavailability and the most important, patient compliance due to reduction in oral units of drug administered.

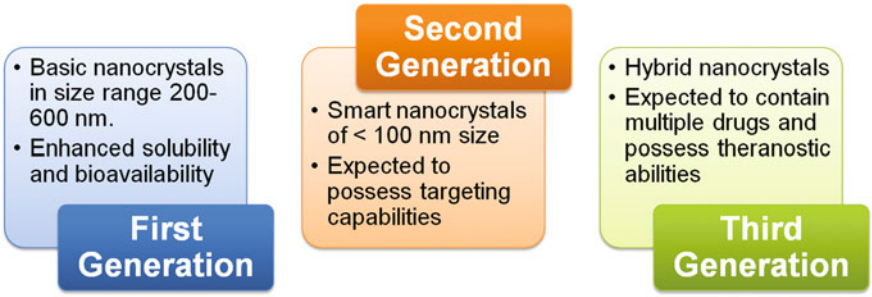

Fig. 1 Classification of nanocrystals

It is remarkable that these systems have entered pharmaceutical market in less than 10 years when compared to liposomes which took nearly 25 years to reach the market. Nanocrystals have demonstrated commercialization potential with a blockbuster product Tricor® whose annual sales are more than 1 billion $ in US with number of other products in pipeline that are about to enter markets in near future.

Tracking the progress of nanocrystals to date and anticipating future possibilities, the developmental journey of nanocrystals can be categorized into three generations as represented in Fig. 1. Literature available to date reports two generations of nanocrystals. First-generation nanocrystals are basic versions, mostly in the size range of 200–600 nm, intended for solving bioavailability and solubility issues of poorly soluble drugs (Patravale and Kulkarni 2004). Second-generation nanocrystals are smart crystals with a particle size less than 100 nm and possess targeting capabilities (Keck et al. 2008). Considering the remarkable progress achieved by nanocrystals during the past few decades, we forecast the development of a third generation nanocrystals representing hybrid systems containing multiple drugs and/possessing theranostic capabilities (Lu et al. 2015).

2 Advantages of Nanocrystals

Nanocrystal possesses some unique features like enhanced saturation solubility, improved dissolution velocity, enhanced bio-adhesiveness to cell membranes and cell surfaces which mainly helps in tackling many biopharmaceutical issues associated with poorly soluble drugs such as low bioavailability, large injection volumes, low dermal penetration and large propensity of side effects. Enhancement of saturation solubility by nanocrystal can be proven through Ostwald-Freundlich equation, which states that saturation solubility is inversely correlated with particle size, and found to be more pronounced as particle size is below 1 μm, as is the case with nanocrystal. However, enhancement of dissolution velocity can be explained from Noyes–Whitney equation. It can be easily confirmed that size reduction to nanometer scale leads to an increase in surface area and ultimately increase dissolution velocity as it is directly proportional to surface area. Enhanced bioadhesion

of nanocrystal can be explained because the particle size reduction to nano level helps in easy penetration into gastric mucosa. Various benefits offered by nanocrystals are depicted in Fig. 2.

Nanocrystallization as a solubilization strategy avoids use of solvents, surfactants, and oils. Of all the nanotechnology-based products, nanocrystals are reported to have highest drug loadings. Significant reduction in therapeutic doses is also observed due to enhanced bioavailability. Enhanced physical and chemical stability of drugs is seen when compared to amorphous forms and other nanotechnology-based products. Nanoscale crystallization helps in passive targeting through enhanced permeation and retention effect (EPR) and active targeting can also be achieved by conjugating with various peptides, antibodies, etc. Additionally nanocrystals are given "New Drug Product" status by USFDA and are very cost effective.

Fig. 2 Advantages of nanocrystals

3 Formulation

Formulation of nanocrystals involves a poorly soluble drug and a stabilizer. Optimal benefits of nanocrystallization are seen with drug molecules possessing high molecular weight (paclitaxel, sirolimus, etc.), high melting point (high crystal lattice energy like telmisartan, hydrochlorothiazide, etc.), and a solubility of less than 0.2 mg/mL (albendazole, celecoxib, itraconazole etc.), because the advantages gained due a smaller particle size are the highest with these types of compounds (Rabinow 2004). Brick dust drugs, which are very difficult to formulate can be easily formulated using advanced nanocrystal technologies (Chingunpituk 2007). BCS class II drugs with poor solubility and high permeability are ideal candidates for formulation of nanocrystals. Class IV drugs may not be ideal candidates for nanocrystallization, but recent reports reveal permeation enhancements using nanocrystals. Drugs with narrow absorption window would also be ideal for the development of nanocrystals as rapid dissolution of nanocrystals in the absorption window would enhance the bioavailability significantly.

Various methods have been explored for the producing drug nanocrystals. They are categorized as bottom-up, top-down, combinative, and miscellaneous approaches. Bottom-up approaches in which crystals are formed at molecular level as in precipitation, top-down approach where in larger micron sized are broken down to nanosized particles by milling or high pressure homogenization and combinative approaches employing both bottom-up and top-down techniques. In all the above processes, a larger surface area is formed increasing the total free energy of the system. Such systems are thermodynamically unstable and tend to agglomerate. This agglomeration tendency is opposed by the addition of stabilizers (Rabinow 2004). Various processes used for the preparation of nanocrystals are depicted in Fig. 3 (Van Eerdenbrugh et al. 2008b; Borchard 2015; Lu et al. 2015).

3.1 Bottom-Up Approaches

Bottom-up approaches include crystallization/precipitation methods. It involves addition of an anti-solvent to drug solution with or without stabilizer. Optimal control of process parameters to promote crystal nucleation and allow crystal growth in nanometer range is a pre-requisite for development of nanocrystals using this approach. This process is critical and can result in formation of polymorphs. The bottom-up approaches require the use of solvents that are usually difficult to remove completely. Presence of residual solvents is one of the major concerns with these processes as use of class 1 and 2 solvents may lead to harmful effects and organic residues present may lead to physical and chemical instability. In addition, needle shaped particles are usually produced in bottom-up approaches due to rapid growth in one direction. This tends to influence the physical stability of the

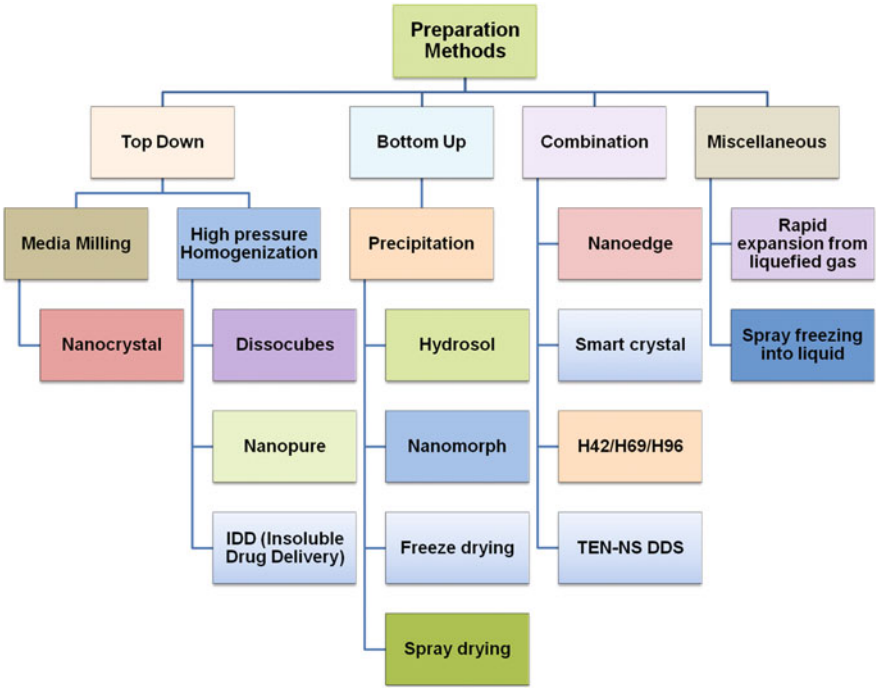

Fig. 3 Technologies used in the preparation of nanocrystals

nanosuspensions negatively (Verma et al. 2009). However, these methods are easier to process on large scale and are suitable for hydrophobic drugs. These methods involve crystallization, filtration and drying of nanocrystals, where input of mechanical energy is minimized compared to top-down methods. Besides conventional crystallization methods, latest technologies operating through high-gravity, supercritical fluids, ultrasonics, cryogenics and microemulsion templates are also utilized for crystallization of the drug nanocrystals. No method is universal, an appropriate choice of crystallization method is vital for the successful production of drug nanocrystals. Crystallization/precipitation process is mainly used. It is an instantaneous process with rapid nucleation kinetics. Mixing is crucial in such processes for determining supersaturation distribution which further determines the particle size distribution. Weakly acidic or basic hydrophobic drugs are ideal candidates for reactive crystallization. Addition of neutralizing solutions (strongly acidic or basic) decreases the solubility inducing crystallization. This method is relatively unexplored. Nanocrystals of few drugs, like crystals of itraconazole (Rabinow et al. 2007) and azithromycin, were obtained using this method, with an average size of 279.3 and 413 nm respectively.

3.2 Top-Down Approaches

Top-down approaches include media milling and homogenization which helps in production of nanocrystals using mechanical forces. These methods have been successful with few FDA approved commercial products on the market. These methods use high energy or pressure to achieve nanosized crystals. They are time consuming with intensive energy use and introduce impurities due to abrasion. Particle size control is inadequate and generates electrostatic effects (Van Eerdenbrugh et al. 2008).

3.2.1 Media Milling

Media milling using high-shear media or pearl mills is being used since long times for the production of nanocrystals. In media milling, the milling chamber is charged with the milling media (zirconium oxide, glass or highly cross linked polystyrene resin), formulation components and then operated at very high-shear rates. Nanosized crystals are produced by the shear forces produced due to impact of the milling media with the drug (Merisko-Liversidge and Liversidge 2011). Drugs with poor solubility in aqueous and organic media can be easily processed using media milling. Scale up is easy with little batch to batch variation and narrow particle size distribution. Contamination due to erosion of milling material is a major problem associated with this technology and this was significantly reduced by the introduction of polystyrene resin beads (Jia 2005). The Nano-crystals® technology developed by Elan Corporation was a core development in the commercialization of nanocrystal products. Nanomill® system was introduced by the same company for lab scale applications. Many products like Verelan PM®, Rapamune®, Focalin XR®, Avinza®, Ritalin LA®, Herbesser®, Zanaflex™, Emend®, Tricor®, Theralux®, Semapimod®, Theodur®, Naprelan® and Megace® ES were successfully commercialized using media milling process.

3.2.2 High Pressure Homogenisation

A high-pressure homogenizer is made up of a high-pressure plunger pump with a relief valve (homogenizing valve). The energy level required for the relief valve is provided by the plunger pump. The relief valve consists of a fixed valve seat and an adjustable valve. The gap conditions, the resistance and thus the homogenizing pressure vary as a function of the force acting on the valve. During the homogenization process, drug particles are fractured by cavitation, high-shear forces and the collision of the particles against each other. The drug suspension in the cylinder is passed through a very narrow homogenization gap. In the homogenization gap, the dynamic pressure of the fluid increases with a simultaneous decrease in the static pressure below the boiling point of water at room temperature. Hence, water starts boiling at room temperature, leading to the formation of gas bubbles, which implode when the suspension leaves the gap (called cavitation) and normal air pressure is reached again. The implosion forces are sufficiently high enough to break down the drug microparticles into nanoparticles (Krause and Muller 2001). Extensive use of energy, pre-micronization step before homogenization, high cost of instrument and

requirement of large number of homogenization cycles to achieve desired particle size are few disadvantages associated with this process. Micro-fluidizer technology (IDD-PTM technology), Dissocubes® technology (SkyePharma), or Nanopure® technology, (Abbott Laboratories) are various technologies developed using high pressure homogenization.

3.3 Combination Methods

Hybrid manufacturing methods were developed to reduce the time consumed for production of drug nanocrystals using regular methods. They are comparatively modern methods and couple crystallization process with high energy top-down techniques. Usually in combination methods, high energy via media milling, high pressure homogenization, ultrasonication, and high energy mixing is imparted post crystallization. Of all the methods, high pressure homogenization is the most popular method which is used in combination with other methods for production of most of the commercial products developed to date. Various drugs and nutraceuticals explored using combination methods are provided in Table 1.

3.3.1 Teniposide Nanosuspension Drug Delivery System (TEN-NSDDS)

TEN-NSDDS is the most recent combination process developed by He et al. In this approach, an anti-solvent sonication–precipitation method was used for the development of TEN nanosuspension. Initially, drug solution in acetone was added to anti-solvent under stirring at 1000 rpm for 10 min. The resulting precipitate was ultrasonicated using bursts for 3 s with a pause of 3 s for every two ultrasonic bursts, at a temperature of 4–8 °C. Residual acetone was removed under vacuum at 35 °C, for 12 h using rotary evaporation. Rod-like TEN nanocrystals with a size of 151 ± 11 nm and a narrow poly dispersion index of 0.138 was obtained. The obtained freeze dried TEN nanosuspensions were stable physically, for 3 months at 4 °C. When tested in rats with C6 tumors, the TEN concentrations in the tumor site was increased by 20-folds when compared to TEN solution at 2 h (He et al. 2015a).

3.3.2 ARTcrystal® Technology

Scholz et al. developed ARTcrystal® technology for producing flavonoid nanocrystals. It is a novel approach involving a rotor–stator pretreatment step with consequent high-pressure homogenization at low pressures for the production of drug nanocrystals. Various process parameters like size of starting material, flow rate, stirring speed, temperature, foaming effects, and valve position from 0° to 45° were studied in detail using an antioxidant rutin. One liter of nanosuspensions containing 5% rutin was produced in 5 min. Post optimization, a minimum pre-milling time of 5 min was recommended. Temperature was found to be a crucial variable affecting the yield and was suggested to be below 30 °C. A milling step with a rotor speed of 24,000 rpm and a flow rate (600 L/h, valve position of 45°) for 5 min at a temperature <30 °C could produce 1 L nanosuspension in 5 min in

Table 1 Drugs/Nutraceuticals explored using combination production technologies

Drug	Category	Technology	Process	Particle size (nm)	Advantages	Disadvantages	Reference
Teniposide	Anticancer	TEN-NSDDS	Precipitation + sonication	151	Small crystal sizes	Less stable product	He et al. (2015a)
Rutin, hesperidin and apigenin	Antioxidant	ARTcrystal®	Premilling + high pressure homogenization	411, 717 and 262 respectively	Faster and easily scalable	Larger particle sizes	Scholz et al. (2014), Scholz and Keck (2015)
Glibenclamide	Anti-diabetic	H96	Freeze-drying + high pressure homogenization	335	Finer particles and suitable for thermolabile products	Lengthy processing times	Salazar et al. (2012)
Apigenin	Antioxidant	CT	Pearl milling + high pressure homogenization	413	Finer particles	Possible contamination due to abrasion of beads	Al Shaal et al. (2010, 2011)
Ibuprofen	Anti-inflammatory	H69	Cavi-precipitation + high pressure homogenization	304	Improvement over Nanoedge®	Solvent residues	Muller and Moschwitzer (2006)
Glibenclamide	Anti-diabetic	H42	Spray drying + high pressure homogenization	236	Finer particles	Thermal degradation and amorphization tendencies	Moschwitzer and Muller (2006)
Itraconazole	Antifungal	Nanoedge®	Precipitation + high pressure homogenization	581	Smaller crystals compared to individual processes	Crystal growth during process and solvent residues	Kipp (2004), Kipp et al. (2003)

continuous circulation mode (Scholz et al. 2014). The proposed method is a fast and an economical process in which initial high-shear stress and subsequent cavitational forces (due to high pressure homogenization) are applied onto the crystals, thus achieving smaller crystal sizes in less amount of time when compared to traditional high pressure homogenization. Mean crystal sizes obtained using this process are in the range of 300–700 nm. Nanocrystals of various antioxidants like rutin (Scholz et al. 2014), hesperetin and apigenin (Scholz and Keck 2015) were successfully produced using this technology.

3.3.3 Combination Technology

Combination technology is a new development to classical bead milling, also known as smartCrystals technology. It consists of bead milling as a pretreatment with subsequent high-pressure homogenization. Shorter pretreatment times are needed in comparison to classical bead milling. Bead milling is carried out to achieve mean particle size of 0.6–1.5 μm followed by 1–3 cycles of high pressure homogenization at reduced pressures. Homogeneity of the intermediate blend obtained post pretreatment helps in reducing the cycle number and operating pressures. Pilot scale up at 3 kg level was successfully carried out achieving a mean particle size of 400 nm. Obtained formulations were stable up to 6 months at 4 °C, room temperature and 40 °C (Al Shaal et al. 2010). Apigenin nanocrystals for commercial applications were successfully developed using this technology. Nanocrystals with a mean size up to 396 nm and low PDI were developed using combination technology (Al Shaal et al. 2011).

3.3.4 H42/69/96 Technologies

These technologies were developed by Moschwitzer et al. exploring the potential of spray drying, freeze-drying and cavi-precipitation in combination with high-pressure homogenization for the production of nanocrystals (Moschwitzer and Muller 2006; Salazar et al. 2013). H42 technology was the initial development in this series combining spray drying with high-pressure homogenization. During the process, organic solution of the drug is added to aqueous solution with or without stabilizer followed by high-pressure homogenization (20 cycles at 1500 bar). Glibenclamide nanocrystals with a mean particle size of 236 nm and spherical morphology were successfully developed using this process. Organic residuals and scope for formation of amorphous phase are the major setbacks of this method (Salazar et al. 2013; Moschwitzer and Muller 2006). H69 technology combines microprecipitation and high-pressure homogenization. In this technology, organic solution of the drug is pumped into the homogenizer gap and anti-solvent is added in controlled manner, by controlled pumping, just before reaching the gap. Once the micro precipitation is initiated, the formed particles are passed through the homogenization gap that subsequently undergoes cavitation. During this process, annealing is applied by high-pressure homogenization to prevent further crystal growth to micrometer range and transform amorphous/semicrystalline form into a more stable crystalline state. This process is controlled by regulating the flow and ratios (Muller and Moschwitzer 2006). Ibuprofen nanocrystals with high degree of

crystallinity and a mean particle size of 304 nm were successfully produced using this technology (Sinha et al. 2013). Another development in this line of combination process is H96 process. In H96 process, drug suspensions are freeze dried, re-dispersed and immediately homogenized using high-pressure homogenization (Moschwitzer and Lemke 2006). This process is comparable to that of spray drying in H42 process, but by employing freeze-drying the process is made more suitable for thermolabile drugs (Teagarden and Baker 2002). Efficient utilization of H96 process was successfully demonstrated by Salazar et al. (2012) comparing it to high pressure homogenization. By freeze-drying, the degree of crystallinity can change tremendously, varying from 7 to 68% depending on the solvent ratio (dimethyl sulfoxide/tert-butanol). Pretreatment using freeze-drying allowed formation of smaller crystals of 335 nm at lower pressures compared to 691 nm using traditional high-pressure homogenization. More efficient results were obtained with pearl milling followed by freeze-drying pretreatment (160 nm compared to 191 nm) (Salazar et al. 2012). Marked reduction in size was attributed to the formation of a less crystalline, porous and brittle intermediate.

3.3.5 Nanoedge® Technology

It was the first combination process to be developed for nanocrystal production combining a microprecipitation and high-pressure homogenization (Kipp et al. 2003). Precipitation and high-pressure homogenization occurs separately in this process. Additional annealing step promotes size reduction of the crystals eliminating amorphous structures and enhancing physical stability (Kipp 2004). Major drawback of this technology is presence of solvent residues and a larger size distribution compared to other combination technologies.

4 Stabilization

Most common problem associated with nanonization is the instability of particles, which tend to aggregate. This results into instabilities like flocculation or sedimentation that are a major hurdle in development of pharmaceutical nanocrystals. Time required for aggregation may vary from seconds to hours or days. Flocculation is a process where destabilized particles conglomerate to form large aggregate. Attraction forces like chemical bonding or van der Waals forces is found to be responsible for aggregation. This physical instability is found to be responsible for loss of solubility and dissolution advantages offered by nanocrystals. Aggregation occurs via three different mechanisms, perikinetic aggregation, orthokinetic aggregation, or differential sedimentation. Perikinetic aggregation is mainly related to the rate of aggregation, which is governed by the frequency of collision of particles and the cohesive bond formation during the collision. Differential sedimentation arises due to different settling rate of the particles due to different sizes and density. Lastly, orthokinetic aggregation is mainly related to occurrence of aggregation due to extensive collision while particles are transported through

colloidal solution. Aggregation can be seen at various stages (production, storage and dissolution) during the developmental process leading to crystal growth and inconsistent dosing. Hence, there is a need to stabilize nanonized particles. Stabilization is predominately achieved by electrostatic repulsion and steric stabilization. Electrostatic stabilization is achieved by the formation of an electrical double layer around nanocrystals by adsorption of ionic charges resulting into generation of repulsive forces. Ionic strength of the medium has a significant influence on the repulsive forces. Due to its low cost and simplicity, this method of stabilization has been widely used but it is applicable to aqueous medium and not effective in solid form. Alternative technique available to electrostatic mechanism is steric stabilization in which non ionic amphipathic polymer is attached or adsorbed on the surface of nanocrystals. These polymers are mutually repulsive and hence prevent aggregation of particles. Advantages offered by steric stabilization mechanism over electrostatic, includes stabilized particles are re dispersible, influence of ionic strength of medium is ruled out and formulation with high concentration of nanocrystals can be obtained. Ionic-polymers which display unique properties of both polymers and surfactants impart electrostatic repulsion (surfactant property) and steric stabilization (polymeric property) (Shete et al. 2014). Various stabilizers used in the development of nanocrystals are enlisted in Table 2.

4.1 Selection Criteria for Stabilizers

Extensive literature is available regarding relationship between stabilization efficacy and properties of stabilizers. Various parameters related to drug, stabilizer and dispersion medium should be carefully assessed before choosing the stabilizer (Shete et al. 2014).

4.1.1 Drug-Related Parameters

Solubility of drug in stabilizer has significant impact on stabilizer selection. It is suggested that stabilizer in which drug has minimum solubility is mostly preferred as Ostwald ripening will occur at the expense of smaller particles which solubilize

Table 2 List of various stabilizers used in nanocrystal development

Type	Examples
Polymers	Povidone, polyvinyl alcohol, polyethylene glycol, carboxymethylcellulose sodium, hydroxypropyl cellulose, hydroxyethyl cellulose, hypromellose, decyl glucoside, etc.
Surfactants	Sodium lauryl sulfate, docusate sodium, tween 80, poloxamers (188, 338, 407), D-α-tocopheryl polyethylene glycol succinate, etc.
Food proteins and biopolymers	Zein, polylactic acid, whey protein isolate, soybean protein isolate and β-lactoglobulin
Amino acids	Phenylalanine and leucine

rapidly and crystallize around large particles. Another important drug-related parameter is zeta potential. It is the electrokinetic potential of colloidal system. It measures the interaction between colloidal particles. Zeta potential is an indicator of stability of colloidal system, and as it increases electrostatic repulsion increases. For a colloidal system to remain stable, zeta potential should be ±30 mV. George et al. reported that drug and stabilizer with nearly similar log P will form a stable nanocrystal suspension (George and Ghosh 2013).

4.1.2 Stabilizer-Related Parameters

High molecular weight stabilizers are preferred because long chain length would help in overcoming the van der Waals forces of attraction. Enough steric repulsion is not offered by short chain lengths and stabilizers with short chain lengths tend to promote aggregation. Polymers stabilizers with molecular weight ranging from 5000 to 25000 g/mol are generally used in the preparation of nanocrystals. Studies reported the influence of hydrophobicity of stabilizers on stability, which concluded that hydrophobic stabilizers are suitable candidates for stabilization of nanocrystal of hydrophobic drug as they are easily adsorbed on drug's surface. Concentration of stabilizers in media have significant impact on stability of nanocrystal medium as an optimum concentration of stabilizer is required to completely coat/cover the drug surface for efficient steric repulsion and formation of a stable system. However, some literature pointed out that efficiency of stabilizer is lost when its concentration exceeds critical micellar concentration. Another important stabilizer related parameter that has significant influence on stability of nanocrystal is viscosity. Positive correlation between viscosity and stability has been found as per Strokes–Einstein equation. This equation postulates that high viscosity ensures colloidal stability by reducing diffusion velocity of drug molecules. Other stabilizer related parameters such as surface energy and particle-stabilizer affinity have also proved their importance toward stability of colloidal system of nanocrystal.

4.1.3 Dispersion Medium-Related Parameters

pH and temperature play a significant role in electrostatic and steric stabilization. pH of aqueous medium affects stability of stabilizer performance mainly for ionizable polymers. Temperature affects the affinity between nanocrystal and stabilizer and hence leads to destabilization of the system. Cooling or heating of colloidal system of nanocrystal may lead to flocculation. Furthermore increase in temperature may lead to alteration of dynamic viscosity and diffusion coefficient.

5 Characterization and Evaluation

Different parameters affecting the quality of nanocrystal products are classified based on the colloidal nature of nanocrystals, bulk colloidal drug suspensions, stabilizer and dispersion media interactions, particle-stabilizer and dispersion media interactions and presence of contaminants. Various properties like content, presence

of impurities, size range, morphology, solid state properties, and thermal behavior should be carefully considered and evaluated to develop a stable nanocrystal formulation. Stabilizer adsorption, dissolution, conformation and dynamics of interaction should be addressed carefully. While dealing with bulk suspensions, electrokinetics, rheological parameters, sedimentation and agglomeration tendencies should be appropriately evaluated (Borchard 2015; Juhnke and John 2014).

Particle size distribution and zeta potential These parameters can be obtained using photon correlation spectroscopy (PCS) (Gulari et al. 1979), laser diffractometry (Baudet et al. 1993) and coulter counter analysis (Hurley 1970). A polydispersity index (PDI) value of 0.1–0.2 signifies a narrow size distribution, whereas a PDI value greater than 0.5 indicates a very broad distribution. A minimum zeta potential of ±30 mv is recommended for electrostatically stabilized nanosuspension, while a zeta potential of ±20 mv is required for a combined electrostatic and steric stabilization.

Crystallinity and morphology The changes in the physical state and the extent of the amorphous content can be determined by Terahertz spectroscopy, X-ray diffractometry (XRD), differential scanning calorimetry (DSC), modulated-DSC and scanning electron microscopy (SEM).

Dissolution Various factors to be considered to understand dissolution outcomes are composition of formulation, shape of crystals, surface area, size distribution, exposed planes, surface chemistry, crystallinity, media exposure, storage conditions, etc. Dissolution can be carried out as per compendia requirements. Apart from the USP Apparatus II paddle method, various other methods like supernatant-assay or dialysis, in situ monitoring of drug particle size reduction by turbidity measurement, pressure separation by liquid chromatography or field-flow fractionation followed by HPLC or UV spectroscopy, monitoring particle dissolution by Dynamic light scattering or UV fiber optic spectroscopy, etc., are being used to understand dissolution of drug nanocrystals (Borchard 2015).

Toxicology studies Hydrophobic interaction chromatography (HIC) can be employed to determine surface hydrophobicity, whereas 2-D PAGE can be used for quantitative and qualitative measurement of protein adsorption post IV injection (Gao et al. 2008). Haemolytic tests play a vital role when considering nanocrystal formulations for IV application (Liu et al. 2010). Various animal models can be employed to study organ distribution and toxicity.

6 Biopharmaceutical Aspects

Nanonization as a formulation strategy would help in bioavailability enhancement of poorly soluble actives as a function of particle size. Nanocrystals can achieve faster T_{max} and higher C_{max} proportionally increasing AUC. Minimal fed/fast state variability is observed with nanocrystals. Recent literature reporting bioavailability enhancements by nanocrystallization are reported in Table 3.

Table 3 List of recently published nanocrystals along with their biopharmaceutical outcomes

Drug	Therapeutic application	Method	Stabilizer	Particle size (nm)	Biopharmaceutical outcome	Reference
Satranidazole	Anti-protozoal	High-pressure homogenization	Span 20, HPMC E-5	208.8	Nanocrystals exhibited twofolds enhancement in bioavailability	Dhat et al. (2016)
Protopanaxadiol	Anticancer	Anti-solvent precipitation	TPGS	90.44 ± 1.45	Decreased T_{max} and increased C_{max} and AUC of nanocrystals when compared to physical mixture	Chen et al. (2016)
Lovastatin	Anti-hypertensive	Sonoprecipitation and bead milling	Mannitol and glucose	503.2 ± 20.4	Sevenfold and fourfold increase in C_{max} of rod-shaped nanocrystals and spherical nanocrystals respectively	Guo et al. (2015)
Nelfinavir mesylate	Anti-retroviral	Ultra sonication	PVA and poloxamer 407	236 ± 19.23	PVA formulation had decreased T_{max} and increased C_{max} and $AUC_{0\text{-}24}$ compared to pure drug	Naga Naresh et al. (2015)
Paclitaxel	Anticancer	High pressure homogenization	Pluronic- chitosan (Pl-g-CH) copolymer	192.7 ± 9.2	12.6-fold enhancement in relative bioavailability was observed with nanocrystals compared to that of Taxol™	Sharma et al. (2015)
Hesperetin	Antioxidant	Spray drying	Mannitol	137.3 ± 90.0	Nanocrystals have shown 1.79 and 2.25-fold increase in C_{max} and oral bioavailability respectively	Shete et al. (2015)
Puerarin	Vasodilator	High pressure homogenization	Sodium dodecyl sulfate	229	4.47-fold enhancement in bioavailability was observed when compared to pure drug	Yi et al. (2015)
Lacidipine	Anti-hypertensive	Bead milling	SDS and HPMC E5	623	2.05-fold increase in $AUC_{0\text{–}24}$ h compared to marketed product	Fu et al. (2015)
Saquinavir	Antiviral	High pressure homogenization	Poly(sodium 4-styrenesulfonate)	205.9 ± 3.74	2.16 and 1.95-fold increase in C_{max} and AUC respectively compared to that of coarse crystalline SQV suspension	He et al. (2015b)
Mebendazole	Anti-helminthic	Wet milling	PVA	210	C_{max} and $AUC_{0\text{-}8}$ were increased by four and threefold respectively	Hashimoto et al. (2015)
Cefdinir	Antibacterial	Wet milling	Poloxamer 407	224.2 ± 2.7	Threefold increase in oral bioavailability was demonstrated	Sawant et al. (2015)

7 Applications

7.1 Cancer Chemotherapy

To date, cancer remains as one of the most life-threatening disease resulting in 8.2 million deaths. A 45% raise in cancer related deaths is projected by 2030 as per WHO reports. IV administration is still preferred route for cancer chemotherapy due to poor solubility and limited oral absorption of most anticancer therapeutics. No significant improvements in this situation are expected as > 40% of cancer therapeutics in development display poor aqueous solubility. In the said scenario, nanocrystals with their unique features, as discussed earlier, would offer a potential platform for the development of safer and effective formulations for cancer chemotherapy. Improved pharmacokinetics and biodistribution can be expected due to uniform and stable physical nature of nanocrystals (Lu et al. 2015). Passive targeting can be expected through EPR effect and active targeting can be achieved by ligand conjugated nanocrystals (Wang et al. 2016; Pawar et al. 2014). Ye et al. recently developed injectable nanocrystals of brick dust drug niclosamide using wet media milling. Tween 80 was used as stabilizer achieving an average particle size distribution of 235 nm. Pharmacokinetics of nanocrystal formulations at a dose of 2 mg/kg were comparable to that of drug solution for anticancer effects in EC9076 cell line (Ye et al. 2015). Ntoutoume et al. developed cyclodextrin-cellulose nanocrystal complexes of curcumin and have shown enhanced cytotoxicity against PC-3, DU145, and HT-29 cell lines (Ntoutoume et al. 2015). Dong et al. developed injectable nanocrystals of anticancer agent SNX-2112 using wet media milling technique. Poloxamer 188 was used as a stabilizer and the particle size was 203 nm. Drug nanocrystals were rapidly absorbed showing comparable pharmacokinetics to drug-cosolvent system. Plasma concentrations, systemic clearance, distribution in heart, lung, kidney and intestine were comparable to that of cosolvent formulation. Accumulation of drug in liver and spleen was observed during initial 1 h due to particulate uptake (Dong et al. 2015). Pawar et al. prepared docetaxel nanocrystals using high-pressure homogenization employing pluronic F-127 as stabilizer. Nanocrystals have shown enhanced G2-M arrest when compared to the free drug and Taxotere® formulation. Enhanced safety of drug nanocrystals compared to the marketed formulation was successfully demonstrated by acute toxicity studies and hemolytic tests (Pawar et al. 2015). Growing literature suggests safety and efficacy of nanocrystals especially in cancer chemotherapy when compared to existing products. This opens potential avenues for the development of nanocrystal based delivery systems for cancer chemotherapy.

7.2 Targeted Drug Delivery

Nanocrystals offer potential platform for targeted drug delivery as their surface properties and invivo behavior can be easily tailored. Fuhrmann et al. have

reviewed targeting possibilities and limitations of injectable nanocrystals. Numerous possibilities for surface orchestration of nanocrystals provide enough scope for enhancing cellular uptake and tumor accumulation. Sub 100 nm size particles are known to penetrate tumors, which can be achieved by nanocrystals. Smart nanocrystals and hybrid nanocrystals which are in sub 100 nm range could thus find potential applications in targeted drug delivery. Modulation of drug release and identifying stimuli responsive stabilizer coatings can help in development of hybrid nanocrystals which can accumulate in disease sites. In addition, conjugation strategies would offer active targeting as seen with other nano carriers (Fuhrmann et al. 2014). Composite nanocrystals of gemcitabine and magnetite resulted in enhanced tumor accumulation providing stimuli responsive delivery through magnetic activation (Arias et al. 2008). Co-administration of tumor-penetrating peptides along with anticancer drugs may help in increasing vascular and tissue permeability leading to increased accumulation of drug at tumor site (Sugahara et al. 2010). Dong et al. synthesized folic acid conjugated cellulose nanocrystals for targeting folate receptor positive cells which are over expressed in breast, colon and ovarian cancer etc. Uptake of the nanocrystals was dependant on the type of cells. In DBTRG-05MG and C6 cells, nanocrystals were internalized via caveolaemediated endocytosis whereas in H4 cells, they were internalized via clathrin-mediated endocytosis (Dong et al. 2014). Wu et al. synthesized magnetic bioceramic hydroxyapatite (mHAP) nanocrystals by wet chemical precipitation process. mHAP nanocrystals were conjugated to hyaluronic acid to achieve targeting using PEG spacer arm. Hyaluronic conjugation helped in targeting MDA-MB-231 cell whereas superparamagnetic properties of nanocrystal composites helped in achieving intracellular hyperthermia for effective tumor eradication (Wu et al. 2016). Li et al. developed folate-chitosan conjugated nanocrystals on bexarotene using precipitation-high pressure homogenization method with a mean particle size of 631.3 ± 2.7 nm. Nanocrystals have shown threefold increase in AUC and 1.5-fold increase in Cmax when compared to drug suspension (Li et al. 2016). Nanocrystals were also reported to enhance drug delivery to brain. Chen et al. reported that surface modification of nanocrystals with efflux inhibitors and functional stabilizers helped in enhancing drug accumulation in brain (Chen et al. 2016). Combination of nanocrystals with various other ligands and functional materials can thus create new platforms for targeted drug delivery (Boles et al. 2016).

7.3 Theranostic Applications

A theranostic platform involves combination of diagnosis and subsequent therapy. From a material perspective, nanocrystals offer a potential for theranostic applications as multiple functionalities can be combined in one nanocrystal. Combining imaging agents with the host nanocrystals of anticancer agents will help in simultaneous tumor therapy and bio-imaging. Evolving generation of nanocrystals

called hybrid nanocrystals possess theranostic capabilities. Inorganic nanocomposites based systems provide a good platform for theranostic applications. Preparation methods may typically involve dissolution of fluorescent dyes, such as rhodamine B, fluorescein and FPR-749, to anti-solvent water (anti-solvent) followed by addition of drug solution in organic solvents as seen in precipitation-ultrasonication method (Lu et al. 2015). Amiri et al. developed polyethylenegylcol fumarate (PEGF)-coated superparamagnetic iron oxide nanoparticles (SPIONS) for theranostic applications with good contrast comparable to that of Endorem® (It is an MRI contrast medium containing aqueous suspension of superparamagnetic iron oxide with dextran for IV administration). The authors successfully loaded tamoxifen citrate and doxorubicin into nanocrystals and evaluated the biocompatibility of PEGF-coated SPIONS (Amiri et al. 2011). Hollis et al. prepared hybrid nanocrystals of paclitaxel using anti-solvent method incorporating two flourophores, MMPSense® 750 FAST and Flamma Fluor® FPR-648. The developed nanocrystals have shown effects similar to that of paclitaxel solution along with bioimaging (Hollis et al. 2014). Poulose et al. recently developed Cu_2S based nanocrystals for trimodal imaging and photothermal therapy. Cu_2S nanocrystals were prepared by reactive crystallization at high temperatures followed by coating with lipid-polymer conjugates. Synergistic effects were observed along with multimodal imaging by photoluminescence of Cu_2S, folate targeting and chemotherapeutic effects of doxorubicin. Photoexcitation at 488 nm helped in drug release from nanocrystal-drug conjugate from the treated cancer cells within 10 min of exposure (Poulose et al. 2015). Amphiphilic plasmonic nanocrystals are composed of soft shell of amphiphilic polymers grafted on to hard metallic core nanocrystals. The hydrophobic shell and the hydrophilic aqueous cavity help in loading of therapeutic agents and diagnostic aids (photo sensitizers, florescent proteins, etc.) which in turn help in stimuli responsive delivery and synergistic therapeutic effects. Loading of photo sensitizers will help in concurrent photothermal and photodynamic therapy. In addition, excellent surface-enhanced Raman scattering and photo acoustic imaging of plasmonic vesicles helps in sensitive detection of cancer cells if they are appropriately targeted to cancer cells. (Song et al. 2015). These evolving classes of drug and metallic nanocrystal conjugates generate tremendous opportunities in guided chemotherapy and for site specific controlled drug delivery with imaging capabilities.

7.4 Safety and Efficacy

Ever increasing awareness of nanotechnology and its implications on human body and environment has lead to serious rethinking about their safety and efficacy. The parameters that determine tolerability and potential toxicity of nanosystems should be carefully considered. Mainly, size and biodegradability are two parameters that determine interactions of this system with cells and hence their fate inside the

biological system should be systematically evaluated. While looking at the "size" parameter, one thing is clear that the benefits like improved saturation solubility and dissolution by developing nanocrystals or any other nanosystems is mainly attributed to their size which is less than 1000 nm. Particles in size in range of 100–1000 nm can be taken up by cell through phagocytosis. Hence, these particles can be taken up by macrophages that are present in limited number and can be considered safer. However, particles whose size is less than 100 nm can be internalized through endocytosis by any cell. This indicates that particles below 100 nm possess higher toxicity risk as large amount of cells get exposed to these particles. Hence, particle size has been considered as a major factor while devising the nanotoxicological classification. Another important parameter is biodegradability; particles that degrade and are eliminated from the body were found to be less toxic as compared to non-biodegradable particles. This suggests the need for inclusion of biodegradability as criteria for nanotoxicological classification system.

Nanotoxicological classification as represented in Fig. 4 contains four classes after considering size and biodegradability as important parameters regarding safety of nanosystems. These classes are defined based on increasing toxicity/risk. Green patterns as depicted in Fig. 4 indicate low risk, yellow indicate medium and red signifies higher risk. Class I possess less risk as particles size is in the range of 100–1000 nm and are biodegradable in nature. When we move from class I to Class II persistency increases, means particle size is same as that of class I but these systems are non-biodegradable. However, class III nanosystems are biodegradable but particle size is less than 100 nm. Both these classes (class II and III) as represented in yellow pose medium risk. Class IV particles are non-biodegradable nature with size below 100 nm indicating that it belongs to a red colored nanotoxicological class with highest toxicity (Keck and Muller 2013). Safety is one of the prime concerns associated with medicines, thus toxicity studies are part of the most important data to be submitted for registration of new therapeutics. Safety might be a more critical aspect when dealing with the poorly soluble drugs. Large amount of

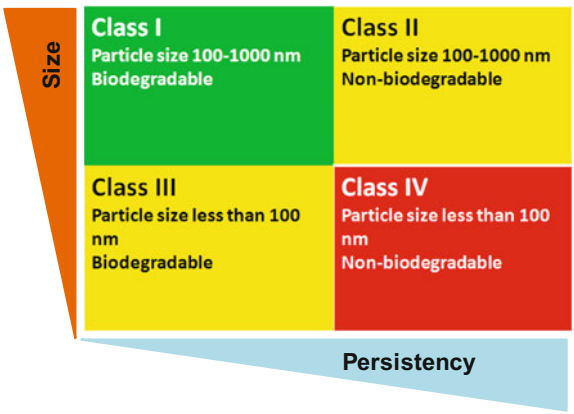

Fig. 4 Pictorial representation of Nanotoxicological classification system

solubilizers and organic cosolvents added to enhance solubility of drugs may lead to various undesired effects like hypersensitivity, nephrotoxicity, and neurotoxicity as seen with Cremophor-EL in Taxol® (Rowinsky et al. 1993; Kim et al. 2001) and renal injury with injectable formulations of itraconazole due to high amount of cyclodextrins (Rabinow et al. 2007).

8 Market Status

Nanocrystal technology competes with other advanced technologies and traditional approaches for formulating drug candidates with poor developability, since it can be readily performed in-house. They remain the most successful of all nanotechnology enabled products for drug delivery. Gris-PEG® developed using the co-precipitation was the first marketed nanocrystal product. Significant changes in the regulatory framework of drug nanocrystals are expected with the ongoing discussions revolving around quality, efficacy and safety of the nanotechnology-based products. As mentioned before, nanocrystal suspensions are stabilized by adsorption of stabilizers to the particle surface. Stabilization mechanisms and role of stabilizers used are to be clearly understood as EMA reflection paper addresses concerns related to variation in opsonization patterns due to engineered surfaces (Ehmann et al. 2013; EMA 2013). Drug nanocrystals had an estimated market size of 596 million USD by 2010 accounting for 44% of the total nanotechnology-based drug delivery market of 1.3 billion USD. Nanocrystals market is projected to increase to 60% of all nanotechnology-based products with a market capture of 82 billion USD by 2021. Lack of experience and sophisticated manufacturing facilities for scale up nanocrystal preparation has been one of the major bottlenecks for limited number of marketed products despite a convenient regulatory framework. Recent surge in marketed products and greater market capture amongst all nanoparticulate systems emphasizes the need for further development of nanocrystals (Borchard 2015). Drug nanocrystals which are currently marketed and further in development are enlisted in Table 4.

9 Concluding Remarks

Nanocrystal technology offers an efficient platform to formulate poorly soluble drugs and provide better dissolution properties with enhanced oral bioavailability. With increase in number of NCEs posing dissolution and bioavailability issues, nanocrystal technology is expected to play a significant role in drug delivery market in coming years. Simplified processes, minimal utilization of excipients, potential for large-scale manufacturing and biopharmaceutical advantages of end products makes them an ideal strategy to deal with various poorly soluble actives especially “brick dust drugs”. Nanocrsytals are versatile and can be successfully formulated

Table 4 Products of drug nanocrystals which are marketed and in clinical trials (Lu et al. 2015)

Drug	Therapy	Product	Company	Route	Technology	Method	Year
Griseofulvin	Antifungal	Gris-Peg®	Novartis	Oral	Bottom–up	Co-precipitation	1982
Verapamil	Anti-arrhythmia	Verelan PM®	Schwarz Pharma		Top-down	Media milling	1998
Sirolimus	Immunosuppressant	Rapamune®	Wyeth				2000
Dexmethylphenidate hydrochloride	Anti-psychotic	Focalin XR®	Novartis				2001
Morphine sulfate	Anti-chronic pain	Avinza®	King Pharm				2002
Methylphenidate hydrochloride	Anti-psychotic	Ritalin LA®	Novartis				2002
Diltiazem	Anti-angina	Herbesser®	Mitsubishi Tanabe Pharma				2002
Tizanidine hydrochloride	Muscle relaxant	Zanaflex™	Acorda				2002
Aprepitant	Anti-emetic	Emend®	Merck				2003
Fenofibrate	Anti-hypercholesterolemia	Tricor®	Abbott				2004
Nabilone	Anti-emetic	Cesamet®	Lilly		Bottom–up	Co-precipitation	2005
Megestrol acetate	Appetite stimulant	Megace® ES	Par Pharma		Top-down	Media milling	2005
Fenofibrate	Anti-hypercholesterolemia	Triglide®	Skye Pharma			High-pressure homogenization	2005
Naproxen sodium	Anti-inflammation	Naprelan®	Wyeth		Top-down	Media milling	2006
Theophylline	Bronchodilator	Theodur®	Mitsubishi Tanabe Pharma				2008
Paliperidone palmitate	Anti-depressant	Invega Sustenna	Johnson & Johnson	IM		High-pressure homogenization	2009
2-Methoxyestradiol	Anticancer	Panzem®	EntreMed	Oral		Media milling	Phase II
Guanylhydrazone	Anti-inflammation	Semapimod®	Ferring	IV			
Paclitaxel	Anticancer	Paxceed®	Angiotech Pharmaceuticals	IV	Unknown		
Thymectacin		Theralux®	Celmed BioSciences	–	Top-down	Media milling	
Silver	Antibacterial	Nucryst®	Nucryst Pharmaceuticals	Oral	Bottom-up	Reactive magnetron sputtering	

for drug delivery using oral, pulmonary, parenteral, ocular and topical routes, etc. Despite all the advantages of nanocrystal technology, it may not be suitable to tailor biopharmaceutical aspects of all the poorly soluble drugs. Nanocrystal may not offer an efficient solution with drug molecules which are rapidly metabolized and display poor permeation properties. Moreover, issues related with intercellular uptake, role of stabilizers with P-gp inhibitory effects in bioavailability enhancement, stability concerns due to phase transformations during solidification process are inadequately addressed to date. Looking at growing number of marketed products of drug nanocrystals one would be optimistic to foresee a very bright future in the field of nanocrystal technology.

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Inorganic Nanocomposites—A New Paradigm in Drug Delivery

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Abstract

Inorganic nanocomposites are nanosized inorganic material, which are intended for drug delivery and molecular diagnostics purposes. In last few decades, they have attracted more attention for their biomedical application. However, as drug delivery system, they provide significant advantage over traditional drug nanocarriers due to their ultra-high surface area and easy surface functionalization. Inorganic nanocomposites that are used for drug delivery purpose mainly include metal, metal oxides, metal oxide ceramics, non-oxide ceramics, silicates, nano-rods, inorganic fullerene, mesoporous silica, and semiconductor nanocrystal (quantum dots). In this chapter, an overview of these inorganic nanocomposites has been provided with a major emphasis on their utility for drug delivery. However, a detailed summary on drug delivery application of some of the selected inorganic nanocomposites like mesoporous silica, quantum dots, metal oxide, and metal oxide ceramics has also been included along with overview of synthesis strategies used for fabricating inorganic nanocomposites, in vitro–in vivo biocompatibility, and their potential uses in drug delivery and molecular diagnostics. This chapter also highlights significant achievement made by inorganic nanocomposites in targeted drug delivery to cancer cells.

Keywords

Mesoporous material · Silica · Surface area · Drug delivery · Porosity · Surface functionalization

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Abbreviations

GRAS	Generally recognized as safe
INM	Inorganic nanomaterials
MRI	Magnetic resonance imaging
MSM	Mesoporous silica nanoparticles
MCM	Mobil composition of matter
QD	Quantum dots
SPION	Superparamagnetic iron oxide nanoparticles
SBA	Santa barbara amorphous

1 Introduction

The term "nanotechnology" describes manipulation of matter at atomic, molecular, and supramolecular level (Rao et al. 2006). It provides tools to understand, create, and use the nanosized structures. Size reduction of material to nanolevel results in a product with different atomic and molecular level properties. Nanomaterials possess unique properties like ultra-small size and large ratio of surface area to mass and high reactivity (Chen 2006). Exploration of materials on a nanometer (nm) range generates unlimited possibilities, and the benefits provided by nanoscale technologies are estimated to have significant impact on more or less all areas of science and technology (e.g., medicine, energy, electronics, plastics and aerospace) (Chan and Nie 1998). Combination of nanotechnology and medicine gave birth to nanomedicine, a new sub-discipline of nanotechnology. Main objective of nanomedicine is to improve drug delivery, for therapy, and diagnosis (Jain 2008; Gaur and Bhatia 2008). Nanoparticles present in size range of 1–100 nm form an important bridge between bulk materials and atomic and molecular structure. Nanoparticles comprising of inorganic materials and inorganic nanomaterials (INMs) possess combination of unique properties of inorganic material along with the advantages of nanosize. Unique properties of INMs render them best suitable for medical and biomedical applications. Inorganic structures also provide scaffolds for the presentation and encapsulation of drugs, biomolecules, and imaging agents, generating delivery systems with structural and dynamic properties complementary to more conventional polymeric and lipid-based carriers. However, its importance to pharmaceutical industry is not new. Use of iron oxide and gadolinium nanoparticles for imaging in magnetic resonance imaging (MRI), and presence of gold nanoparticles in pregnancy kit for detection of pregnancy related hormone from woman's urine are well established. Currently, INMs have gained popularity for drug delivery and diagnostics (Liong et al. 2008; Jain et al. 2008). Metals, metal oxide, metal oxide ceramics, non-oxide ceramics, quantum dots (QDs), mesoporous silica, silicates, nanorods, nanowires, inorganic fullerenes, and bio-nanohybrid

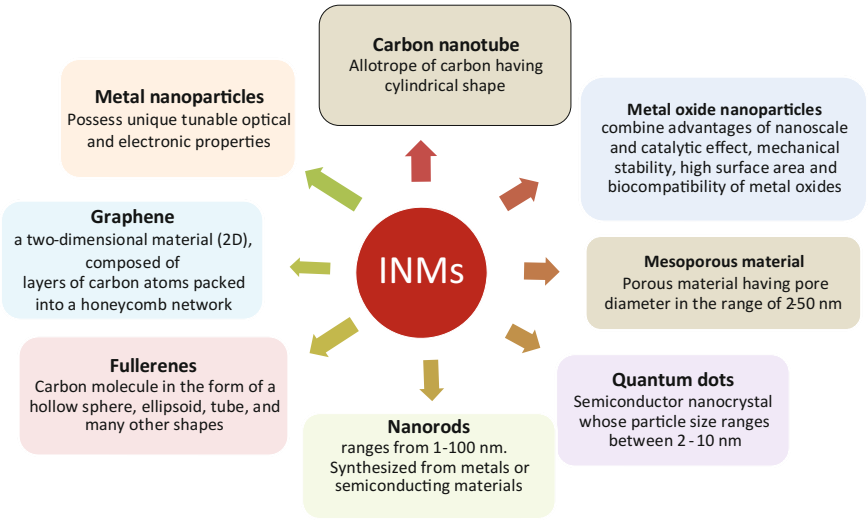

Fig. 1 Examples of INMs

systems or inorganic–organic hybrids are representative examples of INMs described in Fig. 1. However, practical utility of INMs in drug delivery is still in infancy (Sekhon and Kamboj 2010a).

This chapter mainly deals with drug delivery application of INMs. Different INMs have been discussed along with their unique properties, advantages, preparation techniques, applications, and biocompatibility issues. However, major discussion has been focused toward mesoporous silica including mesoporous silica nanoparticles (MSNs), QDs and metal oxide nanoparticles comprised of superparamagnetic iron oxide nanoparticles (SPIONs). In addition, special emphasis on market and clinical status of INMs has been provided.

2 Advantages of Inorganic Nanocomposites

Nanoscale dimension makes INMs different from their bulk forms. Proportion of surface atom increases as size of material reduces to nanorange which directly affects reactivity (Kango et al. 2013). INMs possess unique optical, electronic, magnetic, and mechanical properties (Rotello 2008). These properties are found to be responsible for different advantages offered by INMs. Some of the advantages are shortlisted below.

(a) Quantum confinement property present in some INMs irresponsible for optical properties like fluorescence or luminescence. Use of QDs in live cell imaging and diagnostics is mainly based on quantum confinement property of INMs (Qi and Gao 2008a).

(b) Easy fabrication of INMs for magnetic properties is derived from its nanoscale dimensions which make them the first choice for designing any nanodevice for the purpose of MRI, imaging, cell tracking, targeting, and multimodal contrasting (McCarthy and Weissleder 2008).
(c) INMs can be engineered to different arrays of morphology and shape. Mesoporous silica and silicates with tunable pore size and volume provided platform for improving dissolution of poorly soluble drugs, controlling the drug release and in targeted drug delivery through functionalization, e.g., mesoporous silica. Its application in imaging has been well demonstrated (Wang 2009).
(d) INMs provide flexibility for controlling interface between INMs and their environment. It means easy functionalization of INMs to explore new applications such as targeted drug delivery, cell tracking, diagnostic devices, and in vivo imaging (Kango et al. 2013).
(e) Inorganic materials offer diversified chemical compositions, structure, and textures which decide its affinity for biological systems and determine its stability within the biological system (Urie and Rege 2015). INMs possess slow degradation property inside biological system which is beneficial for increasing residence time of drug inside the body (Chen et al. 2016).
(f) Along with slow degradability, INMs offer advantages of higher biocompatibility, good availability, and low cost (Chen et al. 2016).

3 Different Classes of Inorganic Nanocomposites

Unique physical, chemical, magnetic, electrical, mechanical, and optical properties of INMs make them a preferred carrier system for drug delivery, imaging and diagnostics. Different INMs have been explored to date; a brief summary of INMs is discussed in Table 1 along with their unique properties and applications.

4 Applications of Inorganic Nanocomposites

INMs offer broad range of applications in optical communications, material sciences, electronics, and biological systems. However, INMs are an emerging field in the area of diagnostics (imaging) and drug delivery (Urie and Rege 2015). In last few years, interest in this area has increased tremendously because nanotechnology offers advantage of assembling, encapsulating, or loading drug inside and/or on the surface of INMs by tailoring surface chemistry or by modifying shape and size (Yang et al. 2012b). INMs can be used as probes in biological system for identification, visualization, and quantitation at nanometer scale range. Various techniques like fluorescence Raman microscopy, resonant Raman spectroscopy, MRI, scanning microscopy, Rayleigh light-scattering microscopy, X-ray absorption, and computed tomographic imaging have benefited by use of INMs (Sekhon and

Table 1 Different inorganic nanocarrier systems for drug delivery, imaging and diagnostics applications (Yang et al. 2012a; Portney and Ozkan 2006; Alkilany et al. 2012; Hudson et al. 2008; Urie and Rege 2015; Zrazhevskiy et al. 2010; Sekhon and Kamboj 2010a; Wang 2009; Chen et al. 2016)

S. No.	Inorganic nanocomposites	Properties	Applications
1	Carbon nanotubes	High surface area, easy functionalization for improving aqueous solubility and targeting at specific target receptor	Drug delivery and targeting
2	Fullerenes	Functionalization using hydrophilic ligand improves aqueous solubility	Targeted drug, proteins, and gene delivery
3	QDs	Inorganic nanocrystals (2–10 nm) with unique physical and optical properties, core coated by shell to improve its performance. Capping improves aqueous solubility	Tumor targeting and imaging, potential intravascular probes for drug delivery and imaging
4	Mesoporous silicon/silica	Tunable pore size, pore volume, free silanol group on the surface for functionalization, high surface area, and biocompatibility	Drug and gene delivery, targeting and imaging
5	Nanowires	Good conductivity and good biocompatibility make them ideal for sensing	Biomolecular nanosensors
6	Nanorods	Optical, thermal and electrical properties	Live cell imaging, biomedical applications, and drug delivery
7	Graphene	High surface area, planar structure, electrical and optical properties	Drug delivery and targeting; live cell imaging
8	Metal nanoparticles: gold	Tunable optical and electronic properties, good biocompatibility, easily modified to a desired size range (1–200 nm); easy functionalization of the surface by using thiol ligand	Drug delivery, diagnostics, and biomedical imaging
	Silver	Bactericidal property, photo-physical characteristics, and electrochemical-luminescence useful for sensing applications	Nanosilver-based wound dressings, biosensors, and anti-HIV activity
9	Metal oxides	Catalytic effect, redox potential, high surface area, high mechanical stability, and biocompatibility	Drug delivery, targeting, biomedical imaging, sensing, and therapeutic biology
10	Metal oxide ceramics	Inert behavior, good strength, minimum thermal, and electrical conductivity	Bioactive bone implants, biosensors, drug delivery, protein purification, and enzyme immobilization
11	Silicates	Large internal volume, high surface area, straight narrow channels, and good biocompatibility	Drug delivery, cell tracking, drug targeting, gene transfection, and tissue engineering

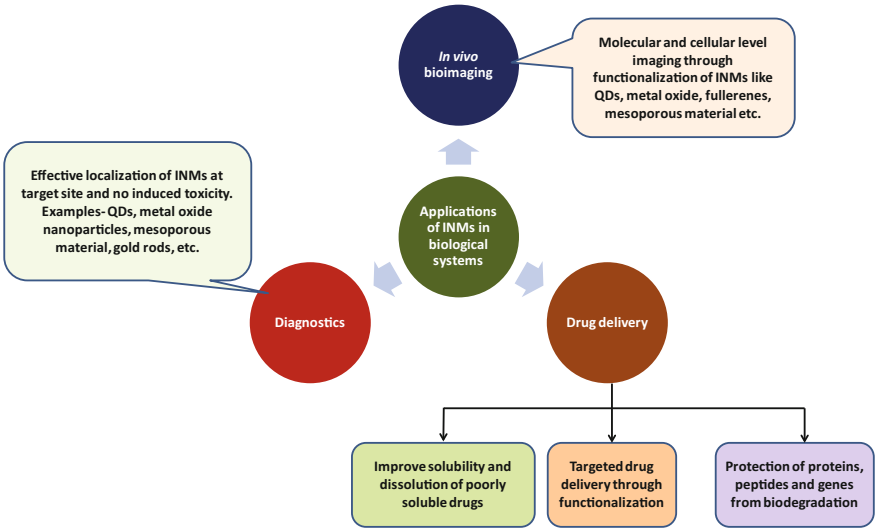

Fig. 2 Applications of INMs in biological systems

Kamboj 2010a, b). INMs are reported to improve solubility, dissolution, bioavailability and chemical stability of drug, protein, and genes. Bioavailability of BCS class II and IV drugs are mainly hampered by poor solubility, which creates hurdles in development of drug products. This problem can be solved by encapsulating such drug molecules in hydrophilic carrier systems. INMs have proven their potential as hydrophilic carrier by increasing solubility and dissolution of such molecules (Williams et al. 2013). Figure 2 describes wide range of applications of INMs in biological systems.

5 Mesoporous Silica

According to IUPAC system, porous materials are classified as microporous (pore diameter less than 2 nm), mesoporous materials (pore diameter in the range of 2–50 nm), and macroporous (pore diameter greater than 50 nm) (Kumar et al. 2014; McCarthy et al. 2015).

5.1 Types of Mesoporous Silica

There are many varieties of mesoporous silica; MCM-41, SBA-15, Syloid, Sylysia, etc. They are categorized as ordered and disordered mesoporous material depending on the mesostructure arrangement (Fig. 3). In disordered mesoporous materials, pores are disordered and not fully opened. In such materials, pore size is widely

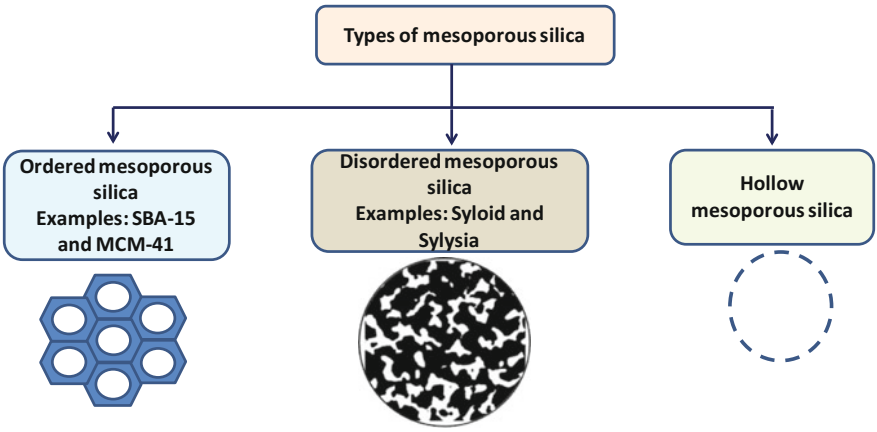

Fig. 3 Different types of mesoporous materials

distributed and difficult to control. Hence, molecule does not get easy access to pores. Sylysia and Syloid belong to disordered mesostructure material. MCM-41, MCM-48, and SBA-15 are known as ordered mesoporous material because they possess large and uniform pore size, highly regular nanopores, and large surface area (McCarthy et al. 2015). This difference in mesostructure architecture is mainly attributed due to difference in manufacturing conditions of mesoporous materials like temperature and surfactant concentration. Use of mesoporous materials in biomedical field is well established. However, in drug delivery it has gained popularity due to the discovery of synthetic techniques to develop uniform-sized silica. Ordered mesoporous silica show promising outcome in drug delivery due to their ability to modify/control the drug release and improve/alter dissolution. Advantages offered by mesoporous materials include exceptionally high surface area that can exceed beyond 1000 m^2/g for mesoporous silica, flexibility for modifying pore size, volume, and surface functionalization which help in loading different payloads of single or multiple therapeutics simultaneously (Wang 2009; Tang et al. 2012). Similarly, these mesoporous structures have high stability toward pH, temperature, and mechanical stress. These properties of mesoporous material thus help in improving solubility of poorly soluble drugs. Mesoporous silica also provides advantage of improved dissolution of poorly soluble drugs. When a drug is converted to an amorphous state to improve dissolution, entrapment of amorphous drug into mesoporous structure impose restriction on conversion of amorphous form to crystalline form as it hinders formation of critical nucleation size (Sliwinska-Bartkowiak et al. 2001). The most critical concerns regarding applications of mesoporous silica for drug delivery are related to safety issues and the scale-up of the production (Xu et al. 2013). Limited literature available on in vivo experiments, also raise concern about functionality of these materials in drug delivery even though several publications claim improvement in dissolution through in vitro studies.

5.2 Synthesis of Mesoporous Silica

Since the discovery of mesoporous material, different attempts have been made to synthesize this type of the material. In 2001, first report on synthesis of MCM-41 (Mobil Composition of Matter No. 41) appeared, later extensive literature were published in the area of synthesis of mesoporous silica (Vallet-Regi et al. 2001). All new fabrication routes are modified versions of MCM-41 synthesis. Usually, these methods are based on formation of micellar aqueous solution due to polymerization of inorganic source followed by removal of the surfactant. Mesoporous silica is usually synthesized by self-assembling silica (tetraethoxysilane or sodium silicate) (Vallet-Regi et al. 2001; Tang et al. 2012) and a surfactant (usually quaternary ammonium salts like cetyl trimethylammonium bromide) (Pang and Tang 2005) micelles used as structure directing agents. The cooperative action between the negatively charged silicate species and the positive-charged quaternary ammonium micelles leads to an ordered structure of these materials. The surfactant is removed by calcinations or extraction, leaving a porous silicate network. Tetraethoxysilane is hydrolyzed to obtain silanol groups. Presence of the silanol groups depends on the pre-condensation of the silica. Higher the precondensation, lower will be chances to get ordered mesoporous silica. Factors that are responsible for condensation of silanol include pH, temperature of the reaction, presence of ions, etc. Condensation of silanol groups with other silanol groups or alkoxysilane groups leads to formation of siloxane bridge (Si–O–Si). Each silicon atom has capacity of formation of four siloxane bridges. In this process, some terminal silanol groups on the surfaces of mesoporous silica are retained and are found to be responsible for interactions with loaded drug molecule (Tang et al. 2012).

MCM-41 preparation method is a liquid crystalline template method in which different surfactants are used to control the final pore size of the mesoporous silica material. Main difference between this novel preparation method and other methods is that liquid crystalline template method focuses more on controlling final pore size and structure rather than controlling process parameters. In this method, aqueous micellar solution is formed using cetyl trimethylammonium bromide (Kresge et al. 1992; Beck et al. 1992). The –OH groups of the surfactant orients toward the aqueous phase on the surface of the micelles, reacts with silica, and is precipitated. This precipitate is washed after filtration to remove excess surfactant present in final product and then dried at high temperature 500–600 °C. The final product that is MCM-41 has a cylindrical pore shape with a very narrow pore size distribution. The pore shape and size of the material can be modified and controlled by proper selection of the surfactant with appropriate chain length, organic and inorganic additives, and hydrolysis or condensation of the silica (Wan and Zhao 2007). Surfactant like Pluronic 123 is commonly used for synthesis of material like Santa Barbara Amorphous type mesoporous silica (SBA)-15 and SBA-16. Addition of co-solvent or co-surfactant mainly affect morphology of silica by lowering surface curvature energy, which results into formation of mesoporous silica with wide variety of the pore structure like sphere, discoid, gyroid, and cylindrical (Xu et al. 2013). This method is popularly known as pore tailoring method. Addition of

organic solvents like trimethylbenzene, hexane into final stage of synthesis leads to expansion of pore volume of mesoporous silica.

Surface functionalization offers diverse application in mesoporous silica. Co-condensation and post-grafting are the two most commonly used methods for surface functionalization. In co-condensation method, silica source and organic silane are mixed together in presence of surfactant. An advantage offered by this method is homogeneous distribution of functional species into framework. However, drawback of this method is that mesoporous structure may lose its pore shape, pore size, and pore volume. Post-grafting method utilizes mixing of silica and organic silane in anhydrous solvent, which leads to surface adsorption of the functionalized species. Benefit of this method over co-condensation method is preservation of mesoporous structure (Yang et al. 2012b).

5.3 Methods of Drug Loading

Selection of appropriate method for loading of drug into mesoporous material is necessary as it affects performance of final product in terms of stability of drug and release rate of drug from mesoporous material. Degradation of drug during drug loading into mesoporous carrier is the most critical issue which has to be considered while selection of loading method (McCarthy et al. 2015). Choice of drug loading methods related to thermal stability of drug molecule, hence, before selection of the method considerable attention should be given to physicochemical properties of the drug (Andersson et al. 2004). Similarly, attention should be paid on selection of proper solvent and carrier as they have significant impact on performance of final product. In this respect, the proper choice of the solvent and appropriate understanding of the surface chemistry of the carrier are essential (Charnay et al. 2004). Roughly, loading methods have been classified under two broad categories like methods involving use of organic solvent and those without the use of organic solvent.

5.3.1 Using Organic Solvent

Organic solvents are mostly preferred solvents over water for loading of poorly soluble drugs into mesoporous silica. Solvent immersion and incipient wetness methods are preferred methods of drug loading in presence of organic solvent. The common steps involved during drug loading by these methods include first immersion of carrier in concentrated drug solution where drug is filled inside the pores through capillary action. Next, diffusion of the drug from the surface into the pores and finally recovery of the drug loaded mesoporous material (Charnay et al. 2004). Amount of the drug dissolved in the organic solvent is close to the solubility of the drug in the same solvent. Usually volume of the drug solution is equal to the pore volume of the mesoporous silica in incipient wetness impregnation method. This is the main difference in organic solvent immersion method and incipient wetness impregnation method (Lehto et al. 2014). The excess solvent in the immersion method is removed by filtration whereas in impregnation method the

solvent is removed by drying. In both the methods, drug is drawn through capillary action. Impregnation method is useful when less amount of the drug is available. Though high drug loading can be achieved with this method (Charnay et al. 2004), it suffers various setbacks like non-uniform distribution of drug in carrier and surface crystallization of drug on solvent evaporation due to residual drug.

5.3.2 Without Using Organic Solvent

Melt Method

In melt method, drug and carrier are mixed and heated above the melting point of the drug. This method is only suitable for thermally stable drug molecules. This method is similar to impregnation method. This method is not suitable for drugs, which cannot withstand melting without degradation (McCarthy et al. 2015). In addition, if the viscosity of the melt is high, it affects diffusion of drug in pore and release is hampered during dissolution. Drug loaded by melt method will not be distributed homogenously in carriers (Mellaerts et al. 2008). Crystallization tendency of the drug on the surface of carrier is more by this method (Mellaerts et al. 2008).

Co-grinding Method

This is a mechanical activation process; crystal structure is broken through grinding without any significant degradation. Mesoporous material and drug are taken in specific proportion and ground together. Some reports claim amorphization of drug during co-grinding (Bahl et al. 2008). In one study, impact of mesoporous carrier on amorphization of drug was studied by grinding indomethacin in presence and in absence of neusilin (mesoporous carrier) and evaluated for phase transformation. It was observed that only in presence of neusilin, crystalline indomethacin was transformed into amorphous form, while in absence of neusilin, it remained in crystalline form. The authors claimed that the rate of amorphization increased as the amount of neusilin increased. Phase transformation during co-grinding with neusilin was attributed due to mechanical energy provided by neusilin through increased shear, which was absent when indomethacin was ground alone. This intimate shearing resulted in rapid disruption of the crystalline structure, which led to amorphization of drug. Another reason stated was lowering of activation energy in presence of neusilin (Bahl and Bogner 2006).

5.3.3 Loading with Supercritical Fluid Technology

Supercritical fluid technology is the most advanced technique in drug loading and offers benefit of minimizing solvent variation, which can be achieved by adjusting temperature and pressure. Specific properties of supercritical fluids make them a better choice as impregnating agents such as similar viscosity as that of gases, density close to that of liquid, minimum interfacial tension, and diffusivity higher than liquids. This enhances transportation of the solute solubilized in supercritical fluid into mesoporous silica. Benefits offered by supercritical fluid technology include even distribution of solute inside mesoporous silica and reduced processing duration.

Another major advantage is that the final product may be free of organic solvent traces (Bouledjouidja et al. 2016). Supercritical CO_2 has been extensively used as a solvent to load drugs into high surface area carriers (Ahern et al. 2013), such as mesoporous silica. There are numerous studies reporting improvement in dissolution kinetics of drug by loading them on mesoporous silica using supercritical CO_2 (Sanganwar and Gupta 2008; Smirnova et al. 2004; Li-hong et al. 2013). Reasons behind selection of CO_2 as supercritical fluid are its unique properties like low critical point (7.4 MPa and 37.2 °C), lesser cost, recyclable, and environmental friendly (Bouledjouidja et al. 2016).

5.4 Factors Influencing Drug Release from the Mesoporous Carrier

5.4.1 Effect of the Pore Size and Pore Volume

Role of pore size of the mesoporous silica is crucial in drug loading as well as drug release (Charnay et al. 2004; Izquierdo-Barba et al. 2005). Increase in pore size leads to increase in drug loading. If the pore diameter to molecular size ratio is greater than 1, then sufficient drug loading will be achieved. Ratio higher than 3 indicate complete utilization of total surface area leading to high drug loading. Few studies have been conducted to study impact of pore size on drug loading and drug release. Nearly 19% loading of ibuprofen was achieved in MCM-41 (pore size 3.6 nm) in comparison to mesoporous carrier with a pore size of 2.5 nm (Andersson et al. 2004). Similarly, loading of the telmisartan increased from the 49 to 60% when the pore size increased from 3.6 to 12.9 nm (Horcajada et al. 2004). Close packing of the drug molecule in the smaller pores may hinder further entry of the drug molecule resulting in surface deposition of drug crystals or poor drug loading. In addition to impact on drug loading, pore size shows an effect on drug release. Increase in pore size of the mesoporous material generally leads to enhanced drug release. Pore size also have significant influence on the solid state stability of the drug molecule as numerous studies have claimed that amorphous form of the drug remained stable for longer duration after loading into mesoporous silica (Shen et al. 2010). Loaded drug did not crystallize in porous material due to small pore diameter that hinder the formation of critical nuclei which is a prerequisite for crystallization (Shen et al. 2010; Mellaerts et al. 2010; Rengarajan et al. 2008).

5.4.2 Effect of Surface Area and Surface Chemistry

Adsorption of drug on mesoporous carrier may form monolayer film or a multi-laminar layer. Large surface area of mesoporous silica is found to be suitable for a monolayer formation, however when the pore size and surface area are small then it leads to formation of a multilayer resulting in poor drug loading. A multilayer film can be formed after immersion of carriers in highly concentrated drug solution. Amount of drug loading onto mesoporous material can be increased by varying the surface area, pore size, and pore volume wherein pore volume and surface area plays a significant role.

Surface chemistry affects drug loading and drug release (Balas et al. 2006). Affinity of drug molecules for the functional groups present on silica surface also contributes toward multilayer formation on silica surface (Chavan et al. 2015). Functionalization of mesoporous silica surface helps in improving drug loading and drug release. In silica mesoporous material, free silanol group present on the surface can interact with $–NH_2$, $–SO_2NH_2$, –OH, –COOH groups of drug molecule. These interactions may be responsible for alteration in drug release and drug loading. In addition, formation of chemical or physical interactions between drug molecule and functional groups present on silica surface may be responsible for the changes in dissolution profile. Hydrogen bonding, hydrophobic bonding, or electrostatic interactions are reported to be responsible for the change in dissolution (Doadrio et al. 2006). Functionalization of mesoporous silica with hydrophobic ligand may result into reduction in loading, while the drug loading may increase with hydrophilic ligands (Song et al. 2005).

5.4.3 Effect of Solvent

Polarity of solvent and dissolution media plays a crucial role in drug loading and dissolution. As mentioned earlier, hydrogen bonding is the most prevalent type of interaction between drug molecule and unmodified mesoporous carrier. Affinity of polar solvent toward unmodified surface of silica is more due to presence of free silanol groups. This results into reduction in drug loading due to stronger adsorption of polar solvent on surface of silica as that of drug. Influence of the polarity of the solvent on the drug loading was studied in case of ibuprofen where drug was dissolved in solvents with varying polarity like dimethylformamide, acetone, hexane, ethanol, and dimethylacetamide (Charnay et al. 2004). Almost no ibuprofen was loaded into the MCM-41 when dimethylacetamide has been used as solvent, however nearly 37% of drug was loaded when hexane was used as solvent. Selection of solvent also depends upon the surface functionalization. On a silica surface is functionalized with hydrophobic group, nonpolar solvent is the best choice for drug loading (Wan and Zhao 2007).

5.5 Application of Mesoporous Silica

Application of mesoporous materials in drug delivery is mainly based on its physical and textural properties (Wan and Zhao 2007). Loading of drug into empty mesoporous material is conventionally utilized for improving dissolution of poorly soluble drug. In recent years, new techniques have been explored to improve applicability of mesoporous material in drug delivery and diagnostics which includes functionalization of mesoporous material (Qu et al. 2006) and stimuli responsive drug delivery system (Mal et al. 2003b). Some representative examples are described in Table 2.

Table 2 Partial list of conventional and functionalized mesoporous systems

S. No.	Drug	Molecular weight (gm/mol)	Carriers	Property improved	Reference
1.	Atazanavir	704.9	Nanoporous folic acid-template material (NFM-1) andanionic surfactant-template mesoporous silica (AMS-6)	Bioavailability	Xia et al. (2012)
2.	Carvedilol	406.5	SBA-16	Improved dissolution	Hu et al. (2012)
3.	Erythromycin	733.93	Hydrophobic group modified SBA-15	Improved dissolution	Doadrio et al. (2006)
4.	Celecoxib	381.4	Carbon	Improved dissolution and reduction in gastric irritation	Zhao et al. (2012)
5.	Ezetimib	409.4	SBA-15	Oral bioavailability	Kiekens et al. (2012)
6.	Fenofibrate	360.8	SBA-15 and MCM-41	Improved bioavailability	Van Speybroeck et al. (2010)
7.	Furosemide	330.7	Thermally carbonized mesoporous silicon and thermally oxidized mesoporous silicon	Improved dissolution with reduced pH dependency and improved permeation	Kaukonen et al. (2007)
8.	Griseofulvin	352.8	Thermally carbonized mesoporous silicon and thermally oxidized mesoporous silicon	Improved solubility	Salonen et al. (2005)
9.	Itraconazole	705.6	Thermally carbonized mesoporous silicon and SBA-15	Influence of pressure on drug release (increased pressure will reduce porosity and hence drug release)	Kinnari et al. (2011)
10.	Indomethacin	357.8	Syloid-244 and MCM-41	Influence of pore size on dissolution	Limnell et al. (2011)
11.	Ibuprofen	206.3	Fe_3O_4 functionalized SBA-15 and hollow MSNs capped with polycation poly(allylamine hydrochloride/ sodium polystyrene sulfonate multilayers	Improved dissolution and pH responsive release	Huang et al. (2008)

(continued)

Table 2 (continued)

S. No.	Drug	Molecular weight (gm/mol)	Carriers	Property improved	Reference
12	Phenanthrene	178.23	Coumarin-modified MCM-41	Photo-stimuli-controlled release	Mal et al. (2003b)
13	Ibuprofen	206.3	Polyelectrolyte coatings of sodium polystyrene sulfonate and the polycation poly(allylamine hydrochloride) on HMS	pH responsive controlled drug release	Zhu et al. (2005)
14	Vancomycin	1449.3	Dimethyldiallylammonium chloride coated SBA-15	Drug released in controlled manner in mild pH	Yang et al. (2005)

5.5.1 Mesoporous Drug Delivery System for Enhanced Dissolution

Rate limiting dissolution and low bioavailability are two major challenges associated with poorly soluble drugs. Adsorption of such drugs on high surface area carriers helps in improving dissolution of poorly soluble drugs. Generation of amorphous form of drug inside the mesopores helps in improving dissolution of poorly soluble drug via reduction in lattice energy and improving the wettability. Steps involved in release of drug from mesopores include entry of dissolution media into mesopores by capillary action, drug dissolution in media inside mesopores, diffusion of dissolved drug against concentration gradient, and finally diffusion and convectional transport of drug in dissolution media (Wang 2009). In addition, mesoporous system also helps in reducing pH dependency during dissolution. Extensive studies on mesoporous system in improving biopharmaceutical performances of poorly soluble drugs are available in literature. Partial list of some of the examples has been depicted in Table 2. As mentioned earlier, several factors affect release rate from mesoporous system as the release is controlled by diffusion. Proper understanding of release kinetics from mesoporous system is important while selecting an appropriate carrier for poorly soluble drugs.

5.5.2 Functionalized Mesoporous System

Limitations of conventional mesoporous systems include, failure to determine exact mechanism behind targeting when using a mesoporous system and tracking of drug release in biological system (Yang et al. 2012b). Functionalization of surface silanol group with magnetic material or luminescent material has gained popularity in last few years. These functionalized mesoporous system have proven their potential in diagnostics and cell imaging. However, in drug delivery, only few studies are reported where surface functionalization has been used for achieving targeted drug delivery. Folate or transferrin functionalized mesoporous systems have been reported for targeting specific receptors and increasing therapeutic efficacy of anticancer drugs like doxorubicin (Lu et al. 2012), paclitaxel (Cui et al. 2013), etc.

5.6 Mesoporous Silica Nanoparticles

Mesoporous silica nanoparticles (MSNs) is a nanocarrier system with unique mesostructure which possess large surface area and pore volume, and has been explored for effective drug delivery (Vallet-Regi et al. 2001; Vallet-Regí 2006). These properties make them a suitable candidate for encapsulating diverse therapeutic agents and deliver them at their target site. Fabrication of MSNs is cost effective, simple, and scalable. The first silica-based diagnostic nanoparticles "C-dots" are approved by FDA, which is in phase I clinical trial. This highlights important step toward acceptance of silica-based nanoparticles (Benezra et al. 2011). Unlike plain drug, MSNs may dissolve, aggregate, and react with cells and tissue depending upon their size, shape, and surface characteristics. MSNs offer several advantages over other drug delivery systems (Slowing et al. 2008) such as

1. **Tunable particle size**: Particle size of the nanoparticles can be tuned from 50 to 300 nm by employing different methods for the preparation of MSNs or by surface functionalization, with a wide range of pore size by using different surfactants.
2. **Stable and rigid framework**: MSNs are stable at wide range of temperature, pH, and mechanical stress.
3. **Uniform and tunable pore size**: Pore size distribution can be tuned from 2 to 6 nm, which helps in loading wide variety of actives. Formulations can be designed for immediate or sustained release by varying the pore size of the MSNs.
4. **High surface area and large pore volume**: MSNs offer high surface area up to 900 m^2/g and about 0.9 cm^3/g pore diameter of carrier for higher drug loading.
5. **Unique porous structure**: Delivery systems like liposomes and dendrimers have interconnecting pore system hence perfect capping is necessary in order to avoid premature release. MSNs comprises of honeycomb like porous structure running from one end to another end without any interconnection, as a result, the chances of leaking are almost nil in a uncapped system. This is the most important and unique feature of MSNs over other porous drug delivery system.
6. It offers high flexibility for surface functionalization, which can be efficiently adopted for achieving targeted drug delivery.

5.6.1 Application of MSNs

First report on use of MSNs in drug delivery came in 2001 (Vallet-Regi et al. 2001), wherein anti-inflammatory drug ibuprofen was loaded in mesoporous silica. Enhanced drug loading and sustained release pattern were observed with this MSNs. Extensive literature on MSNs is available which has demonstrated the advantages of MSNs in improving and controlling the drug release of a wide range of therapeutic agents (Table 3). Mesoporous structure and high surface area offer benefits of higher capacity for accommodating drug molecules and improved release in physiological media. Ordered pore structure with tailored pore size and its geometry help in homogeneous incorporation of drug molecule into carriers. Simple and cost effective manufacturing process makes it suitable method for fulfilling future clinical demand and commercialization.

Drug, Protein, and Gene Delivery

In last few decades, research in the area of MSNs-based drug delivery has increased exponentially. It has proved advantageous over other drug delivery approaches due to its above-mentioned unique properties. MSNs with tunable and flexible pore structure can be a preferred drug delivery option for a diverse range of drug molecules that differ in hydrophobic/hydrophilic nature, molecular weight, and therapeutic applications (Gu et al. 2010; Chen et al. 2009; Lu et al. 2007). Proteins and peptides which are emerging as potential therapeutic agents in various medical conditions usually face problem in delivering them to target site due to large molecular structure and fragile nature (MaHam et al. 2009). MSNs due to its porous

Table 3 Partial list of applications of MSNs in drug, gene and protein delivery, targeted and stimuli responsive drug delivery

Sr. No.	Drug used	Carrier used	Property improved	Reference
1	Epirubicin	–	Stimuli responsive release and improvement in antitumor efficacy of epirubicin	Hanafi-Bojd et al. (2015)
2	Epidermal growth factor	–	Functionalization of MSNs help in improving loading, release, targeting and protecting therapeutic moiety from endosomal entrapment	Mackowiak et al. (2013)
3	siRNA	Co-condensation derived core–shell nanoparticles with medium-sized pores	MSNs capped with cationic block polymer helped in gene knockdown while novel stellate core shell MSNs proved their utility for delivering siRNA due to their versatile surface properties	Moeller et al. (2015)
4	Cytochrome C	MCM-41-type	MSNs help in efficient delivery of proteins like cytochrome C to cells without endosomal entrapment	Slowing et al. (2007)
5	Camptothecin	Fluorescent MSNs prepared by base catalyzed sol gel method	Improved loading and controlled release has been achieved through MSNs formation	Lu et al. (2007)
6	Bevacizumab	–	Antibody conjugated MSNs showed enhancement in intracellular concentration of drug and improved anticancer activity in ovarian cancer cells	Zhang et al. (2015)
7	Ibuprofen	MCM-41-phosphate	Chitosan coated functionalized MSNs showed pH responsive and improved release	Popat et al. (2012)

(continued)

Table 3 (continued)

Sr. No.	Drug used	Carrier used	Property improved	Reference
8	Camptothecin	Folic acid-conjugated MSNs	Enhancement in tumor-suppressing property of camptothecin	Lu et al. (2012)
9	Doxorubicin	Multifunctional envelope-type MSNs	Improved drug loading and glutathione responsive drug release at target site	Zhang et al. (2013)
10	Felodipine	Hollow mesoporous silica	Polyelectrolyte coated mesoporous silica nanospheres successfully developed for delivering poorly soluble drug like felodipine and controlling its release	Hu et al. (2015)
11	Telmisartan	–	Spherical MSNs effective in delivery of drug however drug transport was found to be time, concentration and size dependant	Zhang et al. (2012)
12	Doxorubicin	MCM-41	GSH-responsive PEG-capped MSN showed enhancement of payload of doxorubicin and also help in targeting and controlling the release	Giménez et al. (2015)
13	Celastrol	–	Selective and effective delivery of triterpenoid drug celastrol to cancer cells	Niemelä et al. (2015)
14	Doxorubicin and Bcl-2 siRNA	MCM-41	Simultaneous delivery of doxorubicin and Bcl-2 siRNAinto cancer cells help in enhancing anticancer activity of doxorubicin through reduction of non-pump resistance	Chen et al. (2009)
15	Doxorubicin and p-glycoprotein	–	Enhancement in intracellular delivery and anticancer activity of doxorubicin by co-delivering it with PgP siRNA through functionalized MSNs	Meng et al. (2010)

(continued)

Table 3 (continued)

Sr. No.	Drug used	Carrier used	Property improved	Reference
16	Doxorubicin, paclitaxel, camptothecin, tamoxifen, and curcumin	MCM-41 MSN, MCM-48 MSN	Improvement in loading of multiple drugs in single carrier, enhancement of colloidal stability and controlled release of drug from MSNs help in improving therapeutic efficacy of the drug	Palanikumar et al. (2015)
17	Paclitaxel and gemcitabine	Polyethylenemine/PEG-coated MSNs	Improved loading of gemcitabine, colloidal stability and synergistic effect in tumor suppression	Meng et al. (2015)
18	Doxorubicin and siRNA	Hollow MSNs	Simultaneous delivery of doxorubicin and siRNA by pH responsive hollow mesoporous silica nanoparticles, with improvement in anticancer activity of doxorubicin	Ma et al. (2013)
19	Doxorubicin and anti-angiogenic agent (combretastatin A4)	–	Improvement in therapeutic efficacy through combination of anti-angiogenesis and chemotherapeutic agent	Li et al. (2016)
20	Doxorubicin and ssDNA	Thiol-modified nanoparticles	Functionalized MSNs effective in controlling release of both therapeutic agents	Ma et al. (2012)
21	Fluorescein and soybean trypsin inhibitor type II-S	–	Improved therapeutic efficacy of soybean trypsin inhibitor type II-S	Baeza et al. (2012)

nature and stable configuration provide best option for protein and peptide delivery. It protects protein and peptides from degradation and also prevents premature release (Sood and Panchagnula 2001).

MSN is a safer alternative as a non-viral carrier for gene delivery. It offers benefits over other carrier systems like protecting gene from nuclease enzyme by entrapping in the pores (Torney et al. 2007), providing high loading efficiency by surface functionalization of MSN with cationic polymer like polyethylenimine for accumulation of electronegative nucleic acid. Surface functionalization with polyethylenimine provides additional benefits of escaping from endosome through proton sponge effect. Combinational therapy is desired for achieving synergistic or complementary effects especially in cancer therapy. Property that plays crucial role for co-delivery of therapeutics is tunable pore size. Co-delivery can be achieved by loading drug inside pore and on the surface of MSNs. Sequential drug delivery also possible by using MSNs (Chen et al. 2009). For example, co-delivery of a chemotherapeutic drug (doxorubicin) and anti-angiogenic agent (combretastatin A4) by using MSNs was found to be beneficial in obtaining combined effect of anti-angiogenesis and chemotherapy (Li et al. 2016). Some of the examples where MSNs have proven its potential in delivering drug, protein, and gene have been depicted in Table 3.

Stimuli Responsive Drug Delivery System

Drug encapsulation and release can be controlled or modified by using various organic molecules or drug delivery systems, which can be named as gatekeepers. In such type of drug delivery systems, "zero premature release" can be achieved, which is useful in cases where therapeutic agents such as anticancer agents have various deleterious effects. This approach has demonstrated its utility in achieving precise control over release timing and location. Stimuli responsive controlled release offers great platform to accomplish this objective. Change in pH and redox potential are most commonly used stimuli for site specific delivery using MSNs (Tang et al. 2012). First report of stimuli controlled MSNs came in 2003, when Tanaka and coworkers developed photosensitive drug delivery system containing MCM-41. In this study, they attached coumarin to free silanol groups of MCM-41, which resulted into formation of cyclobutane coumarin dimer that obstructed the entry of drug inside the pores of MCM-41. Pores were only opened reversibly, when it was irradiated by UV light at 250 nm (Mal et al. 2003a). Numerous other ligands like coumarin (Lin et al. 2010), azobenzene derivatives (Angelos et al. 2007), thioundecyl tetraethyleneglycolester-o-nitrobenzylethyldimethyl ammonium bromide (Vivero-Escoto et al. 2009), have been utilized for photosensitive drug delivery system.

Other stimuli like redox (Wan et al. 2010), temperature (Angelos et al. 2009), pH (Park et al. 2007), ultrasound (Kim et al. 2006), magnetic field (Hu et al. 2008), electric field (Zhu et al. 2010), and enzymes (Schlossbauer et al. 2009) have also been attempted for developing stimuli responsive drug delivery using MSNs. Apart from drug delivery, MSNs have been employed for cellular imaging (Lin et al. 2010). To date, literature was more focused on in vitro studies, however recently,

in vivo reports are also emerging (Rim et al. 2011) indicating a need for extensive study on successful management of MSN-based stimuli responsive system in vivo.

Targeted Drug Delivery System

MSNs have higher blood distribution and blood to liver ratio as that of other PEGylated nanocarriers system like polycyanoacrylate nanoparticles and solid lipid nanoparticles. PEGylation of MSNs helps in reduction of reticuloendothelial system accumulation and enhances passive targeting (Wang et al. 2010). Coating of cationic polymer on MSNs is a novel alternative where insignificant increase in accumulation of MSNs can be achieved at tumor site as that of PEGylated MSNs. Additionally, physical stability of MSNs is increased due to steric hindrance provided by cationic polymer (de Wolf et al. 2007). In recent times, bioconjugation of specific targeting ligand with MSNs has been explored for achieving active targeting. Ligands that have been incorporated for active targeting include folate for targeting folate receptor (Rosenholm et al. 2010), cyclic-RGD for targeting $\alpha v\beta 3$ integrin, transferrin for targeting transferring receptor (Ferris et al. 2011), antibody for targeting human epidermal growth factor receptor-2 receptor over expressed in breast or lung cancer (Tsai et al. 2009), and aptamer sgc8 for targeting human protein tyrosine kinase-7 over expressed in colon cancer (Zhu et al. 2009). Selection of ligands for active targeting should take into consideration the following,

1. Ligand should have high specificity with suitable affinity for a particular target site as it may result into low targeting efficiency due to suboptimal affinity of ligand for target site.
2. Bioconjugation should not have any influence on dispersity of MSNs.
3. Interaction between ligand and MSNs should be stable until it reaches target site.
4. Care should be taken toward grafting density of ligands on MSNs surface, as it may lead to multiple interactions between ligand and receptor, resulting into immunogenic reaction (Ruoslahti et al. 2010).

5.6.2 Biocompatibility Issues of Mesoporous Material

Despite availability of numerous publications in support of positive biocompatibility, there is a serious concern about systemic toxicity. Use of nondegradable carrier in intravenous administration is still questionable as it is not considered safe until it is demonstrated that it does not accumulate in the body after repetitive administration (Tang et al. 2012). Hudson et al., compared biocompatibility of polymeric carrier with MSNs and concluded that they have good biocompatibility when administered subcutaneously in rats (Hudson et al. 2008). However, intraperitoneal and intravenous injection in SV129 mice had resulted into death or euthanasia in animals. The authors in their study further stated that functionalization of mesoporous silica with PEG helps in reducing systemic toxicity. There are few reports which claim that MSNs may promote malignant melanoma growth because of their antioxidant effect after subcutaneous administration (Huang et al. 2010).

Hence, there arises a need of extensive research to investigate dose dependent toxicity of mesoporous material in terms of solubility, surface area, geometry, surface chemistry, method of administration, chemical composition, and biodistribution (Lu et al. 2010). Another severe problem associated with biocompatibility study of mesoporous material is lack of correlation between the in vitro and in vivo studies. Moreover, standardization of techniques for analyzing biocompatibility of mesoporous material with good correlations needs to be established before any conclusion on biocompatibility can be made.

6 Quantum Dots

In the area of biomedical research, nanotechnology has shown most promising applicability, mainly in molecular diagnostics and high throughput bioanalytics, wherein nanoscale sensors are more popular when compared with macroscale modalities like MRI, single photon emission computed tomography, positron emission tomography, sonography, and optical imaging for molecular imaging purpose. Nanoscale probes are found to be more promising in visualization, characterization, and quantification of biological processes at the molecular level within living systems. Among nanoscale probes, QDs have emerged as an essential tool in biomedical research. QDs are crystalline particles whose size lies in the range of 2–10 nm. They are also known as semiconductor nanocrystals. Unique optical properties discriminate QDs from other conventional organic dyes and fluorescent proteins. The distinctive brightness of QDs stems from an amalgamation of proficient light absorption, high quantum yield (ratio of number of photons emitted to number of photons absorbed) and a large extinction coefficient. Resistance toward photobleaching makes it more stable when compared to conventional fluorophores. They provide long observation times and high sensitivity during detection by fluorescence microscopy. Additionally they possess narrow emission spectra along with large Stokes shift (difference between the wavelength of absorbed and emitted light) which help in easy separation of fluorescence signal of QDs from light of excitation source. Multicolor imaging is an added benefit provided by QDs even in absence of crosstalk between different detection channels in fluorescence microscopes. Excitation of QDs of different emission maxima from single wavelength eliminates requirement of several excitation sources in the instrumental setup. QDs provide long fluorescence lifetime (20–50 ns) which enables time-resolved detection of the QD-fluorescence and help in increasing signal-to-background ratio (by a factor of 15) compared to cell auto fluorescence. Advances in surface chemistry of nanoparticles have created a platform for the improvement of polymer-encapsulated probes toward stable and highly fluorescent probes under complex biological conditions. This has resulted into generation of water-soluble QDs which solve issues associated with ligand exchange based QDs like quantum yield decrease, short shelf life, and chemical sensitivity. This advancement has laid foundation for generation of new opportunities in ultrasensitive and multicolor imaging of molecular targets in

living cells and animal models. QDs enlarge the capabilities of fluorescence imaging. In addition, they provide a suitable platform for engineering of multifunctional nanodevices with capabilities of exploiting multiple imaging modalities or merging imaging and therapeutic functionalities within a single nanoparticle.

Unique photo-physical and chemical properties of QDs have proven their potential in solving issues raised by biomedical researcher. Nowadays, QDs have been promoted for the development of multifunctional nanocomposites, traceable drug delivery vehicles and novel imaging probes. This has resulted into an increase in publications related to QDs by 300% since 2000.

6.1 General Procedure of Preparation of QD

Extensive research is going on in the area of synthesis; solubilization and stabilization of QDs. Core of QDs are made up of semiconductor nanoparticles, which include hundreds to thousands of atoms of group II and VI elements or group III and V elements. In bulk form, these materials have small band gap of 4 eV between valence bands and conduction bands. Hence, they behave like insulators at ambient conditions and behave like semiconductor only under external stimulations. Movement of electrons from valence band to conduction band by supplying energy results into exceeding of band gap. In certain cases, band gap energy is released in the form of light (fluorescence) due to relaxation of an electron. It is postulated that if the physical size of the nanoparticles is smaller than the exciton Bohr radius then it will result into a 3-D (three-dimensional) quantum confinement of charge carriers within the QD, thus giving nanoparticles unique properties which are absent in bulk materials (Rossetti et al. 1983; Alivisatos 1996; Brus 1984). Inorganic nanoparticles core has been responsible for development of QD probes hence manipulation of its chemical composition, size, and structure control the photo-physical properties of the probe. However, bare inorganic nanoparticles do not possess any biological functionality as it cannot interact with biological systems. Therefore, while designing biocompatible probe with controllable physicochemical properties, care should be taken during encapsulation by designing proper coating materials. Attachment of biofunctionality to probe enables interaction with biological systems.

Step 1: Design of QD core

Core is the main component of QD, which is responsible for its optical properties. QD core must possess properties like precisely controlled nanosize distribution, chemical composition, surface chemistry, higher stability, and geometry. Initially, synthesis methods were reported in aqueous solution which resulted into large size variation and poor fluorescence efficiency (Zheng et al. 2007). In last few decades, advancements in synthetic procedures and surface chemistry have led to the production of water soluble QDs with narrow size distribution and higher quantum yield. However, still it suffers from poor photo-physical and chemical properties. In 1993, Bawendi and his co-workers developed method for synthesis of uniform

colloidal CdSeQDs by using high-temperature organometallic procedure (Murray et al. 1993). This method has wide applicability for QDs synthesis. During synthesis, core organometallic precursor undergoes pyrolysis, which generates nanocrystal, and the reversible conversion of nanocrystal into bulk semiconductor is prevented by trioctyl phosphine/trioctyl phosphine oxide. These stabilizers form coordination complex with unsaturated atoms of metallic core and prevent reverse reaction. However, presence of these stabilizers on the surface prevents photon emission and reduce quantum yield. Hence, new stabilizers like alkyl amine can be used as an alternative. Alkyl amine do not hinder photon emission ultimately increasing quantum yield (Talapin et al. 2001). During scale-up of QDs synthesis use of Cd precursor (dimethyl cadmium) imparts restrictions on QD core designing, equipment, and reaction conditions due to its toxicity. Dimethyl cadmium by CdO in large scale synthesis can solve this problem (Peng and Peng 2001). Advances in synthesis and surface passivation technologies have made QDs an appealing platform for engineering of biological probes with the advantages of enhanced photostability, improved brightness, tunable fluorescence, and single-source multicolor excitation.

Step 2: Design of organic shells

QDs must be present in water soluble form for biological use. Core of QDs synthesized in step 1 by organic phase synthesis produces hydrophobic QDs. Hence, different solubilization approaches are used to make QDs soluble and stable in water and biological buffers. In addition, QDs should retain its entire chemical and photo-physical properties (Zrazhevskiy et al. 2010). Approaches that are used to produce water-soluble QDs include replacement of hydrophobic group from surface by hydrophilic group through ligand exchange mechanism (Chan and Nie 1998) or surface cross-linking of QDs with DL-Cysteine and poly(allylamine) (Sukhanova et al. 2004) or formation of polymerized silanol shell on the surface of QDs through ligand-based approach (Gerion et al. 2001; Bruchez et al. 1998) or encapsulation of hydrophobic QDs by phospholipids or amphiphilic polymers (Wu et al. 2003; Dubertret et al. 2002). However, some lacunae exist in all these approaches. Hence, there exist a huge potential for engineering novel coating materials, which will satisfy the requirements of biomedical applications (i.e., compactness of ligand and protective features of encapsulation procedures).

Step 3: Design of surface ligand

Inert nanoparticles are biofunctionalized for advanced uses of QDs like bioimaging, drug delivery application, and diagnosis. Functionalization of QDs surface with different ligands like peptides, proteins, nucleic acids, and other biomolecules has become necessary for achieving specific interactions with living systems. Surface engineering of QDs combines useful properties of nanoparticles (optical property) and ligands (biological function via specific interactions with living system). Various approaches used for bioconjugation are listed below.

1. Covalent bond formation between reactive functional groups (like carbodiimide-mediated amide formation, ester maleimide-mediated amine and sulfhydryl coupling and amine-sulfhydryl coupling) (Zrazhevskiy et al. 2010)
2. Coordination linkage between biomolecules and metal atoms of QDs core (Medintz et al. 2005)
3. Non-covalent self-assembly of engineered proteins on the surface of QDs with preserved organic shell to prevent direct access to inorganic QD core that exhibit minimal effect on the photo-physical properties (Goldman et al. 2002)

6.2 Biocompatibility of QDs

In last few decades, varieties of QDs were synthesized and most of them differ in chemical composition. Different properties of various compositions have created confusion during toxicity studies. Each QD has its own unique physicochemical properties and hence behave differently in biological systems. Several factors that are involved in QDs generation were found to be responsible for the discrepancies in toxicity data present in the existing literature. Limited literature is available on specific toxicological assessment like dose, mechanism of action, and frequency of exposure. Hence, while summarizing QD toxicity, properties pertaining to core, core shell, and coating toxicities should be taken into consideration.

Potential cause of toxicity related to QDs is Cd present in core. Derfus et al. found that Cd released from the surface of core due to biodegradation could cause cytotoxic effect in rat pheochromocytoma cells. These results were in line with the effects produced due to Cd ion toxicity. This indicates that toxicity induced by core could contribute significantly in QDs toxicity (Derfus et al. 2007). Photoactivation of QDs by UV or visible light is responsible for formation of free radicals, which may damage DNA and may produce detrimental effects on tissues. Encapsulation of core reduces toxic effect associated with the core material. Capping of core by using CdTe/ZnS shell or CdTe capped with mercaptopropionic acid, n-acetylcysteine, and cysteamine minimize the toxicity caused due to generation of free radical from the core material (Bakalova et al. 2005). In addition to the core, special investigation should be carried out to study toxic effect of capping material or shell on nontarget cells or tissues. Uncoated CdTe QD injected in rats produced inconclusive results relating to toxicity and organ damage. The results were inconclusive due to absence of thorough histological evaluation. Disturbances in motor function were visible after injection of uncoated QD, indicating potential toxic effects on neural functions (Zhang et al. 2008). Future investigation should focus in this area.

Potential ways to solve toxicity issues associated with QDs involve use of ultra-stable QDs (coated with nondegradable material) or applying a biodegradable coating material with controlled degradation rate to slow down QDs degradation. Designing quick excretion QDs that can be rapidly excreted after short exposure to target tissues, or use of other core material than Cd which are less toxic can also be attempted to solve toxicity related problems.

6.3 Applications of QDs

Due to its specific chemical and photo-physical properties, biomedical application of QDs are more focused on imaging and diagnostics purposes. However, some of its properties that make it a suitable candidate for drug delivery are,

1. The small size QDs that increase its drug delivery efficiency
2. High surface to volume ratio providing larger surface for linking multiple functionality on single QD
3. Scope of embedding hydrophilic therapeutic agents or targeting moiety between core and coating material
4. Application of in real-time trajectory monitoring as QDs also emit detectable signal

Current applications of QDs in drug delivery are focused on two major areas, using QDs as carriers and labeling therapeutics or drug carriers with QDs (Qi and Gao 2008a). QD as carriers was reported by Yamamoto et al. by preparing captopril conjugated QDs. They claimed equivalent reduction in rat blood pressure as that of captopril alone (Manabe et al. 2006). Baqalkoat et al, reported QDs wherein the targeting moiety (prostate specific membrane antigen) was attached to the core through RNA aptamers A10. Anticancer drug doxorubicin was also embedded onto the core. This design permitted targeted delivery of doxorubicin along with a sensing mechanism for drug release (Bagalkot et al. 2007). Functionalization of QDs for targeting has also been reported. Folic acid conjugation on QDs core helped in accumulation of drug embedded nanodevice into target tissues. In comparison, use of QDs in traceable drug delivery is more straightforward as it involves labeling of conventional drug delivery system with QDs. For tracing drug release from conventional drug delivery, organic fluorophores are frequently used, but these fluorophores face serious drawback of photobleaching. This has created scope for use of QDs in traceable drug delivery as QDs are devoid of photobleaching problem and possesses unique spectral property. Some of the reported studies where QDs were used in drug delivery or for traceable drug delivery are summarized in Table 4.

7 Metal Oxides

Metal oxide nanoparticles are emerging as promising carrier system due to their unique physical and chemical properties. Metal oxide nanoparticles are composed of different materials like zinc oxide (ZnO), zirconium dioxide (ZrO_2), iron oxide (Fe_3O_4), titanium dioxide (TiO_2), aluminum oxide (Al_2O_3), copper oxide (CuO), silver dioxide (Ag_2O), magnesium oxide (MgO), manganese dioxide (MnO_2), etc. (Mallakpour and Madani 2015). Metal oxide nanoparticles combine unique advantages of nanoscale, catalytic effect, redox potential, mechanical stability, high

Table 4 Partial list of quantum dots used for drug delivery and imaging purpose

Sr. No.	Vehicle	Model drug	Purpose of study	Reference
1	CdSe/ZnS	Doxorubicin	Targeted delivery and imaging	Bagalkot et al. (2007)
2	CdSe/ZnS	siRNA	Efficient delivery and real-time imaging in live cells	Qi and Gao (2008b)
3	ZnO QDs	Camptothecin	Tumor-targeted drug delivery system	Chen et al. (2013)
4	QDs	Diphtheria toxoid vaccine	Transdermal vaccine delivery	Upadhyay (2006)
5	QDs	Captopril	Biodistribution studies	Manabe et al. (2006)
6	QDs 655 Poly (lactic-coglycolicacid)	Paclitaxel	Multimodal imaging, hyperthermia, and targeted drug delivery	Cho et al. (2010)
7	CdSe/ZnS	siRNA	Efficient delivery and real-time imaging in live cells	Derfus et al. (2007)

surface area, and biocompatibility of metal oxides. These properties make them popular in the field of biomedical imaging, therapeutics, biosensing, and in vivo imaging (Tuli et al. 2015). They have also shown promising pharmacological activities such as antimetastatic, anti-inflammatory, anti-proliferative, antimicrobial, in neuro-degenerative disorders, etc. Their pharmacological activity may be increased when combined with other drugs/therapeutics (Tuli et al. 2015). For drug delivery purpose, to date Fe_3O_4, TiO_2, Ag_2O, and ZnO nanoparticles have been explored. The following section focuses on application of iron oxide nanoparticles in drug delivery.

7.1 Iron Oxide Nanoparticles

Size reduction of iron oxide to nanoscale leads to formation of particles with superparamagnetic properties, as each particle have one magnetic domain and the thermal energy that can exceed the barrier of magnetic flipping. SPIONs were used for labeling specific cells or tissues to enhance MRI. In multifunctional imaging and phototherapy, SPIONs have demonstrated their superiority over other imaging reagents. They are highly biocompatible and biodegradable. Even though they are one of the few nanomaterials that can be readily injected and incorporated into natural metabolic pathways, the doped magnetic elements are however vulnerable to oxidation and hence toxic. Coating with gold or silica may offer protection from oxidation. Conjugation of antibodies on gold nanoshell coated MRI-visible SPIONs were used for studying the biodistribution, in mice with A431 tumors (Ji et al. 2007). Coating with copper sulfide shell onto ultra-small magnetic iron oxide nanoparticles demonstrated its use in photothermal ablation of cancer cells along

with in vitro and in vivo imaging. Some of the studies report that "Cu" content controls photothermal effect of iron oxide nanoparticles. Such photothermal treatment can be used in suppression of tumor growth. Recently R Urie et al. reported 'nanogrenades' composed of iron oxide nanoparticles functionalized with pH responsive ligand which aided in visualization of small and heterogeneous tumors in mice (Urie and Rege 2015). SPIONs have also been explored for multifunctional imaging and delivery of drug and nucleic acids. Iron oxide nanotubes also showed its potential in imaging and drug delivery. For example paclitaxel loaded iron oxide nanotube with pH responsive ligand was capable of guiding its distribution in target cell through magnetic activation and releasing the drug through pH activation in carcinoma cells (Yue et al. 2011). Partial list of iron oxide nanoparticles, which are mainly utilized for drug delivery, has been described in Table 5.

7.2 Preparation Method

Preparation methods of metal oxide nanoparticles have significant impact on their size, composition, and structure, which indirectly affects their applications in various fields. Hence, considerable precaution should be taken while selecting method for their preparation. They are mainly produced by chemical or physical approaches. Numerous methods available for their preparation include liquid phase, gas phase, sol–gel, co-precipitation, microemulsion/direct/inverse micelle approach, hydrothermal and template/surface derived methods. The sol–gel and hydrothermal methods are aqueous-based methods, which offer considerable advantages over other methods in terms of high yield, uniform products, lowered air pollution, simple manipulation, easy control, and low energy consumption. Precise control over preparation conditions is necessary for obtaining high quality product (Tuli et al. 2015). Another major hurdle with these nanoparticles is agglomeration. It mainly occurs due to increased reactivity as size reduction leads to increase in surface atoms. Functionalization of nanoparticles can address this problem of agglomeration and ultimately improve the stability of this system (Mallakpour and Madani 2015). Size modifications of metal oxide nanoparticles not only help in their entrance into cells but also improve their retention by the EPR effects. Altering the surface chemistry can enhance its therapeutic activity, for example, cationic nanoparticles get access into the cells more easily because of their interaction with the membrane component as that of the anionic particles.

7.3 Biocompatibility and Toxicity

Biocompatibility and toxicity testing of metal oxide nanoparticles are intended for studying acute toxicity, toxicity of degradation products, stimulation of cells with subsequent release of inflammatory mediators, and toxic effects on each organ (Neuberger et al. 2005). A first sign of toxicity can be obtained by studying tissues from the cell cultures after incubation with nanoparticles. S. Maassen et al., reported

Table 5 Reported examples of metal oxides for drug delivery and imaging purposes

Sr. No.	System	Drug	Observation	Reference
1	Fe_3O_4 microspheres coated with silica and functionalized by the YVO_4:Eu^{3+} phosphorous	Ibuprofen	Targeted drug delivery and controlled release	Yang et al. (2009)
2	Magnetic molecularly imprinted polymer by dopamine	5-fluorouracil	Improved antitumor activity	Hashemi-Moghaddam et al. (2016)
3	PEG-b-poly (4-vinyl benzylphosphonate) coated magnetic iron oxide nanoparticles	Doxorubicin	Stimuli responsive release of drug	Hałupka-Bryl et al. (2015)
4	Multifunctionalized magnetic nanoparticles with antiCD44	Gemcitabine	Selective drug delivery	Aires et al. (2016)
5	Magnetic nanohybrids comprising of smart block copolymers and mixed ferrite nanoparticles	Doxorubicin	Improvement in entrapment efficiency and release of drug	Bhattacharya et al. (2016)
6	Iron oxide nanoparticles immobilized with gemcitabine, chlorotoxin, and hyaluronic acid	Gemcitabine	Easy penetration into GBM cells with good cytotoxicity, prolonged blood circulation time and easy excretion via renal system	Mu et al. (2016)
7	Poly paclitaxel/cyclodextrin-SPION nano-assembly	Paclitaxel	Improved anticancer activity both in vitro and in vivo	Jeon et al. (2016)
8	Drug and dye loaded, multifunctional PEG-chitosan iron oxide nanocomposites	Methotrexate	Combined application of magnetic resonance and fluorescence with improved antitumor activity	Lin et al. (2015)
9	Paclitaxel/ SPION-loaded PLGA-based nanoparticles	Paclitaxel	Improved cellular uptake and anticancer activity	Schleich et al. (2013)

(continued)

Table 5 (continued)

Sr. No.	System	Drug	Observation	Reference
10	Fe_3O_4nanoparticles coated with fluorescent SiO_2 and PAA shells	Doxorubicin	Cell imaging and pH-responsive drug delivery	Schleich et al. (2013)
11	ZnO nanoparticles	5-fluorouracil	Improvement in antitumor activity	Al-Ajmi et al. (2016)
12	Fe_3O_4nanoparticles coated with poly-n(isopropylacrylamide)	Doxorubicin	Targeting, hyperthermia, and controlled drug release	Bakrudeen et al. (2015)
13	Human serum albumin coated iron oxide nanoparticles loaded with doxorubicin	Doxorubicin	Enhanced cellular imaging and anticancer activity	Xie et al. 2010

that the cytotoxicity was generally much higher in vitro in comparison with in vivo (Neuberger et al. 2005). This was attributed to toxicity induced by accumulation of degradation products at application site during in vitro study, which may not reflect in vivo study due to their continuous elimination from application site. This fact clearly indicates the limitations of in vitro toxicity study. Many alternatives have been proposed for studying toxicity of metal oxide nanoparticles. In one of such toxicity tests where nanoparticles were administered intraperitoneally in mice, mutagenicity, LD_{50} dosage and organ toxicity were studied by means of histological analysis. FDA has granted GRAS (generally recognized as safe) status to some of the metal oxides but it is limited to materials in micrometer range. Size reduction to nanometer level may develop new toxic actions on body. This indicates the need of advanced evaluation tools for studying the toxicity of nanomaterials as well as identifying means to reduce unwanted toxicity. Approaches that are mostly used to improve biocompatibility of metal oxide nanoparticles include coating of nanoparticles and surface functionalization (Rasmussen et al. 2010). Conversely repeated administration of stable and biocompatible metal oxides nanoparticles may cause toxicity due to accumulation in body (Wang et al. 2012). Impact of size and surface chemistry of nanoparticles on coagulation and complement activation in human blood was studied by Mayer et al. They studied it by using nanoparticles with different chemistry and concluded that with positively charged nanoparticles, complement system was activated, while with negative charge, hematotoxicity increased significantly. In addition, they concluded that particles with size less than 60 nm were responsible for hemolysis. Hence, size of nanoparticles and surface chemistry should be taken into consideration while designing such systems.

A comparative assessment of toxicity of different metal oxide nanoparticles (Fe_3O_4, Fe_2O_3, Fe_2O_4, CuO, TiO_2, and ZnO) on human lung epithelial cells (A549 cell lines) revealed that all nanoparticles except Fe_3O_4 and Fe_2O_3 cause damage to DNA. Extent of toxicity was high with CuO nanoparticles; which was also reported to cause oxidative lesions. Extensive study by exposing A549 cells to CuO nanoparticles confirmed that CuO nanoparticles are potentially genotoxic and act through reduction of cell viability, production of glutathione, increase in oxidative stress, stimulation of lipid peroxidation, and catalase (Ahamed et al. 2010). Toxicity assessment of Ce_2O_3, TiO_2, and Fe_2O_3, revealed that iron oxide nanoparticles are safest with very low toxicity whereas Ce_2O_3 nanoparticles are toxic even at very low concentration. This may create problems in its medical applications and photocatalysis. TiO_2 nanoparticles are relatively inert with very low toxicity. However, recent reports of tumorigenic effect of titanium oxide nanoparticles in lung cells have led to it classified as Group 2B (possible carcinogenic to human) by International Agency for Research on Cancer. Iron oxide nanoparticles are hence preferred in wide variety of applications because of their low toxicity. It has also found application as a negative control while performing in vitro and in vivo particle toxicological studies.

8 Market and Clinical Status

INMs are used for a wide range of applications such as enhancement of radiotherapy, live cell imaging, and drug delivery. Among all INMs, iron oxide nanoparticles have demonstrated its application in diagnostics. Some of the iron oxide based products are in clinical trials for imaging of tumors. NanoTherm® is an aqueous colloidal dispersion of iron oxide nanoparticles for thermal ablation of tumor by applying alternating magnetic field. This applicability of iron oxide nanoparticles for magnetic hyperthermia has also gained popularity for treatment of glioblastoma leading to a marketing approval for NanoTherm® in several European countries for treatment glioblastoma. Hafnium oxide nanoparticle has completed its first phase of clinical trial as radiosenstizers in patients with soft tissue sarcoma. Its phase 2 clinical trials have begun in 2014. Although to date not a single INMs has been approved for drug delivery, few are in early clinical testing such as PEGylated colloidal gold—Tumor Necrosis Factor α particles for treatment of cancer and parental peptide delivery by using silicon nanocarriers. Ongoing clinical trials for INMs are listed in Table 6.

Table 6 Clinical status of INMs

Sr. No.	Product (company)	System	Indications	Applications	Clinical status
1	Targeted SNP (memorial sloan kettering cancer center)	Silica nanoparticles	Melanoma	Lymph node imaging	Phase 0
2	NanoXray (Nanobiotix)	Hafnium oxide nanoparticles	Solid tumors	Radiotherapy	Phase 1
3	AuroLase (nanospectra)	Gold coated silica nanoparticles	Melanoma	Photothermal ablation	Phase 1
4	Ferumoxytol (MD Anderson)	Iron oxide nanoparticles	Melanoma and lymph node cancer	MRI	Phase 1
5	Aurimune (cytimmune sciences)	Colloidal gold	Solid tumors	Tumor Necrosis factor delivery	Phase 1/2
6	Ferumoxtran-10 (Guerbet)	Iron oxide nanoparticles	Prostate cancer	MRI	Phase 3
7	Feridex®	SPION coated with dextran	–	MRI	FDA 1996
8	Feraheme™	SPION coated with dextran	Iron deficiency anaemia	Mononuclear phagocytic system targeting	FDA 2009
9	NanoTherm®	SPION coated with aminosilane	Glioblastoma, prostate, and pancreatic cancer	Thermal ablation	Europe 2013

9 Conclusion

In this chapter, we have discussed some of the interesting aspects of INMs in relation to drug delivery. Different inorganic carriers have been addressed with respect to their unique physicochemical properties. Unique properties that made INMs different from their bulk material properties have been explained in detail. Some of the important aspects of the selected INMs, including mesoporous silica- and silicon-based nanomaterials, QDs, and metal oxide nanoparticles were addressed.

INMs have shown great potential in drug delivery, diagnostics, and in vivo imaging. Functionalization of these engineered nanomaterials can boost drug efficacy by effective localization of carrier system at target site and improve targeting specific receptor in body ultimately resulting into reduced side effects. For example, functionalization of mesoporous silica, QDs, and metal oxide nanoparticles with different types of ligands aid in achieving targeted drug delivery and also help in tracking the release of drug at target tissues by attaching fluorescent dye to them.

Apart from targeting, surface functionalization has been employed for advancing cellular imaging, cell tracking, or stimuli responsive drug release. Although INMs provide good option for drug delivery, cellular imaging, and diagnostics, several issues mainly related to side effects, long-term effects on biodistribution and accumulation of nonbiodegradable material inside the body are a matter of concern and needs to be address for the carrier system to be used for drug delivery. Toxicity of these carrier systems needs to be extensively studied, as INMs are chemically more active and hence show greater chances of interacting with body cells. Current achievements of INMs in the field of drug delivery and imaging are quiet encouraging but extensive research is required on the interaction of these INMs with biological systems from the point of view of safe use and for the conversion of these nanomaterials to clinical therapy.

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Green Synthesized Gold Nanoparticles for Future Biomedical Applications

Sudip Mukherjee, Susheel Kumar Nethi and Chitta Ranjan Patra

Abstract

Nanobiotechnology is an emerging field of biological and engineering sciences. The design and development of green chemistry approach for the synthesis of biocompatible nanoparticles is always better selection due to eco-friendliness. The green chemistry approach for the synthesis of metal nanoparticles has several advantages (simple, safer, energy efficient, fast, mostly one-pot processes, inexpensive, and less toxic routes toward synthesis) over conventional synthetic procedures. Among various biologically synthesized metal nanoparticles, noble nanoparticles (gold, silver and platinum) especially gold nanoparticles (AuNPs) are exceptionally attractive in biomedical application due to presence of unusual physicochemical properties, ease of synthesis and surface modification in the nanoscale range, biocompatibility, and several other advantages. Recently, several researchers including our group have intensely focused to explore the green synthesis and potential applications of AuNPs in biology and medicine. In this chapter, we discuss about the (i) synthesis of AuNPs using rational utilization of various bioresources, (ii) their prospective applications toward various disease therapy, (iii) in vitro and in vivo toxicity, (iv) biodistribution studies, and (v) future challenges and opportunities.

Keywords

Green chemistry approach · Biosynthesis · Gold nanoparticles · Drug delivery · Disease theranostics · Biomedical applications

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1 Introduction

Nanotechnology is the most promising area of modern science where the size of the matter can be controlled (~1–100 nm) in the atomic, molecular, or macromolecular range with fundamentally new properties and functions (Whitesides 2005). In addition to size, higher surface to volume ratio, surface energy, and aspect ratio of the nanomaterials aids in exhibiting several dramatic properties (physical, chemical, biological, and optical) compared to their bulk scale. Nanotechnology has developed wide applications toward various fields like catalysis, electronics, optics, solar cells, food sector, engineering, textiles, paints, drug delivery, diagnostics, therapeutics, and imaging and many more (Daniel and Astruc 2004; Dastjerdi and Montazer 2010; McEuen 1998; Parveen et al. 2012; Sozer and Kokini 2009; Yella et al. 2011). Since all the pathological consequences in the biological systems initiate at a molecular level, it is best to address them at the nanoscale range using nanomedicine approach. Due to very small size, nanoscale materials can easily enter the cells (size: 10–30 μm) and interact with cellular components (DNA: ~2.5 nm; proteins: ~1–20 nm; cell surface receptors: ~10 nm; cell membrane width: ~6–10 nm) (Kim et al. 2010; Lynch et al. 2009; Seo et al. 2015). Altogether, these characteristic properties of nanoparticles support the promising applications of nanotechnology in biology and medicine. The first FDA approved nanomedicine was introduced in the year 1990 and since then several nanomedicine-based therapeutic agents (~175) have been commercially approved (Noorlander et al. 2015). Various nanomaterials such as inorganic nanoparticles (gold, silver, platinum) (Jain et al. 2008), carbon nanotubes (Baughman et al. 2002), fullerenes (Jensen et al. 1996), dendrimers (Mintzer and Grinstaff 2011), liposomes (Lasic 1998), and polyplexes (Ulasov et al. 2011), have been extensively investigated for their nanomedicinal applications. Recently, the scientists all over the world have mainly concentrated to explore numerous applications of gold nanoparticles (AuNPs) toward drug delivery, gene delivery, imaging, photothermal therapy, CT imaging, biosensing (Deng et al. 2014; Giljohann et al. 2010; Han et al. 2007; Huang et al. 2006; Kang et al. 2015; Khlebtsov et al. 2013; Kodiha et al. 2015; Patra et al. 2008b, 2010; Sanpui et al. 2015; Xia et al. 2011; Zhang et al. 2013) and others due to many advantages (Jain et al. 2008) However, the major drawback of AuNPs toward biological applications is the chemical synthetic route involving the use of toxic precursors, organic solvents and harsh conditions. This has led to the advancement of alternative eco-friendly green synthetic procedures (Dahl et al. 2007; Hutchison 2008).

The green synthesis of nanoparticles is a simple, clean, eco-friendly and efficient procedure, which minimizes the use of hazardous chemicals, has many advantages over the conventional methods (Duan et al. 2015; Kharissova et al. 2013). It involves the use of various biological sources (plant and microbes), which act as proficient reducing or stabilizing agents (Singh et al. 2016). Numerous metal nanoparticles such as gold (AuNPs) (Mukherjee et al. 2016), silver (AgNPs) (Mukherjee et al. 2014), platinum (PtNPs) (Attard et al. 2012), copper (CuNPs)

(Salvadori et al. 2014), along with quantum dots (Sturzenbaum et al. 2013) have been reported for their green synthesis and several biomedical applications. However, in this present book chapter we will discuss about the potential biomedical applications of biosynthesized AuNPs (b-AuNPs) and their future perspectives.

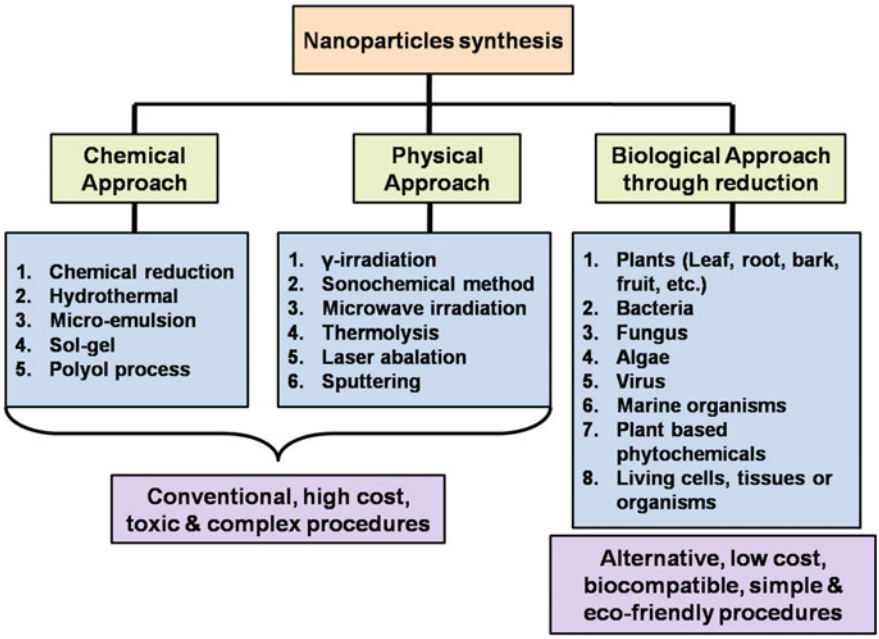
2 Gold Nanoparticles (AuNPs) History

Gold nanoparticles (AuNPs) and gold compounds have a long history in the field of medicine and technology in both Eastern and Western traditions (China, India, Greece, and Egypt) from ancient times. The Lycurgus cup anciently used by the Romans during the fourth century AD represents an excellent nanotechnology developed at ancient times. It owes its red ruby transmission color to the presence of colloidal AuNPs (Freestone et al. 2007). The red colloidal gold is still in use today in several parts of India. In ancient India Ayurvedic physicians, prescribed these nanoparticles for rejuvenation and revitalization in old age under the name of Swarna Bhasma (red gold) (Mahdihassan 1985, 1988). In India cinnabar-gold is known as "Makaradhwaja". Makaradhwaja means emblem of god of fertility, a drug for vigor of youth. Numerous publications demonstrated the history of medicinal use of AuNPs (Frick er 1996; Higby 1982; Patra et al. 2010). Biological properties of nanoparticles directly depend on its size, shape, and morphology that can be controlled by synthesis procedures. Because of vast biomedical applications of AuNPs (Deng et al. 2014; Giljohann et al. 2010; Han et al. 2007; Huang et al. 2006; Kang et al. 2015; Khlebtsov et al. 2013; Kodiha et al. 2015; Patra et al. 2008b, 2010; Sanpui et al. 2015; Xia et al. 2011; Zhang et al. 2013), ideal selection of synthesis method is one of the important criteria before its use in biological systems. Therefore, several investigators are concentrating in designing the development of new nanoparticles with different shape and size using various synthetic routes so that these nanomaterials could be useful for theranostics applications.

3 Synthesis Strategies of AuNPs

In general, synthesis of AuNPs is mainly classified into three categories described in Scheme 1:

1. Chemical approach
2. Physical approach
3. Biological approach



Scheme 1 Representation of the synthesis and methodologies of nanoparticles using various approaches

3.1 Chemical Approach

In this process, AuNPs are produced by the reduction of chloroauric acid ($HAuCl_4$) using citrate, borohydride, phosphorous, or other reducing agents in aqueous or organic solvents. In 1857, Michael Faraday first time demonstrated the synthesis of AuNPs by the reduction of gold chloride using phosphorous as reducing agent in carbon disulfide (CS_2) solvent (Faraday 1857). Later on, several groups demonstrated the synthesis of stable 10–20 nm AuNPs using sodium citrate or sodium borohydride as reducing agents (Brust et al. 1994; Turkevich et al. 1951). Apart from the reduction process, often various stabilizing agents or capping agents [poly ethylene glycol (PEG), 11-mercaptoundecanoic acid (MUA), poly ethylene imine (PEI), poly (N-vinylpyrrolidone)] were used to make the AuNPs stable for longer time (Guo et al. 2012; Hartono et al. 2010; Mendoza et al. 2010; Park et al. 2013).

3.2 Physical Approach

Physical methods for the synthesis of AuNPs generally involve various techniques like γ-irradiation method (El-Batal et al. 2013), electrostatic interaction (Ishida et al. 2015), sonochemical method (Yusof and Ashokkumar 2015), microwave

irradiation method (Augustine et al. 2014), thermolysis (Hsu and Lin 2009), laser ablations (Wender et al. 2011).

3.3 Biological Approach

Green synthesis is a simple eco-friendly procedure for the synthesis of AuNPs in which parts of a plant such as leaf (Mukherjee et al. 2013), root (Suman et al. 2014), seed (Fazal et al. 2014), bark (Daisy and Saipriya 2012), flower (Das et al. 2011) or whole plants (Ahmad et al. 2003, 2006; Chandran et al. 2006; Mandal et al. 2006; Shankar et al. 2003, 2004, 2005) are used for biosynthesis. The plant-based phytochemicals (including gums, resins) (Husen and Siddiqi 2014; Iravani 2011; Narayanan and Sakthivel 2011) are also employed for nanoparticle biosynthesis. Along with plant bioresources, microbial sources such as algae (Annamalai and Nallamuthu 2015), fungus (Mukherjee et al. 2001), bacteria (Ahmad et al. 2003; He et al. 2007), virus (Nam et al. 2012), several other microorganisms (Hulkoti and Taranath 2014), marine organisms (Asmathunisha and Kathiresan 2013) and living cells or tissues (in situ) (Haveli et al. 2012; Wang et al. 2013) are used. Proteins, polyphenols, carbohydrates, and sugars present in biosources help in the in situ single step reduction process. Also, the use of large environment pool of bioresource (plants and plants-based phytochemicals) simply makes the large-scale production as cost-effective. Apart from this, the minimal use of toxic chemicals can avoid the ecotoxicity of nanoparticles.

4 Size, Shape and In Vitro Stability Studies

AuNPs were used enormously in the recent past for several biomedical applications. The morphological characteristics (size and shape) along with the stability parameters are very critical to extend these b-AuNPs for various biomedical applications. Varying the volume and concentration of bioreducing agents (e.g., sugar, protein, polyphenols, carbohydrate, etc.) makes possible to modulate the size, shape and stability of b-AuNPs (Chandran et al. 2006; Mandal et al. 2006; Mukherjee et al. 2012; Nune et al. 2009a; Shankar et al. 2003, 2005; Shukla et al. 2008). In recent times, several groups synthesized a variety of b-AuNPs with diverse size and shape by altering the concentrations of bioreducing agents. The b-AuNPs have a wide size range between 2–300 nm, confirmed by transmission electron microscopy (TEM), scanning electron microscopy (SEM) and dynamic light scattering (DLS) techniques. Similar to size, the modulation of the biosynthesis process results in wide range of b-AuNPs with different shapes (spherical, hexagonal, triangular, decahedral, icosahedral, microcubes, microwires, nanorods, nanoprisms, dumble, pentagons, polygonals, nanoplates, quasispherical, nanowires, icosahedral multiple twinned, decahedral multiple twinned, tetrahedral, octahedral, dodecahedral, etc.) (Ahmad et al. 2003; Chandran et al. 2006; Mandal et al. 2006;

Mukherjee et al. 2012; Nune et al. 2009a; Shankar et al. 2003, 2004, 2005; Shukla et al. 2008). The generation of various size and morphologies of b-AuNPs is attributed to the slow reducing property of the bioreducing agents. The b-AuNPs generally tend to possess high negative surface charge or zeta potential signifying the well dispersive characteristics of b-AuNPs. Many research groups thoroughly investigated and demonstrated the extreme stability of b-AuNPs in various physiological buffers and biological fluids (phosphate buffered saline having pH ~6–8 and fetal bovine serum) (Mukherjee et al. 2012; Shukla et al. 2008). The high stability and versatility of the size and shape parameters of b-AuNPs facilitate their potential applications in health care system.

5 Biomedical Applications of Biologically Synthesized AuNPs

Biologically synthesized nanoparticle has been extensively used for several biomedical applications. The biologically synthesized AuNPs using different bioresource, their sizes, shapes and theranostics (therapeutic + diagnostic) applications are provided in Table 1.

Table 1 Potential theranostic applications of green synthesized gold nanoparticles for various diseases

S. No.	Bioresource	Morphology and size (nm)	[a]Biomedical applications	References
1	*Theobromo cacao* (cocoa) seeds	Triangular (150–200)	PT therapy, CT imaging	(Fazal et al. 2014)
2	Cinnamon phytochemicals	Spherical (15–20)	Photoacoustic/ CT imaging	(Chanda et al. 2011)
3	*Olax scandens* leaf	Spherical, rod, dumble, etc. (5–100)	Anticancer, Fluorescence imaging	(Mukherjee et al. 2013)
4	Cancerous cell (i.e., HepG2, K562, leukemia cell line (In situ)	Nanoclusters (2–3)	Tumor specific biomarkers	(Wang et al. 2013)
5	Human hair (In situ)	(2–3)	Red fluorescence, hair dyeing	(Haveli et al. 2012)
6	*Peltophorum pterocarpum* leaf	Spherical (5–10)	In vitro and in vivo drug delivery	(Mukherjee et al. 2016)
7	Culture supernatant of *Delftia* sp.	Spherical (11.3)	In vitro drug delivery in cancer	(Kumar et al. 2014)
8	Gellan Gum	Spherical (13–14)	In vitro drug delivery in cancer	(Dhar et al. 2008)
9	*Eclipta alba* leaf	Spherical, triangular (5–200)	In vitro drug delivery in cancer	(Mukherjee et al. 2012)

(continued)

Table 1 (continued)

S. No.	Bioresource	Morphology and size (nm)	[a]Biomedical applications	References
10	Sophorolipid with gellan gum	Spherical (17)	In vitro drug delivery in cancer	(Dhar et al. 2011)
11	*Butea monosperma* leaf	Spherical (10–30)	In vitro drug delivery in cancer	(Patra et al. 2015)
12	*Punica granatum* plant	Spherical (70–80)	Targeted in vitro drug delivery in cancer therapy	(Ganeshkumar et al. 2013)
13	Pullulan stabilized gold nanoparticles	Spherical, triangular (20–166)	FA targeted drug delivery in cancer therapy	(Ganeshkumar et al. 2014)
14	*Mappia foetida* leaf	Spherical (20–50)	FA targeted drug delivery in cancer therapy	(Yallappa et al. 2015)
15	*Sesuvium portulacastrum* L leaves	Spherical (37)	Anticancer (A549)	(Ramalingam et al. 2016)
16	Culture supernatant of *Streptomyces clavuligerus*	Spherical (8.2)	Anticancer, biocompatible	(Kumar et al. 2015)
17	*Lantana montevidensis* leaf	Spherical (10–20)	Anticancer, biocompatible	(Mukherjee et al. 2015a)
18	*Abelmoschus esculentus* (L.) pulp	Spherical (14)	Anticancer, antibacterial	(Mollick et al. 2014)
19	Guava leaf (*Psidium guajava*), Clove bud (*Syzygium aromaticum*)	Spherical, triangular (30–50)	Anticancer	(Raghunandan et al. 2011)
20	*Argemone mexicana* leaf	–	Anticancer	(Sellappa et al. 2015)
21	Pathogenic fungus *Helminthosporium solani*	Spherical, triangular, star, etc. (2–70)	In vitro toxicity	(Kumar et al. 2008)
22	*Hamelia patens* leaf	Spherical (25–50)	Angiogenesis	(Nethi et al. 2014)
23	Gallic acid, protocatechuic acid, and isoflavone	Spherical (20)	Antioxidant	(Lee et al. 2011)
24	Polyphenol (epigallocatechin-3-gallate (EGCG), resveratrol (RSV), and fisetin (FS)	Spherical (10–25)	Antioxidant	(Sanna et al. 2014)
25	*C. sinensis* (green tea) and *P. fulgens*	Spherical (100)	Antioxidant	(Bhaumik et al. 2015)
26	Cell free supernatant (CFS) of *Gordonia amicalis*	Spherical (5–20)	Antioxidant	(Sowani et al. 2015)
27	*C. fistula* aqueous stem bark	Spherical (50–100)	Antioxidant in in vivo mice model, hypoglycemic agents	(Daisy and Saipriya 2012)

(continued)

Table 1 (continued)

S. No.	Bioresource	Morphology and size (nm)	[a]Biomedical applications	References
28	*Bacillus licheniformis* biomass	Spherical (50)	Antidiabetic in in vivo mice model	(Barathmanikanth et al. 2010)
29	Guavanoic acid, phytochemical of *Psidium guajava* (Pg)	Spherical (12)	PTP 1B inhibitory effects, antidiabetic	(Khaleel Basha et al. 2010)
30	*A. comosus* fruit	Spherical, triangular (5–20)	Antibacterial	(Bindhu and Umadevi 2014)
31	*Nigella sativa* essential oil	Spherical (15–30)	Antibacterial, antibiofilm	(Manju et al. 2016)
32	*Mentha piperita* (Lamiaceae) leaf	Spherical (90)	Antibacterial	(MubarakAli et al. 2011)
33	*Euphorbia hirta L.* leaf extract	Spherical (10–50)	Antibacterial	(Annamalai et al. 2013)
34	Seed extract of *Abelmoschus esculentus*	Spherical (45–75)	Antifungal	(Jayaseelan et al. 2013)

[a]Abbreviations of the terminology mentioned in the above table. *FA* Folic acid; *PT* Photothermal; *CT* Computed Tomography

5.1 Biomedical Imaging and Cancer Diagnostics of b-AuNPs

Nanoparticles are growing as impending tools for biomedical imaging due to their unique versatile properties (magnetic, tunable absorption, and emission properties) (Nune et al. 2009b). Tracking the growth of cancerous tissues inside the body using various fluorescence and bioimaging techniques helps us to monitor the stage of cancer progression. AuNPs are well established for diagnostics and cellular imaging purposes owing to their characteristic applications in the fields of X-ray imaging (Cole et al. 2015), computed tomography (CT) (Popovtzer et al. 2008), photoacoustics (Li and Chen 2015), and fluorescence imaging (Boisselier and Astruc 2009; Curry et al. 2014). Several groups demonstrated the cancer cell imaging ability of b-AuNPs using CT and fluorescence imaging techniques. For example, Fajal et al. demonstrated excellent X-ray contrast properties of anisotropic AuNPs, synthesized using *Theobromo cacao* (cocoa) seeds extract, observed by CT imaging (Fazal et al. 2014). According to the United States Department of Agriculture, Cocoa contains high amount of oxygen radical absorbance capacity (ORAC). The high ORAC content justifies the facile reduction capacity of $HAuCl_4$ by the cocoa extract (U.S. Department of Agriculture 2010). The as-synthesized b-AuNPs (size: 150–200 nm) exhibited higher CT contrasting properties compared to clinically approved standards (Omnipaque). Quantitative analysis showed nearly 1.5 times higher signal intensity for b-AuNPs compared to Omnipaque, featuring its greater clinical significance. The reason for producing superior X-rays contrast of anisotropic AuNPs was probably due to the higher atomic number of gold, which attenuates X-rays efficiently than iodine (Aydogan et al. 2010). Chanda et al. showed

cinnamon phytochemicals mediated synthesis of 15–20 nm sized biocompatible AuNPs (Cin-AuNPs), which can be used as effective CT/photoacoustic contrast enhancement agents for tumor detection (Chanda et al. 2011). PC-3 cells incubated with Cin-AuNPs (100 µg/mL) showed photoacoustic signal under irradiation, whereas untreated PC-3 cells showed a linear response. The selective internalization of Cin-AuNPs inside cancer (PC-3) cells provides a tumor selective threshold nanoparticle concentration for contrasting purpose to detect and diagnose cancer cells specifically. Interestingly, Cin-AuNPs exhibited excellent X-ray CT contrast properties along with consistent enhancement of attenuation coefficients with increasing concentrations (Fig. 1). From the figure, a linear relationship can be drawn between the concentration of Cin-AuNPs used and the AHU (HU-Hounsfield Units-scale for CT imaging). Thus, Cin-AuNPs can be used as in vivo CT molecular imaging agent for the detection of cancerous tissues and lesions. Recently, our group demonstrated the biosynthesis of AuNPs using *Olax scandens* leaf extract that exhibited bright red fluorescence inside A549 (human lung cancer) cells (Mukherjee et al. 2013). The red fluorescence observed might be attributed to occurrence of any fluorescent phytochemicals or natural compounds in the leaf extract of *Olax scandens,* which has to be further explored. The intense red fluorescence of b-AuNPs can be utilized as imaging facilitator or in cellular imaging studies. The biosynthesized silver nanoparticles (b-AgNPs-OX) using the same leaf extract of *Olax scandens* show fluorescence properties inside the cancer cells (Mukherjee et al. 2014). Wang et al. reported for the first time the in situ biosynthesis of gold nanoclusters which

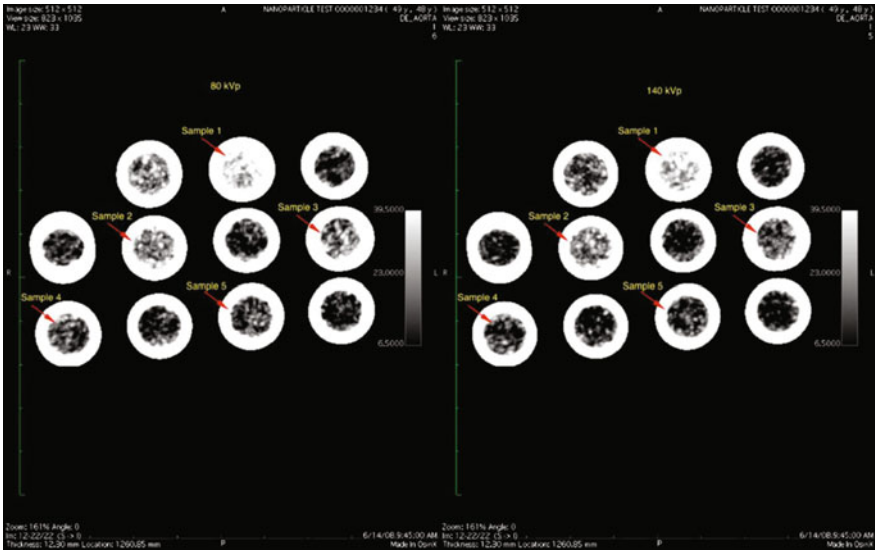


Fig. 1 Maximum intensity projection (MIP) CT images of the samples at **a** 80 and **b** 140 kVp. Vials containing Cin-AuNPs with the concentrations of 880 (*Sample 1*), 704 (Sample 2), 528 (Sample 3), 352 (Sample 4), and 176 (Sample 5) µg/mL. Reprinted with permission from (Chanda et al. 2011). Copyright @ Springer

function as efficient fluorescent bioimaging agents for the detection of tumors (Wang et al. 2013). The human hepatocarcinoma cells (HepG2) and human leukemia cells (K562) were incubated with chloroauric acid ($HAuCl_4$) solution for 24–48 h, which showed excellent green fluorescence observed by Laser confocal fluorescence micrographic studies. The L02 (human embryo liver cell strand) cells were used as control cells, which showed none to minimal fluorescence after incubation with $HAuCl_4$ solution suggesting the specificity of gold nanocluster biosynthesis to cancerous cells. Further, they extrapolated the bioimaging application to in vivo xenograft tumor model in BALB/c athymic nude mouse by subcutaneous innoculation of HepG2 or K562 cells followed by subcutaneous injection of $HAuCl_4$ solution (10 mmol/L). The in vivo fluorescence imaging studies showed the clear bright fluorescence around the tumor which was observable even after 72 h of $HAuCl_4$ injection suggesting the persistent fluorescence of in vivo biosynthesized gold nanoclusters (Fig. 2). The above study portrait the promising biomedical applications of b-AuNPs for specific and sensitive imaging of tumors (Wang et al. 2013). Another recent report demonstrated the one-pot nucleation and growth mediated green synthesis of red fluorescent AuNPs inside human hair, achieved by the treatment of hair with alkaline $HAuCl_4$ (Haveli et al. 2012). The as-synthesized b-AuNPs (2–3 nm) were located on the shaft of the hair cortex. This red fluorescent b-AuNPs can be utilized for the applications in biosensing, bioimaging and hair-dyeing industries. On the whole, the above potential advancements forecast the green synthesized AuNPs/nanoconjugates as novel, cheap, and cost-effective tools for biomedical imaging and cancer theranostics in near future.

5.2 Drug Delivery

Drug delivery is the process by which a drug is formulated as cargo and loaded/conjugated to a carrier vehicle and administered to the patient. It results in enhanced therapeutic efficacy of the drug with lowered side effects. The nanoparticle systems have been widely investigated by many researchers as delivery systems to carry drugs, proteins, nucleic acids such as DNA and RNA (Ding et al. 2014; Han et al. 2007; Yahia-Ammar et al. 2016; Yih and Al-Fandi 2006). The nanotechnology has revolutionized the field of cancer therapeutics by using the drug delivery approach (Cho et al. 2008). Over the past decade, several groups including our group demonstrated the application of nanotechnology in cancer therapy. AuNPs provide promising scaffolds for drug and gene delivery. The unique features of AuNPs (tunable core size, monodispersity, large surface to volume ratio, and easy functionalization) encourage their application for drug delivery in cancer therapeutics and diagnostics. Green synthesized AuNPs also play a crucial role for the delivery of drugs by possessing several advantages over conventional chemically modified drug delivery systems (DDSs). As biomolecules like proteins, lipids, carbohydrates and other several phytochemicals involved in biosynthesis process, create an external biomatrix or protein corona around AuNPs surface, which aids in easy and effective attachment or adsorption of drug molecules. This avoids the use of any external

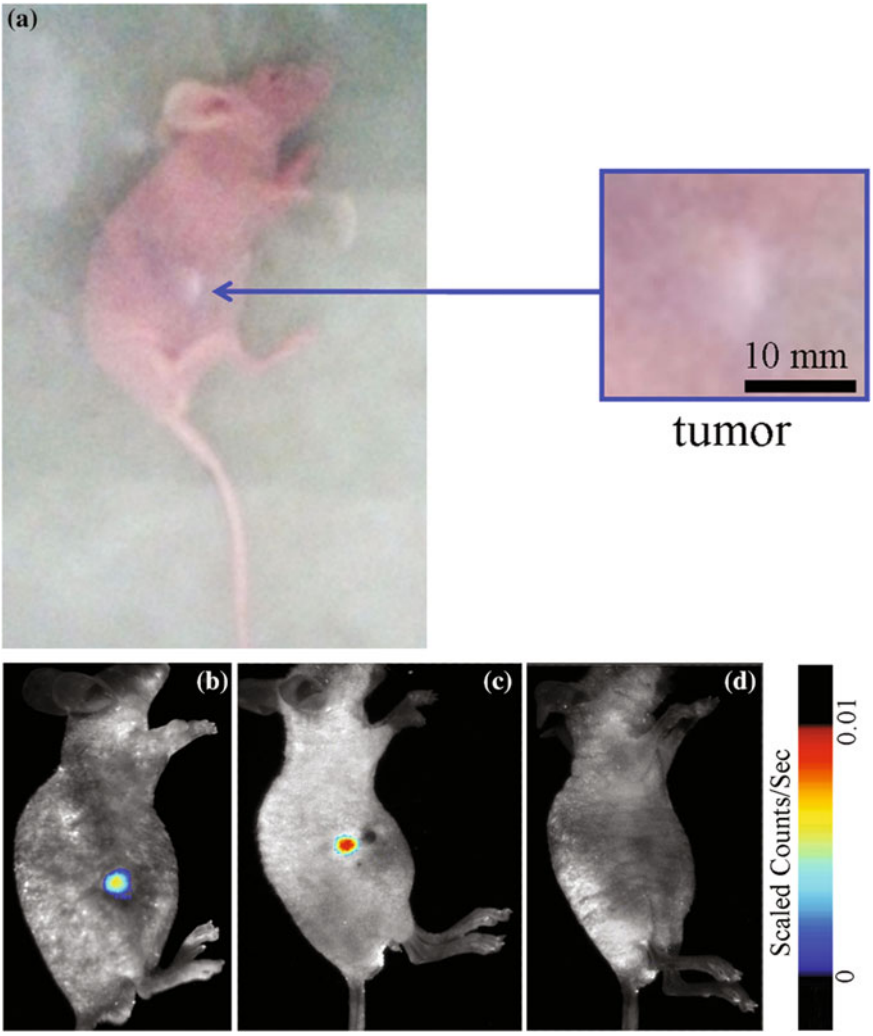


Fig. 2 Representative xenograft tumor mouse models of hepatocellular carcinoma observed in normal light (**a**) or by in vivo fluorescence imaging (**b**) 24 h after a subcutaneous injection of 10 mmol/L HAuCl4 solution near the tumor. In (**a**), the *inset* shows an enlarged view of the xenograft tumor. Xenograft tumor mouse models of chronic myeloid leukemia observed by in vivo fluorescence imaging (**c**) 24 h after a subcutaneous injection of 10 mmol/L HAuCl4 solution near the tumor. **d** Control mouse observed by in vivo fluorescence imaging 48 h after a subcutaneous injection of 10 mmol/L HAuCl4 solution in the right side of their abdomen. Reprinted with permission from (Wang et al. 2013). Copyright @ Nature Publishing Group

chemical hazards such as capping agents or stabilizers for drug binding. Dhar et al. successfully conjugated a FDA approved anticancer drug doxorubicin (DOX) with b-AuNPs derived from gellan gum and sophorolipid-gellan gum. The biosynthesized

nanogold-DOX conjugate showed more prominent cytotoxic effects compared to pristine DOX in human glioma cell lines (LN-229 and LN-18) and human glioma stem cell line (HNGC-2) (Dhar et al. 2008, 2011). The high attachment of DOX (~75%) might be attributed to the hydrogen bonding and electrostatic interactions between free –OH groups of DOX and sugar groups of gum present on the surface of AuNPs. Confocal microscopic images showed clear apoptosis of glioma cancer cells treated with b-AuNPs-DOX, that supports the enhanced anticancer activities of DOX conjugated AuNPs (Fig. 3) (Dhar et al. 2008, 2011). Recently, our group demonstrated the synthesis of AuNPs using plant (*Eclipta alba*: Bhringaraj) leaf extract and further conjugated with DOX (Mukherjee et al. 2012). The as-synthesized nanoconjugates were successfully applied in breast cancer cell line (MCF-7), which showed enhanced inhibition of cancer cell proliferation than free DOX in a dose-dependent manner after 48 h of treatment (Mukherjee et al. 2012). In another report by our group, the efficient in vitro and in vivo delivery of DOX using b-AuNPs derived from *Peltophorum pterocarpum* (PP) leaf extract was demonstrated (Mukherjee et al. 2016). The nanoparticles obtained were highly crystalline, 5–15 nm in size and highly stable in various physiological buffer solutions, whereas the binding of DOX with the biocompatible b-AuNPs-PP was comparatively low. FTIR and zeta potential studies revealed the interaction between DOX and b-AuNPs-PP as weak electrostatic and dative bonding between phenolic-OH groups of DOX and nanogold surface. A sustained release of DOX was observed for 7 days. The nano-DOX conjugates exhibited enhanced therapeutic efficacy compared to naked DOX treatment in various cancer cells (A549 and B16F10) and in vivo mouse subcutaneous melanoma tumor model at an alternative day dosing for 2 weeks. A significant reduction of tumor volume was observed 2 weeks post-treatment of

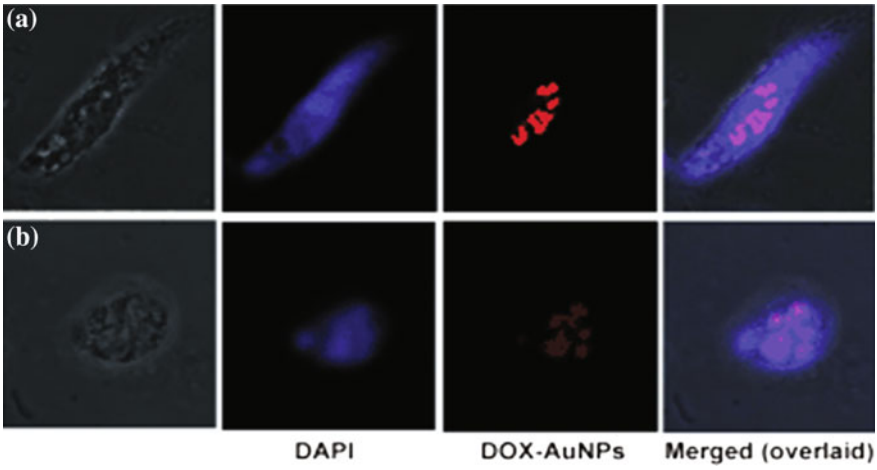


Fig. 3 **a** LN-18, **b** LN-229. Confocal microscopy images to demonstrate the apoptosis induced by DOX-loaded gold nanoparticles. Cells were initially cultured for 24 h, followed by addition of DOX nanoparticles, then continually cultured for 24 h before examination. Reprinted with permission from (Dhar et al. 2008). Copyright @ WILEY-VCH

b-AuNPs-DOX compared to untreated mouse and free DOX treated mouse. Mechanistic studies revealed significant up-regulation of tumor suppressor protein p53, which attenuates cell cycle arrest in G0/G1 phase for A549 cell line and sub-G1 phase for B16F10 cell lines. Together, these results indicate the possible therapeutic potential of biosynthesized gold nanoparticle-based drug delivery system for the development of an alternative cost-effective treatment strategy for cancer therapy. Kumar et al. recently reported the delivery of resveratrol (a natural phenol used as anticancer agent) by using the culture supernatant of a motile bacterium *Delftia* sp. strain KCM-006 in A549 lung cancer cells (Kumar et al. 2014). The as-synthesized b-AuNPs were crystalline in nature with an approximate size of about 25 nm and highly stable in various physiological buffers for up to 3 weeks. The binding of resveratrol was determined to be approximately 20%, whereas rapid release of resveratrol ($\sim$95%) at pH = 5.2 compared to only 26% at pH 7.4 was noticed by drug release studies. This characteristic drug release by b-AuNPs at low pH conditions forecasts the tumor environment specific drug release promoting tumor cell killing and low nonspecific toxicity toward normal tissues. The resveratrol-loaded b-AuNPs (RSV-AuNPs) was reported to exhibit enhanced anticancer activity against A549 cells compared to free resveratrol and untreated cells in a dose-dependent manner as evident from the lower IC_{50} values. Immunofluorescence studies revealed the inhibition of tubulin polymerization, where high disruption of microtubule organization was observed in case of RSV-AuNPs treated cells. Similarly, our group recently showed the DOX delivery using b-AuNPs and b-AgNPs synthesized from the leaf extract of *Butea monosperma* (BM) (Patra et al. 2015). A delivery system was designed by conjugating DOX to these nanoparticles. Even in this case, both nano-DOX conjugates exhibited augmented anticancer activity toward various cancer cells (B16F10 and MCF-7). Another research group headed by S Anil kumar reported the biosynthesis of gold nanocrystals of size range 2–70 nm using a plant pathogenic fungus called *Helminthosporium solani* (Kumar et al. 2008). Further they conjugated the smallest fraction of nanoparticles with DOX and demonstrated its delivery to human embryonic kidney cells (HEK293) by microscopic imaging studies (Kumar et al. 2008). Taken together, all the recent reports firmly encourage the in vitro and in vivo delivery of several drugs and biomolecules using b-AuNPs toward the treatment of cancer and other diseases in near future.

5.3 Targeted Drug Delivery

In spite of the excellent caliber of noble metal nanoparticles to deliver therapeutic biomolecules in most of the cases their mode of delivery is nonspecific. This nonspecificity leads to various unwanted side effects and necessity of repeated dose-regimen for desired therapeutic efficacy. Recent reports suggest the development of smart targeted inorganic metal nanoparticles (NPs) especially AuNPs (AuNPs) that can specifically deliver therapeutic molecules to primary and advanced metastatic tumors with better efficacy and lower toxicity in a systematic way (Patra

et al. 2008b, 2010; Yallappa et al. 2015). Ganeshkumar et al. illustrated the rapid biosynthesis of highly stable monodispersed AuNPs using the fruit peel extract of *Punica granatum* (Ganeshkumar et al. 2013). The as-synthesized AuNPs functionalized with 5-fluorouracil (5-FU) (a FDA approved anticancer drug) and folic acid (targeting moiety) could successfully induce enhanced cytotoxicity toward MCF-7 breast cancer cells (over expressed folate receptors) with much lower IC_{50} values and arrests the cancer cells at G0/G1 phase of cell cycle, suggesting the targeted drug delivery capability of b-AuNPs. Further these b-AuNPs were hemocompatible toward human blood samples, which demonstrates their advancement toward clinical applications. At molecular level, these biosynthesized conjugates activated MAPK cascade proteins such as ERK and p38 MAPK (p38 mitogen-activated protein kinases) in tumor cells. Inhibition of the PI3 K/Akt/mTOR signaling pathway by these green synthesized gold nanoconjugates lead to the inhibition of cancer cell proliferation. In another report by the same group, pullulan (a polysaccharide polymer) stabilized AuNPs (P-AuNPs) were prepared using microwave irradiation method (Ganeshkumar et al. 2014). The toxicity studies of the P-AuNPs in zebra fish embryo model demonstrated their high biocompatibility compared to untreated controls. Similar to the previous report, they conjugated 5-FU and folic acid to P-AuNPs for in vitro targeted drug delivery in HepG2 (Hepatocellular carcinoma) cells. The concentration of 5-FU required to attain 50% growth inhibition of HepG2 cells was much lower in the P-AuNPs decorated with 5-FU and folic acid formulation compared to free 5-FU. The biodistribution studies of P-AuNPs performed in male Wistar rats showed the maximum accumulation of P-AuNPs in liver compared to other organs such as lungs, kidney, and spleen signifying the liver targeting ability of modified P-AuNPs. Altogether, the above observations strongly support the wide applications of b-AuNPs essentially in cancer therapy using targeted drug delivery approach.

5.4 Anticancer Agents

The conventionally synthesized AuNPs/nanoconjugates by chemical methods have been rarely used unmodified as anticancer agents because they barely possess any therapeutic value (Joshi et al. 2012; Priya and Iyer 2015), whereas several groups have well established the anticancer activities of green synthesized AuNPs (Kumar et al. 2015; Gatea et al. 2015; Manivasagan and Oh 2015; Mollick et al. 2014; Mukherjee et al. 2013, 2015a; Raghunandan et al. 2011; Ramalingam et al. 2016; Sellappa et al. 2015; Vaikundamoorthy et al. 2016). The main reason for the anticancer activity of b-AuNPs is due to the presence of medicinally active anticancer phytochemicals (flavonoids, polyphenols, and isoflavones, etc.) in the plant bioresources used for biosynthesis. Hence, some of these anticancer phytochemicals attach over the nanogold nanosurface during biosynthesis, and impart the pharmacological anticancer property to the b-AuNPs. Recently our group demonstrated the anticancer activity of in situ one-pot synthesized b-AuNPs by employing leaf extract of *Olax scandens*. Further, it was interesting to note that these

nanoconjugates showed better anticancer therapeutic efficacy than pristine *Olax* extract at various concentrations in A549, MCF-7 and COLO 205 (human colon cancer) cells (Mukherjee et al. 2013). Therefore, the b-AuNPs show high therapeutic efficacy at cost-effective synthesis processes. In another report, our group showed the anticancer activity of b-AuNPs, synthesized using *Lantana montevidensis* (LM) leaf extract (Mukherjee et al. 2015a). LM contains several active anticancer ingredients (eupatorine, cirsilineol, apigenin, hispidulin, eupafolin, b-caryophyllene, etc.) (Makboul et al. 2013; Mukherjee et al. 2015a; Sousa et al. 2011) that help in accelerating the anticancer potential of b-AuNPs toward cancer cells, at similar concentration of LM extract employed. Cell cycle analysis showed the G_2/M arrest in A549 cell lines and sub-G1 arrest in case of MCF-7 cells lines possibly leading to cancer cell cytotoxicity. Further the detailed mechanistic studies revealed that b-Au-LM NPs inhibit the cancer cell proliferation through the reactive oxygen species (ROS) mediated apoptosis pathway. In another recent report, Kumar et al. demonstrated the in vitro anticancer activity of b-AuNPs synthesized from culture supernatant of *Streptomyces clavuligerus* (Kumar et al. 2015). The b-AuNPs showed biocompatibility with normal human lung cell lines (MRC-5), whereas exhibited cytotoxic activity toward different cancer cells (A549, HeLa, and DU145). The b-AuNPs induced the G2/M arrest and inhibited microtubule assembly in DU145 cells. Mechanistic studies revealed that anticancer activity of b-AuNPs was due to formation of ROS, reduction of mitochondrial membrane potential (MMP) and decrease in glutathione (GSH) levels. Crucial evidence revealed that b-AuNPs upregulated various apoptotic proteins such as caspases-3, 8, and 9 and induced apoptosis through intrinsic pathway. Mollick et al. showed that spherical b-AuNPs synthesized using *Abelmoschus esculentus* (L.) pulp extract exhibited excellent anticancer property against Jurkat leukemia cancer cells (Mollick et al. 2014). Detailed mechanistic studies demonstrated significant elevation of intracellular ROS upon treatment with b-AuNPs and further reduction of MMP. These results confirmed the involvement of intrinsic apoptotic pathway in cancer cell death. In another recent study, Manju et al. demonstrated excellent antibacterial, antibiofilm and cytotoxic effects of b-AuNPs (NsEO-AuNPs) derived from *Nigella sativa* essential oil (Manju et al. 2016). The b-AuNPs exhibited significant anticancer activity toward A549. An IC_{50} value of the *N. sativa* oil conjugated with AuNPs was much lower than both the oil and AuNPs alone, suggesting the enhanced efficacy of the nanoconjugates. All these recent studies confirmed that biosynthesized AuNPs/nanoconjugates could be utilized for the future therapeutic application in cancer therapy in vitro and in vivo.

5.5 Photothermal Therapy in Cancer

Photothermal effect has gained huge importance due to its potential in curing localized tumors by irradiating the destructive heat selectively to the tumors. The application of various heating nanoparticles in assistance with photothermal therapy for both in vitro and in vivo therapeutic applications was well documented

(Jaque et al. 2014). AuNPs were long used for their photothermal therapy application in cancer (Deng et al. 2014; Huang et al. 2006; Khlebtsov et al. 2013). This is because the absorption and scattering radiative properties of AuNPs are enhanced due to the unique surface plasmon resonance, due to which the incident radiation could be strongly absorbed and quickly converted to heat energy (Huang and El-Sayed 2010). Until late, there were no reports on b-AuNPs, acting as photothermal agents. But very recently, Fajal et al. demonstrated excellent photothermal properties in epidermoid carcinoma A431 cells of anisotropic AuNPs; synthesized from *Theobroma cacao* (cocoa) seeds extract (Fazal et al. 2014). The biosynthesized anisotropic AuNPs possessed near-infrared (NIR) absorbance at 700–1000 nm, which facilitated excellent photothermal therapy. The density gradient centrifugation using various concentrations of sucrose was employed to obtain maximum concentration of anisotropic AuNPs having NIR absorbance, important for superior photothermal activity. Differential interference contrast (DIC) images of A431 cells treated with AuNPs for 24 h followed by laser irradiation exhibited extensive morphological changes. Noticeable changes such as cellular appendage withdrawal and cell membrane damage, leading to cell blebbing and leaching of cytoplasmic fluid associated with maximum damage at the periphery of the cell colony was observed. Again, confocal fluorescence images of live/dead A431 stained cells after nanoparticle treatment followed by laser irradiation showed

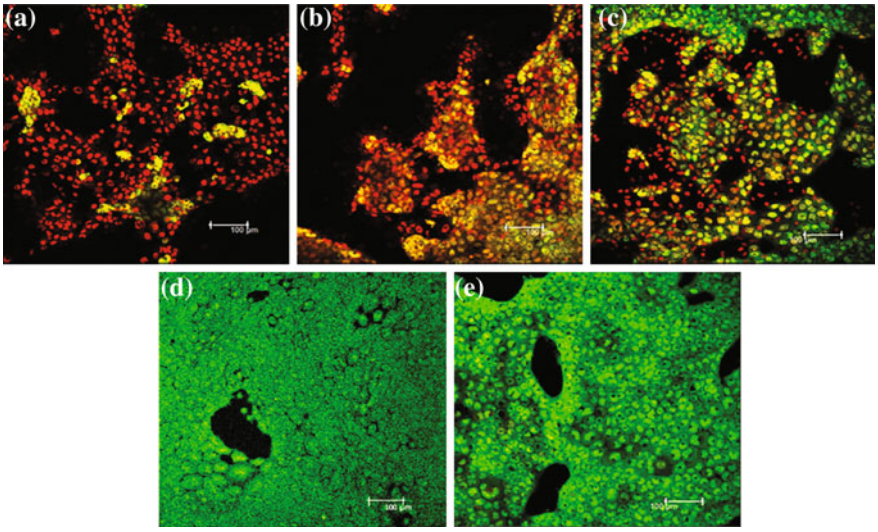


Fig. 4 Live/dead staining of A431 cells after 800 nm laser irradiation for 10 min at power = 36 mW. Nanoparticle concentration in panels **a**, **b**, **c**, and **d** are 200, 100, 50, and 0 μg/mL respectively. Decreasing amounts of red fluorescence is obtained with lower concentration of nanoparticles which indicates the photothermal activity of the nanoparticles in cell killing. **e** Live/dead staining of A431 cells treated with 100 μg/mL nanoparticle concentration without laser treatment. Reprinted with permission from (Fazal et al. 2014). Copyright @ American Chemical Society

enhanced cell death (Fig. 4). Contrary in case of untreated A431 cells no cytotoxicity was observed after exposure with laser irradiation due to lack of heat generation in absence of b-AuNPs. Interestingly, the laser power density of 6 W/cm^2 used in this work was much lesser than the previously published reports using chemically synthesized AuNPs such as gold nanorods (10 W/cm^2), gold-silica nanoshells (80 W/cm^2), gold-gold sulfide nanoparticles (25 W/cm^2) (Huang et al. 2006; Loo et al. 2005). Altogether, the above literature strongly supports the feasibility of using anisotropic b-AuNPs with NIR absorbance for photothermal applications in cancer therapy at low fluences and reducing local healthy tissue damage.

5.6 Angiogenesis

Angiogenesis is a physiological process of generation of new blood vessels from already existing vasculature. It plays a vital role in growth and development of fetus, wound healing, remodeling of damaged tissues such as the skin and bones, rheumatoid arthritis, diabetic retinopathy, post-ischemic vascularization of the myocardium, and atherosclerosis (Carmeliet and Jain 2000, 2011). The process of controlled angiogenesis aimed for the therapy of cardiovascular, ischemic diseases, and tissue regenerations is known as therapeutic angiogenesis (Simons and Ware 2003). Conventional treatments with growth factors like VEGF, PDGF, and b-FGF have several limitations including thrombosis, fibrosis, nonspecific angiogenesis and tumorigenesis. In order to overcome the limitations of conventional treatment strategies, alternative therapeutic treatment strategy is urgently required. Nanomedicine plays an important role for the development of alternative methods for induction of therapeutic angiogenesis (Gupta 2011; Mulder and Fayad 2008). Several groups including ours reported the therapeutic pro-angiogenic properties of different nanomaterials including europium hydroxide nanorods (EHNs), AuNPs, cerium oxide, zinc oxide nanoflowers (ZONF), graphene oxides (Barui et al. 2012; Das et al. 2012; Mukherjee et al. 2015b; Nethi et al. 2014; Patra et al. 2008a, 2011; Thomas et al. 2014). Certain research groups demonstrated the anti-angiogenic properties of biosynthesized nanoparticles (b-AgNPs) (Baharara et al. 2014; Gurunathan et al. 2009). Since our focus in this chapter is regarding the biomedical applications of green synthesized AuNPs, only the reports pertaining to b-AuNPs are highlighted. The AuNPs synthesized by conventional methods possess anti-angiogenic nature (Arvizo et al. 2011; Mukherjee et al. 2005). This property highly limits the utility of AuNPs in the field of therapeutic angiogenesis. Our group demonstrated the pro-angiogenic properties of b-AuNPs observed by several in vitro and in vivo assays (Nethi et al. 2014). The AuNPs were synthesized from *Hamelia patens* (HP) leaf extract that acted as a reducing as well as stabilizing/capping agent. HP shrub is being long used in Mexico and Central America for the treatment of wound healing and menstrual disorders in traditional medicine, which encouraged us to apply this shrub for AuNPs biosynthesis. The size of the biosynthesized AuNPs (b-Au-HP) were 25–50 nm and mostly spherical

in shape. This b-Au-HP aided the growth and migration of the human umbilical vein endothelial cells (HUVECs) in a dose-dependent manner. Furthermore in vivo generation of new blood vessels in chicken embryonic assay (CEA) and promoted wound healing in HUVECs were noticed (Fig. 5). Controlled generation of ROS and activation of phospho-Akt (cell survival and proliferation protein) might be the

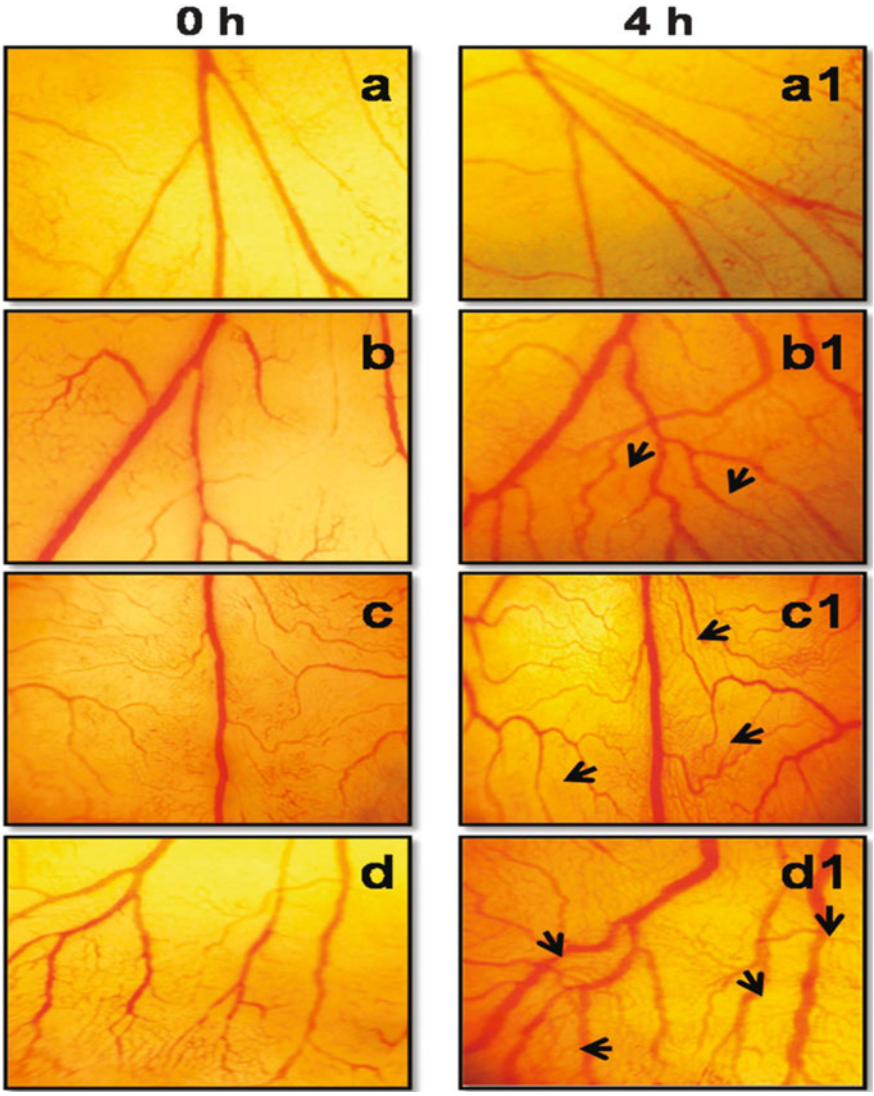


Fig. 5 Chick embryo angiogenesis (CEA) assay: in vivo angiogenesis assay in a chick embryo model incubated with **a**-**a1** control, **b**-**b1** HP 10 mg, **c**-**c1** b-Au-HP 10 mg and **d**-**d1** VEGF, respectively. The results demonstrate the pro-angiogenic nature of the b-Au-HP nanoparticles. Reprinted with permission from (Nethi et al. 2014). Copyright @ The Royal Society of Chemistry

plausible mechanism of angiogenesis induced by b-Au-HP. Mass spectrometry and chromatographic analysis revealed apigenin as one of the major ingredients present in the HP leaf extract responsible for the pro-angiogenic property. Therefore, this present study highlights the importance of ecologically designed b-AuNPs for the therapeutic application toward cardiovascular diseases and ischemia-related diseases where angiogenesis plays a vital role.

5.7 Antioxidants

Antioxidants play a major role to maintain the enzymatic balance in biological system. Any slight imbalance in this homeostasis may produce oxidative stress inside body. Several groups were extensively worked on the antioxidant properties of b-AuNPs (Lee et al. 2011; Sanna et al. 2014; Barathmanikanth et al. 2010; Sowani et al. 2015; Bhaumik et al. 2015). Lee et al. demonstrated the excellent cytoprotective activity of phytochemical-induced (gallic acid + isoflavone or protocatechuic acid + isoflavone) b-AuNPs in L-929 cells, observed by fluorescence spectroscopy and fluorescence microscopic techniques (Lee et al. 2011). They determined the antioxidant properties of b-AuNPs indicated by the decreased green fluorescence intensity of DCF (produced when DCFH is oxidized by the cellular oxidants) in cells treated with b-AuNPs. Sanna et al. reported the polyphenols [epigallocatechin-3-gallate (EGCG), resveratrol (RSV), and fisetin (FS)] facilitated b-AuNPs exhibited good antioxidant properties, determined by 2, 2-diphenyl-1-picrylhydrazyl (DPPH) and 2, 2′-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) cation (ABTS) radical-scavenging assays (Sanna et al. 2014). It was found that the radical-scavenging activity of AuNPs was directly proportionate to the concentration of b-AuNPs and polyphenols. Recently, Bhaumik et al. showed the antioxidant activities of b-AuNPs determined by ABTS and DPPH assay (Bhaumik et al. 2015). The b-AuNPs, synthesized by *C. sinensis* (green tea) and *P. fulgens* leaf extract displayed high radical-scavenging activities, similar to positive control (ascorbic acid). All these studies demonstrate the excellent antioxidant activities of b-AuNPs and ideally support their clinical use as antidiabetic or cytoprotective agents in near future.

5.8 Antidiabetic Agents

The AuNPs fabricated using conventional methods were seldom used as antidiabetic agents due to lack of therapeutic efficacy (Venkatachalam et al. 2013). It is well known that oxidative stress could be one of the reasons for the multifaceted metabolic disorders (Golbidi et al. 2011; Koya et al. 2003). During the last two decades several studies were carried out proposing antioxidants as a complementary therapeutic approach for the treatment of diabetes (Golbidi et al. 2011; Koya et al. 2003). The antioxidant properties of b-AuNPs were previously discussed in this chapter. The b-AuNPs play a vital role in the treatment of diabetes. BarathManiKanth et al. demonstrated the antidiabetic effect of b-AuNPs derived

from *Bacillus licheniformis* biomass (Barathmanikanth et al. 2010). The b-AuNPs exhibited persistent control over the blood glucose level, serum biochemical profiles and lipids in streptozotocin-induced diabetic mice compared to the untreated controls, by effective utilization of blood glucose. Beside this, the histopathological and hematological evaluations exhibited the biocompatible and cytoprotective nature of the b-AuNPs toward the vital body organs. Surprisingly, following 45 days of the study, the liver of diabetic mice showed lymphocytic infiltrations, high fatty cells content and lobular inflammation. However, the mice treated with b-AuNPs exhibit normal liver without major morphological change and disruptions, indicating the protective effect of b-AuNPs against hyperglycemic conditions. Detailed mechanistic studies revealed that b-AuNPs have control over different antioxidant enzymes (OD, catalase, GSH, and GPx) in diabetic mouse by inhibition of ROS generation and lipid peroxidation during hyperglycemia. These are the striking evidences of the antioxidant activity of b-AuNPs facilitating in the recovery of hyperglycemia. Daisy et al. reported the antidiabetic properties of AuNPs developed using aqueous stem bark extract of *C. fistula* (Daisy and Saipriya 2012). The following remedial indications such as reduced blood glucose, increased body weight, improved total protein content (transaminase activity), increased levels of high-density lipoproteins (better lipid profile), and reversed renal dysfunction were observed in streptozotocin-induced diabetic mice administered with b-AuNPs. These observations firmly support the application of phytochemically synthesized b-AuNPs as therapeutic hypoglycemic agents in the treatment of diabetes mellitus. Protein tyrosine phosphatase 1B (PTP 1B) is a promising therapeutic target for the treatment of type II diabetes as it can regulate insulin receptors signaling pathways by dephosphorylation and control the blood glucose levels (Hu et al. 2007; Pei et al. 2004). Basha et al. illustrated AuNPs synthesized using guavanoic acid [a phytochemical of *Psidium guajava* (Pg)] exhibited significant PTP 1B inhibitory activity (Khaleel Basha et al. 2010). Thus, by reducing the levels of PTP 1B, the b-AuNPs enhanced the activity of insulin. The authors demonstrated that b-AuNPs exhibit excellent PTP 1B inhibitory effects. Even though thorough investigation is further needed, yet taken together the results of the above studies indicate the potential applications of b-AuNPs toward the treatment of diabetes, hyperglycemia in the coming future.

5.9 Antibacterial, Antibiofilm, and Antifungal Activity

Antibacterial agents play an important role in the water disinfection, textile industry, healthcare, medicine, and food packaging. Organic compounds or drugs used for bacterial disinfection have several disadvantages, including high cost, toxicity to the human body, drug resistance, and others. Therefore, the development of alternative and low cost metal nanoparticles (AuNPs, AgNPs) has gained huge importance (Manju et al. 2016; Mukherjee et al. 2014). The antibacterial and antifungal activities of b-AuNPs were well explored by various groups (Annamalai et al. 2013; Basu et al. 2015; Bindhu and Umadevi 2014; Jayaseelan et al. 2013; Manju et al. 2016;

Mollick et al. 2014; MubarakAli et al. 2011; Rajathi et al. 2012; Wang et al. 2015). Bindhu et al. demonstrated the antibacterial activity of AuNPs synthesized from *A. comosus* fruit extract against Gram-positive *Pseudomonas aeruginosa* and Gram-negative *Staphylococcus aureus* bacteria (Bindhu and Umadevi 2014). Mollick et al. showed that AuNPs synthesized using pulp extract of *Abelmoschus esculentus* (L.) exhibited prolific antibacterial activity against various Gram-positive and Gram-negative bacteria such as *Escherichia coli*, *Bacillus subtilis*, *Micrococcus luteus*, *Bacillus cereus*, and *Pseudomonas aeruginosa* (Mollick et al. 2014). Very recently, Manju et al. demonstrated the antibacterial, antibiofilm and cytotoxic effects of b-AuNPs (NsEO-AuNPs) derived from *Nigella sativa* essential oil (Manju et al. 2016). NsEO-AuNPs were characterized to be face centered cubic crystalline in nature with a size of around 15–30 nm and spherical shape. The NsEO-AuNPs exhibited profound antibacterial activity against Gram-positive bacteria [*S. aureus* (16 mm)] compared to Gram-negative bacteria [*V. harveyi* (5 mm)]. Also, the nanoparticles successfully inhibited the biofilm formation of *Vibrio harveyi* and *Staphylococcus aureus* bacteria by decreasing the hydrophobicity index (46 and 78% respectively) in a dose-dependent manner, confirmed by the light and confocal laser scanning microscopy techniques (Fig. 6). Both the bacteria showed disintegrated and recalcitrant biofilm structure at higher doses, probably due to the inhibition of exo-polysaccharide synthesis in presence of biometallic nanoparticles. Annamalai et al. demonstrated the biosynthesis, detailed characterization and antimicrobial activity of AuNPs using leaf extract of *Euphorbia hirta* (Annamalai et al. 2013). The attained nanoparticles were spherical in shape with an average size

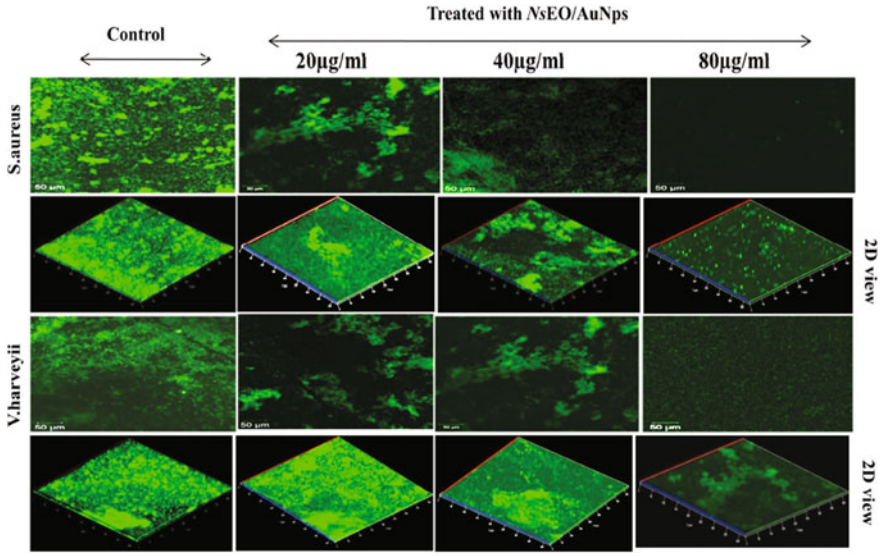


Fig. 6 Confocal laser scanning microscopic images showing the antibiofilm activity of *NsEO*-AuNPs at different concentrations (2D view). Reprinted with permission from (Manju et al. 2016). Copyright @ Elsevier

of 10–50 nm. These nanoparticles exhibited dose-dependent antimicrobial activity in various bacterial strains such as *E. coli*, *P. aeruginosa* and *K. pneumonia*. Minimum inhibitory concentration (MIC) values were determined to be around 88% for *E. coli*, 86% in case of *P. Aeruginosa* and approximately 94% for *K. pneumonia*. The antibacterial property of *Euphorbia hirta* is previously documented and the enhanced antibacterial activity of b-AuNPs may be due to the synergistic effect of surface adhered *Euphorbia hirta* phytochemicals around AuNPs surface (Abubakar 2009). These all published reports demonstrated antibacterial, antibiofilm and antifungal activities of b-AuNPs, which could be effectively utilized for several clinical applications associated with bacterial or fungal infections.

6 Toxicity, Biodistribution and Cellular Internalization of b-AuNPs

6.1 In Vitro Cytotoxicity

For the successful biomedical application of b-AuNPs the most important criteria is to show high in vitro and in vivo biocompatibility. Several groups demonstrated that b-AuNPs were biocompatible in in vitro conditions. Nune et al. showed the in vitro biocompatibility of b-AuNPs derived from tea phytochemicals in MCF-7 breast cancer cells and PC-3 prostate cancer cells up to high gold concentrations (Nune et al. 2009a). Shukla et al. demonstrated that soybeans phytochemical mediated AuNPs were biocompatible toward fibroblast cell lines up to 165 μM of gold concentration (Shukla et al. 2008). Our group recently showed that b-AuNPs from *Eclipta alba* plant leaf extract did not show any significant cytotoxicity toward MCF-7 and MDA-MB-231 breast cancer cell lines, indicating biocompatibility (Mukherjee et al. 2012). Kumar et al. reported that the b-AuNPs synthesized from plant pathogenic fungus, *Helminthosporium solani*; exhibited around 80% cell viability of kidney normal cell lines indicating high biocompatibility (Kumar et al. 2008). These studies showed the biocompatibility nature of b-AuNPs that can be securely used toward different biomedical applications including drug delivery, imaging, antibacterial activity, and many more.

6.2 In Vivo Toxicity and Biodistribution Studies

Prior to promote the AuNPs for clinical applications, it is very important to understand their in vivo toxicity and biodistribution. Even though much work has not been done in this area, there are reports, which state the non-toxic and biocompatible nature of b-AuNPs. Our group demonstrated that b-AuNPs synthesized from leaf extract of *Peltophorum pterocarpum* (PP), the "yellow flame tree" did not exhibit any significant changes in various serum biochemical parameters (SGPT, SGOT, total glucose, cholesterol, urea nitrogen, triglycerides and uric acid)

compared to control group nanoparticles after intraperitoneal (IP) injections for a period of 7 days (Mukherjee et al. 2016). Similarly, histopathological evaluation of various organs (lung, kidney, liver, heart, spleen, brain, uterus, and thymus) of treated mouse did not show any toxicity indication (such as necrosis, inflammation, etc.). However, toxicity was observed in the mouse treated with chemically synthesized gold (c-Au-PEG), indicating biocompatibility of b-AuNPs over c-AuNPs (chemically synthesized). In another study, our group showed that mouse injected with AuNPs synthesized from leaf extract of *Lantana montevidensis* (LM) did not exhibit significant in vivo toxicity compared to untreated mouse (Mukherjee et al. 2015a). Both serum biochemical parameters and histopathological evaluation were normal and did not exhibit any symptoms of toxicity. All the in vivo toxicological results revealed that the b-AuNPs were non-toxic in animal models and thus can be safely applied for biomedical applications. Chanda et al. checked the in vivo biodistribution of cinnamon phytochemicals-derived biocompatible AuNPs in mouse model (Chanda et al. 2011). They demonstrated the accumulation of intravenous administered b-AuNPs majorly in lungs after 4 and 24 h post-treatment indicating the lung targeting ability of b-AuNPs. Besides, the spleen and liver also exhibited the accumulation of b-AuNPs gradually with time. It was noticed that the blood plasma contained very less percentage of Au suggesting the minimum binding of b-AuNPs with the proteins present in the blood plasma. All the above observations inferred the high in vivo stability of b-AuNPs to the effective coating or capping of the gold nanosurface by the cinnamon phytochemicals. Ganeshkumar et al. demonstrated the in vivo biodistribution of b-AuNPs after intraperitoneal injection (i.p.) in male Wistar rats, where the major accumulation of these nanoparticles was noticed in liver and spleen followed by kidneys and lungs (Ganeshkumar et al. 2014). All the above-published reports demonstrated that b-AuNPs are potentially safe up to high concentrations in vivo and can safely be utilized for several biomedical applications in healthcare and medicine.

6.3 Cellular Internalization

Internalization is an important phenomenon essential for any nanoparticles to elicit its biological response. Cellular internalization of green synthesized AuNPs can be analyzed by TEM or confocal microscopy. AuNPs synthesized by green methods can easily internalize into the cells mainly by nonspecific endocytosis and pinocytosis. The cellular internalization of b-AuNPs cannot happen by phagocytosis or receptor-mediated endocytosis (RME) because there is no specific ligand decorated with this kind of AuNPs. Lee et al. showed that the AuNPs synthesized using phytochemicals such as gallic acid, protocatechuic acid, and isoflavone can penetrate and disperse all over the L-929 cells (murine fibroblast cells) without any aggregation to specific areas of cytoplasm or mitochondria (Lee et al. 2011). Nune et al. demonstrated that tea-phytochemical-derived AuNPs could enter the prostate (PC-3) and breast (MCF-7) cancer cells by processes including phagocytosis, fluid-phase endocytosis, and receptor-mediated endocytosis as tea phytochemicals

impart the cell penetrating property. Also, the viability after post internalization in those cell lines indicates the biocompatibility or non-toxic nature of the as-synthesized green AuNPs (Nune et al. 2009a). Till date, there is no report of b-AuNPs internalization inside the nucleus of the cancer cells. Dhar et al. showed that sophorolipid-gellan gum-gold nanoparticle conjugates labeled with Texas red can enter in the cytoplasm and perinuclear region of the human glioma cell line LN-229 through endocytosis (Dhar et al. 2008). Kumar et al. confirmed the same observation by reflectance and fluorescence confocal microscopic studies. They showed that fungus mediated b-AuNPs conjugated with FDA approved anticancer drug doxorubicin (DOX) cannot enter inside the nucleus of HEK293 cells and appeared to be associated with intracellular membranous organelles. It was confirmed by the fact that lysosomal areas of Au–DOX treated cells showed much greater red fluorescence than Au particle scattering. While DOX can easily enter to the cellular nucleus whereas, AuNPs-DOX remains in the cytoplasm and other organelles. Thus, by TEM and confocal microscopy the cellular internalization and localization of b-AuNPs was shown (Kumar et al. 2008). The effect of nanoparticle shape and size on cellular internalization has been well demonstrated (Bannunah et al. 2014). However, the information regarding cellular internalization and fate of AuNPs is limited and detailed investigation is emergently needed regarding the size and shape-dependent cellular internalization and interactions of green synthesized AuNPs.

7 Mechanism of Green Synthesis of AuNPs

Several published literature demonstrated the green synthesis of AuNPs using bioresources. However, the exact mechanism for the synthesis of b-AuNPs was not thoroughly explored. Earlier published reports provide some valuable clues and information regarding the mechanisms of biosynthesis. It is well known that plant and plant-based phytochemicals are rich in the amount of various polyphenols, aldehydes, acids, and alcoholic compounds. These polyphenols or other phytochemicals can help in the reduction of chloroauric acid ($HAuCl_4$) by following reaction.

$$HAuCl_4 + R{-}OH = Au^0 + R{=}O \quad (1)$$

The reason behind the facile reduction process is the higher oxidation potential of alcohol to the ketone–aldehyde system ($E^0_{ROH/RCO/RCHO}$ = 1.80 V) than the highly favorable positive standard reduction potential of Au^{3+}/Au^0 ($E^0_{Au3+/Au0}$ = 1.50 V) (Korchev et al. 2005). Beside this several flavonoids, alkaloids, proteins, amino acids, polysaccharides, heterocyclic compounds, and secondary metabolites (such as gallic acid, ellagic acid, cyclic peptide, euphol, flavin, phyllanthin, etc.) play vital role for the reduction of metal salts (Duan et al. 2015). Apart from this, several research groups demonstrated the role of proteins and amino acids present

in plants or microorganisms. Our group including others, showed the involvement of plant leaf proteins for the synthesis of b-AuNPs, observed by SDS-PAGE analysis and FTIR analysis (Mukherjee et al. 2012, 2013, 2015a; Shukla et al. 2008). Various small and high molecular weight proteins are responsible for the reduction process through the free radical mechanism. Newman et al. proposed the role of proteins for the reduction of metallic precursors by following the mechanistic route (Newman and Blanchard 2006).

$$HAuCl_4 + 3NR_3 = Au^0 + 3NR_3^{+\cdot} + H^+ + 4Cl^- \quad (2)$$

Beside this bacterium, fungi, or other microorganism also play very important role for the green synthesis of AuNPs. Several investigations suggest the main mechanism of bacteria mediated reduction process involves nitrate reductase enzymes (Li et al. 2011). Other mechanisms behind the mycosynthesis of metal nanoparticles include the involvement of electron shuttle quinines and α-NADPH-dependent reductase enzymes (Alghuthaymi et al. 2015; Castro-Longoria et al. 2011). All the above studies give an overall idea about the green synthesis of metal nanoparticles, although detailed mechanistic studies need to be carried out to understand the mechanism clearly.

8 Advantages and Disadvantages of Green Synthesis

8.1 Advantages

- The mode of synthesis is simple, rapid, cheap, and eco-friendly with ease of scale-up technology.
- The synthesis procedure does not involve the requirement of stabilizers or capping agents.
- Most of the time, it is one-pot synthesis and does not need any special requirements.
- The b-AuNPs are pharmacologically active which is attributed to the potent phytochemicals/biomolecules conjugated around the nanogold surface during the biosynthesis process.
- The b-AuNPs have ease of functionalization, which helps in decorating them with coupling agents, proteins, nucleic acids or drug molecules.
- The b-AuNPs are monodispersed and highly stable compared to the chemically prepared AuNPs.

8.2 Disadvantages

- Identification of the exact chemical component present in plant extracts or microorganisms responsible for the b-AuNPs activity is not easy as the bioresource extracts are a mixture of huge sets of biomolecules.
- Excessive/large-scale biosynthesis of b-AuNPs may lead to an ecological imbalance due to the over utilization and imbalance in the biological species such as plants or microbes.
- All the metal NPs might not be prepared using the green synthesis procedure, which is possible using the physical or chemical synthesis methods.
- The reduction process of standard metal salts by the plant-mediated extracts cannot be controlled.
- Due to seasonal change or climate change, the concentrations of the active biomolecules present in the plant may change, thus affecting the overall synthesis or activity.

9 Future Opportunities and Challenges

Nanomedicine is the upcoming science, which is expected to tremendously contribute to the challenges in the field of biology and medicine. A serious need has developed for the alternative approaches to the conventional synthetic procedures for the nanoparticle fabrication. In this aspect, the biosynthesis process for nanoparticle preparation such as gold using the green synthesis approach has proven to be cheap, eco-friendly, and efficient process with high scalability and preparation of stable AuNPs. Numerous researchers including our group have reported the wide therapeutic applications of the b-AuNPs. The toxicity concerns of the AuNPs have also been observed to be low in case of b-AuNPs. Beside this, biodegradability and clearance of AuNPs also play a decisive role to avoid potential effect of long term toxicity. Recently, Regan et al. showed the biodegradability and slow clearance of liposomal-gold nanoparticles through renal and hepatobiliary routes (Rengan et al. 2015). AuNPs were degraded metabolically in liver and hepatocytes and were found to be excreted through feces and urine; detected by ICP-MS analysis. In vivo biodistribution studies confirmed the gradual decrease of gold deposition in kidney, liver and blood plasma with time. This study gives a nice idea regarding the degradability and clearance of AuNPs. However, there are no available reports presenting the clearance, degradability, long term toxicity of b-AuNPs. Thus, detailed studies need to be carried out before understanding the exact mechanistic pathways of clearance and biodegradability of b-AuNPs. Although the mechanistic pathways underlying the activity of these b-AuNPs remain unclear, the potential biological response shown by these nanoparticles demonstrates their high biomedical applications. Rigorous in vitro and in vivo characterization followed by preclinical and clinical analysis can establish the

b-AuNPs as efficient candidates toward the therapy of various dreadful diseases in near future.

10 Conclusions

The day-to-day progressions in increase of disease occurrence rate and disease tolerance mechanisms raise an emergent need for the improvement of conventional modes of treatment strategies. Nanotechnology has recently introduced numerous advancements in disease diagnostics and therapeutics using nanomedicine approach. The AuNPs have pioneered the nanomedicine treatments, which can be evidently understood from the progressions of several AuNPs-based nanomedicine in clinical phase trials. In this context, the b-AuNPs have overcome the conventional chemically prepared AuNPs as novel nanosystems with reduced systemic toxic effects and increased therapeutic efficacy. Even though, the mechanism of AuNPs biosynthesis and their and mode of action is not yet clear, detailed investigation is needed. Therefore, the enormous potential biomedical applications of b-AuNPs demonstrated by various researchers as described above in-detail establish these NPs as efficient and effective promising candidates for theranostics applications in biology and medicine in near future.

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Cationic Polyelectrolyte Vectors in Gene Delivery

M. Caroline Diana Sherly, S.S. Priya and M.R. Rekha

Abstract

Gene therapy holds great promise and potential for treating diseases arising from genetic disorders whether it is acquired or inherited. Cationic polymers are widely used for gene delivery applications as they can form nanoplexes readily with DNA, an anionic polyelectrolyte that can be delivered intracellularly with ease. Till date a variety of numerous cationic polymers of varying molecular weight, structure, functional groups, composition and charge density are developed for gene delivery applications. These polymers can be broadly classified into synthetic, natural and graft polymers. The major barriers associated with non-viral vectors include its toxicity, evading immune system, hemocompatibility, efficient intracellular delivery at target site, intracellular unpacking and ultimately the gene expression. The first report on the usage of cationic polymer as gene delivery vector dates back to three decades. Though cationic polymers are now well established as a successful gene delivery vector, those reaching to clinical trials are still limited owing to above mentioned barriers. The chapters of this book will focus on the most successful cationic polymeric systems of both natural and synthetic origin, design concerns and also the recent advances in the polymer design approach for a successful gene transfer.

Keywords

Non-viral gene delivery · Redox sensitive polymers · Graft polysaccharides · Cationic polymers

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1 Introduction

Millions of people are affected by various genetic disorders be it a hereditary or acquired one. Gene therapy is a promising approach to replace the faulty genes with or introduce a functional one so as to cure or treat a particular clinical condition. In gene therapy gene is the medicine that needs to be delivered intracellularly. To introduce a foreign gene into a living system is not a simple task and needs careful designing of vectors for a successful therapeutic application. Initially when the concept of gene therapy was introduced viruses were chosen as gene delivery vectors owing to their high transfection ability. Various viral vectors used for clinical trials include adenovirus, adeno-associated virus, retrovirus, lentivirus, herpes simplex virus and so on (Verma and Somia 1997; During 1997; Vile et al. 1996). In spite of their advantage as suitable gene carrier, there is large number of problems associated with both in vivo and in vitro applications of these vectors. This mainly include the induction of immune response by the viral proteins, insertional mutagenesis, recombination with wild type viruses, limitation in the size of DNA to be carried and difficulty in the large-scale production of vectors (Wolfert et al. 1996; Persidis and Tomczyk 1997; Sheridan 2011). Apart from that poor toxicological profile and incorrect intracellular site of action together with incidence such as adenoviral mediated stimulation of immune response have aggravated the search for an alternative approach for gene transport. Hence, there is an increasing interest in the use of non-viral gene delivery vectors for safer and efficient gene delivery (Niidome and Huang 2002). Two major classes of non-viral vectors have been distinguished, i.e. lipid-based and polymer-based; the complexes formed upon interaction with DNA formed lipoplexes and polyplexes, respectively. The cellular transport system especially endocytosis and macropinocytosis generally take these complexes into the cells, transported to the site of action mostly to the nucleus where they release the gene to be expressed. However, the cationic polymers have gained much attention in terms of efficient non-viral gene delivery vectors due to their high DNA loading ability, ease of fabrication to avoid immunogenicity and compliancy for chemical modifications (Ambrose et al. 1958; Moroson 1971). In comparison with viral vectors non-viral vectors are safer, easy to handle but with limited transfection efficiency. Till now a clinically successful polymeric vector for gene delivery is not reported. Hence, there is a continuing need to develop newer polymers towards this application which can ultimately be used clinically.

The concept of gene therapy was introduced in 1970s and even after five decades this treatment modality still faces many hurdles. The scientific advancement in the past few decades have matured the field of nanotechnology, drug delivery involving proteins and nucleic acids to this extent that the accumulated information can be utilized for designing better carriers for delivering biological therapeutics. Having a good understanding on the physiological barriers put up by the body will provide an insight on how to design the vectors.

Table 1 Barriers in gene delivery that need to be addressed for designing polymeric vectors

Barriers in gene delivery that need to be addressed	
Extracellular	Sequestration in lung capillaries
	Blood cells
	Protein interactions/opsonization
	Complement activation
	Clearance by macrophages and or RES
	Targetability
	Barriers mounted by extra cellular matrix which includes diffusion barriers, contact probability with the target cell
Intracellular	Endosomal escape
	Avoid lysosomal degradation
	Cytoplasmic endonucleases
	Mobility of the nanoplexes through cytoplasm
	Vector unpacking
	Nuclear transport
	Gene expression and or integration

As mentioned above gene is the molecule of medicine and introducing a foreign gene and integrating it to a host genome possess several barriers. A wide number of specialized reviews are there in literature dealing with various aspects of intracellular and extracellular barriers. To mention briefly, the main extracellular barriers starts right from the systemic delivery where the delivery systems comes in contact with various blood components, protein adsorption, macrophage or RES mediated clearance, stability of the nucleic acid, enzymatic degradation, non-target sites, targetability, etc. (Table 1). The intracellular barriers include endosomal escape, avoid lysosomal degradation, vector unpacking, cytoplasmic endonucleases, proper nuclear transport and efficient gene expression.

Over the past two decades polycations or cationic polymers have emerged as a promising dene delivery vectors owing to their versatile nature leading to development of numerous tailor made polymers with successful gene transfection rate. The major polymers include polyethyleneimine (PEI), poly-L-lysine (PLL), PDMAEMA, chitosan and other cationically modified polymers. In this chapter, we are dealing with cationic polymers for gene delivery which ultimately forms a nanoplex with the nucleic acid to be delivered. These being nanocarriers bearing positive charge, the chances of adverse interactions with the blood components are higher and need careful designing of the vectors (Table 2).

Cationic polymers that are explored for gene delivery includes both synthetic and natural as well as the hybrid graft polymers. In this chapter recent developments in polymeric gene delivery will be addressed.

Table 2 Design requirements for nucleic acid delivery

Physical	Ability to bind nucleic acids to form a stable nanostrucutre
	Should have an optimal positive charge so as to stably bind the nucleic acid and enable cellular uptake
	High loading capacity
	Easy manufacturing of the vectors without harmful steps
	Protect from endonucleases
	Should remain stable in systemic circulation where it comes in contact with numerous anionic proteins
	Enhance cellular uptake
	Enable endosomal escape and avoid lysosomal degradation
	DNA/RNA should be released intracellularly so as to facilitate the gene expression
Physiological	Nontoxic
	Non-immunogenic
	Non-thrombotic, hemocompatible
	Avoid RES or macrophages
	Longer circulation time
	Targeting ligands to the site of need
	Nuclear transport

2 Polysaccharide-Based Vectors

Polysaccharides are a class of natural polymers that are widely investigated for various biomedical applications including drug delivery, tissue engineering and currently gene delivery. These polymers are available naturally from various living sources. The main polymers include starch and cellulose from plant sources, chitin and chitosan from crustaceans, alginate from algae, pullulan, dextran, etc. from microbes such as fungus and bacteria, respectively. These polymers have wide acceptability towards biomedical applications mainly because of their nontoxicity, biocompatibility, biodegradability, non-immunogenicity and versatility which enable surface modifications as per the end need. Numerous review articles on various polysaccharide-based vectors are published. Here we will be focusing on graft polymers for gene delivery.

2.1 Graft Polymers Based on Polysaccharides

Graft copolymers are segmented polymers in which numerous similar or different side chains (branches) attached to the main polymer chain (the backbone) most predominantly by covalent bond and that gives pendant in a chain like appearance. The components and composition of main polymer chain may differ from that of the side chains. There are three different approaches for the synthesis of graft

copolymers and that includes grafting-through, grafting-from, grafting-onto with/without covalent interactions (Feng et al. 2011; Zhang and Muller 2005). In grafting-onto approach, the main and the side chain polymers are synthesized separately and then connected by means of coupling reaction. Advantage of this approach includes tunability of chain length since these polymers synthesized separately. Functional group for coupling reaction can be introduced either to the main chain or in the side chain, by either in cooperating in the monomer units or after polymerization by post functionalization. Grafting density depends on the efficiency of the coupling method and also the removal of unreacted side chains (Hadjichristidis et al. 2001; Moses and Moorhouse 2007; Gao and Matyjaszewski 2007; Fu et al. 2008). Second one is the grafting-through approach and in this technique, the main polymer chain synthesis and side chains grafting occurs simultaneously by using macromonomers with polymerizable end group. Difficulty to remove the unreacted monomers from those of functionalized one is the major drawback of using this technique (Shinoda and Matyjaszewski 2001; Hawker et al. 1997; Matyjaszewski et al. 1998). Third approach is grafting-from and here the main polymer chain also called macroinitiator as it contains the initiator for the formation of side chains. This functional group for side chain initiation can be incorporated to the main chain either by using monomer containing initiation group or by functionalization of the precursor main chain after polymerisation. Cross linking of multifunctional macrointiator was the major drawback of this approach which can be minimised by carrying out the polymerization reaction in higly dilute environment (Inoue et al. 2004; Paik et al. 1998; Percec and Asgarzadeh 2001).

Grafting is one of the versatile techniques to combine the synthetic and natural polymers and thereby getting the advantages of both. Grafting with suitable moieties improve the biodegradability and biocompatibility of the polymers, and also the transfection efficiency, blood circulation time, nuclear and cell targeting of the polymers that intended for gene delivery. By grafting, we can adjust hydrophilic to hydrophobic ratio and also can synthesize stimuli-responsive polymers. Natural polysaccharides are inexpensive, renewable, nontoxic, biodegradable, biocompatible materials and the ease to tailor their properties makes them most attractive in the field of drug and gene delivery. In the following section, we will discuss about various grafted copolymers of natural polysaccharides for gene delivery purpose.

2.2 Grafted Copolymers of Chitosan

Chitosan is a naturally occurring cationic polysaccharide and the only one of its kind. Chitin a polymer of *N*-acetyl glucosamine linked by β (1 → 4) linkage is the main component of exoskeleton of insects and crustaceans. Chitosan is obtained from chitin by its deacetylation leading to formation of a polymer composed of glucosamine and *N*-acetyl glucosamine. Due to these biological properties chitosan is being extensively studied for drug delivery (Khor 2001). But one major limitation is that the chitosan is soluble only at acidic pH and pKa of chitosan is 6.5.

Molecular weight and degree of deacetylation also determines the solubility of chitosan. But due to its versatile properties chitosan can be modified easily to suit the purpose. Chitosan has reactive amino groups, which can be chemically altered by mild reactions, and thus its physical and chemical properties can be adjusted as required (Muzzarelli et al. 2012).

Molecular weight and degree of deacetylation of chitosan determines its toxicity, solubility, hydrophobicity and also their ability to condense DNA. The DNA binding capacity of chitosan and thereby their use in the field of gene delivery was first explored by Mumper et al. (1995). But, in general chitosan as such is not an excellent transfecting agent owing to its high molecular weight, low transfection efficiency, poor solubility at physiological pH. The major hurdle for use of this polymer in the field of gene delivery was their lower transfection efficiency and poor solubility at physiological pH (Thakur and Thakur 2014). The chitosan is attractive for this application because of its biocompatibility, biodegradability and the various modifications that can be achieved to make it a successful gene delivery vector. The presence of different functional groups in their back bone like amino, hydroxyl and acetamido groups make this polymer most attractive for grafting with suitable moieties that improve transfection ability and also solubility and thereby rectifying their drawbacks (Estevinho et al. 2013; Muzzareli et al. 2012). Appropriate functional groups, ligands are attached to improve its applicability as a gene delivery vector. Chitosan is degraded by lysozymes and the degradation products are generally nontoxic in nature which makes it an ideal choice of vector. However, to improve its ability as a gene delivery vector many modifications are carried out and there is a need to evaluate whether such modifications affects its native biocompatible nature.

Failure to form tight complex with nucleic acid was one of the reasons for the lower transfection efficiency of CS. Various research groups tried to address this issue by providing cationicity to CS through the modification with PEI using different methods. In a study conducted by Wong et al. (2006) CS-g-PEI was synthesized by carrying out cationic polymerization of aziridine with CS through chain transfer reaction. Chain length of the PEI grafted to CS can be controlled by adjusting feed molar ratio of aziridine to amine in CS. This CS-g-PEI shows similar amine substitution as that of hyperbranched PEI and also their transfection efficiency was higher than that of 25 kDa PEI which was evaluated both in vitro and in vivo studies using pDNA containing luciferase gene. In vitro studies were carried out in three different cells lines—HeLa, HepG2 and primary hepatocytes while in vivo studies carried out in Wistar rat liver tissue by directly injecting nanoplexes into the bile duct.

Similarly Jiang et al. (2007) synthesized CS-G-PEI by grafting CS with PEI (1.8 kDa) through imine reaction and studied its transfection efficiency in three different cell lines namely 293T, HeLa and HepG2. They observed higher transfection efficiency in 293 T cell lines and it was also higher than that of 25 kDa PEI at higher N/P ratio (35). In another study, they further modified CS-g-PEI with galactosylated PEG to target hepatic cells and to increase blood circulation time of the polymer. They carried out both in vitro and in vivo studies with reporter genes,

luciferase and GFP (Green flourescent protein), respectively, to evaluate transfection efficacy and target specificity of the synthesized polymer. This GPCS-g-PEI shows higher gene transfection efficiency in HepG2 cells than with HeLa cells as the former express more ASGPR (Asialoglycoprotein receptor) that facilitates greater cellular uptake of nanoplex through receptor mediated endocytosis due to the presence of galactose in the grafted polymer. In vivo studies carried out in BALB/c mice further confirms the liver targetability of this grafted polymer (Jiang et al. 2008). Recently Chen et al. (2015) introduced new method for the synthesis of CS-g-PEI where grafting density of PEI (1.8 kDa) to CS was controlled by using ionic solvent 1-butyl-3-methyl imidazolium acetate ([BMIM]Ac) as reaction medium and CDI as coupling agent. The advantage of using this ionic solvent includes solubility of large molecular weight CS, acceleration of nucleophilic substitution reaction by enhancing amines nucleophility and stability of activated intermediates which lowers the reaction time and also controls the grafting density.

Hydrophobic to hydrophilic ratio is very important for polymers that intended for drug and gene delivery as it determines cell surface adsorption and subsequent internalization, vector unpacking and the release therapeutic drug or gene, stability in blood during systemic circulation and cytotoxicity (Kurisawa et al. 2000; Xu et al. 2010). Influence of hydrophobicity on transfection efficiency of CS was studied by various groups by modifying with bile acids like deoxy cholic acid, cholic acid, fatty acids like stearic acid, palmitic acid, hydrophobic amino acids and with various alkyl groups. In a study conducted by Layek and Singh (2013), CS grafted with various non-polar amino acids like L-alanine, L-valine, L-leucine and L-isoleucine were synthesized and evaluated for their ability to transfect HEK 293 cells. They found that transfection efficiency of CS significantly increases with increasing hydrophobicity of grafted amino acid and this may due to the weakening of the electrostatic interaction of polymer with pDNA that causes the release of pDNA once the polyplex get internalized into the cell. In another study, they synthesized amphilic CS by grafting with hexanoic acid for providing hydrophobicity and PEG to increase water solubility and stability in blood circulation. Transfection efficiency of this amphilic polymer was evaluated in HEK 293 cells using two reporter genes pGFP and pβ-Gal. They found that transfection efficiency initially increases with increase in grafting percentage of both hexanoic acid and PEG and reaches peak and after it get declines. This dual-grafted chitosan showed best transfection efficiency with 54.5% hexanoic acid and 7.6% PEG substitution at optimized weight ratio 20 (polymer to pDNA). On comparison with unmodified CS, grafted polymer shows about 10-fold rise in GFP expression and 27.5–50. 4-fold rise in β-galactosidase expression in HEK 293 cells (Layek et al. 2014).

In another study conducted by Tang et al. (2014a, b), trimethyl CS (TMC) of 15 kDa molecular weight was grafted with Poly (ε-caprolactone) (PCL) in order to provide amphilic nature to the polymer that in turn helps in the formation of self-assembled nanoparticles in aqueous media. In this graft polymer, TMC forms tight complex with DNA while hydrophobicity of PCL facilitates the cellular entry of nanoparticle by interacting with plasma membrane. Even though nanoplex formed from TMC-g-PCL vector shows higher cellular uptake in human embryonic

kidney cell line HEK293T than that of PEI, transfection efficiency was higher for later than with former. Transfection efficiency of the graft polymer found to be increasing in the presence of chloroquine, the lysosomotropic agent that prevents the fusion of endosome with lysome. This observation suggests the entrapment of the nanoplex within the lysosomal compartment which may be due to the reduction in buffering capacity of the polymer by quarternization.

Amphoteric CS was synthesized by Shi et al. (2012) by grafting with carboxyl and imidazolyl groups that improve the water solubility and transfection efficiency of unmodified CS. They grafted carboxylmethyl groups at 6-O-site of the 2-amino-2-deoxy-D-glucopyranose ring of CS in the presence of $ClCH_2COOH$. This modification improves the water solubility of the polymer over wider pH range (4–10) as both amino and carboxyl groups present on the same moiety imparts ampholytical characteristics to the polymer. On the other hand grafting of imidazolyl group improves the transfection efficiency of the polymer as it contains secondary and ternary amino groups which bind more tightly with therapeutic DNA and it also helps in the endosomal escape.

Targeted gene delivery can be achieved by conjugating chitosan with various ligand like transferrin and folate whose receptors over expressed in cancer cells, mannose and galactose to target dendritic cells and kupffer cells, respectively (Duceppe and Tabrizian 2010; Mao et al. 2010). In a study conducted by Chan et al. (2007) CS was modified with folate using PEG as spacer for targeted gene therapy. Presence of folate helps to target cancer cells where its receptor expressed in abundance while PEG helps to increase blood circulation time. They observed that water solubility of CS increased with increasing PEG grafting percentage and also these modifications did not affect the cell viability considerably even at higher N/P ratio of polyplex (up to 20) in HEK 293 cells. Similar study was carried out by Lin et al. (2007) where lactobionic acid was used as ligand to target liver cells. Lactobionic acid is a disaccharide containing galactose and gluconic acid linked via ether bond. Galactose in the lactobionic acid helps to target liver cells through interaction with ASGPR while carboxyl group in the gluconic acid helps to form amide bond with chitosan. PEGylation prevents the aggregation of particles and also increase transfection efficiency up to 19.8% while grafting with lactobionic acid further increase the transfection efficiency of the grafted polymer to 45.3% through receptor mediated endocytosis.

Synthesis and development of stimuli-responsive nanocarrier attains greater attention in the field of drug and gene delivery since the release of pharmaceutical agent can be controlled in time, site and dose dependent manner and thereby evading adverse side effects. Such temperature and pH responsive polymer was synthesized by Bao et al. (2010) by grafting CS with two hydrophilic moieties viz PDMAEMA and PNIPAM through ATRP and click chemistry. This graft terpolymer could form different structures ranging from unimer to miscellar aggregates with distinct core and shell structures by modulating temperature and pH of the system.

Dextran is a branched polysaccharide of bacterial origin having α-1, 6 glycosidic linkages between glucose units in straight chain while α-1, 3 linkages between glucose units that connect branches to the main chain. They have wider application in medical field such as antithrombotic agent, plasma volume expander, etc. but their use as gene delivery agent was first studied by Pagano and Vaheri in 1965. They found that infectivity by Poliovirus RNA increases in the presence of Diethylaminoethyl-dextran (DEAE) both in primate and non-primate cell lines and also the presence of DEAE dextran protects the RNA from ribonuclease degradation. But still transfection efficiency of this cationized polymer was lower than that of viral counter parts. To address this issue, Onishi et al. (2007) modified DEAE dextran with methyl methacrylate by using ceric ammonium nitrate and observed 16-fold rise in transfection efficiency in HEK 293 cell line than the parent chain due to the formation of more stable complexes which protect the nanoplex from DNase and dextran sucranase degradation.

In another study, Tang et al. (2014a, b) synthesized oligopeptide grafted dextran and studied the effect of molecular weight and grafting percentage on transfection efficiency in ovarian carcinoma cell line, SKOV-3. They grafted NH2-RRRRRHHHHHC-COOH (R5H5) to methacrylate modified dextran of 3 different molecular weights (10, 20 and 70 kDa) by thiol-acrylate Michael type reaction and found that lower molecular weight dextran with higher grafting density transfect cells more efficiently than the higher molecular weight with lesser grafting density.

2.3 Grafted Copolymers of Pullulan

Pullulan, the natural polysaccharide synthesized from Aureobasidium *pullulans* (Leduy et al. 1988; Tsujisaka and Mitsuhashi 1993) has wider application in the field of medicine, cosmetics and food industry due to their biocompatible nature (Mishra et al. 2011). It is a polymer of maltotriose units linked by α (1 → 6) glycosidic bond while the glucose units present within the maltotriose unit are linked by α (1 → 4) linkage (Catley and Whelan 1971; Catley et al. 1986; Taguchi et al. 1973). Liver targeting effect of pullulan was studied by various groups (Kaneo et al. 2001; Yamaoka et al. 1993). Yang et al. (2014) synthesized biocleavable pullulan by modifying hydroxyl groups with cystamine to impart bioreducible di sulphide linkage followed by grafting with poly (glycidyl methacrylate) which was then functionalized with ethanolamine via atom transfer radical polymerization (ATRP). This biocleavable pullulan shows excellent hemocompatibility as compared with that of PEI and also their cellular uptake was higher in HepG2 cells than with Hela cells that show their liver targetability. In our lab, we found that grafting of PEI with pullulan reduces the cytotoxicity to greater extent without compromising transfection efficiency of the former and also liver targetability of pullulan not get affected by this modification (Rekha and Sharma 2011a). In another study

we conducted, modification with transferrin increases the cellular uptake of pullulan PEI in C6 glioma cells (Rekha and Sharma 2011b).

Juan et al. (2007) synthesized cationized pullulan incorporated 3D matrices of 2 mm thickness for gene delivery purpose in endoluminal vascular cells. They grafted pullulan with chloro–*N, N*-diethylamino ethyl hydrochloride and characterized for the delivery of pSEAP (plasmid containing cDNA coding for secreted form of alkaline phosphatase) on cultured rabbit SMCs. They then incorporated this cationized pullulan in hydrogel containing pullulan and dextran in order to concentrate gene near target site and also to eliminate loss during systemic circulation. Higher gene expression was observed in SMCs cultured in the presence of matrix impregnated with pSEAP than with plasmid alone and this may due to the sustain release of the plasmid from the matrix.

2.4 Grafted Polymers of Other Polysaccharides

Cellulose is the most abundant polymer of natural origin and it is a linear polysaccharide of β-D-glucopyranose units joined by β(1 → 4) linkage (Qin et al. 2008; Gürdağ, Sarmad 2013). Comb shaped cationic copolymer was developed by Xu et al. (2009) by grafting bromoisobutyryl-terminated hydroxypropyl cellulose with Poly ((2-dimethyl amino) ethyl methaacrylate) via ATRP (atom transfer radical polymerisation). They further quaterinized poly ((2-dimethyl amino) ethyl methaacrylate) in the parent chain with quaternary ammonium group and compared their transfection efficiency in HEK293 cells. Even though the quaternized polymer form more tight complex with the DNA, the transfection efficiency was lower as compared to that of parent chain and this may due to the higher cytotoxicity and lower buffering capacity that makes lysosomal escape difficult for quaternized polymer.

In a study conducted by Yamada et al. (2014) starch was grafted with PEI (0.8 kDa) by using 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methyl-morpholinium chloride as conjugating agent. They found that transfection efficiency depends on grafting percentage of PEI as well as the molecular weight of the starch backbone. Transfection efficiency similar to 25 kDa PEI was observed for higher molecular weight starch with 30% PEI. Moreover, this grafted polymer exhibit good enzymatic degradation and low cytotoxicity over bPEI that makes this promising for gene delivery purpose.

3 Redox Responsive Vectors

As mentioned in the introductory section, the success of cationic polymer-based gene delivery largely depend on various factors such as DNA condensation ability of polymer, successful transportation of gene in the extracellular medium, targeting

to desired cells, cellular internalization, endosomal escape, efficient unpacking to release DNA, nuclear transport of genetic material and subsequent expression of gene (Colin et al. 2001). Among them, the major dilemma is such that the stability of polyplex in the extracellular milieu and effective release of nucleic acid at the intracellular target site. Many of the previous studies have shown that the delivery efficacy of the polyplex largely depends on the chemical structure and molecular weight (MW) (El-Aneed 2004; Cosco et al. 2014). It is well known that the polycations with high molecular weight tightly bind with nucleic acids to form small compact structures and facilitate the cellular uptake via adsorptive pinocytosis. However, the high MW of the polycation leads to the inefficient release of nucleic acid at the target site and creates cytotoxicity by interacting with the anionic cellular membrane. On the other hand, the low MW polycation result in the unstable polyplex formation due to minimal interaction with negatively charged nucleic acids and hence poor transfection efficiency but lower cytotoxicity. In addition, it has also been reported that the cationic polymers are easily removed from the circulation via RES by interacting with the negatively charged blood components (Dar-Li and Huang 2009). Hence, a successful strategy to solve this dilemma is to include additional characteristic to the delivery vector such as integration of stimuli-responsive attributes to the polycation in order to respond to the diverse physiological environment of extracellular and intracellular domain (William and Nicholas 2012). The formation of bioresponsive polymers have been a better choice which act with respect to difference in pH, temperature, redox gradient, ATP and enzymes. The use of biodegradable polymer is an important strategy as degradation of polymer occurs in response to changes in pH or temperature which include ester, phosphoester and acetal bonds (Wolfert et al. 1999). They have been reported to show good transfection ability by the effective release of genetic material from polyplexes and minimal cytotoxicity apart from forming nontoxic and easily removable degradation product of polymer. However, the major disadvantage of biodegradable polymer is their considerable instability in the aqueous extracellular medium. Hence, the conflicting issue of condensation and release of nucleic acid in the extracellular and intracellular region can be well addressed by the exploitation of redox gradient in these compartments. The intracellular redox environment has been mainly maintained by the cellular glutathione (GSH) (Magdalena and Tak 2008). The differences in the concentration of reducing agent, glutathione between the compartments can be well used for the reduction of disulphide linkages in the polyplexes for the effective release of therapeutic genes in the intracellular site while maintaining their stable transport in the extracellular medium.

3.1 Biological Redox Environment

Redox environment of the cell comprise of many redox couples which form the reduced species and its corresponding oxidized form and this reductant/oxidant couple indicate the biological status of the cell. The various redox couple inside the

cell include thioredoxin (TRXred/TRXox), nicotinamide adenine dinucleotide phosphate (NADPH/NADP), glutathione (GSH/GSSG) (Moriatry-Craige and Jones 2004). The reductant/oxidant ratio varies with the energy status of the cell and different redox couple have different redox potential and reducing capacity. The redox potential is concerned with the ability of chemical substances such as ions, molecules and atoms to acquire energy and it is an intrinsic property of each molecule. Redox potential is expressed in millivolts (mV), the more positive the redox potential of a substance, greater its affinity towards electron. The regulation of redox potential is crucial in maintaining the cellular homeostasis. The redox potential of redox couples can be calculated from the equation

$$E = E_0 + (RT/nF) \times \mathrm{In}([\text{oxidized reductant }]/[\text{reductant}]^x)$$

where E is the standard half cell potential, R stand for gas constant, T indicate absolute temperature, n is the number of moles and F is the faraday constant and x is 2 for GSH and Cys and 1 for Trx (Jensen and Elving 1984).

Among the redox couples, GSH/GSSG form the most abundant redox pair with approximately 500–1000 fold higher in concentration compared to TRX and NADPH (Follmann and Haberlein 1995) GSH consists of serine, cysteine and glutamate and is synthesized in almost all the cells with the help of two consecutive enzyme activity such as gamma glutamylcysteine synthetase, which joins glutamate and cysteine, followed by the attachment of serine residue by glutathione synthetase. GSH is an important antioxidant and has role in regulating protein structure and function, detoxification of xenobiotics, cell proliferation, signalling and apoptosis. It is also to be noted that the concentration of glutathione and redox ratio varies in different subcellular compartments, indicating its diverse role in different sites. The concentration of GSH in intracellular and extracellular compartments varies greatly with almost 3–10 mM in the intracellular region against approximately 2.8 μM in the extracellular region (Kirlin et al. 1999). This is far higher than other redox couples in the cell, hence the measurement of total redox environment of the cell is estimated by the level of GSH/GSSG. The intracellular concentration of glutathione is kept reduced by the combined activity of glutathione reductase and NADPH. Glutathione is thus considered to be the major thiol-disulphide redox buffer system in the intracellular environment. This along with the enormous differences in the concentration of GSH in both the compartments is exploited in the redox responsive gene delivery. Very low concentration of GSH in the extracellular plasma provides less opportunity for the disulphide bond to get reduced and hence the delivery system shows a high stability outside the cell. On the other hand, the exposure of the delivery system to high concentration of GSH triggers rapid release of genetic materials such as DNA, siRNA and peptides and drugs in the intra cellular region by the selective cleavage of disulphide bonds (Sies 1999). Though glutathione plays a protective role in cancer by the removal and detoxification of carcinogens, it has a profound influence on cell survival as well. Also, the level of glutathione is almost fourfold higher in tumour cell when compared with normal

cell, rendering the tumour specific delivery of genes and drugs more feasible using disulphide cross-linked conjugates (Trachootham et al. 2008).

4 Preparation of Bioresponsive Polyplexes

The presence of disulphide linkage in polyplexes enhances the relative stability in the extracellular site and easy degradability in the intracellular region make it a suitable candidate for gene delivery purposes. There are different methods to include disulphide linkages into the polyplex structure. This mainly include use of (a) sulfhydryl group containing polycations (b) disulphide group containing polymers (c) disulphide containing cross-linking agent (Manickam and Oupicky 2006a, b). The first method was initially put forward by McKenzie and colleagues in which used peptides containing different numbers of cysteine residues in the backbone and during the formation of polyplex, disulphide bonds are formed by aerial oxidation (McKenzie et al. 2000a, b). Additionally, this method does not require any external oxidizing agent. Another similar approach showed the addition of Trauts reagent and SPDP in cationic blocks for the introduction of sulfhydryl group and further aerial oxidation resulted in the formation of disulphide bonds in the backbone of the polymer (Miyata et al. 2004). The second method includes the formation of redox sensitive polyplexes using disulphide bond containing polymers. Peng et al. carried out the ring opening reaction of low molecular weight PEI (PEI 800 Da) with methylthiirane, produced thiolated polyethyleneimine with the average number of thiol group on the polymer adjusted by the methylthiirane/PEI ratio (Peng et al. 2008a, b). Further oxidation with DMSO forms the disulphide cross-linked PEI (PEI-SSx). This effectively condense with plasmid to form nano-sized complexes with lower cytotoxicity and superior transfection efficiency. Another study by Takae et al. formed PEG-based polyplex miscelles by using block cationic polymer, PEG-SS-P[Asp(DET)] by inserting disulphide linkage between PEG and polycationic segment of poly(aspartamide) with flanking *N*-(2-aminoethyl)-2 aminoethyl group, P[Asp(DET)] (Takae et al. 2008). The polyplex miscelles from polymer and plasmid DNA forms stable nano-sized structure with minimal cytotoxicity and releases the PEG coat in the intracellular reductive environment. This results in more efficient release of therapeutic gene inside cell with minimal cytotoxicity. Similarly, Xiaojun et al. synthesized the dual stimulus responsive mPEG-SS-PLL glutaraldehyde star polymers, in which the polyplexes of the same showed good intracellular uptake and rapid release of DNA by the simultaneous cleavage of PEG in presence of GSH and the acid induced escape to accelerate the release of genetic payload (Cai et al. 2015). Another method of forming polyplexes with disulphide bonds is to insert disulphide containing cross-linking agent to the exiting polymer. This is the simplest method of forming stable disulphide bonds in the polyplex backbone. For example polyplex of PLL was efficiently stabilized by cross linking with dimethyl 3-3 o-dithiobispropionimidate (DTBP) via the amino group of PLL. Similarly, several report suggested the cross linking of LMW PEI (800 and 1800 Da) with disulphide

containing cross-linking agents such as cystamine bisacrylamide (CBA), dithiobis (succinimidylpropionate) (DSP), dimethyl-3,30-dithiobispropionimidate-2HCl (DTBP), to form superior transfection efficiency and significantly lower toxicities as compared with the commercial transfection agents such as Lipofectamine, JetPEI and SuperFect (Kim and Kim 2011; Kakizawa et al. 1999)

5 Reduction Sensitive Polymers and Intracellular Gene Delivery

5.1 Bioreducible PEI Polymers

PEI-based non-viral vectors have drawn enormous attention in gene delivery due to its high DNA binding ability and superior transfection efficiency. PEI is known for proton sponge effect and the corresponding endosomal swelling and release of polyplex to the cytosol. Though high molecular weight PEI (HMW) provide excellent condensation of DNA and stability in the extracellular medium, possess high cellular cytotoxicity and poor or inefficient release of nucleic acid in the cytosol (Wang et al. 2006). On the other hand, LMW PEI possess minimal cytotoxicity but very low transfection, thus is not suitable for gene delivery. Hence, in order to circumvent the problems of cytotoxicity and transfection efficiency, the LMW PEI have been converted to HMW PEI by the insertion of biodegradable linkages such as acetals, esters, amides and disulphides (Peng 2008a, b). Out of which the disulphide linkages addresses both cytotoxicity and stability and release of DNA intracellularly in the presence of GSH. One of the initial works on bioreducible polymers were carried out in PEI. In this way, Shen et al. cross-linked PEI (1800 Da) using DTBP containing inbuilt disulphide bonds, and the corresponding polyplexes of N/P ratio 10 showed significantly reduced cytotoxity and release DNA in presence of 3 mM GSH (Wang et al. 2006). Many in vitro experiments on disulphide cross-linked PEI showed remarkable transfection efficiency compared with branched PEI (25 kDa). Similarly, Lee and coworkers (1999) used the endo osmolytic protein called listeriolysin O (LLO) from Listera monocytogens to conjugate PEI via disulphide linkage (LLO-ss-PEI) and the corresponding LLO-ss-PEI/DNA polyplex showed enhanced transfection efficiency (Choi and Lee 2008). The LMW PEI via click reaction between disulphide containing dialkyne cross linker and azide functionalized LMW PEI formed a high molecular weight disulphide cross-linked PEI (PEI-SSCL) (Liu et al. 2010). PEI-SSCLs were shown to be less toxic and more effective in terms of transfection both in the presence and absence of serum than PEI (25 kDa). PEI has been known to gain attention in the delivery of siRNA. Miriam et al. reported a disulphide cross-linked PEI (ss PEI) as carrier of siRNA which promoted its efficient release into the cytoplasm (Miriam et al. 2007). It was demonstrated that compared with linear chain PEI, combination of branched chain PEI and reducible disulphide linkage could be efficient in terms of cellular uptake and release of siRNA. In order

to increase stability of PEI and prolonged circulation time of carrier, the addition of PEG was carried out to form PEG-PEI diblock and PEG-PEI-PEG triblock polymer followed by cross linking of the polyplexes with DSP and the in vivo experiment showed prolonged half life (Zou et al. 2012). Another interesting study of disulphide cross-linked PEI, indicated the efficiency in microRNA(miRNA) delivery technique. Do won et al. developed brain specific rabies virus glycoprotein (RVG) labelled SSPEI nanoparticle to deliver miR-124a to brain in vivo. The RVG-SSPEI along with mannitol showed more efficient delivery of miR-124a to brain, where the latter help in the disruption of blood brain barrier and the carrier readily released the cargo in the cytosolic region (Hwang et al. 2011). The polyplex injected via the tail region of rat also showed superior accumulation of the polyplex in the brain. Hence the RVG-SSPEI polymeric system can be a potential candidate for the delivery of variety of oligomers for the treatment of neurological diseases.

5.2 Bioreducible Poly(amido)amines (PAA)

Polyamidoamine is a class of dendrimers containing highly branched structures of amides and amines. Michael type poly addition using varied primary, secondary and tertiary amine group with bisacrylate monomers provide an excellent opportunity to add various functional group in the side chain of the polymer (Lin and Engbersen 2008). Many studies indicate the water solubility, low cytotoxicity and long term biodegradable nature of PAA and this highlight its suitability as gene carrier. Moreover, addition of cystamine bisacrylamide to primary amine groups of PAA formed linear bioreducible poly (amidoamines) containing multiple disulphide bonds (SSPAA). Studies showed that many of the SSPAA condense DNA very strongly and formed nano-sized structure of <200 nm. The buffering capacity, endosomal escape efficacy and in vitro transfection of SSPAAs were also superior to that of branched chain PEI (25 kDa). It is interesting to note that the polymers with protonable nitrogen group such as pDMEA and pHIS show high DNA condensation ability and buffering capacity than those that lack protonable nitrogen group such as pABOL (Lin et al. 2007). Another study by Xue et al. showed the introduction of dendritic poly (amidoamines) to the side chains of disulphide containing poly(amidoamines) by Michael addition, which led to the formation of bioreducible poly(amidoamines) grafted with dendritic poly (amidoamines). It showed superior DNA binding ability, high buffering capacity and low cytotoxicity and the DNA released under reductive environment conditions. Apart from that, the bioreducible poly (amido amine) with grafted dendrimer showed greatly improved transfection efficiency in 293T and HeLa cells in comparison with original disulfide containing poly (amidoamine) with aminoethyl side chain (Xue et al. 2010). In another study, eight different bioreducible PAAs were prepared by Michael addition of *N*,*N*′-bisacrylolylpiperazine (BP) with cystamine (CYST) and *N*,*N*′-cystaminebisacrylamide (CBA) with *N*,*N*′-ethylenediamine (EDA) or *N*,*N*′-dimethylethylenediamine (DMEDA). Both linear and hyperbranched PAA showed polyplexes with the size around 200 nm and zeta

potential ranging from +10 and +22 mV with remarkably low cytotoxicity (Piest et al. 2008). It is interesting to note that the hyperbranched CBA containing PAA showed higher gene transfection in COS-7 cells compared with the linear counterparts and the transfection efficiency was observed to be higher in presence of serum indicating its suitability in in vivo studies. These observation state that the incorporation of disulphide linkage not only enhance the release of DNA and degradability of polymer but also influences size, zeta potential, DNA binding ability and buffering ability (Federico et al. 2012). Another study by Rubina et al. synthesized poly (amido amine disulphide) by the Michael addition between amines and biacrylamide (Parmar et al. 2013). A combination of poly (amido amine disulphide) with acid labile masking group, whose lytic property remain masked at the physiological pH where as it can restore at the endosomal pH can be demonstrated as an efficient delivery vehicle for siRNA. This showed efficient mRNA knock down for apolipoprotein B for mouse liver. In another study, reducible poly(amido ethylenimines) were synthesized by Michael addition between CBA and ethylenediamine (EDA), diethylenetriamine (DETA) and triethylenetetramine (TETA) (Christensen et al. 2007a, b). These polymers showed appropriate biophysical properties and in vitro transfection studies in different cell lines such as primary bovine aortic endothelial cell (BAEC) and rat aortic smooth muscle cell line (A7R5) revealed that these polymers showed superior transfection efficiency compared with branched chain PEI (25 kDa) (Christensen et al. 2007a, b). The polyplex formed between the vascular endothelial growth factor plasmid (VEGF) and reducible poly (amido ethylenimine) produced significantly high expression of VEGF both in vitro conditions and in vivo models, where rabbit myocardial infarct model showed high VEGF protein expression at the region of infarct (Jeong et al. 2007). Yu et al. has introduced several strategies to improve the efficiency of gene delivery by the introduction of ligand to reducible polymer to target the tumour site and thereby enhance the target site specificity (Yu et al. 2012). In order to achieve high efficacy in cancer delivery, tumour targeting bioreducible polymer were developed by conjugating cyclic RGDfc peptides to the polymer to form PA-PEG_{1k}-RGD, which efficiently transfer deliver plasmid DNA into integrin expressing cancer cells such as MCF-7 breast cancer and PANC-1 pancreatic cancer cell (Kim et al. 2008). The VEGF siRNA expressing plasmid delivered to the cancer cells showed 50–71% reduction in the VEGF expression and revealed the viability as less than 43%.

The low molecular weight (LMW) peptides have been utilized for gene delivery due to low side effects and ease of modification. However, the poor DNA condensation ability of the polymer remains a hurdle. To overcome this, Rice and coworkers have developed reducible LMW peptide by imparting disulphide cross linkage to the backbone. They formed this by incorporating one cysteine residue to four lysine residues of Cys-Trp-Lys18 and the subsequent oxidation of thiol group of cysteine form the inter peptide disulphide linkage. This reversibly cross-linked peptide obtained stable polyplex and showed 4–60% enhancement in gene expression in HepG2 cells compared with the uncross-linked counterparts (McKenzie 2000b).

In the following study, the incorporation of his residue for Lys in Trp-$(Lys)_{17}$-Cys yield peptide showing improved buffering capacity and successful gene expression in HepG2 cells in the absence of chloroquine (Mckenzie 2000b). In a similar study, the reduction sensitive peptide, poly(oligo-D-arginine) (rPOA), condenses siRNA against VEGF more strongly and dissociate the complex more rapidly in the cytoplasm. The rapid release of siRNA from the polyplex without losing bioactivity was used successfully both in vitro and in vivo animal models (Kim et al. 2014). The suppression of tumour growth in animal model proved the bioactivity of siRNA in siRNA/rPOA delivery system. In order to enhance plasma half life, Kwok et al. used both reducible and non-reducible peptide such as PEG-SS-CWK18 and PEG-VS-CWK18, respectively. Both condenses DNA into smaller size polyplexes of <100 nm with positive zeta potential (+10 mV), where as there was remarkable differences in the gene transfection efficiency, i.e. the reducible peptide showed a gene expression of over two orders of magnitude higher than the non-reducible counterpart in the HepG2 cells (Kwok et al. 1999). Functional peptides such as TAT peptides and NLS also have also attracted considerable attention as gene delivery vehicle. Cell penetrating peptides composed of 40–50 aminoacids, have the ability to translocate the cell membrane in a receptor independent manner and the sequence of NLS helps the transportation of gene vehicle to cross the nuclear membrane. TAT is the cell penetrating peptides used commonly for the transport of gene, however, the application is limited by the instability of TAT/DNA complex as well as poor release in the endosome (Räägel et al. 2013). In order to circumvent the issue, Wang and Lo used the cysteine and Histidine residue in the TAT sequence so as to stabilize the complex by the presence of disulphide linkage and also endosomal buffering ability by the presence of histidine (Lo and Wang 2008). The addition of 10 histidine residue and two cysteine residue over normal TAT sequence resulted in several fold increase in the transfection efficiency with gene expression close to 25 kDa. Manickam et al. synthesized peptides which is histidine rich with terminal cysteine residues (HRP, CKHHHKHHHKC, Mr 1431) and nuclear localization sequence (NLS, CGAGPKKRKVC, Mr 1274) formed reducible copolypeptides which condense with DNA to form nano-sized polyplexes and superior transfection efficiency in all cell lines tested (Manickam and Oupicky 2006a, b). However, it was observed that the incorporation of NLS sequence has little influence on the transfection efficiency of the copolypeptides and this is due to the early release of NLS sequence from the polyplex in the reductive environment conditions.

5.3 Other Reduction Sensitive Polymeric System

Several reducible liposomes have been synthesized as a vehicle for gene delivery. Balakirev et al. synthesized lipoic acid-based cationic amphiphilic reducible polymer, which could successfully bind with DNA in the oxidative condition whereas rapidly release the same in the reductive intracellular environment (Balakirev 2000). The transfection studies showed several fold increase in the gene

expression compared with DOTAP and further increase in terms of transfection efficiency was performed by incorporating nucleus targeting peptides to the polymer. Similarly Gabriele et al. have developed ternary DOPC/DOPE/SS14 liposomes with bioreducible cationic *gemini* surfactant, SS14 and observed high transfection efficiency with almost threefold enhancement in transfection compared with commercially available transfection agent, Lipofectamine 2000 (Candiani 2010). Recently bioreducible microcapsules also gained much attention as gene delivery vehicle. Caruso et al. synthesized disulphide cross-linked polymer microcapsule, which composed of 18 mol% thiolated polymethacrylic acid (PMASH) and poly (vinylpyrrolidone) (PVPON) onto silica particles, along with subsequent oxidation of thiol group in PMASH and removal of silica particles leads to the formation of microcapsule with disulphide cross linkage (Zelikin et al. 2006). These microcapsules were stable at the physiological pH but were rapidly degraded in presence of DTT. These could able to encapsulate DNA with varied size without causing degradation. The released DNA maintains the functionality and structural integrity as evidenced by various enzymatic reactions. Again reducible polyion complex (PIC) miscelles were synthesized based on poly(ethylene glycol)-SS-antisense oligodeoxynucelotide conjugate (PEG-SS-asODN) were revealed to achieved high levels of antisense effect by the enhanced cleavage of disulphide linkage in the presence of intracellular glutathione level compared with non-reducible PIC micelle system (Takae et al. 2008).

6 Conclusion

Non-viral vectors came into existence about three decades back. However, even with enormous efforts for developing a vast library of cationic polymers with varying properties to improve the transfection efficiency, till date clinically established polymeric vectors are not reported yet. Being cationic the non-viral vectors can pose various levels of compatibility or toxicity problems. Hence, it is very essential that the vectors employed for delivering gene to the cell should be either compatible and could be cleared from the physiological system for which various molecules can be grafted and designed to meet this need. Biodegradable or bioreducible systems that respond to various physiological conditions can fulfill the task without any adverse reactions. Many polymeric gene delivery systems have been developed recently. Of which, reduction sensitive polymers and conjugate sought special attention due to its unique characteristics, such as close packing of the gene carrier in the oxidative environment and easy release of the cargo in the reductive intracellular medium. Thus, it provides high stability of the carrier along with having additional advantage of low cytotoxicity and increases gene transfection efficiency. Hence it is particularly suitable for the delivery of gene and siRNA to the target site, together with the attachment of ligand on to the surface of the nano-sized delivery system further enhance the therapeutic efficacy of the attached nucleic acid drug.

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Nanoparticulate Immunotherapy: An Intelligent Way to Tailor Make Our Defense System

Rituparna Acharya, Suman Saha, Sayantan Ray and Jui Chakraborty

Abstract

The term 'Immunotherapy' refers to a complex process to combat cancer, infections, and other diseases by suppressing, enhancing, or inducing the immune response to the host. The major limitation of the therapy is its inability to produce enough trained immune cells in the system. Currently, researchers have pursued immunotherapy as a treatment protocol, based on training the host's immune system to fight with the diseases. Immune response can be activated by dendritic cell based, T cell adoptive, autologous immune enhancement or genetically engineered T cell or can be suppressed by some drugs that are very much useful in organ transplantation. Immune tolerance refers to a process by which body will not launch an attack to its own cells but helps to stop attacks to its tissue, simultaneously, that occurs in autoimmune diseases, generally. In allergic conditions, immunotherapy is the only treatment option available, in which body can change or modify the immune response by reducing allergen sensitivity. In case of biological application, macro-size possesses numerous drawbacks on account of the smaller size of cellular compartment. Thus advance drug delivery system comprising nanoparticles encapsulating immunologically active compound holds great potential for target specific immunotherapy, in general. Hence, nanotechnology-based immunomodulatory drugs and vaccines help in the improvement in the field of immunotherapy, for the immunological diseases.

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1 Introduction

The immune system is one of the most vital systems of our body that protects us against diseases, and efficiently distinguishes various pathogens from the healthy tissues, with the aid of two of its subsystems, e.g., the innate and the adaptive immunity (Shao et al. 2015; Goldsby et al. 2000). The innate immune system combats the microbes at the infection site by its cells and proteins. The components which are important of the innate immune system are (1) dendritic cells, (2) phagocytic leukocytes, (3) epithelial barriers, (4) circulating plasma proteins, and (5) natural killer (NK) cell. The adaptive/acquired immune system is active against pathogens that overcome innate immune defenses. Its components are generally silent, although, on activation, they 'adapt' in the presence of infectious agents by activating, proliferating, and creating potent mechanisms for eliminating the microbes. They also have a special ability to recognize specific pathogens and thus, create an immunological memory for an enhanced encounter with the same (Grasso et al. 2002). The adaptive immunity is further classified as humoral immunity, mediated by antibodies produced by B lymphocytes, and cell-mediated immunity, controlled by cytotoxic T lymphocytes (Alberts and Johnson 2002; Janeway et al. 2001a, b, 2005).

In response to the changing requirements of the internal and external environment, our immune system can be suppressed or stimulated. Of these, immunosuppression is a condition that lowers our body's defense against infection and thereby leads to reduction of the activity of the immune system. Immunosuppression can be both deliberate and nondeliberate in nature: the deliberately induced category is necessarily immunosuppressive drug/biomolecule/environmental toxins (Starnes 1992) based that are used to combat excessive immunostimulatory conditions, e.g., autoimmune disorder, allergy, allotransplantation etc., whereas the nondeliberate category can be due to malnutrition, ageing, chronic infection leading to acquired immune deficiency syndrome (AIDS) and many types of cancer (Alberts and Johnson 2002; Janeway et al. 2001a, b, 2005). On the contrary, immunostimulation is the mechanism of enhancement of the activity of the components of the immune system, e.g., granulocyte macrophage colony-stimulating factor. It is further categorized into specific and nonspecific immunostimulants depending on the antigenic specificity (Kumar et al. 2011; Wira et al. 2004; Lang 2004; Moriyama et al. 1999). Autoimmune disorder that comprises around 80 different types of autoimmune diseases (e.g., type I diabetes mellitus, rheumatoid arthritis, multiple sclerosis, psoriasis, etc.) is a condition when our own immune

system identifies our healthy tissues as foreign entity, becomes much activated and attacks them, to cause abnormal organ growth or to alter the specific organ function. On the contrary, immune suppressive disorder (such as leukemia, lymphoma, multiple myeloma, AIDS etc.) is a condition leveraging decreased function of the immune system, resulting in increased susceptibility of pathogens.

In the above conditions, immunotherapy is a treatment procedure that can be adapted by two mechanisms: either by suppressing the immune response, broadly known as suppression immunotherapy or by amplifying the immune response, called activation immunotherapy or immunostimulation (Good 1972; Caspi 2008). Of these, the suppression immunotherapy is antigen-specific therapy, applied in case of autoimmune diseases, aimed to inhibit common immune cell activation and its pathways that is triggered by cytokine and cytokine receptors and also the costimulatory molecules. Lymphocytes that have escaped controlled mechanism are regulated, eliminated, and tolerized by this mechanism (Chatenoud 2006). Similar to this, allergy/hypersensitivity reaction of the immune system, induced by pollen/food is another condition that requires immunosuppressive therapy. Further, transplant rejection is also an important issue where transplanted tissue is rejected by the recipient immune system, although the transplant rejection can be lessened by immunosuppressive therapy after transplantation. The activation immunotherapy (immunostimulation) or antigen nonspecific approaches on the other hand, are directed against cell surface molecules, receptors or functions that are involved in common activation and effector pathways of the immune system. The amplification of the above pathways is the mechanism in general of the antigen nonspecific therapy (Kappos et al. 2000; Bielekova et al. 2000).

Nanotechnology-based platform in case of immunotherapy contains the application of vaccines, adjuvants, and immunomodulatory drugs that can be delivered to specific target sites, to improve the clinical scenario of immunological disorders (Park et al. 2013). Nanoparticle-based immunotherapy renders a number of special advantages including (a) targeting of immune molecules/cells against immune checkpoint molecules and thereby enhancing immune activation via the use of new stimuli-responsive or immune stimulatory materials, (b) augment the efficacy of adoptive cell therapies along with specificity that in turn reduces the dosage frequency and toxic side effects of the therapeutic payload (c) protecting the therapeutic payload from the surrounding physiological/biological environment (extreme pH condition, bile and pancreatic secretion), thereby increasing the half-life of the same and enhancing its in vitro and in vivo stability, in turn (Zolnik et al. 2010; Dobrovolskaia et al. 2008).

The size, surface charge, shape, hydrophobicity/hydrophilicity, and the steric effects of nanoparticles due to surface coating governs the compatibility of the same with the immune system (Dobrovolskaia et al. 2008; Aggarwal et al. 2009; Dobrovolskaia and McNeil 2007). It is known that a hydrophilic coating of nanoparticle using biocompatible polymer shields them from immune recognition, although, the intelligent adaptive immune system generates specific antibodies on administration of the above nanoparticles that might eliminate it from the blood and hence influence the efficacy and pharmacokinetic profile of the said therapy.

Another area of concern is the toxicity issue of the nanoparticle-based immunotherapy, associated with different administration routes which might be attributed to lack of proper evaluation of physicochemical/immunological properties of the nanoparticle w.r.t density, thickness, surface coating stability under in vivo condition (Dobrovolskaia and McNeil 2007; Descotes 2004).

The interaction of the nanoparticles with the plasma proteins and blood components generate a new complex entity, often different from the originally formulated molecule. This in turn influences the biological response including the cellular uptake that might alter its biodistribution, altogether. Further, being engulfed by the immune cells in the blood stream, e.g., monocytes, platelets, leukocytes, dendritic cells and resident phagocytes in tissues, is a source of potential threat of the nanoparticles used in immunotherapy. Additionally, hemolysis, thrombogenecity, and complement activation might affect the nanoparticle distribution and delivery to the intended target site (Leu et al. 1984; Gref et al. 1994; Goppert and Muller 2005).

Based on the mechanism of action, the nanoparticles used in immunotherapy can be broadly classified as immunosuppressive or immunostimulatory in nature. Of this, immunosuppression is needed for treatment of autoimmune diseases, various allergic conditions and for prevention of rejection of the transplanted organs, whereas, immunostimulation is primarily applied for the treatment of cancer and AIDS. In the present communication, the nanoparticle-based immunotherapy has been categorized and discussed based on the above two mechanisms.

2 Nanoparticle Based Immunosuppression

2.1 Autoimmune Disorder

Autoimmune disorder is one of the top ten leading causes of death and women younger than 64 years are the worst affected, with an estimated prevalence of 7.6–9.4% of the world population (Cooper et al. 2009). These diseases can be devastating, as they are chronic and potentially life-threatening. The current treatment protocol for rheumatoid arthritis (RA), systemic lupus erythematosis (SLE), and multiple sclerosis (MS) which include physical therapy, nonsteroidal anti-inflammatory drugs (NSAIDs), corticosteroids, disease-modifying antirheumatic drugs (DMARDs), anti-cytokine therapies, monoclonal antibodies, biological T cell inhibitory function and B cell inhibition (Rosata et al. 2010), etc., have considerable drawbacks. Further, the nonspecific immunosuppressants, e.g., glucocorticoids, cyclophosphamide and azathioprine are associated with an increased risk for infections (Bernatsky et al. 2010; Atzeni et al. 2012) as well as associated with several other adverse effects (Rosman et al. 2013).

In this scenario, barring the use of drugs, the cellular toxicity of various categories of nanomaterials/nanoparticles, e.g., multiwalled carbon nanotubes (Serra and Santamaria 2015) can be deleterious for dendritic cells, causing immunosuppression (Tkach et al. 2011). Similarly, citrate-coated gold, cerium oxide

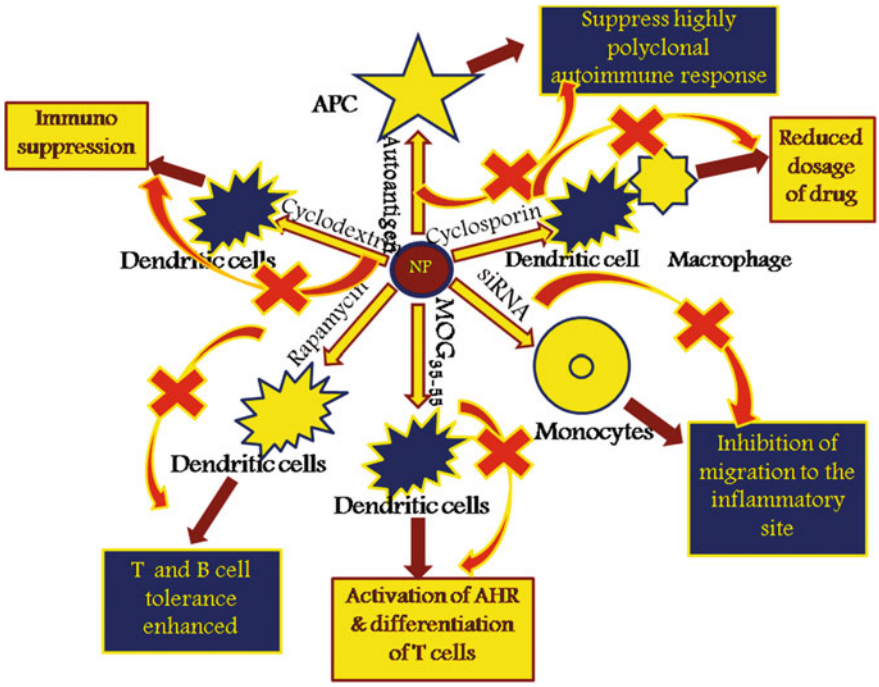

Fig. 1 Mechanism of action of nanoparticle based immunotherapy in autoimmune disorder, based on immunosuppression

(Sumbayev et al. 2013; Chen et al. 2013; Schanen et al. 2013; Pagliari et al. 2012; Hirst et al. 2009), nanoparticles have anti-inflammatory and antioxidative properties, although they have been reported to have organ toxicity to some extent. In this scenario, selective nanoparticles with appropriate functionalization can be loaded with the desired cargo, e.g., immunoregulatory molecules, which enhances the possibility of cellular internalization and improve its biological activity, thereby limiting the toxicity of the bare drug. Hence, nanoparticulate immunotherapy comprising surface with tailor-made physical and chemical properties (Janus nanoparticles) (Kaewsaneha et al. 2013) for autoimmune disorder targeting (Fig. 1) the antigen-presenting cells and T cells, follows the mechanisms as given below.

2.1.1 Delivery of Anti-inflammatory Molecules

Polymeric (polylactide) (Azzi et al. 2010) and liposomal (Schweingruber et al. 2011) nanoparticles have been used to deliver the anti-inflammatory drugs, cyclosporine A, glucocorticoid to dendritic cells/macrophages that leads to sustained release of the immunosuppressant drugs and aids to reduce the dosage of the same. Importantly, nanoparticle-based delivery as above is instrumental in modifying the pharmacodynamics of the payloads, promoting immunosuppression of the macrophages.

For cellular targeting, studies have established the efficacy of functionalized dendrimers (covalently bonded folic acid) to target activated macrophages in type II collagen-induced model of arthritis in rat to deliver methotrexate and promote its possible cure (Thomas et al. 2011). In another work, azabisphosphonate capped dendrimer has been used to induce anti-inflammatory phenotype in monocytes in rheumatoid arthritis in murine model (Hayder et al. 2011).

The delivery of small interfering RNA (siRNAs) to inflammatory monocytes using lipid-like nanoparticles (dia: 70–80 nm) as vehicles, helps to downregulate the monocytes and inhibit its migration to the inflammatory site (Leuschner et al. 2011). The same molecule has also been delivered in vivo, using various nanovehicles including cationic polymers, peptides and inorganic nanoparticles, e.g., gold, quantum dots (Hong and Nam 2014). Further siRNA has been found to render a defined role in allogeneic pancreatic islet cell transplantation (Serra and Santamaria 2015). For inhibition of rejection, caspase-3 specific siRNAs have been used in conjugation with magnetic nanoparticles (Wang et al. 2011, 2012).

2.1.2 Delivery of Autoantigens

An autoantigen is a complex of proteins or a normal protein and might be DNA or RNA recognized by the immune system with specific autoimmune disorder. When administered, such molecules have a major role of modulating the immune system to induce hyporesponsiveness to the existing self-antigens, which is the primary cause of the origin of autoimmune disorder related to the disruption of the immune tolerance of the same (Ngo et al. 2014). In other words, uptake of autoantigens by tolerogenic dendritic cells and macrophages lead to inactivation and eradication of the T cells that are responsible for immunostimulatory responses and in this regard, the advantages of nanoparticle-based delivery of the therapeutic payload is well known (Dobrovolskaia and McNeil 2007; Descotes 2004; Leu et al. 1984; Gref et al. 1994; Goppert and Muller 2005).

Gold nanoparticles (dia: 60 nm) loaded with epitope, myelin oligodendrocyte glycoprotein (MOG 35-55), have been targeted to dendritic cells, helps in the activation of aryl hydrocarbon receptor transcription factor (AhR) that promotes the differentiation of specific type of T cells (Wang et al. 2011). In another work, polymeric nanoparticles have been used to co-deliver autoantigen with an immunosuppressant drug, rapamycin, targeted to dendritic cells, that in turn helps to promote T and B cell tolerance (Yeste et al. 2012; Maldonado et al. 2015; Taner et al. 2005; Hester et al. 2012). For the delivery of cyclodextrin macromolecules, lipid bilayer nanostructures have been used for targeting dendritic cells (Haxhinasto et al. 2008). The effective role of these nanostructures as delivery vehicle of the immunosuppressant, mycophenolic acid, in partially suppressing the onset and progression of lupus by modulating the dendritic cell phenotype has been tested using murine model (Serra and Santamaria 2015).

2.1.3 T Cell Targeting

Here the concept lies in the fact that $CD8^+$ T cell deletion promotes the expansion of autoregulatory T cell memory. Autoregulatory $CD8^+$ T cells targeted to

autoantigen loaded APCs (antigen-presenting cells) helps to suppress the highly polyclonal autoimmune responses. pMHC (multimeric peptide major histocompatibility complex) and nanoparticle conjugate, targeted to T cells produce antidiabetogenic memory in transgenic non obese diabetic (NOD) mice (Look et al. 2013; Tsai et al. 2010; Shameli et al. 2011, 2013). Further, it was observed that another conjugate comprising IGRP206-214 H-2Kd nanoparticles (NPs) could restore normoglycemia in the newly diabetic mice models as above. Hence, the autoantigen loaded APCs are suppressed and killed by the expanded $CD8^+$ T cells through pMHC class I nanoparticle-conjugate therapy (Look et al. 2013; Han et al. 2005; Clemente-Casares and Santamaria 2014; Clemente-Casares et al. 2012).

2.2 Allergy

Immunotherapy is one of the most efficient treatment options in allergic conditions and is widely used for the same. It acts to make the host tolerant to specific allergens, the mechanism lies in inducing allergic symptoms initially, followed by an immediate phase and late phase allergic reduction (Clemente-Casares et al. 2011). In summary, immunotherapy helps to reduce the ratio of Th2/Th1 (Th:T helper cell) and such a change increases the production of interleukin (IL)-10, that in turn activates the B cells to produce immunoglobulins (Ig) G and A, thereby suppressing the secretion of IgE, which is the key molecule for allergic response (Akdis and Akdis 2009). Henceforth, debarring IgE, IgG and IgA effectively binds to the allergen, reasonably reducing the influence and the number of the lymphocytes, basophil, eosinophil, and the neutrophils, that in turn also reduces the inflammatory response.

Despite the above simple mechanism, patients treated using classical immunotherapy comprise inoculation of repeated dose of allergen for a prolonged period, for suppressing the formation of IgE (Gamazo et al. 2014; Broos et al. 2010). Further, subcutaneous immunotherapy has several disadvantages including local allergic reactions, anaphylaxis, etc. (Steinke and Lawrence 2014; Ragusa and Massolo 2004). In this scenario, attempts for safer and more convenient strategies are needed and for the same, nanoparticle conjugates are being used as adjuvants for delivery of proteins and allergen encoding genes for allergy immunotherapy (Amin et al. 2006; Niederberger et al. 2004; Weiss et al. 2013).

The important parameters that decides the efficacy of the nanoparticle to generate a specific immune response in allergic condition depends on its size, chemical composition, selection of the polymer vehicle, surface modification of the same, etc. Hence, a myriad of polymers, including poly (D,L-lactide-co-glycolide) (PLGA), cationic cross-linked polysaccharides, biopolymers (lipid, starch, chitosan etc.) are used for antigen delivery without any structural modification (Weinberger et al. 2013; Kim et al. 1999; Porporatto et al. 2005; Torres et al. 2011; Petersen et al. 2011; Ulery et al. 2009). Using these nanoparticles, the reduction of dosage frequency via oral route has been achieved by coating the nanoparticle with polyethylene glycol (PEG), popularly known as PEGylation, leading to

improvement of its bioadhesive property which helps the residence of the nanoparticles in the mucosa (Ulery et al. 2011). Other benefits of PEGylation includes faster immune response compared to its bare counterpart. Further, higher density of PEGylation leads to smaller size of the nanoparticle that transports the payload across the nasal mucosa, conveniently, compared to the non coated/coated with PEG having lower density (Yoncheva et al. 2005; Bharali et al. 2008). Another category of polymer-based nanoparticles induce the innate immune system and lead to the upregulation of a class of specific proteins of the dendritic cells, e.g., CD40, CD80 and CD86, etc. (Vila 2004a, b; Tamayo et al. 2010).

The role of biodegradable and non-biodegradable polymer-based delivery systems (nanoparticulate) in allergy vaccines is significant (Camacho et al. 2011; Francis and Durham 2004). Of these, the biodegradable nanoparticles can have the morphologies of nanocapsule/nanospheres, depending on the method of synthesis (Wheeler and Woroniecki 2001; Couvreur et al. 2002), augments the immunopotentiating effect of the encapsulated antigens, thereby exhibiting an enhanced efficacy (Juliana et al. 2012). Various natural (chitosan, albumin, gelatin, carrageenan, collagen etc.) and synthetic polymers and copolymers e.g., polylactic acid (PLA), poly(lactide-co-glycolic acids) (PLGA), polymethyl methacrylate (PMMA), poly-ε-caprolactone (PCL), poly(alkylcyanoacrylates) (PACA) can generate the biodegradable vehicles, although, preference is given to the natural polymers only, on account of the possibility of unwarranted byproducts from the later, leading to various side effects. Further, the degradation rate of the natural polymers is very high, leading to faster release of the payload, compared their synthetic counterparts (Madan et al. 1997; Bramwell and Perrie 2006).

The monomer: copolymer ratio of 75:25, in case of PLGA, is the ideal one for a sustained release of antigens and target it to the APCs and in this job, its efficacy has been found to be much higher compared to the bare antigens (Irache et al. 2011; Spiers et al. 2000; Igartua et al. 1998; Eldrige et al. 1991). A less known polymeric system comprising poly (D,L-lactide-co-glycolide) has been effectively used for generating allergen-specific vaccines (Combadiere and Mahe 2008; Batanero et al. 2002).

Compared to the polylactide group of delivery systems, the anhydride group generates less toxic byproducts and as a result, are more acceptable for the purpose (Partidos et al. 1999). The copolymers, methyl vinyl ether and maleic anhydride have an excellent ability to develop strong bioadhesive interactions with the components of the gut mucosa (Mallapragada and Narasimhan 2008), facilitating its biodistribution, that in turn influences its affinity towards the intestinal mucosa. Hence, this category of nanoparticle is suitable for oral immunotherapy and this observation is authenticated by the work of C. Gamazo et al., who had obtained an increased level of Th1 and Th2 markers in animal model (Arbos et al. 2003; Gomez et al. 2006, 2007, 2009), when administered via oral route. Several other advantages of using the above biodegradable polymeric system includes (a) patient compliance (b) safe and viable route of administration (c) comprises a reactive copolymer that facilitates the system to link with different immunostimulants, e.g., proteins/lipopolysaccharides (Irache et al. 2010).

Analogous to the above, a polypeptide, poly (γ-glutamic acid) (γ-PGA) has been for entrapment of proteins, e.g., ovalbumin which is nontoxic against the corresponding cell line (Ochoa et al. 2007) and at the same time activates the adaptive humoral immune response (Akagi et al. 2005).

Chitosan, a biodegradable, biocompatible natural polymer is worth mentioning, on account of its versatility in structure, function, and properties that not only helps to maintain the immunogenicity of the antigens, when used as a delivery vehicle, but also makes it a suitable candidate for various vaccine formulations (Soppimath et al. 2001; Illum et al. 2001; Bowman and Leong 2006; Masotti and Ortaggi 2009; Nagpal et al. 2010; Van der Lubben et al. 2001). These nanoparticles are being developed widely for targeted delivery of gene, plasmid DNA, and RNA (Borchard 2001; Jiang et al. 2007; Rudzinski and Aminabhavi 2010; Il'ina and Varlamov 2005) along with soluble antigens, applied for tetanus toxoid and diphtheria in general, in the form intranasal vaccination (Vila et al. 2002, 2004a, b). Despite the above, a matter of concern in relation to this nanoparticle-based delivery vehicle is its lack of solubility at physiological pH condition, leading to poor permeability and absorption of the encapsulated payload (Domard et al. 1986; Kotze et al. 1999). Hence, alternative derivatives of chitosan/conjugation of the same with biodegradable polyelectrolyte polymers are being explored for mucosal vaccination (Borges et al. 2005, 2007, 2008).

A few nondegradable nanoparticles that exhibit advantages technically include both organic and inorganic materials (Combadiere and Mahe 2008; Peek et al. 2008; Ho et al. 2011). Of significance is the group of polymers that can be surface functionalized, leading to the conjugation of a variety of antigens that play a key role in the improvement of the immunogenicity for an extended period of time (Combadiere and Mahe 2008; Peek et al. 2008; Fifis et al. 2004). Despite the above advantages, importantly, the toxicity of nondegradable inorganic/polymeric nanoparticles, aggregation at the site of action, and lack of overall efficacy in cross presenting the antigens, makes them poor candidates compared to their degradable counterparts, both in in vitro and in vivo conditions (Kwon et al. 2005a, b).

2.3 Allotransplantation

The key role played by the human leucocyte antigen (HLA) to identify pathogens and display them for the recognition of appropriate T cells that in turn is proactive in determining the compatibility of donors for organ transplant via a defined mechanism (Janeway et al. 2001a, b), is a complex and less understood phenomenon. Here, the tolerance or rejection of the transplanted graft depends on a few factors associated with the host immune system, e.g., APCs, antibodies, helper and cytotoxic T cells, signaling mechanisms and cytokines released by them. Any mismatch in the above leads to mortality of the recipient. Further, for a reduced risk, patients are needed to undertake immunosuppressive drugs for a long period of time that leads to major threats (Fisher et al. 2015; Niethammer et al. 1999; Naesens 2009; Errasti et al. 2010). Hence, the current trends of nanoparticle-based drug

delivery/immunotherapy to the target tissues is an effective means to enhance the efficacy of the appropriate drugs along with reduced side effects and prolonged survival of the allotransplant (Fisher et al. 2015).

The chemical composition, molecular weight, degree of cross linking, size, etc., are some of the most significant parameters that influences cellular uptake and thereby its efficacy in mitigating the problem. The surface modification of inorganic/polymeric nanoparticles is easy, that allows covalent bonding of antibodies/receptors of the target cells/tissues, leading to delivery of the therapeutic payload at the site of action, exactly (Danhier et al. 2012; Kohane 2007; Glowacki et al. 2014; Balmert and Little 2012). In allotransplantation, the use of nanoparticle based therapeutics is primarily applied encompassing the drugs that have severe toxic side effects/poor bioavailability (Naesens et al. 2009; Hebert 1997).

2.3.1 Calcineurin Inhibitor-Nanoparticle Conjugate

Calcineurin is a calcium and calmodulin dependent serine or threonine protein phosphatase that can be blocked by immunosuppressive drugs and potentially activates the T cells. It upregulates the gene expression of IL-2, which stimulates the growth and differentiation of the T cells. Hence, inhibition of calcineurin function with the aid of suitable drugs leads to immunosuppression, which is a prerequisite in allotransplantation procedure.

PLGA nanoparticles were the first of its kind to entrap cyclosporine (a calcineurin inhibitor), although the release profile is essentially size dependent, when tested in vivo via subcutaneous injection, using murine model (Sánchez and Alonso 1993; Sánchez et al. 1995; Sanchez and Alonso 1995). The same nanoparticle has been extensively used for prolonging the release of cyclosporine drug up to a period of 30 days (Yoshikawa 1996). Interestingly, using nanocarrier, there is substantial enhancement in the delivery of immunosuppressant drugs, sometimes up to a level of 20 times or more, compared to the conventional route of administration (Yoshikawa 1996). Among the other polymers, PLA has been used as carrier of cyclosporine drug that exhibited a sustained release profile for a period of 30 days, for treatment of arthritis in animal model (Urata and Nakano 1999). Further the advantage of PEG coupled PLA nanoparticles is enhanced hydrophilicity when conjugated with cyclosporine drug, could inhibit T cell proliferation, the mechanism lies in phagocytosed nanoparticles as above, by dendritic cells that has been shown to suppress T cell proliferation further (Balmert and Little 2012; Azzi et al. 2010). Importantly, it has been seen that natural counterparts (chitosan/fibrin etc.) have much better properties w.r.t genotoxicity, cytotoxicity and hemocompatibility compared to the synthetic nanoparticles (Praveen et al. 2012; De et al. 2001). In this regard, specifically, for the prevention of liver rejection, PLGA-based nanoparticles have been used for controlled and sustained delivery of tacrolimous (calcineurin inhibitor), commonly called as FK506, on account of its potency and superiority compared to the other drugs of similar category (Fung et al. 1996; Abou-Jaoude et al. 2005; Miyamoto et al. 2004; Wang et al. 2004; Lamprecht et al. 2005a, b). In another application, for prevention of hindlimb allograft transplant rejection,

triglycerol monostearate (TGMS) loaded with tacrolimous (sustained release) has been used in rat model and the result is promising (Gajanayake et al. 2014).

2.3.2 Rapamycin-Nanoparticle Conjugate

These systems are multifunctional in nature that they play a pivotal role in inhibiting T cell proliferation and has an important role in dendritic cell maturation and others (Saunders et al. 2001; Fischer et al. 2009; Hackstein et al. 2003; Turnquist et al. 2007; Glowacki et al. 2015). Delivery of rapamycin using poly (ethylene glycol)-b-poly (epsilon-caprolactone) (PEG-PCL) for a prolonged period has been described in injectable form (Forrest et al. 2006). Also, targeted delivery of the above drug to the dendritic cells, using PLGA-based nanoparticles has been carried out (Jhunjhunwala et al. 2009). Similar work has also been reported using smaller PLGA nanoparticles that has much higher efficacy compared to free rapamycin in that they reduced the immunostimulatory capacity of dendritric cells (Haddadi et al. 2008; Das et al. 2008). Some other research groups have synthesized rapamycin loaded chitosan-PLA nanoparticles that has been used for prevention of rejection of corneal allotransplantation, and showed superior properties (Yuan et al. 2008).

2.3.3 Mycophenolate Mofetil (MMF)-Nanoparticle Conjugate

This specific antiproliferative immunosuppressant has been used in prolonging full thickness murine skin transplants via PD-L1 mediated mechanism, despite a much reduced dosage compared to rapamycin, along with no detectable toxicity (Shirali et al. 2011). In another work, comparison between PLGA-MMF nanoparticle and MMF nanogel has been carried out and it has been seen that MMF nanogel is taken up by the dendritic cells and PLGA-MMF nanoparticle have similar immunosuppressive effect, in expense of higher dose (Look et al. 2014).

2.3.4 Genetic Material-Nanoparticle Conjugate

The evolving interest in gene therapy for immunomodulation/immunosuppression can be extended using specific nanoparticles to have siRNA/oligonucleotide conjugate to downregulate the appearance of specific genes in the inflammatory pathway. Research groups have used antisense CD40, CD80, and CD86 oligonucleotides directed to dendritic cells in the treatment of type 1 diabetes mellitus that progressed far, uptil clinical trial (Machen et al. 2004) and much more promising results are expected on extended work based on the above mechanism.

3 Nanoparticle Based Immunostimulation

3.1 Cancer

Since long, immunotherapy has been used and explored as a possible therapeutic approach to combat cancer, despite some disappointing failures in the above

treatment strategy on account of the lack of proper understanding (Machen et al. 2004; Fan and Moon 2015). Very recently, the dendritic cell based vaccines (Park et al. 2013) and immune checkpoint blockade agent have come up in a big way to boost our immune system largely to eliminate the possibility as well to eradicate tumour formation (Mellman et al. 2011; Couzin-Frankel 2013). However, the above approaches, used by conventional treatment procedures have often failed to produce sufficient immunostimulation and thereby has limitation (Rosenberg et al. 2004). Hence, newer strategies are required to deliver the tumour antigens and adjuvants to the APCs that can aid in up regulation of the immune responses to eradicate the tumour cells. In this aspect, the role of nanoparticular delivery of conventional chemotherapeutics targeted to the tumour site has been established widely (Kumar and Mohammad 2011; Fan et al. 2013; Hu et al. 2013). Further, nanoparticles conjugated with immune stimulating components including co-delivery of tumour antigens and adjuvants to the APCs also elicit diverse promises over the conventional therapeutics in cancer immunotherapy (Moon et al. 2012; Irvine et al. 2013; Goldberg 2015). The superiority of nanocarrier-based immunotherapy lies in (i) co-delivery of the vaccine components to the target APCs (ii) tailor-make size and surface chemistry for tissue-specific targeted delivery (iii) generation of artificial APCs for T cell immune response by surface engineered nanoparticles (iv) design of delivery systems to reverse immune suppression, by targeting the immune checkpoint molecules (v) nanoparticles loaded with active components can undertake T cell stimulation thereby improving antitumor efficacy (Leleux and Roy 2013; Silva et al. 2013; Dewitte et al. 2014; Nune et al. 2011; Swartz et al. 2012; Irvine et al. 2015; Sahdev et al. 2014; Sunshine and Green 2013; Shao et al. 2015).

The size of the nanoparticles plays a key role in modulating the immune response of cancer patients, in the way that it influences the cellular uptake and lymphatic trafficking when in the size range of 5–100 nm. The larger particles having size >500 nm remains trapped within the extracellular matrix, subsequently taken up by the APCs followed by passing on to the draining lymph nodes (Irvine et al. 2013; Manolova et al. 2008). Further, the action of the nanoparticles in the lymph nodes is also size dependent in that, the larger particles remain taken up by the macrophages mostly whereas the smaller particles are accessible by the dendritic cells residing in the lymph nodes (Irvine et al. 2013). This corroborates the finding of research groups who found higher lymphatic drainage and lymph node retention of poly (propylene) disulphide nanoparticles having size <50 nm (Reddy et al. 2006). Studies have also shown the influence of the morphology/shape along with size of the nanoparticles to elicit specific immune response (Mueller et al. 2015). Another important criteria to be considered for nanoparticle-based immunotherapy includes particle surface charge that influences the lymphatic drainage and retention (Hartwell et al. 2015; Zhan et al. 2012). The cationic nanoparticles interact with the anionic proteins in the extracellular matrix and are ultimately phagocytozed by the APCs to reach the lymph nodes (Hartwell et al. 2015). On the contrary, anionic/neutral nanoparticles can be easily targeted to the draining lymph nodes via lymphatic vesicles or can be engulfed by the dendritic

cells (Rahimian et al. 2015). Importantly, as it is evident that the immune cells in general identify the pathogens from their surface architecture, hence, mimicking the same, the surface engineering to impart similar surface characteristics as above, e.g., highly repetitive surface patterns can lead to activation of a complementary cascade pathway leading to an enhanced immune response (Bachmann and Jennings 2010).

The mechanism of immunosuppression by tumour cells by downregulation of tumour antigens and thereby evading immune responses is well known (Seliger et al. 2000; Zou 2005). Tumour cells secrete the immunosuppressive cytokines, IL-10, tumour growth factor (TGF)-β that in turn inhibit the proliferation of cytotoxic T lymphocytes (CTLs) and promotes the differentiation of regulatory T cells (TREGs) that has an active role in deactivation of CTLs in conjugation with macrophages and myeloid-derived suppressor cells (MDSCs) (Lindau et al. 2013). Hence, the tumour cells successfully create an immunosuppressive microenvironment where CTLs cannot invade. To combat such situation, there is a need of immunostimulation or enhance the immune activation, which can be achieved via the use of nanoparticle-based immunotherapy (Fig. 2).

3.1.1 Delivery of Epigenetic Modulator

Considering the limitation of the epigenetic modulators in immune modulation in tumour cells on account of their low solubility, short half-life, prompt degradation in circulation, poor cellular uptake and nonspecific toxicity, appropriate delivery systems is the need of the hour. In this regard a popular epigenetic modulator,

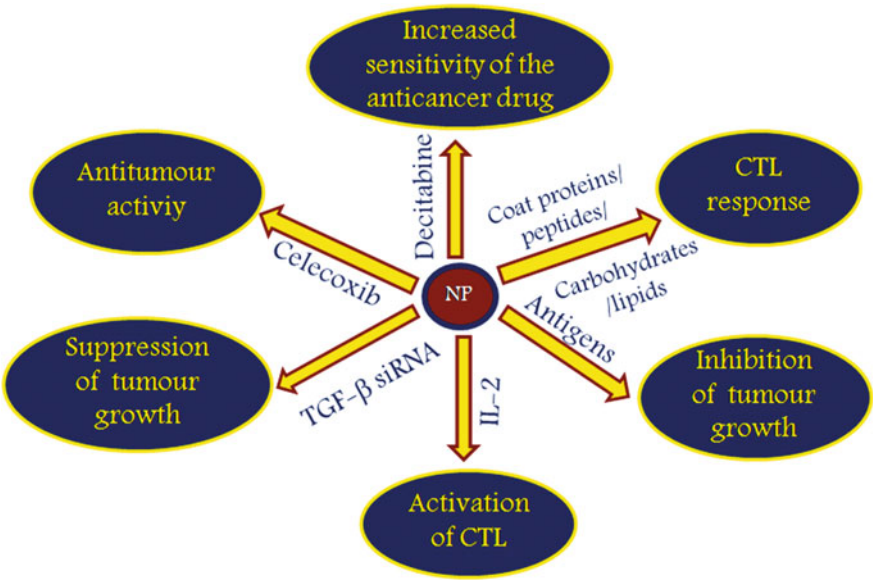

Fig. 2 Mechanism of action of nanoparticle based immunotherapy in cancer, based on immunostimulation

vorinostat that inhibits histone deacetylase to modulate the gene expression and for the treatment of cutaneous T cell lymphoma, has been conjugated with poly (ethylene oxide) (PEO)-polylactic acid (PLA) that improved the pharmacokinetic and dynamic properties of the therapeutic to a large extent (Mohamed et al. 2012; Tran et al. 2014). In addition to this, it has been observed that efficacy of the modulators as above to sensitize tumours to chemotherapy is enhanced multifold on conjugation with nanoparticles, e.g., nanoparticle-decitabine conjugate that acts as a DNA-methyltransferase enzyme inhibitor increases the sensitivity of anticancer drug doxorubicin to breast cancer cells (Su et al. 2013).

3.1.2 Delivery of Vaccine

In soluble form, the components of pathogenic beings like bacterial coat proteins, carbohydrates peptides and lipids, administered to excite the immune system is known as vaccination. They have limitations of poor immunogenicity and short half lives in vivo, and hence are unable to reach the target cells. Hence to overcome the above disadvantages and enable the delivery of the antigens to the cytosol through a specific pathway, that activates the CTLs in cancer immunotherapy. In this regard, an organic–inorganic hybrid nanoparticle (lipid-calcium phosphate, LCP) delivers the cargo as above and hence induces potent CTL response. In another attempt, the same nanoparticle has been surface modified to deliver different impermeable antigens targeted to the lymph node and as a result obtained higher concentration of the payload and thereby higher efficacy pertaining to inhibition of tumour growth in specific cases of cancer (Xu et al. 2013). There is also a mention of a cationic lipid nanoparticle that has been conjugated with antigenic peptide to activate the lymphocytes which in turn infiltrates into the tumour site (Vasievich et al. 2012). Such nanoparticle-based delivery systems also exhibit superiority in tumour regression in an effective manner when it has been used to deliver multiple peptide/antigen epitopes, e.g., tyrosinase-related protein (Trp-2), glycoprotein (gp 100), retroviral envelope protein (p15E), etc. (Tan et al. 2014). In another approach, PLGA nanoparticles have been conjugated with antibodies to target various surface receptors of dendritic cells which in turn produce IL-12 and helps in the activation of $CD8^+$ T cells which produces interferon (IFN-γ) cytokine. As evident, the above surface modified nanoparticles demonstrated much better activation of CTLs compared to similar nanoparticles without surface modification (Cruz et al. 2014). Further, in one more case, PLGA nanoparticle, conjugated with antibody (anti CD40) to target dendritic cells for co-delivery of OVA (ov albumin) with toll like receptor 3 (TLR3) agonist (Rosalia et al. 2015). In another work, siRNA conjugated with cationic lipid nanoparticles and also PEI/PG/siRNA complexes demonstrated promising result in suppressing the peptides of antigen (Rosalia et al. 2015; Hobo et al. 2013; Roeven et al. 2015; Cubillos-Ruiz et al. 2009).

3.1.3 Delivery of Cytokines

The cytokines having high immunostimulatory nature, TNF-α, IFN-γ and IL-2 are rapidly excreted through urine and are enzymatically degraded when used in high concentration, which is necessary for appropriate therapeutic effect. In this, the

toxic effect of the higher dose can be taken care of by introduction of lipid- and polymer-based nanoparticles (Teo et al. 2015) that helps in targeted delivery, prevents degradation and improves pharmacological properties of the active molecule (Christian and Hunter 2012; Anderson et al. 1991; Kedar et al. 1997, 2000). The delivery of liposome in conjugation with IL-2 and IL-12 activated cytotoxic T lymphocytes against the tumour cells and demonstrated the suppression of tumour metastasis (Van der Veen et al. 1998; Shah and D'Souza 1999). However, the PEGylated liposomes help to increase the circulation half-life, along with decreasing the opsonization to the target site. Nanoparticles have a significant role which reduces the tumour growth in comparison to soluble cytokines when it is not conjugated with the same (Loeffler et al. 1991). When cancer vaccine and cytokines have been co delivered, it increased the activity of T cells and regression of the tumour growth in phase I clinical trial (Kedar et al. 1994; Neelapu et al. 2007).

3.1.4 Nanoparticle-Based Targeted Delivery to the Tumour Microenvironment (TME)

Nanolipogels of cyclodextrin demonstrated inhibitory action of tumour growth factor (TGF)-β in tumour microenvironment by the delivery of IL-2. Using a liposome based nanoparticle in conjugation with TGF-β siRNA, co-delivering with cancer vaccine was able to suppress the TGF-β expression. Such combined therapy activates the $CD8^+$ T cells and suppress the tumour growth in comparison with vaccine treatment alone (Neelapu et al. 2004).

The disadvantages of small molecule nitric oxide (NO) donors meant for targeting myeloid derived suppressor cells (MDSCs) are manifold including their poor permeability and retention, along with their toxicity. All these have been taken care of by the use of silica nanoparticle based formulation comprising an NO releasing derivative that inhibits the growth of specific cancer cells (Xu et al. 2014). In this regard, the limitation of ursolic acid (UA) that has multiple actions in that they are antiinflammatory, antitumour and hepatoprotective in nature, can be conjugated with methyl-PEG (mPEG)-PCL methyl-poly (ethylene) glycol poly caprolactone block copolymers) exhibits significantly increased apoptosis of gastric cancer cells along with enhanced inhibition of cyclooxygenase (COX-2) activity compared to bare UA (Stevens et al. 2010). Using a biodegradable nanoparticle comprising PLGA and a potent COX-2 inhibitor, celecoxib, improved the antitumour activity against specific tumours, e.g., glioma tumour (Zhang et al. 2013). In another work, all trans retinoic acid (ATRA) is a potent differentiating agent of cancer stem cell that can be co-delivered with doxorubicin in the form of nanoemulsion, helps in cancer stem cell differentiation and subsequently could suppress the tumour growth (Kim et al. 2011). Using pH-sensitive dendrimers of poly (amidoamine) (PAMAM), when delivers ATRA, it inhibits the proliferation of specific carcinoma cell lines (Sun et al. 2015).

PEGylated single-walled carbon nanotubes when conjugated with specific ligand to target intratumoral Tregs demonstrated high accumulation of single-walled carbon nanotubes in the intratumoral nonTregs/splenic Treg (Wang et al. 2014).

The disadvantages of using bare bisphosphonates for inducing apoptosis in cancer cells is manifold: short half-life of the drug along with fast accumulation in

the bone (Sacchetti et al. 2013). These have limited their application as antitumour agent which can be taken care of with the help of nanoparticulate delivery vehicle, e.g., clodronate has been encapsulated in lipid vesicles exhibiting superior properties in inhibition of tumour growth (melanoma) that has been studied using animal model (Baay et al. 2011). In another work, depletion of macrophages was found by using zoledronate, a PEGylated self assembled nanoparticle, targeted to red blood cells (Gazzaniga et al. 2007). Mannose receptor of macrophages can also be targeted for the delivery of the payload using PLGA nanoparticles with sheddable PEG that demonstrated an improved accumulation of nanoparticles in tumour-associated macrophages in comparison to its non-sheddable counterpart for obvious reasons (Sabatino et al. 2014).

3.2 Acquired Immune Deficiency Syndrome (AIDS)

AIDS is a global pandemic, taking into account the sub-Saharan Africa which is the most affected one (Zhu et al. 2013), since the appearance of the disease in the year 1981 followed by identification and documentation of the virus in 1983 (Cohen et al. 2008; Blattner et al. 1988; Gallo 2002; Gallo and Montagnier 2003). As of 2014, approximately 37 million people have been infected by this deadly virus worldwide with the number of new infections being about 2 million each year and is the leading killer disease of adults worldwide (Montagnier 2002). With innumerable cases being added every year, the disease has caused tremendous social and economic damage worldwide, specifically in the developing countries, sub-Saharan Africa is most heavily affected.

HIV (human immunodeficiency virus) is a retrovirus and a member of the genus lentivirus. It is the cause of a series of diseases known as AIDS (acquired immune deficiency syndrome) that primarily infects and destroys $CD4^+$ T cells, macrophages and dendritic cells of the immune system (Furin et al. 2008). Lentivirus is responsible for prolonged illness with long incubation period (Alimonti et al. 2003). These viruses are transmitted as single-stranded, positive-sense, enveloped RNA viruses. When it enters into the target cell, the retroviral genome is converted to DNA by a viral reverse transcriptase which is transported together with the viral genome in the virus structure. The resulting viral DNA is intercalated into the nucleus of the cell and is incorporated into the cellular DNA by a viral integrase enzyme and host co-factors (Lévy 1993). Once integrated, the virus avoids detection by the host's immune system as it becomes dormant (Smith and René 2006).

The HIV lentivirus has two different (hybrid) mechanisms of spreading: cell-free and cell-to-cell. Of these, in the first route, the virus particles erupt from an infected T cell and thereby enter into the bloodstream or extracellular fluid of the host and hence, infect another T cell following an encounter with it (Martínez 2010). Without the intervention of any extracellular fluid as above, the virus can also be transmitted from one cell to another, directly to follow the cell to cell spread

(Zhang et al. 2015; Jolly et al. 2004). Such spreading mechanism explains the ongoing replication of virus against antiretroviral therapies (Sattentau 2008).

There are two types of virus that has been characterized: HIV1 and HIV2, of which the former one is much more virulent and infective, being cause of majority of the HIV infections globally (Sigal et al. 2011).

On entering, the virus rapidly multiplies and becomes abundant in the peripheral blood of the infected person, followed by a marked drop in the number of $CD4^+$ T cells, which is associated with the activation of $CD8^+$ T cells that in turn is involved in seroconversion or killing of the HIV infected cells and subsequent production of antibodies and is important in controlling the virus level. A good response of the $CD8^+$ T cell is thought to slow down the disease progression, although, it is not capable of the virus elimination (Gilbert et al. 2003).

The mechanism of $CD4^+$ T cell depletion takes place via acute and chronic phases (Pantaleo et al. 1997). On being infected, major loss of theses specific cells takes place during the first four weeks of infection, specially in the intestinal mucosa that comprises majority of the lymphocytes found in the body. HIV uses C-C chemokine receptor type 5 (CCR5), a protein expressed by the mucosal $CD4^+$ T cells, as co-receptor to enter the cells and destroys them (Mehandru et al. 2004; Brenchley et al. 2004). This is followed by a vigorous immune response/activation, leading to release of pro inflammatory cytokines that in turn induces ongoing HIV replication, ultimately causing complete breakdown of the immune surveillance system of the gastrointestinal mucosal barrier. Such condition weakens the immune system and allows opportunistic infections (Olson and Jacobson 2009; Aliberti 2011) including influenza or mononucleosis-like illness.

It was not until the mid-1990s that a treatment of AIDS that could improve the quality of life of the patients could be achieved. It was none other than 'highly active antiretroviral therapy' or HAART comprising combination of three or more classes of different drugs that had revolutionized the AIDS treatment procedure (Brenchley et al. 2006; Walensky et al. 2006), although, the major difficulty in this is the failure of the treatment on account of lack of patient compliance in many cases (Richman et al. 2009). It has been observed that patients fail to maintain the prescribed daily dosage of the drug and hence, the therapeutic index of the drug is not reached causing replication of the lentivirus (Harrigan et al. 2005; Richman et al. 2009; Chun et al. 1999). Another major issue is the patient specific resistance to the combination drugs of HAART treatment procedure, on account of the continuous mutation leading to vast genetic diversity of the HIV1. Such a situation is taken care of by individualized therapy, by identifying a combination of drugs that will have higher efficacy for every patient (Marsden and Zack 2009). Further, the side effects and toxicity of the drugs administered is also a major issue of concern (Harrigan et al. 2005).

The most modern approach of the treatment procedure also could not remove the virus completely, on account of its presence as 'latent reservoirs' at specified zones of $CD4^+$ T cells (Harrigan et al. 2005; Chun et al. 1999). Apart from being a resource as above, the macrophages play an important role in enhancing the mutation procedure by genetic recombination (Sax et al. 2007). In this regard,

nanoparticulate delivery of antiretroviral drugs has come up as an entirely new solution to the existing treatment procedure, using the same drugs by modulation of their pharmacokinetic properties ($t_{1/2,}$ k_{el}, k_a, V_d, AUC, MRT, etc.) to a large extent. This has also aided in targeted delivery of the above drugs to specific cells, e.g., $CD4^+$ T cells and macrophages along with the latent reservoirs (Lamers et al. 2009; Vyas et al. 2006).

It is well known that a massive immunosuppression occurs due to the depletion of the $CD4^+$ T cells in acute HIV infection that has a vital role in retaining the normal immune functions of the body. In such condition, on the contrary, $CD8^+$ cytotoxic T cells remain normal, although, the primary activity of B cell, which is antibody production, gets slowed down (Amiji et al. 2006). In chronic stage, along with the $CD4^+$ T cells, $CD8^+$ T cells also become depleted leading to the loss of activity of the B cells, natural killer cells and macrophages (McMichael et al. 2010). Hence, fortification of the immune system can be a good treatment option via immunotherapy for HIV infected patients (Pett 2009; Cohen 2007; Rinaldo 2009).

The potent immunomodulatory agents including various cytokines, e.g., IL-2, IL-7, IL-15, antigens and vaccine formulations are being used in the immunotherapy approach as above (McMichael et al. 2010; Pett 2009). In this, the delivery of the above immunogenic factors have been made generally through virus or ex vivo dendritic cells, which involves various risk factors along with high cost and complicated procedure, when considered in clinical studies (Gandhi and Walker 2002). Hence, there is an immense scope for nanoparticle-based targeted delivery of the immunomodulators to the dendritic cells in vivo (Pett 2009).

Polymeric nanoparticles have been found to be suitable as nanodelivery vehicle for small molecule, protein and DNA targeted to the dendritic cells for immunotherapy. In this regard, poly propylene sulphide nanoparticle stabilized with PEG have been used and was accumulated in the dendritic cells in the lymph node (Tacken et al. 2007). In another work, a cross-linked multifunctional polymeric nanoparticle have been used to target the dendritic cells for the delivery of protein and small molecules cargo (Reddy et al. 2006). Ovalbumin, a protein antigen is delivered to the dendritic cells that activate the $CD8^+$ T cells and $CD4^+$ T cells, demonstrated in fluorescence microscopy.

The use of PLGA in delivery of antigens to bone-marrow derived dendritic cells in vitro demonstrated the immunotherapy (Hori et al. 2008). In another study, PLA nanoparticle conjugated with HIV p24 protein was efficiently delivered to the dendritic cells, ex vivo (Elamanchili et al. 2004) for maturation of the same. This work can be extended in in vivo models for targeting of the dendritic cells, eventually.

Among all the above nanotechnology based approaches adapted for HIV immunotherapy, the most significant and successful attempt that needs mention is the 'Derma Vir' patch. It comprises polyethyleimine mannose (PEIm), glucose conjugated with HIV antigen coding DNA plasmid having size $\sim$100 nm, is applied as a patch on the skin. The nanoparticles permeate via the porous subcutaneous layers for their onward delivery to the epidermal langerhans cells which subsequently proceed to the lymph nodes, inducing the cellular immunity via an

established mechanism (Aline et al. 2009). Importantly, this is the first nanotechnology based treatment procedure that has completed Phase I clinical trial successfully and the Phase II trial is under progress.

The most important issue that needs to be considered for generation of AIDS preventive vaccine are the vast diversity of the virus strain that hinders to produce the humoral and cellular immune responses, e.g., formation of the neutralizing antibodies and the cytotoxic T cells (Tewodros et al. 2010). Here, the role of protein antigens is obvious (Barouch 2008), and the delivery of exogenous antigens to the APCs requires 'cross presentation' in order to activate the $CD8^+$ cytotoxic T cells (Guermonprez et al. 2002). The simultaneous action of the cytosolic delivery of antigens and its cross presentation inhibits the generation of HIV intracellular antigen vaccine, altogether (Trombetta and Mellman 2005).

The potential advantage of nanoparticle-based vaccine delivery over the conventional systems is that, the former can be administered via both oral and nasal routes to enhance the mucosal immunity compared to the later, where bare intramuscular administration only is possible (McHeyzer-Williams and McHeyzer-Williams 2005). Importantly, detailed in vivo studies have revealed that a monovalent encapsulated antigen is unable to impart sufficient immune response for generation of an effective HIV vaccine (Csaba et al. 2009; Singh and Bisen 2006; Wagner et al. 2007; Watson et al. 2009; Letvin 2006) and hence, there is enough scope for improvisation in this regard.

In another attempt, MF 59, an oil-in-water emulsion based nanoparticle (size <250 nm) has been used for delivery of specific DNA molecules in order to generate stronger antibodies and cellular responses, in comparison with bare DNA molecule (Burton et al. 2004; Ott et al. 1995; Copland et al. 2005; Tritto et al. 2009). The combination therapy of the DNA plasmid for the proteins gp 140, group-specific antigen or gag, twin-arginine translocation or tat demonstrated complete protection against HIV infection, as observed in in vitro bioassays (Leung et al. 2004). Further, immunization with Cpg oligonucleotide, targeted to envelop proteins and MF 59 demonstrated higher efficacy compared to the bare nanoparticle (Brave et al. 2007). Similarly, another oil-in-water emulsion based nanoparticle delivered gp 120 protein (nasal route) generated significant antibody and cellular immune response (Burke et al. 2009).

A polymer-based system comprising PLA, coated with HIV p24 protein produced substantial antibody reaction in several animal models and cytotoxic T lymphocyte response in mice (Bielinska et al. 2008). The suitability of PLA surface to functionalize has been made use of by co-adsorption of a couple of HIV proteins to generate multivalent vaccine for appropriate antigen–antibody reaction (Ataman-Onal et al. 2006). In continuation a myriad of polymer-based nanoparticles comprising PLGA, polystyrene, poly-γ-glutamic acid (Wang et al. 2008) and many others have been used after multiple conjugation/coating to elicit the requisite immunomodulatory action (Guillon et al. 2007; Miyake et al. 2004; Kawamura et al. 2005; Akagi et al. 2007; Wang et al. 2007), although major effort should focus on the fabrication of an appropriate system to boost up the immune cells to combat the invasion by the HIV pathogen.

4 Conclusions

As evident, our body is fortified with an in situ policing system that safeguards us from all kind of unwarranted external invasions and internal anomalies. A minute slip in any of the components of the same might lead to major disorders as has been discussed in detail. In such situations, immunomodulation or tailor-making our own immune system is the need of the hour and herewith, the invincible nanotechnology has a key role to play. It has been observed that nanoparticle-based delivery of potent drugs or biomolecules offer to reduce dosage and hence dose-related toxicity in general, apart from activation of the dendritic cells and in turn improvement in the T and B cell tolerance so to induce immune suppression in autoimmune disorders. On the contrary, in the deadly diseases of cancer or AIDS, upregulation of the immune response or immunostimulation by delivering the tumour antigens and adjuvants to the APCs is a prerequisite for complete eradication of the tumour cells. In this regard, nanoparticles with their vast diversity in surface characteristics, size, shape, hydrophobicity, hydrophilicity, steric effects, etc., conjugated/equipped with the suitable immunosuppressants/immunostimulants have been playing a key role in tailor-making our immune system, as needed for the treatment of the diseases that originate on account of disruption of the normal functions of the immune system. The nanoparticles used for the above purpose are primarily polymeric in nature (natural/synthetic, biodegradable/nonbiodegradable) with a few exceptions of a few dendrimers and inorganic metal oxide/metallic nanocarriers. In both immune suppression/stimulation, they have been designed and orchestrated to reach the target site to deliver the cargo, exhibiting high efficacy with minimum or null toxicity, enhancing the pharmacokinetic properties to a large extent compared to the bare drug. Hence, as discussed, the treatment option of 'nanoparticulate immunotherapy' for some of the chronic and deadly diseases of twenty-first century paves a room for hope and promises for lot more exploration before it turns into a standard procedure applicable en masse.

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