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ADVANCES IN NANOMEDICINE FOR THE DELIVERY OF THERAPEUTIC NUCLEIC ACIDS

EDITED BY SURENDRA NIMESH, RAMESH CHANDRA, NIDHI GUPTA

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Advances in Nanomedicine for the Delivery of Therapeutic Nucleic Acids

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Advances in Nanomedicine for the Delivery of Therapeutic Nucleic Acids

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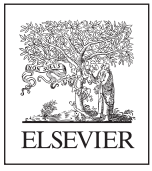

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Foreword

The book, *Advances in Nanomedicine for the Delivery of Therapeutic Nucleic Acids*, edited by Dr. Surendra Nimesh, Dr. Nidhi Gupta and Prof. Ramesh Chandra (Nimesh *et al*., 2017), is the second book by these authors on this subject. This new book is a reflection of the rapid development in this area. It contains complete updates and an overview of every aspect of this area since the previous book.

This book begins with an introduction on nanomedicine for therapeutics, including the properties of nanoparticles and nanoparticle-based strategies for overcoming *in vitro* and *in vivo* barriers. The more general aspects of nanoparticles as nucleic acid delivery vectors and methods for the characterization of nanoparticles are then described. This is followed by a brief account of various nanoparticles employed for *in vitro* and *in vivo* gene delivery studies.

The chapters that follow describe various specific approaches in detail. This includes nanoparticle-mediated studies of *in vitro* and *in vivo* gene silencing for gene therapy. Gene silencing is a naturally occurring mechanism that takes place in the cytoplasm of the cell to knock down the function of malfunctioning genes. The next area is the use of nanoparticles for the delivery of locked nucleic acids for gene therapy. This is followed by a discussion on nanotechnology-based vectors for the delivery of therapeutic DNAzymes. A DNAzyme is a catalyst for the breakdown of target mRNA sequences.

The last part of the book covers three important areas. (1) The first is pharmacokinetics and the biodistribution of nanoparticles. This chapter outlines the important topic of strategies for detection and estimation of toxicity of nanoparticles employed for nucleic acid delivery. (2) The next chapter describes the regulatory aspects of nanoparticulate-mediated nucleic acid delivery systems. (3) The book ends with an important chapter on the update of studies undergoing clinical trials, followed by a conclusion and future prospects.

The initial publication (Chang, 1964) has evolved into many different areas of artificial cells (Chang, 2005, 2007) including nanomedicine (Chang, 2013). *Advances in Nanomedicine for the Delivery of Therapeutic Nucleic Acids* is a detailed description and update of one of the many areas of nanomedicine. The editors have contributed greatly in the research and development in this specific area. This book reflects their experience and expertise.

TMS Chang

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Preface

Nanotechnology has been constantly gaining pace in multidisciplinary fields, attributing to its wide range of beneficial properties that has most significantly led the way toward health and medicine. The application of genes as drugs originated in the United States in the 1970s with its inception for the treatment of genetic disorders such as cystic fibrosis, muscular dystrophy, haemophilia and several other disorders. The emerging trend of gene therapy as a regimen has been implemented in different diseases apart from genetic disorders. Therapeutic nucleic acids, for the replacement or silencing of the target gene, demand specificity and accuracy in design as well as delivery strategies. Nucleic acids tend to degrade in the physiological environment. Hence, nanotechnology-based nonviral vectors can be employed for the delivery of therapeutic molecules that shields against enzymatic degradation and enhances cellular uptake. The major advantage of employing nanoparticles is its tailorability and the ability to deliver biomolecules to treat diseases with minimal side effects. Further, nanoparticles decorated with ligands can specifically bind to target cells. Some widely investigated nanomaterials for the purpose of delivery of nucleic acids (plasmid DNA, antisense oligodeoxynucleotides, siRNA and miRNA) are lipids, polymers such as poly(lactic-co-glycolic) acid, polyethylenimine, poly-L-lysine, dendrimers, chitosan and several inorganic materials.

The present book seeks to give a broad view of nanoparticle-assisted gene delivery, its toxicity, biodistribution and its clinical aspects. The first chapter gives a brief account of nanomedicine for nucleic acid delivery and its properties. This is followed by a discussion of the role of nanoparticles as a vector which considers both passive and active delivery of therapeutic molecules. Further, the synthesis of stable particles of therapeutic value requires thorough characterization steps that measure size, zeta potential, stability and surface morphology, which is discussed in Chapter 3. In general, nanoparticles have been utilized for the delivery of different types of nucleic acids such as DNA, siRNA, locked nucleic acids, ribozymes and DNAzymes. Herein, Chapters 4–8 discuss various polymer-based nanoparticles such as poly(lactic-co-glycolic) acid, polyethylenimine, poly-L-lysine, dendrimers and chitosan involved in the delivery of various nucleic acids *in vitro* and *in vivo.* Moreover, after successful delivery, there arises a need to determine biodistribution, pharmacokinetics and toxicological issues of nanoparticles *in vitro* and *in vivo*, which has been discussed in Chapters 9 and 10, as

nanoparticles of different origin behave differently in physiological conditions. Further, Chapter 11 describes issues related to nanoparticle-mediated nucleic acid delivery and current approaches for regulating pharmaceutical nanoparticles. At a final note, in Chapter 12, development of nanoparticles for gene delivery at the clinical level has been discussed.

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The authors are indebted to their teachers and colleagues for their encouragement, motivation, undying patience and critical evaluation through the various stages of book manuscript preparation. They have been a source of inspiration all along, and supportive with their inexhaustible knowledge. Their valuable input and fruitful suggestions throughout this work is an asset.

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Careful editing of the manuscript requires a lot of effort and work without much appreciation. The authors acknowledge deep gratitude to their students Geeta, R. Mankamna and Nikita for taking on this cumbersome job.

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Dr. Surendra Nimesh, Ph.D.
Prof. Ramesh Chandra, Ph.D.
Dr. Nidhi Gupta, Ph.D.

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Nanomedicine for delivery of therapeutic molecules

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1.1 Introduction

Specificity and accuracy are two important aspects in the design and development of drug delivery strategies. In the initial stages, Paul Ehrlich had led a vision of designing "magic bullets" that would deliver drugs with the utmost specificity to the target tissue or cells. Nanotechnology is one step closer to the reality of this vision that was initiated in the beginning of the 20th century (Kayser *et al.*, 2005). As a beginner, the best way to develop a drug delivery system (DDS) is to understand the mechanism of targeting. One simple, chemical approach for designing DDS is the use of modified forms of active drugs, also known as prodrugs, that can be cleaved enzymatically or chemically to release an active drug. The prodrugs could more easily access the target sites (Higuchi, 1975). Bioconjugate systems are also being developed that employ the use of monoclonal antibodies, polyclonal antibodies, sugars or lectins as targeting moieties, coupled chemically with drug moiety to increase target specificity of the latter. The ligand conjugated to the drug decides the fate of the coupled molecule and underlines its importance in site-specific delivery of a drug.

A number of delivery or carrier systems for drugs have found place in research since 1996, including strategies based on liposomes, nanoparticles, microparticles, and microemulsions (Aynie *et al.*, 1999; Fattal *et al.*, 1998; Forssen and Tokes, 1983a,b; Gregoriadis, 1990; Landfester 2001, 2009; Lee *et al.*, 2003; Rahman *et al.*, 1990; Tan and Gregoriadis, 1990; Zou *et al.*, 1993a,b,c). The purpose of developing all these systems is to maximally avoid interactions with nontarget sites. Several advantages are linked with the usage of carrier-conjugated drug delivery systems, such as

1. prevention of the drug molecule from immunogenic attack and minimal exposure of the drug to normal milieu;
2. minimal renal excretion that increases the half-life of the drug;
3. the drug can be maintained in its active form;
4. at the target site, the required therapeutic concentration can be prevailed;
5. at the disease site, the drug moiety can be activated; and
6. several physiological barriers could be overcome to finally ensure cell-specific interaction.

The foremost goal of controlled drug delivery is to deliver a particular drug to a target site or a specific population of cells within a particular organ or tissue. For

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designing a new system for the target-specific delivery of a drug, the following factors should be taken into consideration:

1. The system should have the capability to differentiate between target and nontarget sites, ultimately minimizing the toxic effects.
2. The system should be free of any toxic compounds on prolonged retention in the system.
3. The carrier system deployed should be biodegradable after its role imparted at the target site, and the degraded components produced should be nontoxic to the system to which it is administered.

The major problem faced during the process of the delivery of polymeric nanoparticulate carriers in the body is the systemic scavenging machinery such as the reticuloendothelial system (RES). Herein, the nanoparticles or any other foreign body entering into the vasculature system get surrounded or coated by various plasma proteins and glycoproteins called opsonins by the process termed "opsonization." Consequently, the RES removes the carrier system from the blood circulation similar to the mechanism followed in the removal of pathogens, dying cells, and foreign bodies from the blood circulation by phagocytosis (Müller and Wallis, 1993). In addition, the Kupffer cells, spleen, lungs, and circulating macrophages also play a significant role in the removal of opsonized particles. It has been observed that the RES mainly depends on the size and surface properties of the particles. As a result, the particulates with greater hydrophobic surfaces get efficiently coated with opsonins and are rapidly cleared from the circulation, whereas hydrophilic particle experiences prolonged circulation as it escapes being recognized by the opsonins (Storm *et al.*, 1995).

In the current scenario, antisense nucleotides such as siRNA have been employed for the treatment of several infectious and inflammatory diseases including cancer (Stephenson and Zamecnik, 1978; Zamecnik and Stephenson, 1978). This nucleic acid (NA) therapeutics is considered to be more efficient compared to conventional therapeutics as it works at the level of gene expression, inhibiting the expression of target protein. Also, NA offers unique prospects in its therapeutic activity on intractable targets, such as regulation of transcription factors for the expression of entire groups of genes. Thus, NA could be either employed for incorporating desired function or inhibition of the function at the molecular level known as gene therapy (Scanlon, 2004). In gene therapy, generally large NA molecules are used for expressing a desired protein, whereas small and specific NA sequences are used for inhibiting target gene function. Though the therapeutic use of NA is considered to display several benefits, it is hampered by challenges such as limited stability and poor cellular internalization. Hence, there is a need of a carrier capable of administering NA *in vitro* and *in vivo* efficiently, safely, and repeatedly that can be attained by applying the use of nanoparticles.

Nanotechnology generally deals with the design, synthesis and application of materials in the nanometer range (usually 100 nm or smaller). Previous studies have shown that nanocarriers could be used for successful delivery of drugs to the target tissue with greater potential, penetration and improved efficacy. Among most of the nanoparticles available, polymeric nanoparticles have evolved as one of the most promising candidates, with rapid progress as vectors for targeted drug and gene delivery

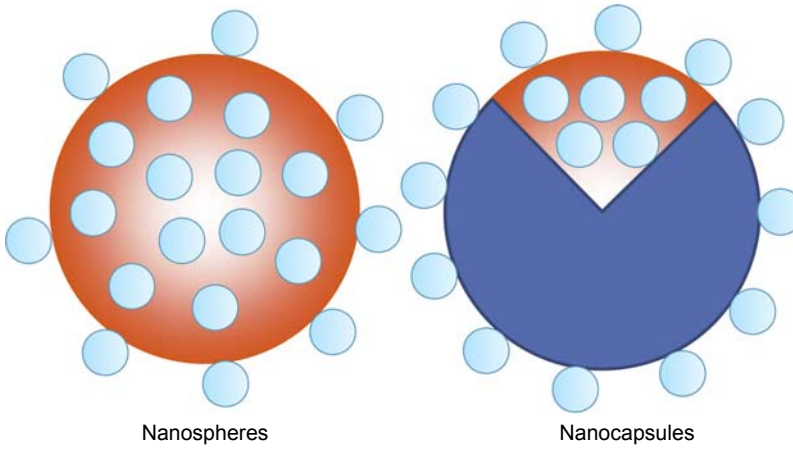

Figure 1.1 Types of nanoparticles.

(Peer *et al*., 2007). Thus polymeric nanocarriers could be exploited for the delivery of several therapeutic agents such as low molecular weight drugs and macromolecules involving proteins or NA. These nanocarriers could be categorized as (1) nanospheres, spherical nanometer range particles where the desired molecules can either be entrapped inside the sphere or adsorbed on the outer surface or both (Fig. 1.1) or (2) nanocapsules, solid polymeric shells with an inner liquid core, and the desired molecules entrapped inside (Fig. 1.1) (Fattal *et al*., 1998; Mao *et al*., 2010; Reischl and Zimmer, 2009). Basically, the fate of nanoparticles within the body is governed by its size, shape, surface charge, and nature (hydrophobic or hydrophilic) of the polymer (Alexis *et al*., 2008). So far, natural and synthetic polymers investigated for NA delivery include chitosan, polyethylenimine (PEI), cyclodextrin-based polycations, and polyethylene glycol (PEG), etc.

1.2 Properties of nanoparticles

Size of particles has been a major concern during the preparation of various formulation systems such as DNA/polymers, lipid complexes, and liposomes. The size of nanoparticles has been observed to be one of the critical factors that significantly influences the transfectivity (Dauty *et al*., 2001; Lee *et al*., 2001; Sakurai *et al*., 2000). A plethora of reports have shown that the particle size significantly affects their cellular uptake, and that the cell lines can take up submicron-sized particles more efficiently compared to larger-sized particles (e.g., Hepa 1-6, HepG2 and KLN 205) (Desai *et al*., 1997; Zauner *et al*., 2001). Similarly, Prabha *et al*. reported that the smaller-sized nanoparticles (mean diameter $= 70 \pm 2$ nm) show a 27-fold higher transfection than the larger-sized nanoparticles (mean diameter $= 202 \pm 9$ nm) in COS-7 cell line and a four-fold higher transfection in HEK-293 cell line (Prabha *et al*., 2002).

Although the small-sized nanoparticles seem to be preferential candidates over the large-sized ones, reports have claimed that the large particles (>200 nm) are also

capable of showing higher transfection efficacy which could be attributed to the combination of several features:

1. their quick sedimentation rate on cell surface as compared to the small particles and
2. destabilization of the cell membrane, thereby gaining entry into the cells due to the availability of free cationic polymers in addition to those complexed with DNA (Luo and Saltzman, 2000; Nimesh and Chandra, 2008).

Furthermore, it is observed that with the increase in incubation time there is an increase in the agglomeration of particles, which alters their effective size presented to the cells. This point is of paramount importance as the process of endocytosis of nanoparticles takes place at the size range of ~20–200 nm in diameter, while above this cut-off, size nanoparticles become more prone to phagocytosis (Xiang *et al*., 2006).

Surface charge of the nanoparticles, termed zeta potential, is another crucial property known to influence activity of the nanoparticles. It is the measure of magnitude of repulsion or attraction between particles. Hence, surface charge of the nanoparticles determines the interaction with the cells as well as its uptake and transfection efficiency. Small-sized nanoparticles with access to the desired endocytic pathway may not be able to deliver the payload successfully until they have sufficient cationic charge to interact with cell membranes. This property could be explained by a well-known polycationic polymer, chitosan, which interacts with the negatively-charged cell membrane by nonspecific electrostatic forces of attraction favored by the presence of high positive surface charge; however, this interaction does not get associated with any cell membrane receptor. The results obtained in one study with chitosan-DNA nanoparticles are also in accordance with this theory, where higher uptake of the complex was found at pH 6.5 compared to its uptake at pH 7.1 and 7.4, as chitosan tends to be more protonated at a lower pH (Nimesh *et al*., 2010; Schipper *et al*., 1997). Also, it was observed that uptake of chitosan nanoparticles was significantly higher compared to that of only chitosan, which could be attributed to the formation of well-defined particles at pH 6.5 in contrast to the use of only chitosan. The cell dependence on the uptake of condensed particles was consistent with the previous report, where chitosan nanoparticles of hydrodynamic diameter 433 ± 28 nm were shown to exhibit higher internalization than chitosan molecules with hydrodynamic diameter of 830 ± 516 nm (Ma and Lim, 2003).

Besides the size and zeta potential of the nanoparticles, shape of the nanoparticles is also known to contribute a significant role on phagocytosis, which was first investigated by Champion *et al*. (2007) using alveolar rat macrophages. This theory was further proved in a study where 20 different polystyrene-based particles differing in their shape, such as spheres, rectangles, rods, worms, oblate ellipses, elliptical disks and UFO-like, in the size range of 1–10 μm were prepared, which showed greater influence on initiating phagocytosis independent of their sizes (Champion and Mitragotri, 2006). The shape of the particle exhibits significant behavior at the point of attachment to the macrophage (Champion *et al*., 2007). For instance, the sharper side of the ellipse would be internalized at a much faster rate by the macrophages in comparison to the dull side of the particles. Moreover, it was observed that the spheres were internalized from any point of attachment independent of their sizes. The size of

particles had a considerable effect on the completion of the phagocytotic process preferentially when the particle volume exceeded that of a cell, though it is known to have a lesser role in the initiation of the phagocytosis. The influence of geometric effect in phagocytosis was quantified by determining the angle between the membrane at the point of initial contact and the line defining the particle curvature at that point (Champion and Mitragotri, 2006). The critical value for this angle is 45 degree, above which the macrophages would tend to lose its ability to entrap particles and hence, attach to the particles in a process similar to spreading. Therefore, the complex actin structure of the cells is required to be rearranged to allow engulfment and ultimately forces macrophages to switch spreading behavior. This study therefore clearly shows the role of phagocytosis and physical properties of the phagocytosed materials.

1.3 Polymeric nanoparticles

Polymers have gained considerable attraction due to their various unique properties with a wide range of applications in diverse fields. These well-defined polymeric nanoparticles can be tailored specifically against a target disease as well as to carry payloads and to encapsulate different molecules such as hydrophilic, as well as hydrophobic, drugs, genes, oligonucleotides, and siRNAs (Akinc *et al*., 2008). The use of biologically approved polymers allows easy construction of nanoparticles that can also be scaled up under regulatory guidelines. Besides, the polymeric nanoparticles also have the capability to gain spatial and temporal control on the release of the encapsulated drug or gene payload, in order to optimize cancer therapy. Developments have shown that there has been parallel synthesis of degradable and biocompatible polymeric material libraries with high-throughput screening methodologies specially done for biomedical applications (Green *et al*., 2008). Out of several applications of nanotechnology in medicine, the main focus has been the target-specific drug/gene delivery and early diagnosis in cancer treatment using nanoparticles (Amass *et al*., 1998).

1.4 Nanoparticle-based strategies for overcoming *in vitro* and *in vivo* barriers

The success of NA-based gene silencing depends mainly on the properties of the delivery vectors which further determine the timely and efficient delivery of NA to the target site. However, NA delivery faces several challenges that ultimately hamper its efficacy rate. Based on the mode of study, the barriers faced during the delivery process can be divided into two broad categories, *in vitro* and *in vivo*.

1.4.1 In vitro *barriers*

Though the significant role of siRNA and of DNA begins in cytoplasm, and in the nucleus respectively, the molecules appear to encounter similar type of barriers,

i.e., starting with cell targeting and followed by internalization and its endosomal escape (Grayson *et al*., 2006; Katas and Alpar, 2006). The electrostatic interaction between the amino groups of the polycationic polymers and phosphate groups of siRNA results in the formation of the siRNA−polymer complex. However, the size of the formed complexes also depends upon various other factors such as siRNA concentration, pH, buffer type and charge ratio of polymer to DNA (N/P ratio), and it has been observed that a 50−200 nm size range of the vector−siRNA complexes can easily gain access inside the cells by means of endocytosis or pinocytosis (Aoki *et al*., 2004; Gao *et al*., 2005). Another issue faced during the process is the aggregation of the complexes in the extracellular environment with a specific size that can be internalized. So, to avoid aggregation, PEG or sugar molecules such as cyclodextrin and hyaluronic acid are incorporated at the surface of nanoparticles (Bartlett *et al*., 2007; Lee *et al*., 2007). The large size (∼13 kDa) of the complex and presence of polyanionic charges on siRNA are other reasons that impede the efficient uptake by the cells. Studies reported that for initial modifications, cell penetrating peptides and cholesterol can be used in order to enhance the uptake efficiency of siRNA (Moschos *et al*., 2007; Muratovska and Eccles, 2004). Several polycationic formulations have been used, including chitosan, polyarginine, polylysine, histidylated polylysine peptides, PEI, and polyamidoamine (PAMAM) dendrimers for the delivery of siRNA (Malhotra *et al*., 2009, 2011, 2013a,b,c).

Once internalized, these result in the fusion of intracellular vesicles carrying complexes with the endocytic vesicles. There have been several hypotheses proposed for the escape of complexes through endosomal degradation. According to the most accepted hypothesis of endosomal escape, the presence of a cationic polymer, for instance, PAMAM dendrimers and polylysine, directly interacts with the negatively charged endosomal membrane, leading to the physical disruption of the negatively charged endosomal membrane, thus escaping the degradation (Zhang and Smith, 2000). Either reduction-sensitive or pH-responsive polymers can be used as other approaches. PEI has ionizable amino groups, and this favors the release of endocytosed polyplexes as explained by the proton sponge effect (Akinc *et al*., 2005; Boussif *et al*., 1995; Thomas and Klibanov, 2002; Thomas *et al*., 2005). Kim *et al*. explained that the pH-dependent protonation of N-(2-aminoethyl)-2-aminoethyl groups in the side chain of polyaspartamide derivative PAsp (DET) results in efficient endosomal escape (Kim *et al*., 2010). For the purpose of siRNA delivery, the block copolymers of dimethylaminoethyl methacrylate and propylacrylic acid (PAA) undergo structural rearrangements due to the acidic pH of endosomes (Convertine *et al*., 2009). The block includes PAA and a cationic complexation component that facilitates a hydrophilic-to-hydrophobic transition at the endosomal pH, leading to the disruption of the membrane. The delivery of siRNA can also be accomplished by the thermosensitive cationic polymeric nanocapsules. These nanocapsules can cause the physical disruption of the endosome due to the induced swelling (∼119 nm at 37°C and ∼412 nm at 15°C) at a specific temperature (Lee *et al*., 2008).

The stability of nanoparticles is highly suitable for the protection of extracellular siRNA but, in the meantime, dissociation of the complex is necessary to release siRNA in order to mediate gene silencing. Hence, a critical balance must be prevailed between

the protection and release of siRNA for efficient silencing of the target gene by the siRNA−nanoparticle complex (Zhang *et al*., 2009, 2010). Bioresponsive nanoparticles have also been used for the release of siRNA as soon as the intracellular stimulus is provided. For example, introduction of acid-labile ketal linkages to PEI increases the transfection efficiency and siRNA-mediated silencing. Ketalized PEI−siRNA polyplexes enable higher gene silencing efficiency than unmodified linear-PEI. This is due to selective cytoplasmic localization of the polyplexes and efficient disassembly of siRNA from the polyplexes (Shim and Kwon, 2009). Polyplexes including reducible polycations are studied for their ability to be used as siRNA delivery carriers. In the redox condition of the cytoplasm, these polycations get degraded. Under the physiological conditions, siRNA condenses with the reducible poly(amido ethylenimine) to form stable complexes and releases the siRNA in the reducing environment (Hoon Jeong *et al*., 2007). Similarly, in another study, it was reported that the siRNA transfection efficiency was 100-fold higher with the polyion complex micellae prepared with a disulfide cross-linked core through the assembly of iminothiolane-modified poly(ethylene glycol)-*block*-poly(L-lysine) [PEG-*b*-(PLL-IM)] in comparison to the noncross-linked polyion complexes (PICs) (Matsumoto *et al*., 2009). The use of PICs allowed the selective release of siRNA in the intracellular milieu as well as protected it from degradation in extracellular milieu and nonspecific clearance. Also, siRNA embedded over poly(aspartic acid) [PAsp(-SS-siRNA)] through a disulfide linkage gave higher siRNA efficiency due to efficient siRNA release from the PIC under intracellular reductive conditions (Takemoto *et al*., 2010).

1.4.2 In vivo *barriers*

On the basis of the targeted disease, the route of siRNA administration can be intravenous, intranasal, intratracheal, subcutaneous, intratumor, intramuscular or oral, thus characterizing the *in vivo* barriers of siRNA therapeutics. One of the most significant biological barriers faced during the administration of siRNA is the degradation by nuclease activity in plasma. The major degradative enzymatic activity occurs by 3′ exonuclease in the plasma, although cleavage of internucleotide bonds can also take place to a small extent. The major challenge is to protect the siRNA in serum with various chemical modifications, including modifications of the sugars or the backbone of siRNA by 2′-O-methyl and 2′-deoxy-2′-fluoro or phosphorothioate linkages (Akhtar and Benter, 2007). Since, polymeric nanoparticle-complexed siRNA does not have sites for 3′ nuclease binding, it therefore can easily avoid this barrier and gets protected from cleavage. Rapid clearance by the RES contributes to another major problem encountered during siRNA delivery. This occurs through the engulfment of endocytosed siRNA as well as their carriers by the Kupffer cells present in the liver and spleen macrophages (Alexis *et al*., 2008). The nanoparticles used to deliver siRNA as foreign particles in the body are recognized by the opsonins, constituting immunoglobulins, complement system proteins, and other serum proteins. During opsonization, these particles are engulfed through the receptors present on the surface of macrophages. The Fc receptors present on the Immunoglobulin G-opsonized particles and that of complement receptors on complement-opsonized particles are internalized,

resulting in their degradation. The modifications of nanoparticle surfaces with hydrophilic polymers, such as PEG, reduce the adsorption of opsonins and clearance by phagocytosis through RES-associated clearance (van Vlerken *et al.*, 2007). The specific recognition and binding to the target mRNA in the cytosol is one of the most critical steps involved in siRNA-mediated gene silencing. The nanoparticle-complexed siRNA delivery can result in some inaccurate and false effects. Some of the observed effects are toxicity, stimulation of immune response, inflammation and undesirable effects on other genes (Aigner, 2006). It has been reported that chemical modifications in siRNA, such as the replacement of uridine bases by their 2′-fluoro, 2′-deoxy or 2′-*O*-methyl modified counterparts, can block immune recognition of siRNAs by toll-like receptors (TLR) (Sioud, 2006). Hence, several strategies have been initiated, such as the use of polymers that can bypass the delivery into endosomes or block TLR signalling. In principle, various factors are required to be taken into account both at the cellular and whole organism level to design and develop appropriate siRNA delivery vectors.

1.5 Book compilation

The book addresses several issues related to the safe and effective delivery of NAs using nanoparticles. A further emphasis is given on the mechanism of delivery of NAs, the barriers encountered, and the strategies adapted to combat them. It provides a detailed description about the delivery of different kinds of NAs being realized with nanomedicine. The book provides a discussion about the delivery issues of less-explored NAs such as Locked nucleic acids, Ribozymes, DNAzymes, etc. On a final note the regulatory aspects of nanoparticle-mediated NA have also been discussed, with a focus on their clinical relevance.

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Nanoparticles as nucleic acid delivery vectors

2

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2.1 Introduction

Nucleic acid (NA)-based therapy is a prominent, novel, and promising area in pharmaceutical and medical sciences due to its potential for treating a variety of genetic disorders, like diabetes, whose treatment is still a challenge (Lai and Lin, 2009). Basically, NA therapy consists of two parts: first, the therapeutic NA itself, with plasmid DNA (pDNA) and different types of small RNA, and second, the carrier which delivers the NA to the desired site, including viral and nonviral vectors (Mesnil and Yamasaki, 2000). Nucleic acids, such as pDNA, antisense oligonucleotide, and RNA molecules, have a great potential to be used as therapeutics for the treatment of various genetic and acquired diseases (Zhu and Mahato, 2010). In fact, the plasmid DNA, oligonucleotide and RNA interference (siRNA, shRNA, miRNA) are the most commonly investigated nucleic acid-based therapies. The biomedical applications and therapeutic outcomes of these nucleic acids may vary depending upon their mechanisms of action. These are typically macromolecules big in hydrodynamic size with a negative charge in an aqueous solution. Therefore several strategies are being explored for their enhanced gene transfer, including design of different synthetic carriers (Wolff *et al*., 1990) or the use of physical approaches (Gill and Prausnitz, 2007). Compared to the drug delivery approaches, gene therapy is emerging as a novel area of research with its striking and advantageous features. Gene therapy is believed to be the therapy in which the root cause of the diseases can be treated at the molecular level. Generally gene therapy helps in the identification of the origin of the disorder instead of using drugs to diminish or control the symptoms. Since 2005, the role of functional genetic materials used in gene therapy has been globally broadened and diversified. Notably, gene therapy emerged as the gold standard when two Nobel prizes were acclaimed by Andrew Fire and Craig Mello, who demonstrated the mechanism and application of RNA interference (RNAi) in 2006 (Fire *et al*., 1998). The application of nucleic acids to treat and control diseases is known as "gene therapy." Usually the gene therapy is mediated by the use of viral and nonviral vectors to transport foreign genes into somatic cells to restorative defective genes (Luo and Saltzman, 2000a). As mentioned earlier, antisense oligonucleotide (AON), siRNA, and pDNA are the important types of nucleic acids for gene therapy (Fig. 2.1) which are principally utilized as potent therapeutic agents to silence the expressions of defective genes. Thus the delivery of intracellular therapeutic agents, DNA and RNA, can be utilized for vaccination and for the treatment of several diseases such as cancer, viral infections

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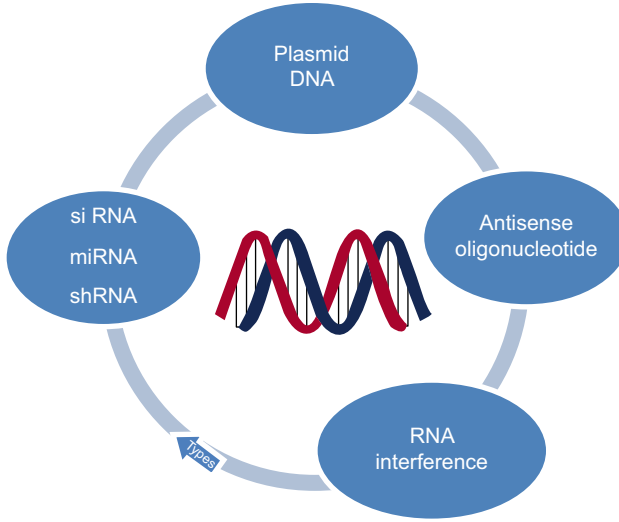

Figure 2.1 Types of nucleic acids used in gene therapy.

and dermatological diseases (Elsabahy *et al.*, 2011). The types of nucleic acid and their characteristics are illustrated in Table 2.1. In the following paragraphs the brief details of all three important types of nucleic acid delivery tools have been discussed.

2.1.1 Plasmid DNA

Plasmid is a unique type of double-stranded DNA, usually circular in shape, used in gene therapy, and it contains the transgene that encodes for a specific protein. The size of the most commonly used pDNA ranges from a few hundred base pairs (bp) to several thousand bp (Yin *et al.*, 2005). Therapeutically, plasmids can be used for the treatment of several diseases, as well as an active constituent for vaccination. Promoter and enhancer sequences in pDNA, as regulatory signals, play an important role in regulating gene expression (Williams *et al.*, 2009). Promoter sequences are derived from viral origins such as cytomegalovirus and roux sarcoma virus, which initiate the transcription process by acting as a recognition site for the RNA polymerase which ensures high transcription efficiency. Enhancers are the regions in the pDNA that can be upstreamed or downstreamed from the promoter region. They act as binding sites for proteins that enhance the initiation of gene transcription. The selection of a suitable enhancer can improve transcription efficiency by several hundred folds (Asoh *et al.*, 1994; Noss *et al.*, 2002).

2.1.2 Antisense oligonucleotide

AONs are typically 18−21 nucleotides, single-stranded deoxy-ribonucleotides possessing a complementary sequence to the target mRNA (Dean and Bennett, 2003). AONs are used to treat certain diseases, especially those cases that are associated with dysregulated gene expression leading to an excessive amount of the protein production in cells. Hybridization of this nucleotide to the target mRNA specifically inhibits gene

Table 2.1 Types of nucleic acids along with their characteristics

Characteristics	Plasmid DNA	siRNA	shRNA	miRNA	Antisense oligonucleotide
Properties	Extra chromosomal circular DNA molecules (Hayes, 2003)	Double-stranded RNA molecules, 20–25 bp in length (Bernstein *et al.*, 2001)	Artificial RNA molecule with a tight hairpin turn (Zhaohui *et al.*, 2011)	Small noncoding RNA molecule (containing about 22 nucleotides (Ambros, 2004)	Short strand of deoxyribonucleotide analog that hybridizes with the complementary mRNA (Chan *et al.*, 2006).
Molecular weight	~0.5–5 kbp	19–22 bp	25–29 bp (stem)	21–24 nucleotides	18–21 nucleotides
Mechanism of action	Express particular gene with replacement of faulty one	RNA interference	RNA interference	RNA interference	Interfere with mRNA maturation or incorrect splicing in the nucleus
Advantage	Can be used to express a missing gene	Required in lower amount, lower cost and toxicity	Required in lower amount, lower cost and toxicity	Required in lower amount, lower cost and toxicity	No nuclear barrier; designing is easier
Disadvantages	Difficult to construct and formulate nuclear barrier	Cannot be used to express a gene of interest	Cannot be used to express a gene of interest	Cannot be used to express a gene of interest	Nuclear barrier can exist for some therapeutic applications
Immune response	TLR9	RNAs are generally recognized by three main types of immunoreceptors: TLR, protein kinase R, and helicases			TLR9 (if there are CpG motifs in the AON sequence) (Rojanasakul, 1996)

expression by different mechanisms: first, by translational arrest through steric hindrance of ribosomal activity and the induction of RNase H endonuclease activity, and secondly, through the RNase H enzyme which cleaves the mRNA in the RNA-DNA hetero duplex, while leaving the AON intact. The second one is the most important mechanism of any AON (Rojanasakul, 1996; Chan *et al*., 2006). Factors that affect the mechanism of action and gene expression are AON sequence and incorporation of certain chemically modified nucleotides to the AON (Sazani and Kole, 2003; Hogrefe, 1999).

2.1.3 RNA interference

RNA interference (RNAi) reveals great therapeutic potential as a posttranslation gene regulation process for a variety of diseases, including genetic disorders, autoimmune diseases, viral infections, and cancer (Williford *et al*., 2014; Bumcrot *et al*., 2006). The important types of RNAs used under the RNAi therapy are as follows.

Small interfering RNA

The small interfering RNA (siRNA) or silencing RNA is a double-stranded RNA molecule with 20−25 bp that has a major role in RNAi pathways, where it interferes with the expression of specific genes with complementary nucleotide sequences (Agrawal *et al*., 2003). RNA interference is a type of regulatory mechanism that most commonly occurs in the cytoplasm of most eukaryotic cells to control gene activity (Fougerolles *et al*., 2007; Grimm and Kay, 2007). In the process of RNA interference, long double-stranded RNA is cleaved into shorter double-stranded RNA segments (siRNA) by the dicer proteins. After cellular entry, the siRNA duplex is assembled into large protein assemblies, called the RNA-induced silencing complexes (RISC) (Fig. 2.2), where the sense strand of siRNA is removed and cleaved by the RNase H-like activity of Ago-2 (cleavage-dependent pathway, member of the argonaut family of proteins) (Preall and Sontheimer, 2005) and is a major component of RISC (Hammond *et al*., 2001). The other members of argonaut family, like Ago-1, Ago-3, and Ago-4, do not have specific endonuclease activity, and they function through a cleavage-independent pathway (Watts *et al*., 2008).

Small hairpin RNA

Small hairpin RNA (shRNAs) consists of stems that can vary from 25 to 29 bases and a loop of 4−23 nucleotides. It is a type of RNA sequence that can induce gene silencing through RNAi (Paddison *et al*., 2002). shRNA is processed in the cytoplasm by the dicer protein to remove the loop to produce siRNA, which then forms RISC (Fig. 2.2), followed by subsequent cleavage of the target mRNA (Chen *et al*., 2008). Bifunctional shRNA has been designed to enhance the efficiency of shRNA. It employs two shRNA, one with the perfect match for cleavage-dependent RISC loading and the second with mismatched sense strands (at the central location) for cleavage-independent RISC loading (Rao *et al*., 2009).

MicroRNA

MicroRNA (miRNAs) are mature and short (20−24 nucleotides) single-stranded, noncoding RNAs that bind to partially complementary sites, called miRNA binding

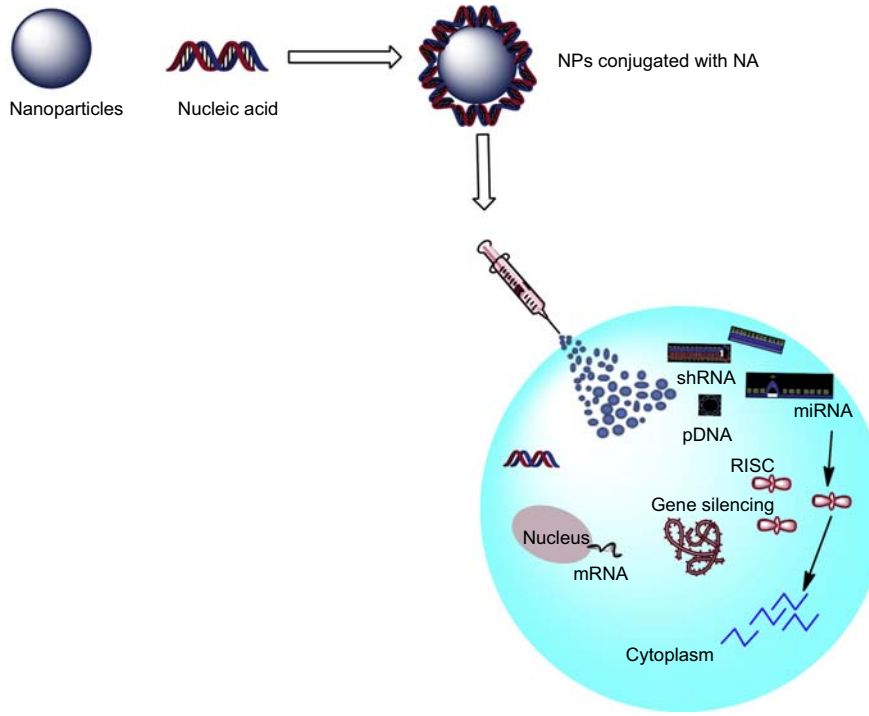

Figure 2.2 Mechanism of nonviral vector-mediated delivery of nucleic acids inside the cell.

sites. miRNA silences gene expression by affecting the stability of target mRNAs or via translational repression. miRNAs are transcribed from DNA initially as long primary transcripts termed as "pri-miRNA" comprised of an RNA hairpin structure which is cleaved from the pre-miRNA by the nuclear microprocessor complex and the endonuclease drosha complex in the nucleus, which subsequently gives rise to precursor miRNA termed as "pre-miRNA" (Lee *et al*., 2002). The pre-miRNA is transported from the nucleus to the cytoplasm by exportin-5 (Zeng and Cullen, 2004) and is further cleaved by the endonuclease dicer into short double-stranded RNA, in which one strand is the mature miRNA and its counterpart is known as the miRNA (Yang and Mattes, 2008).

2.2 Challenges and barriers to the nucleic acid delivery

In the context of efficient delivery of nucleic acids, it should fulfill two of the most important criteria, namely, safety and efficacy (Pathak *et al*., 2009). One of the challenges to systemic delivery of nucleic acid therapeutics is the potential degradation of the NA by endonucleases in physiological fluids and the extracellular space (Yin *et al*., 2014). Among all of the challenges in the delivery of nucleic acids, some important

challenges are described and should be considered while designing the appropriate delivery approach.

2.2.1 Intracellular bioavailability

The introduction of nucleic acids either in naked or encapsulated form in biological fluids faces many physiological or biological barriers leading to altered cellular biodistribution as well as overall intracellular bioavailability. Unmodified DNA and RNA, whether it may be plasmid, siRNA, or shRNA rapidly degrade in biological fluids by various extra- and intracellular enzymes before they can reach the surface of the target cells; this administration ultimately leads to the loss of therapeutic activity of nucleic acid (Harada-Shiba *et al*., 2002) and poses a challenge to the effective delivery.

2.2.2 Induction of immune response

The exogenously administered nucleic acid materials may trigger and activate the immune system, resulting in the release of inflammatory cytokines, which may lead to serious local and systemic inflammatory reactions (Kariko *et al*., 2004). The endosomal toll-like receptors (TLRs) are the most commonly identified mechanisms involved in the release of the proinflammatory cytokines. This immune mechanism can be augmented by the complexation of nucleic acid with cationic polymer or some lipids (Yoshida *et al*., 2009).

2.2.3 Low nuclear uptake

It is evident that there must be translocation between plasmid and nucleic acid for gene expression without considering the types of carrier and internalization pathway. Translocation, which involves both trafficking to the nucleus and penetration of the nuclear membrane through naked DNA or RNA into the nucleus, is usually ineffectual in both dividing and nondividing cells. As per Bieber *et al*. (2002), only a small fraction of DNA/polyethyleneimine (PEI) complexes, which are internalized by the cells and released from the endosomes, successfully reaches inside the nucleus. The characteristic of nuclear membrane permeability and the translocation process of the nucleus allows import and export of specific molecules into and out of the nucleus (Liu *et al*., 2003). Due to the membrane pore size (25 nm), molecules smaller than 40 kDa or complexes with a particle size of 25 nm can diffuse passively, whereas larger molecules cannot diffuse (Roth and Sundaram, 2004).

2.2.4 Extracellular and intracellular barriers in nucleic acid delivery

It is difficult to achieve the desired therapeutic effect of nucleic acid delivery by conventional delivery strategies due to several biological and cellular barriers (Fig. 2.3) present in the body. Before reaching the targeted site, NAs face many obstacles to

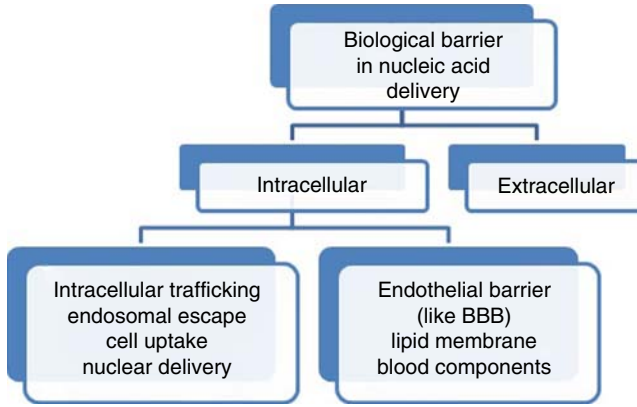

Figure 2.3 Different types of biological barriers in nucleic acid delivery.

go across extracellular, cellular, and intracellular biological membranes (Zhu and Mahato, 2010). Following systemic administration, these molecules have to go across various intracellular barriers such as intracellular trafficking, endosomal escape, and extracellular barriers like lipid bilayer membrane and various endothelial barriers. Systemic administration of drugs is affected by extracellular barriers like extreme pH, proteases and nucleases, the immune defense, and the scavenger system which are present in most of the extracellular environment that influences biodistribution of the administered drug from the point of delivery to the intended target cells (Belting *et al*., 2005).

The most common examples of the extracellular barrier are the gut, airway mucosa, and the skin, which represents the outermost physical barrier. In spite of that, many microbes and their toxins manage to cross these barriers. An interesting example in that particular case is dietary proteins that can even bypass the blood−brain barrier, as exemplified by the prion protein that targets the CNS after ingestion of prion-diseased products (Prusiner, 2001; Blattler, 2002). The delivery of nucleic acids from outside the cell to the nucleus is a challenging task requiring some synthetic vectors which facilitate a number of distinct steps like cell-specific binding, internalization, or endocytosis, escape from endocytic vesicles, transport through the cytoplasm, translocation across the nuclear membrane, and release of DNA for transcription. The next barrier is the lack of techniques to study the intracellular trafficking of polyplexes to probe and elucidate steps that limit transfection efficiency (Read *et al*., 2005). Specific examples of different types of biological barriers and various strategies to overcome those, as reported in the prior literature, are illustrated in Fig. 2.4.

2.3 Vectors in nucleic acid delivery

Vectors are the carriers that act as the delivering vehicles for the drugs, nucleic acid, and various biotechnological active substances. For delivering nucleic acids to the cells, these are broadly divided into two categories named viral and nonviral vectors.

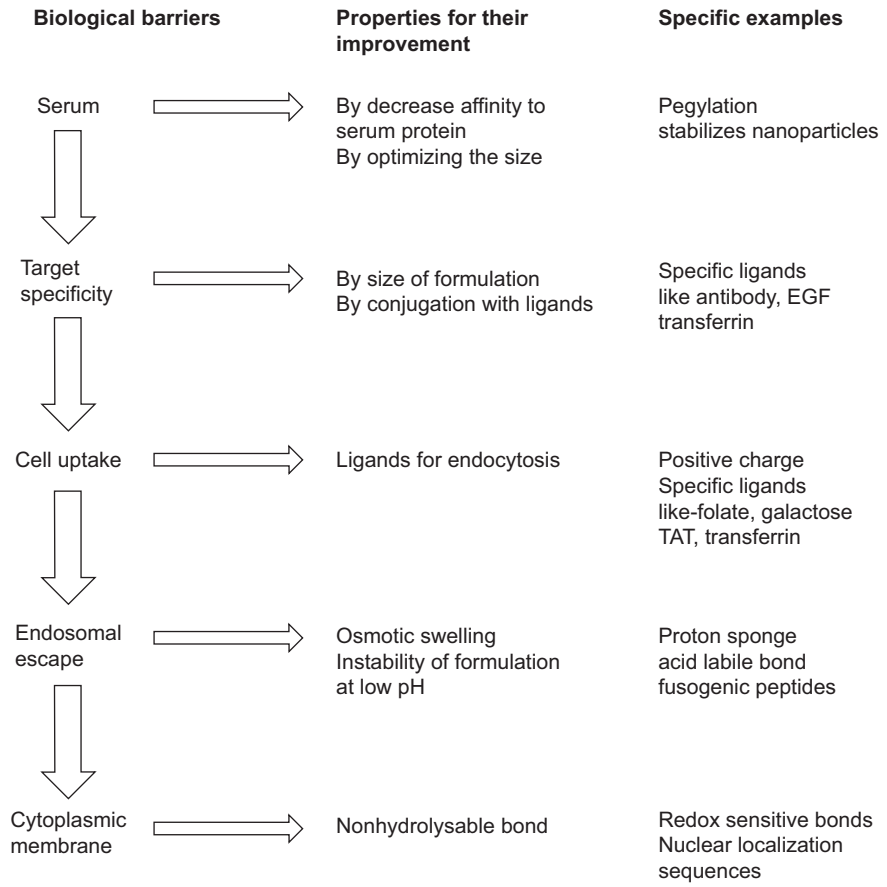

Figure 2.4 Approaches to overcome the biological barriers in the nucleic acid delivery.

2.3.1 Viral vectors

The successful history of nucleic acid and gene delivery is based on viral vectors (Huang *et al*., 2011). In fact, the first concept of the delivery gene was designed with the viral vectors in mind. The viral vectors however offer some disadvantages, such as their noticeable immunogenicity, that may cause the induction of the inflammatory system, toxin production, mortality, and transgenic capacity size (Gardlik *et al*., 2005). Some common examples of viral vectors include retroviral vectors, adenoviral vectors, adeno-associated vectors, pox virus vectors, etc. (Nayerossadat *et al*., 2012).

2.3.2 Nonviral vectors

Nonviral vectors are based on the physical and chemical system (Fig. 2.5); these include either chemical methods like cationic liposomes and polymers or physical methods such as particle bombardment, magnetofection, and ultrasound applications (Hirai *et al*., 1997; Robertson *et al*., 1996).

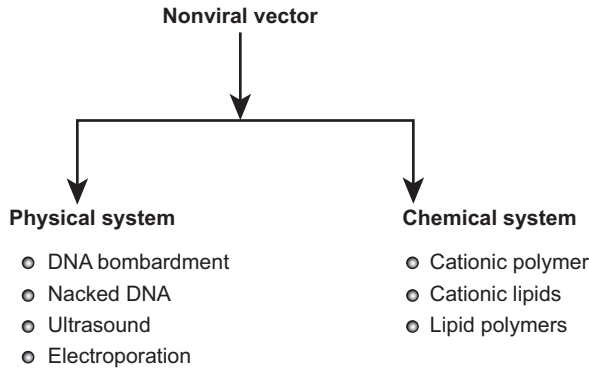

Figure 2.5 Types of nonviral vectors.

2.4 Nanoparticles as nonviral nucleic acid delivery vectors

Nucleic acid as a therapeutic agent is an important tool to treat the more serious diseases which require systemic administration of the gene or nucleic acid substances to enter the target cells (Luten *et al*., 2008). Today, effective gene delivery to the target tissue has become a prominent challenge due to the lack of efficient nanocarriers, which leads these macromolecules to the desired site without altering the therapeutic effectiveness. Naked nucleic acids can be delivered to a specific organ/tissue by various physical methods like electroporation (Heller *et al*., 2005) or hydrodynamic injection (Knapp and Liu, 2004), but these methods have failed in the commercial gene therapy due to their limitations. This led to the search of some gene vector which must be able to access the desired site of disease at the favorable time after parenteral administration (Mahato and Kim, 2002). The success of gene delivery strategies largely depends on the availability of suitable vectors. Viral vectors exerts transfection property in a quality manner both *in vitro* and *in vivo* along with some limitations (Zhang and Godbey, 2006; Gorecki, 2001), such as induction of immune responses against viral protein, possible recombination with wild-type viruses, limitations in the size of inserting DNA, and difficult large-scale pharmaceutical grade production (Lehrman, 1999; Liu and Muruve, 2003; Sun *et al*., 2003). Therefore the researchers showed much more interest in the nonviral vectors like nanoparticles (NPs), liposomes, dendrimers, or any other cationic polymers because of the efficient gene binding and condensation capacity both *in vitro* and *in vivo* (Louise, 2006; Vasir and Labhasetwar, 2006).

Among all the carriers, nanoparticles (NPs) such as gold, silica, lipid, chitosan, and solid lipid (Fig. 2.6) emerge as novel nanocarriers for the nucleic acid delivery to the targeted tissue. Nanoparticles can be defined as solid colloidal particles with macromolecular identity with a size of <1 μm. Initially, NPs (1970) were designed for the vaccination and anticancer therapy (Couvreur *et al*., 1986), but nowadays the applicability of the nanoparticles has been broadened, including use as nonviral vectors. A considerable research has been devoted with NPs for delivery of DNA and other nucleic acids.

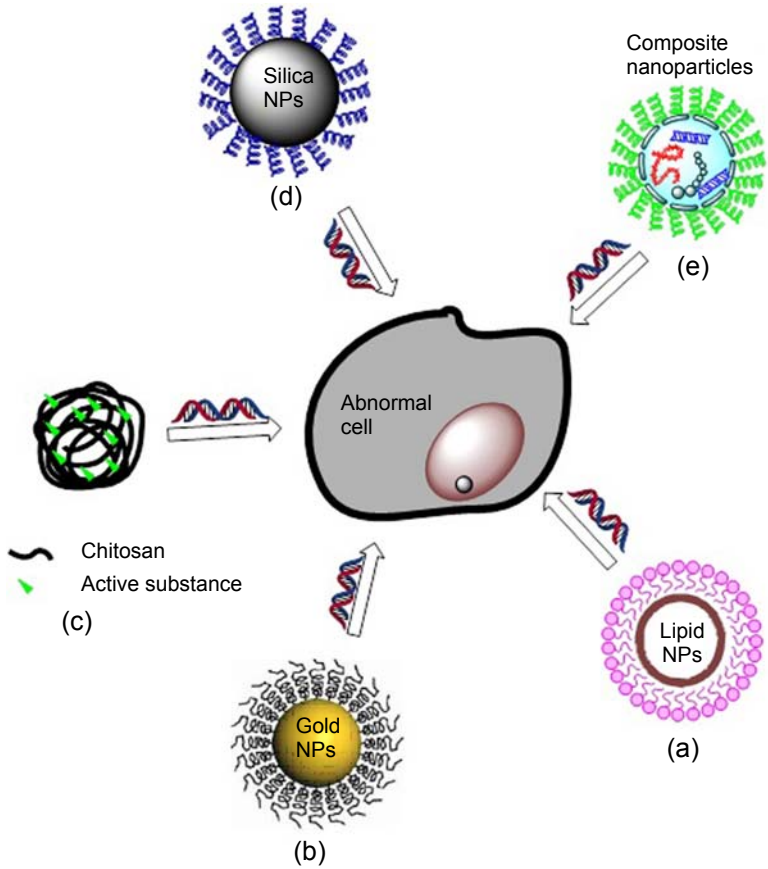

Figure 2.6 Different types of nanoparticles in nucleic acid delivery: (a) lipid nanoparticles; (b) gold nanoparticles; (c) chitosan nanoaprticles; (d) silica nanoparticles; (e) composite nanoparticles.

The potential advantages of NPs as a promising vector for the nucleic acid delivery are due to their ability to encapsulate and entrap various biomolecules or drugs by chemical conjugation or adsorption to the surface (Kumar *et al*., 2004). Ideally a nanoparticle should possess the following properties to act as an effective nonviral vector:

- Good biocompatibility
- Low immunogenicity
- Selective targeting
- Low circulation time
- Efficient penetration to the various barriers like blood−brain barrier, vascular endothelium, as well as self-release without any serious side effects (Nimesh *et al*., 2014)

Different groups of researchers used different types of nanoparticles for effective NA delivery to the desired site. In the following paragraphs the most important classes of the nanoparticles which have been reported to be used as nonviral vectors for gene therapy are discussed.

2.4.1 Lipid-based nanoparticles

The lipid-based nanoparticulate system (Fig. 2.6(a)) as the NA delivery vector has become the most efficient due to the extensive research work in this area. Cationic liposome plays an important role because cationic liposomes can condense DNA into a cationic nanoparticulate system when the two components are mixed together. This cationic lipid/DNA complex, also known as lipoplex, can protect DNA from enzymatic degradation and deliver DNA into cells by interacting with the negatively charged cell membrane. Originally, lipoplex is not an ordered DNA phase surrounded by a lipid layer, but is a partially condensed DNA complex with an ordered substructure and an irregular morphology (Xu *et al.*, 1999; Templeton *et al.*, 1997). Many of the lipid-based nonviral vectors have been synthesized for NA delivery (Mahato and Kim, 2002); among them, a few are in clinical trials (Huang *et al.*, 1999; Edelstein *et al.*, 2004). The cationic-based delivery system has been failing in many circumstances due to its low gene transfer efficacy, low circulation time and toxicity associated with inflammation and complement activation. Origin of the toxicity was not fully understood; meanwhile, the larger diameter of lipoplex and a combination of unmethylated nucleic acid with cationic carrier was found to be completely responsible for the induction of toxicity (Judge *et al.*, 2005; Zhang *et al.*, 2005; Jason *et al.*, 2004). To overcome cationic lipid-based induced toxicity, many researchers tried to encapsulate nucleic acid substances like DNA and other nucleic acid-based drugs into a PEGylated cationic liposomal bilayer using various approaches (Harvie *et al.*, 2000; Li *et al.*, 2005; Jeffs *et al.*, 2005). These cationic-based polymeric systems have a bilayer shell around the nucleic acid with a smaller particle diameter ($<$100 nm) and better stability *in vivo*. These properties are also favoured in prolonged *in vivo* circulation time of the particles as well as an increased fraction of particles (Li and Szoka, 2007). Hope (2014) developed the dialkylamino iL nanoparticles (iLNPs) for enhancing the siRNA delivery at targeted sites upon intravenous administration using amino lipids as core polymers; through this formulation the authors gained accelerated entry of siRNA to hepatocytes. The detailed understanding of amino lipid structure activity relationship and the role of endogenous ApoE in receptor-mediated uptake of iLNPs by hepatocytes has given rise to an advanced generation of siRNA formulations, which were highly potent and well tolerated in humans. Lipid nanoparticles consist of cationic, neutral and anionic lipids, which display their own advantages and disadvantages.

Cationic lipids

Cationic lipids with multivalency on their surfaces are crucial components to condense negatively charged nucleic acid by a charge−charge interaction into a small particle. Most of the NPs, which are used in the delivery of biotechnological products, are based on cationic lipids due to the tendency to condense the DNA. A widely accepted advantage of cationic lipids for NPs is that they can bind the negatively charged human cell membranes and also facilitate the overall uptake of NA into the cell (Semple *et al.*, 2001).

Neutral/helper lipids

Neutral lipids like fusogenic lipids and helper lipids like cholesterol may increase transfection activity of the DNA-containing liposomal particles. DOPE

(1,2-dioleoyl-snglycero-3-phosphoethanolamine) is a widely used cationic lipid which can induce membrane fusion by facilitating the formation of fusion intermediate structures (Siegel and Epand, 1997).

Anionic lipids

Due to common toxicity and rapid clearance of cationic lipids, anionic lipid is an alternative approach to reduce the nonspecific interaction with negatively charged serum proteins such as albumin and the extracellular matrix. Anionic lipids are added into the system to form a surface coat and an anionic or charge-neutral lipid particle (Lee and Huang, 1996; Mastrobattista *et al*., 2001).

PEGylated lipids

It is a biocompatible as well as inert polymer which generally increases the efficiency of gene transfer and circulation time and reduces the toxicity (Fenske *et al*., 2002) with the added advantage of minimization of the interaction between particles and the cell surface (Wheeler *et al*., 1999). The targeting ligand plays a crucial role in the case of low cellular uptake in target cells, especially in the case of PEG-stabilized NPs. Generally the function of the ligand would be expected to improve the binding and uptake of the DNA on internalizing receptors (Ruoslahti and Rajotte, 2000). The aforementioned NPs for NA delivery can be formulated by various methods like direct mixing, detergent analysis, ethanol dialysis, etc. (Li and Szoka, 2007).

2.4.2 Gold nanoparticles

For the last few years, gold nanoparticles (Fig. 2.6(b)) have emerged as attractive polymeric nanocarriers for the delivery of various biomolecules like DNA, siRNA, and other NAs, due to their unique chemical and physical properties along with biocompatibility, monodispersity, ease of synthesis and ease of their surface functionalization (Kim *et al*., 2009; Ghosh *et al*., 2008a). Generally a gold core is inert and nontoxic in nature, so that many biological, as well as biotechnological, products can be uploaded in gold NPs without any harmful effects to the active substances (Connor *et al*., 2005). Nanosized and multifunctional surfaces make them useful nonviral vectors for efficient recognition and targeted delivery of many biomolecular drugs. Many research groups have shown their interest in the delivery of protein, peptides, and nucleic acids like DNA or RNA (Ghosh *et al*., 2008b). In 2001, McIntosh *et al*. reported gold NPs functionalized with cationic quaternary ammonium compounds and then bound to plasmid DNA electrostatically. This unique composition protected the DNA from enzymatic degradation and regulated the transcription of RNA polymerase. The same group also reported in subsequent years about the release of DNA from modified gold NPs after treatment with glutathione (Han *et al*., 2005). Niidome *et al*. (2004) reported the gold NPs fabricated by $NaBH_4$ reduction in the presence of 2-aminoethanethiol formed a complex structure with plasmid DNA containing a luciferase gene. Guo *et al*. (2010) prepared charge-reversal functional gold nanoparticles through a

layer-by-layer technique for the delivery of siRNA and plasmid DNA to the cancer cells, in which measurement of siRNA by polyacrylamide gel electrophoresis confirmed the occurrence of the charge-reversal property of functional gold nanoparticles. These types of NPs also showed the much lower toxicity to cell proliferation.

Via covalent conjugation

The nucleic acid can be delivered via covalent as well as noncovalent conjugation for the treatment of genetic and acquired disease (Miller, 1992). Au NPs have a property to make a covalent linkage with some compounds transporting the NA materials in the cells, in which several polycationic materials are used to condense the DNA and perform the same activity as described above. NA has a property that it can be easily modified with thiols (-SH) for grafting into NPs; in this way, some research group conjugated thiolated siRNA with Au NPs to achieve cellular delivery (Oishi *et al*., 2006). Some oligonucleotide-modified gold nanoparticles, which carry large negative surface potential, are stable against enzymatic degradation (Rosi *et al*., 2006).

Via noncovalent conjugation

Achieving efficient nucleic acid delivery through a carrier should provide some protection of NA from degradation through nuclease (Thomas and Klibanov 2003a). Among all the nonviral vectors, Au NPs provide a high surface-to-volume ratio and maximize pay load ratio, leading to higher efficiency in gene delivery. Noncovalent functionalization through certain cationic quaternary ammonium compounds protect DNA from enzymatic degradation (Han *et al*., 2006) and bind plasmid DNA through electrostatic interactions (McIntosh *et al*., 2001). This noncovalent interaction between Au NPs and NA provides an effective means of gene delivery (Sandhu *et al*., 2002). Gold nanoparticles functionalized with branched polyethylenimine provide hybrid Au NP polymer transfection vectors (Thomas and Klibanov, 2003b).

2.4.3 Chitosan nanoparticles

Chitosan is a linear heteropolysaccharide composed of N-acetyl-D-glucosamine and β-(1, 4)-linked D-glucosamine. Commercially, chitosan is obtained from chitin via alkaline deacetylation, which is performed by decolourizing crab and shrimp shells with potassium permanganate and then boiling them in sodium hydroxide (Paul and Garside, 2000). Due to its biocompatibility, biodegradability, and NA-binding ability, chitosan has been widely investigated as a pDNA carrier. In general, chitosan-based pDNA transfer depends greatly on both the ionic and nonionic interactions between the carbohydrate backbone of chitosan and surface proteins of transfected cells (Venkatesh and Smith, 1998). Chitosan has a primary amine group with pKa value of 6.5. Generally in an acidic aqueous medium, it forms positively charged single helicoidal stiff chains (Berth *et al*., 1998). This positive surface charge of chitosan allows it to interact with macromolecular substances like exogenous NA, negatively charged mucosal surfaces and the plasma membrane (Hejazi and Amiji, 2003).

Chitosan-based gene or NA nonviral vectors could neutralize the negative charge of nucleic acids and reveal significant versatility in terms of rigidity, hydrophobicity/hydrophilicity, charge density, and biodegradability (Martirosyan *et al*., 2014).

Role of chitosan in plasmid DNA transfer

Mumper *et al*. (1995) first investigated the potential of chitosan for *in vitro* pDNA delivery. Chitosan NPs (Fig. 2.6(c)) can be prepared by different methods like coacervation (Mao *et al*., 2001), covalent cross-linking (Ohya *et al*., 1999), ionic gelation (Berthold *et al*., 1996), and desolvation (Hamidia *et al*., 2008). Variation in processing parameters in different techniques and other parameters like NA concentration, charge ratio, pH, and concentration of salt can affect the size of the chitosan−DNA complex and overall transfection efficiency (Janes *et al*., 2001). This is exemplified by the increased transfectability of chitosan in highly differentiated cystic fibrosis bronchial epithelial CFBE4o- cells, and this increased transfection was likely to be associated with the damage of the cell surface and variation of surface charge under a low pH environment (Nydert *et al*., 2008). Along with the carrier of pDNA, chitosan has been found to enhance the transfectability of other transfecting agents like transfection efficacy in HepG2 cells mediated by a cationic emulsion composed of Tween 80, 3β [N-(N′,N′- dimethylaminoethane) carbamoyl] cholesterol, dioleoylphosphatidyl ethanolamine, and castor oil (Lee *et al*., 2005). Chitosan salts like chitosan lactate, chitosan hydrochloride, chitosan acetate, chitosan glutamate and chitosan aspartate were evaluated for their performance in CHO-K1 cell transfection and were found to have superior transfectability than standard chitosan (Weecharangsan *et al*., 2008).

Chitosan in therapeutic small RNA transfer

Chitosan NPs are not only useful in the plasmid DNA delivery, but can also transfer RNA with some advantages like increased cellular uptake and alleviation from degradation of naked mRNA or siRNA both *in vitro* and *in vivo* (Lai and Lin, 2009). In this context, Howard *et al*. (2006) reported the reduced number of enhanced green fluorescence protein-expressing epithelial cells in the bronchiole of mice via daily nasal administration of interpolyelectrolyte siRNA/chitosan complexes; the reduction was 43% and 37% compared to the untreated and mismatch control, respectively. Several attempts in the past have been made in vector modification or derivatization by various means like structural modification or ligand conjugation (Table 2.2) for accelerating the efficiency of RNA transfer with chitosan. The first attempt has been made by Katas and Alpar (2006), who synthesized chitosan nanoparticles with an ionic gelatin of chitosan salts like chitosan hydrochloride or glutamate or sodium tri-polyphosphate instead of using conventional chitosan as a vector. The authors reported that NPs prepared by this method were more efficient than standard chitosan in siRNA transfer in terms of higher RNA binding capacity and loading efficiency. Not only is chitosan alone functioning as an RNA vector, it has been also incorporated with other vectors for RNA delivery such as chitosan coated PLGA-NPs. These NPs have been reported to be successfully bound to the antisense oligonucleotide (2′-O-methyl-RNA) and were taken up by A549 cells after six hours of incubation (Nafee *et al*., 2007). Currently, chitosan-mediated nasal or oral routes of nucleic acid delivery attract biomedical researchers due to their viability in therapeutic efficacy (Allemann *et al*., 1998).

Table 2.2 Different approaches used in chitosan vector derivation along with advantages and disadvantages

Approaches	Advantage	Disadvantage
Structural modification		
Copolymerization	Old-style exercise in polymer chemistry	Limited availability of conventionally used polymeric modifiers
Functional group modification	No significant increase in the size of the nucleic acid carrier after the modification	Require sophisticated consideration of SAR however
Ligand conjugation		
Proteinaceous	Proteins and peptides can possess diverse functionalities and a wide choice of ligands	These may be immunogenic and expensive
Nonproteinaceous	Cheaper than proteinaceous ligands	Choice of conventionally used ligands could be limited

SAR, structure activity relationship.

2.4.4 Silica nanoparticles

The varying size of NPs compared to bulk materials and composition is important in bioanalysis and biotechnological applications. The unique properties like high surface-to-volume ratio, optical properties, and size-dependent qualities offer the added advantages in this regard (Tan *et al*., 2004). The wide utility of silica as a substrate in biosensors and biochips makes them an attractive agent in bioanalytical applications. Silica-based nanoparticles can be prepared through various approaches (Fig. 2.6(d)). The silica nanoparticles are generally affected by the chemical properties of the surface and also based on the silanols and siloxanes present on the surface (Spange, 2000). Among the standard silica, mesoporous silica nanoparticles (MSNs) are prepared by polymerizing silica in the presence of surfactants. These MSNs have many advantages like tunable pore sizes, large surface area and volumes, and encapsulation of drugs, especially for intracellular delivery (Hom *et al*., 2009). To enhance the usefulness of silica NPs, a variety of surface modifications were reported to couple the NPs to various biomolecules such as oligonucleotides, enzymes, antibodies, and proteins. A remarkable advantage noted was the versatility of the silica surface toward different surface modification protocols. The silica surface can be modified to contain sulfide, avidin, amine, or carboxylate groups (Tan *et al*., 2004). Wagner *et al*. (1991) delivered NA materials through the polycation−DNA complexes. This cationic attachment is generally applied to the silica NPs to protect, bind and deliver DNA and other oligonucleotides and RNAs (Fig. 2.7). In an excellent study of surface modification of silica NPs, Kneuer *et al*. (2000a) modified the commercially available silica particles (IPAST) with either N-(2-aminoethyl)-3aminopropyltrimethoxysilane or N-(6- aminohexyl)-3-aminopropyltrimethoxysilane. The size of NPs was between 10 and 100 nm and surface charge potentials from +7 to +31 mV at pH 7.4. Similarly,

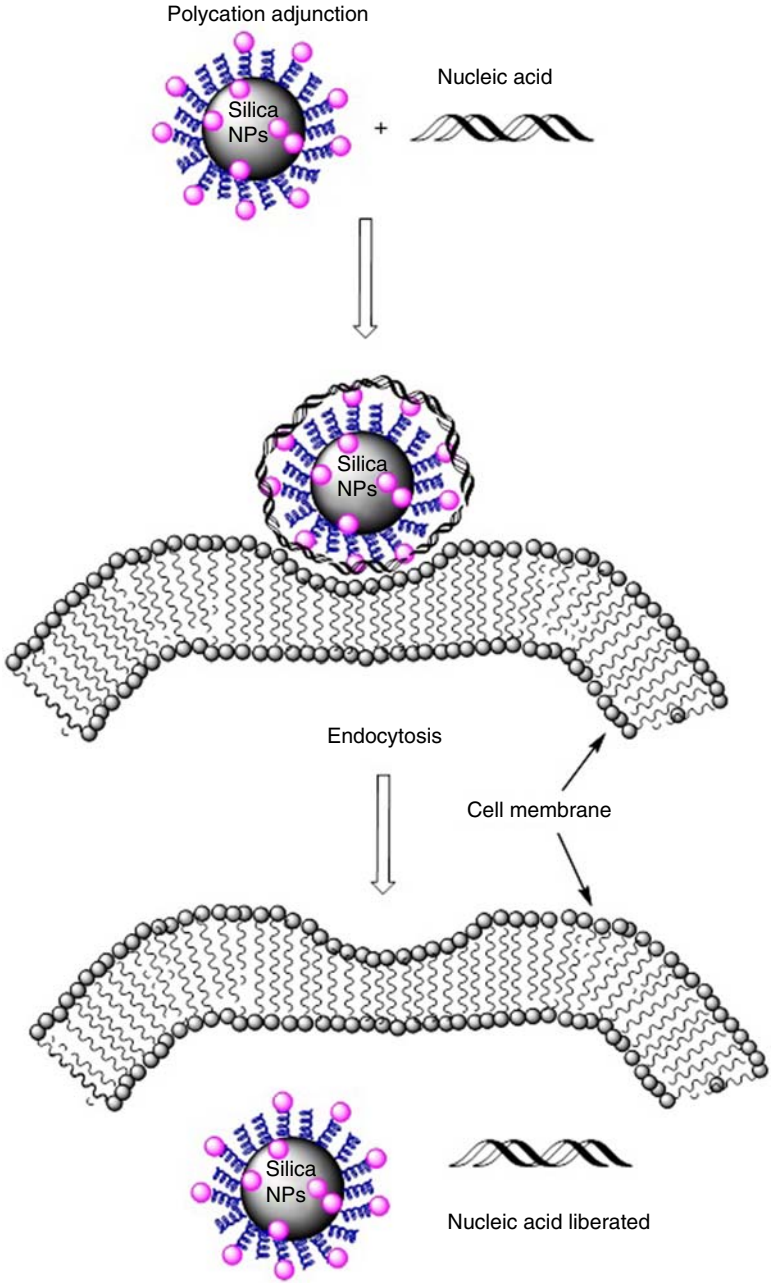

Figure 2.7 Binding of nucleic acids to positively charged silica nanoparticles.

He *et al*. (2003) synthesized amino-modified silica nanoparticles using the synchronous hydrolysis of tetra-ethoxy silane and N-(β-amino ethyl) -γ-aminopropyl-triethoxysilane in water-in-oil microemulsion; size of the NPs was 45 ± 4 nm. Another investigation has shown that colloidal silica particles with covalently attached cationic external surface modifications with amino alkyl silanes could transfect plasmid DNA *in vitro* successfully by using β-Galactosidase as the genetic payload, and its transfection efficiency was measured in Cos-1 cells. The authors also reported that the use of silica−silane−DNA nanoplexes leads to elevated expression of their DNA cargo and transfection rates increased by using the chloroquine conjunction with DNA nanoplexes (Kneuer *et al*., 2000b). Radu *et al*. (2004) prepared polyamidoamine (PAMAM) dendrimer-capped mesoporous silica nanospheres as a nonviral gene transfecting carrier. In their research, PAMAM dendrimers were covalently attached to the external surface of the nanospheres and then were complexed with plasmid DNA. Gel electrophoresis revealed the protection of plasmid DNA from enzymatic degradation. They observed that there is a strong electrostatic interaction binding with negatively charged DNA to positively charged PAMAM nanospheres.

Silica nanoparticles and antisense oligonucleotides

As discussed in the previous sections, AONs are short nucleotide sequences of DNA. These inhibit gene expression at both mRNA and protein levels through Watson−Crick base pairing (Mergny *et al*., 1992). AON shows potential advantages as a therapeutic agent against diseases with fundamental genetic components, such as viral infections and cancer. But like siRNA, they show poor intracellular uptake and stability (Yakubov *et al*., 1989). Generally these deformities lead to limited therapeutic uses. To overcome the above hurdles, Poly-L-lysine-modified silica nanoparticles (PMS-NPs) have been used to bind and protect AON. PMS-NPs were prepared in a microemulsion system using polyoxyethylene nonylphenyl ether/cyclohexane/ammonium hydroxide with a size of 20 ± 2 nm. The results indicated that PMS-NP displayed significantly low cytotoxicity in human nasopharyngeal carcinoma cells and HeLa cells (Zhu *et al*., 2004).

Silica nanoparticles and small interfering RNAs

siRNA, also known as short interfering RNA, generated through the cleavage of longer double-stranded RNA by the action of ribonuclease III family enzymes, is a class of 20−25 nucleotide-long double-stranded RNA molecules that are involved in the RNA interference (RNAi) pathway, where it interferes with the expression of a specific gene (Elbashir *et al*., 2001). Along with potential advantages of siRNA in gene therapy, some obstacles are also there such as poor intracellular uptake, nonspecific immune stimulation, and stability (Filleur *et al*., 2003). NPs have shown to be effective vectors in siRNA and antisense therapeutics. Apart from the uses of silica NPs in siRNA delivery, some synthetic polymeric NPs have also achieved success *in vitro* and *in vivo*, like NPs consisting of PEI that is PEGylated with an Arg-Gly-Asp peptide ligand. The size of the resulting nanoplex was about 100 nm. The purpose of this conjugation was to target tumor expressing integrin and deliver siRNA inhibiting

vascular endothelial growth factor receptor-2, thereby inhibiting tumor angiogenesis (Schiffelers *et al*., 2004).

Silica nanoparticles in enhancing transfection efficiency

A nonviral delivery system can consist of several components including NA, transfection agents, and NPs. These components are designed in such a manner so as to overcome certain obstructions like cellular uptake, transfection efficiency, cytotoxicity, and protein expression (Luo *et al*., 2004). In this approach, silica NPs use transfection enhancers rather than a mode of delivery like dense inorganic silica nanoparticles, which by themselves do not deliver DNA and are able to enhance DNA transfection mediated by other commonly used transfection reagents. In this delivery system, there are three components: silica NPs, transfection reagent and NA materials. Each component serves a different function to overcome the aforementioned barriers. The particular role of silica nanoparticles is to enhance uptake by physical concentration at the cell surface (Luo and Saltzman *et al*., 2000b).

2.4.5 Solid lipid nanoparticles

In the context of drug delivery systems, solid lipid nanoparticles (SLNs) have the ability to trap drugs within the solid lipid matrix, allowing them to not only provide protection against chemical degradation, but also cause a modulation of the drug release profile, named the sustained release profile (Bunjes, 2010). Several properties of SLNs make them suitable carriers for nucleic acid or gene delivery vectors. Certain properties, such as long-term physical stability and high pay load, can perfectly accommodate varying formulations and are readily amenable to surface coupling of specific ligands. Since 2000, they have come to be known as gene delivery vectors (Olbrich *et al*., 2001). They are readily malleable to surface coupling of specific ligands, thus allowing the formation of decorated particles for the specific targeting of specific cells, tissues, or organs, including the brain (Patel *et al*., 2011; Yu *et al*., 2012).

A variety of procedures have been reported for preparing SLNs (Attama, 2011). To optimize the various production methods, it is necessary to evaluate each individual step in SLN production. To accommodate the negatively charged nucleic acids, cationic SLNs are prepared by including a positively charged cosurfactant in their formulation like benzalkonium chloride, cetylpyridinium chloride, cetrimide, N, N-di-(b-stearoylethyl)-N, N-dimethyl-ammonium chloride (Esterquat 1), N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethyl ammonium chloride, or 6-lauroxyhexyl lysinate (Manjunath *et al*., 2005). Regarding the SLN-mediated delivery of nucleic acids, the choice of an appropriate combination of cationic and matrix lipids seems vital, as it may exert a significant effect on the transfection efficiency (Tabatt *et al*., 2004).

Association of solid lipid nanoparticle–DNA complex (lipoplex)

Generally, complexes between nucleic acids and cationic lipids (e.g., SLNs) are prepared by simply adding DNA to preformed cationic lipid-containing nanoparticles,

suspended in aqueous solution (Rehman *et al*., 2013). Lipoplex is a spontaneous process ($\Delta G < 0$) for cationic lipid. A net-positive enthalpy gain is maintained by an increase in ionic strength, resulting in a decrease in the binding enthalpy, regardless of the ionic strength or the order of DNA and lipid addition (Kennedy *et al.,* 2000; Pozharski and Macdonald, 2003). Thus an increase in entropy should be the thermodynamic driving force for the spontaneous formation of lipoplexes. Lipoplex formation (Fig. 2.8) has a significant impact on the structure of both the cationic liposomes and DNA. The vesicular structures formed are completely reorganized due to nucleic acid-induced aggregation and fusion (Oberle *et al*., 2000; Kikuchi and Carmona-Ribeiro, 2000). The interaction between DNA and three-dimensional ordered multilayered structures are described by three different organizations:

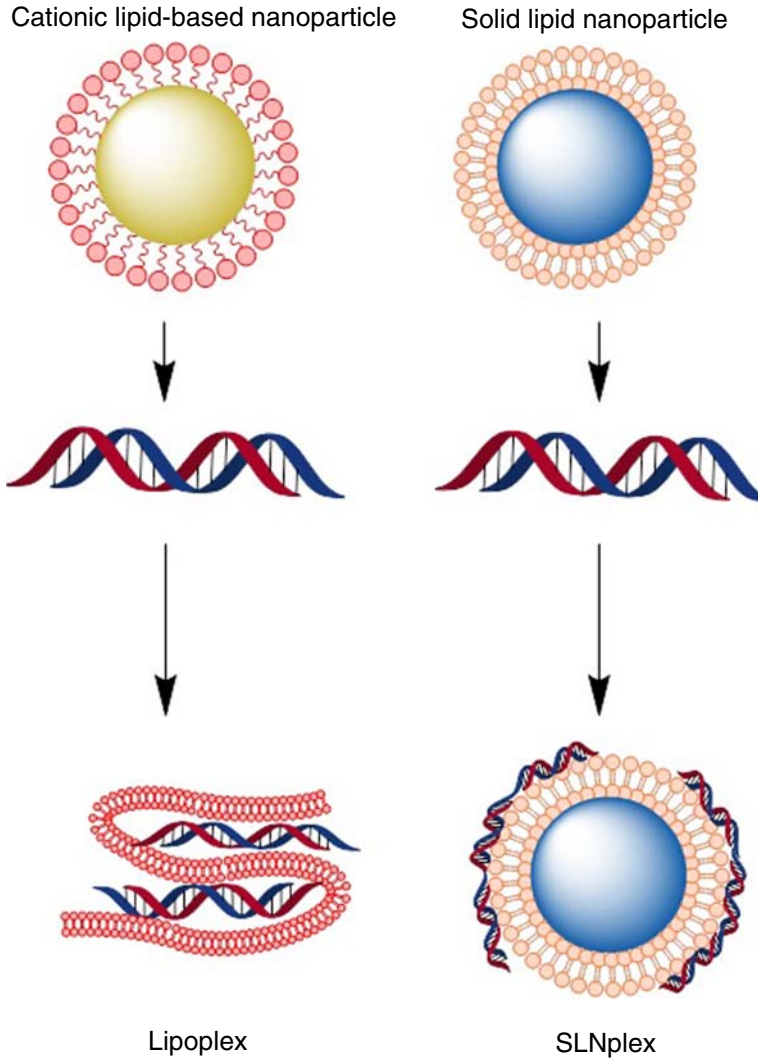

Figure 2.8 Lipoplex and SLNplex formation during the complexation of nanoparticles to DNA.

(1) the intercalated hexagonal structure (HI), characterized by DNA rods surrounded by three cylindrical lipid micelles arranged on a hexagonal lattice; (2) the lamellar structure (LCα), characterized by DNA rods inserted between lipid bilayers; and (3) the inverted hexagonal structure (HII), characterized by DNA rods covered with lipid monolayers organized on a hexagonal matrix (Ilarduya *et al*., 2010; Pector *et al*., 2000).

Association of solid lipid nanoparticle–DNA complex

"SLNplexes," which are complexes formed between solid lipid nanoparticles (SLNs) and nucleic acid, display several features that are similar to those observed for lipoplex formation, i.e., between cationic lipids and nucleic acid, although the final structural organization may be different. Similar to lipoplex formation, SLNplex formation (Fig. 2.8) is also a spontaneous process ($\Delta G < 0$) and ITC measurements showed a positive enthalpy (Rullaud *et al*., 2012). Like electrostatic interactions, hydrophobic interactions also appear to play a major role in the assembly and stabilization of the SLNplexes. In the assembly of SLNplexes, the interaction between nucleic acids and SLNs is mainly through adsorption of the nucleic acid to the particle surface (Jesus *et al*., 2014; Vighi *et al*., 2010).

2.4.6 Composite nanoparticles

Composite nanoparticles (Fig. 2.6(e)) are the carriers, which are fabricated with multiple materials and are furnished with varied functionality. Due to scientific importance, these NPs have attracted much more attention in the material science field. Before the preparation of composite NPs, it is necessary to understand all the physicochemical properties of novel materials that have been exploited to set up reproducible and well-controlled fabrication protocols (Wang *et al*., 2014). Composite NPs can be prepared by various methods with multiple steps due to the complicated composition of NPs such as self-assembly, microfluidic mixing, layer-by-layer and through particle replication in nonwetting templates technology.

Sometimes simple nanoparticles made up of a single material are failing to overcome certain barriers in nucleic acid delivery that have been discussed previously in this chapter. In order to resolve this problem, diverse functional materials are chosen and incorporated into one nanoparticle to overcome these barriers. Nowadays, that type of technology emerged as a very effective modular design in gene delivery. There is much research that has been done on composite NPs to overcome certain barriers in gene delivery; for example, the reticulo-endothelial system takes up the nanoparticles in the blood circulation, the cytoplasmic membrane prevents the nanoparticles from entering the cells, the nanoparticles are trapped in the end/lysosome compartment and the nuclear envelope blocks the DNA from entering the nucleus for transcription.

Lipid-derived composite nanoparticles

Lipids have been extensively used in the fabrication of composite NPs due to their property of easily formulated nanopreparation through electrostatic and hydrophobic interactions. Generally lipid coating provides the liposomal characteristics to composite NPs. The surface of lipid-coated NPs can be easily manipulated or modified by the

well-established protocols, such as PEGylation, or any other targeting ligand or other functional monogram of nanoparticles can be achieved by postinsertion of lipid PEG conjugates.

Different types of cores can be used in the fabrication of lipid-derived composite NPs; these may be polyplex, mesoporous silica, gold, and magnetic NPs.

Polyplex-based core

These are nanosized complexes between a negatively charged polyanion-like nucleic acid and a positively charged polycation such as synthetic polymer (polylysine), natural polymer (chitosan), and polypeptides (histones). These polyplex-based core NPs are widely used in the delivery of NA, siRNA, or DNA. Instability issues and the nonspecific uptake of the reticuloendothelial system after systemic administration of these NPs can be overcome by the coating of the polyplexes with lipids. When polyplexes are mixed with lipids, a lipid bilayer spontaneously forms on the surface of the polyplex. This strategy takes the advantage of low toxicity and immunogenicity of liposomes along with their ability to be PEGylated for extended circulation. An interesting example of the aforementioned approach is the formulation of the core membrane nanoparticles using poly (L-lysine) and protamine to condense pDNA into a negatively charged polyplex (Li and Huang, 1997).

Mesoporous silica-based core

In order to deliver nucleic acid, the surface of silica in mesoporous silica NPs converts to carry positive charges for the binding of DNA or siRNA. This is grasped by the grafting of an amine group on the surface of MSNs (Kneuer *et al*., 2000b). Due to the relatively small pore sizes of MSNs, nucleic acid therapeutics such as DNA and siRNA are usually immobilized on the external surface of the MSNs. Liu *et al.* (2009) developed mesoporous silica-based composite NPs by fusing liposomes on MSNs and simultaneously loading and sealing the cargos inside the particles with the lipid membrane termed as "protocells." Compared to conventional liposomes, protocells have a proven higher drug-loading capacity and stability (Ashley *et al*., 2011).

Gold and magnetic nanoparticle-based core

Gold nanoparticles have been reported as an efficient nucleic acid delivery system due to their biocompatibility, versatility, and facile surface modification through gold−thiol linkages (Ding *et al*., 2014). Barnaby *et al.* (2014) reported the nucleic acid−gold nanostructures and their biomedical applications. They mixed the gold NPs with thiolated siRNA where siRNAs duplexes were allowed to chemisorb via thio−gold bonds. The surface functionality of NA increases the stability of bound materials, and negatively charged, nucleic acid-adsorbed gold NPs showed conceded cellular uptake because of the weak interactions between the cellular membrane and nanoparticles. To overcome this issue, Rhim *et al.* (2008) prepared coated nucleic acid-adsorbed gold NPs with cationic liposomes to enhance the cellular uptake and protect cargos from nuclease-mediated degradation. In the magnetic NP-based core, the magnetic field has been exploited as external energy to increase the transfection efficiency of

nonviral-mediated NA delivery (Duan *et al.*, 2014). Namiki *et al.* (2009) prepared "lipomag," a lipid-coated magnetic nanocrystal. This special class of delivery vector was prepared by oleic acid-coated magnetite nanocrystal cores with cationic lipid in chloroform and then the solvent was removed. Final NPs were generated on top of the nanocrystals via hydrophobic interactions, and finally, siRNAs were then adsorbed on the surface of the lipomag through electrostatic interactions. The delivery of siRNA by the lipomag was observed in a tumor lesion which resulted in the target gene silencing activity.

2.5 Conclusion and future prospects

The delivery of DNA and other nucleotides as therapeutics has continuously been a challenge to researchers. The extracellular, intracellular and other barriers offer added hurdles in the delivery of DNA, RNA, and AON to the affected cells. Another challenge, which has equal impact to the delivery of genes, is the unstable nature of the DNA under the influence of the enzymes DNAase and RNAase. Similar to drug delivery, the encapsulation and/or conjugation of genes to the polymeric carriers has always been advantageous in terms of the effective transfection and delivery to the targeted cells. The size has played a crucial role in this regard and the smaller the size, the more the chance of the vector to be bypassed from the mononuclear phagocytic system of the body which would in turn increase its overall circulation time and stability. Nanoparticles that are colloidal and have a size range of 1−100 nm offer unique advantages and properties as nonviral vectors for the delivery of genes. The tailor-made surface functionality, nonimmunogenicity, availability of a variety of polymers, lipids, and proteins, as well as the different methods of preparation make these carriers ideal vectors for the delivery of DNA or RNA. As mentioned earlier, the different types of nanoparticles such as lipidic, chitosan based, gold, silica, and composite are the important classes of nanoparticles which have proven to be highly efficient transfecting agents. The opportunity to engineer the nanoparticulate surfaces with PEG or other ligands has added another advantage in terms of increased encapsulation and reduced toxicity associated with the cationic charge. The cationic charge associated with the nanoparticles, such as lipid nanoparticles, silica nanoparticles and others, plays a crucial role in the formation of the polyplex, SLNplex, and other nanoparticle/DNA complexes. These polyplexes, through the RISC formation, silence the desired defected genes in a target cell.

Since 1996, gene delivery has emerged as a promising approach for the treatment of complex disorders such as cancer, Alzheimer's, AIDS and related disorders. The possibilities of nanoparticles to act as efficient nonviral vectors have emerged as alternate and effective solutions to gene delivery. Future studies may include the co-delivery of antibodies with the relevant DNA or RNA. The cocktail delivery of drugs with DNA or siRNA can have a dual effect in the amelioration of the disorders. The increasing challenge of the drug-induced resistance can be overcome by silencing the responsible gene expression and simultaneously delivering the drug for which the cells have been sensitized.

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Methods for characterization of nanoparticles

3

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3.1 Introduction and challenges in nanoparticle characterization

Nanoparticles and −based formulations have occupied several important application domains that have direct relevance to our routine. The range of nanoparticle applications extends from drugs and drug delivery to the development of efficient solar cells (Beek *et al*., 2004; Baxter and Aydil, 2005; Law *et al*., 2005; Nakayama *et al*., 2008). Given the nature of their applications, it is crucial to characterize these nanoparticles for several physical, chemical, and biological properties. Various considerable properties that require an adequate characterization are listed in Table 3.1.

In order to characterize nanoparticles for the aforementioned properties, several state-of-the-art tools are present at our disposal (Table 3.2). These tools provide detailed information of the physical and chemical properties of nanoparticles.

Characterizing nanoparticles for biomedical applications, specifically for *in vivo* delivery, is crucial (Mürbe *et al*., 2008; Bajaj *et al*., 2012; Reddy *et al*., 2012). A plethora of characterization techniques have been approved but achieving a complete characterization profile of nanoparticles is still a challenging task. Major bottlenecks in such characterizations are summarized in Table 3.3. An important reason for a faulty or under-characterized nanoparticle formulation is the time-dependent fluctuations of their chemical and physical properties. The simplest example could be a formulation based on DNA-grafted silica nanoparticles (Kar *et al*., 2013; Wu *et al*., 2013) that is completely characterized for the properties described in Table 3.2; however, after a period, these particles will show flocculation due to the interactions of salt or other stabilizing proteins with the surface caused by aging. Now if these particles were to be characterized again without any tempering, their properties will be different from

Table 3.1 Important physicochemical properties of nanoparticles

- Particle shape, size, and distribution
- Particle roughness and topography
- Surface area and surface chemistries
- Stability, dispersion, swelling, agglomeration, and aggregation
- Purity
- Reactivity and hydrophobicity

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Table 3.2 Techniques for nanoparticle characterization

Properties of nanoparticles	Methods of characterization
Morphology (shape/size)	Dynamic light scattering
	Electron microscopy (scanning/transmission)
	Atomic force microscopy
Topography (surface)	X-ray diffraction
	BET
Chemical	UV−visible spectroscopy
	Electron dispersive X-ray spectroscopy
	Fourier transform infrared spectroscopy/attenuated total reflection
Electrical	Electrokinetics (zeta/cyclic voltammetry studies)
Optical	Microscopy
	Double photon correlation spectroscopy
	Raman spectroscopy
	Surface plasmon resonance
Biological	*In vitro* cell viability
	In vivo
	Microbial colony viability

the initial characterization. The best practice would be to create characterization profiles for several time points and under several rounds of lyophilization and later correlate those with each other to get a better understanding of why and how aging affects the physicochemical properties of nucleic acid-grafted nanoparticles.

3.2 Techniques and approaches to nanoparticle characterization

3.2.1 Morphology and topography analysis

Particle shape and size is the most basic set of information collected at the beginning of characterization. Particle size determines the suitability of nanoparticles for a specific application.

3.2.1.1 Dynamic light scattering

Dynamic light scattering (DLS) is the most common approach to analyze hydrodynamic particle size and distribution of the particles over a range of sizes. DLS is

Table 3.3 Challenges in nanoparticle characterization

Property	Challenges	References
Size	Heterogeneity in size. Size-dependent properties vary (charge density, surface area, etc.) Characterization averages out the information thus leading to misrepresentation.	Hoo *et al.* (2008), Lu *et al.* (2011), Mahl *et al.* (2011) and Boyd *et al.* (2011)
Surface	Strong dependence on nanoparticle's microenvironment. Surface-dependent properties vary (functional groups, reactivities, etc.) Correlation between chemical and physical characterization must be done to obtain quantitative details. Stability of nucleic acid-grafted nanoparticles.	Widegren and Bergström (2002), Kirby and Hasselbrink (2004), Brant *et al.* (2005) and Murdock *et al.* (2008)
Aging	Time dependent. Affects size-, shape-, surface-, and stability-based properties, such as swelling, shrinking, adsorption of contaminants on surface, agglomeration/aggregation, multipolar deformations, etc. Characterization variability over a period restricts their usability.	Kang *et al.* (2010), Lee *et al.* (2010), Kuchibhatla *et al.* (2012) and Rossano *et al.* (2014)

the measurement of light interference based on the Brownian motion of nanoparticles in suspension and a correlation of its velocity (diffusion coefficient) with its size using Stokes−Einstein equation (Bizheva *et al.*, 1998; Li *et al.*, 2014). The size distribution range of the particles is depicted as polydispersity index (PDI), which is an output of autocorrelation function. PDI values lie between 0 and 1, where 1 is the highly heterogeneous population and 0 is the highly homogeneous nanoparticle population. This technique also allows for analyzing nonspherical particles, such as rods, using multiangle DLS.

Dispersity is the major limiting factor in obtaining good correlation, and thus an accurate size determination. The reason is that there is no absolute control on the synthesis process, and a chemical reaction to develop nanoparticles is not a homogeneous process. This results in the development of nanoparticles with a variety of shapes and sizes introducing intra- and interbatch variabilities. However, fine optimization and tune-ups can provide near to homogeneous samples. Also, sample preparation is critical while performing DLS. Slight contamination with dust and fibers will result in inappropriate measurements.

3.2.1.2 Electron microscopy

Electron microscopy (scanning EM/tunneling EM) can be employed for revealing the details of the nanoparticle shape and surface. However, sophisticated and laborious sample preparation approaches associated with the biological samples (nucleic acid-coated particles) constitute the major restriction to their use. Biomolecule-coated particles result in poor quality electron microscopy images. Sample preparation causes dehydration and leads to sample shrinking. Drying samples tend to aggregate, thus introducing additional artifacts. In addition, longer analyses times and an inability to calculate dispersity constitute another set of challenges.

3.2.1.3 Other methods

Density gradient and size exclusion centrifugation can also be employed for estimating the particle size and can allow for theoretical calculation of particle size. The key to this approach is that it will separate homogeneous single-sized particle population from the multidispersed samples. This can allow researchers to collect the sample of a specific size that they are interested in as per the application.

Surface area and porosity are other important parameters that can be limiting on the *in vivo* application of nanoparticles. Gas adsorption experiments based on the Brunauer–Emmett–Teller theory and Barrett–Joyner–Halenda analysis are employed for surface area and pore size estimation, respectively. These analyses are crucial for an appropriate particle characterization. Particles with high surface area and porosity will adsorb nonspecific protein *in vivo* that may not be useful at the site of action, or they may coat a big population of nucleic acid on the surface of these particles, thus rendering them useless. Also, particles for *in vivo* applications are mostly dye-doped, and bigger pores will expedite leaching of such doped materials.

Apart from the shape and size of the particles the charge is another very important criterion for achieving highly stable nanoparticles with high-efficiency nucleic acid delivery.

3.2.2 Surface charge analysis (zeta potential measurement)

Zeta potential measurement is a technique for determining the surface charge of nanoparticles in a colloidal solution. Nanoparticles have a charge on the surface that attracts a thin layer of counter ions to the nanoparticle surface (Stern layer). This double layer of ions travels with the nanoparticle as it diffuses throughout the solution. The electric potential at the boundary of the double layer is known as the zeta potential of the particles and has values that typically range from +100 to −100 mV. The magnitude of the zeta potential is predictive of the colloidal stability of the solution (Table 3.4).

In general, nanoparticles with zeta potential values greater than positive 30 mV or less than negative 30 mV have high degrees of stability. Dispersions with less than +25 mV or greater than −25 mV zeta potential value will eventually agglomerate due to interparticle interactions, including van der Waals and hydrophobic interactions, and hydrogen bonding. Zeta potential is an important tool for understanding

Table 3.4 Stability behavior of the colloid based on zeta potential value

Zeta potential value (mV)	Stability behavior
0 to ±5	Flocculation or coagulation
±10 to ±30	Incipient instability
±30 to ±40	Moderate stability
±40 to ±60	Good stability
Greater than ±60	Excellent stability

the surface of the nanoparticle and predicting the stability of the nanoparticle in a solution. Zeta potential analysis is routinely employed to monitor the successful surface functionalization or modifications. It is very important to understand the limitation and strengths of zeta potential measurement when interpreting the results, as measurement results can be easily misinterpreted.

For better zeta potential measurement the samples should be:

- highly monodispersed
- of optimum concentration to effectively scatter the light (633 nm wavelength)
- <1 mS/cm (low salt concentrations) in conductivity
- well dispersed in polar dispersant

Conversely, the majority of the nanodispersion does not meet all of the above criteria and thus may not give good quality zeta potential results. Common challenges in the as-prepared samples are listed in Table 3.5.

3.3 Stability evaluation of nanoparticles

The stability of nanoparticles is defined in terms of their ability to contain their chemical and physical composition over a period of time. The stability of the precursor materials is critical as they form stable or weak breakable chemical formulations. It could also relate to their orientation within their chemical composition because these materials may not chemically degrade, but may physically alter their arrangement/confirmation over time or under different physiological conditions (temperature, light, humidity, polarity, pH, etc.). If the nanoparticles are serving as biomolecule carriers for *in vivo* applications, then it is important to understand the overall stability of the interaction of the carrier particle with the loaded biomolecules under several biophysical conditions mimicking the systemic circulation (blood) matrix. One of the challenges in nanomedicine is engineering and matching up the drug/diagnostics physicochemical properties with nanoparticles to achieve sufficient stability along with an ability to protect the drug/agent (or prevent toxicity) yet allow it to "function," i.e., release the drug or be taken up at target sites.

Table 3.5 Restrictions associated to zeta potential measurements due to sampling restrictions

Properties	Challenges
High conductivity	• Electrode polarization • Degradation
Very low concentration of solution	• Not sufficient light scattering events • Instrument reaches its detection limit • Difficult to stabilize the potential values • Low conductivity
Very high concentration of solution	• Can diminish the intensity of the scattered light • Multiple scattering reduces data quality
Unknown properties of the solution	• Unknown values of viscosity and refractive index • Dissolve solids other than target particles • Residual reactant (when particles are not cleaned properly)
pH of the measuring solution	• pH varies with temperature and concentration
Very small particle size	• Very small sized particles (<20 nm in diameter) have high mobility and low light scattering properties. • Narrow range of sample concentrations

3.3.1 Stability issues related to nanoparticle dispersion and suspensions

Stability issues related with nanodispersion have been widely studied and can be categorized as physical and chemical stability. The common physical stability issues include agglomeration, sedimentation/creaming, change of crystallinity state and growth of crystals.

3.3.1.1 Agglomeration

The large surface area of nanoparticles creates high total surface energy, which is thermodynamically unfavorable (Wu *et al*., 2011). Accordingly, the particles tend to agglomerate to minimize the surface energy. Agglomeration can cause a variety of issues for nanosuspensions including rapid settling/creaming, crystal growth and inconsistent dosing. The most common strategy to tackle this issue is to introduce stabilizers to the formulation. In addition to safety and regulation considerations, the selection of stabilizers is based on their ability to provide wetting to the surface of the particles and offer a barrier to prevent nanoparticles from agglomeration (Gao *et al*., 2008; Van Eerdenbrugh *et al*., 2008). There are two main mechanisms through which colloidal suspensions can be stabilized in both aqueous and nonaqueous mediums, i.e., electrostatic repulsion and steric stabilization (Rabinow, 2004; Nutan and Reddy, 2010). These two mechanisms can be achieved by adding ionic and

nonionic stabilizers into the medium, respectively. The commonly used ionic stabilizers in an aqueous medium include sodium dodecyl sulfate, sodium lauryl sulfate, lecithin, and docusate sodium (Hameed, 2013). The nonionic surfactants used in an aqueous medium are usually selected from Pluronic surfactants, Tween 80, polyethylene glycol, polyvinyl alcohol polyvinylpyrrolidone, and cellulose polymers such as hydroxypropyl cellulose and hydroxypropyl methylcellulose (Foster *et al*., 2013; Giardiello *et al*., 2013).

Despite the proven importance of stabilizers in preventing particle agglomeration, there have been a few studies that generated stable nanosuspensions without stabilizers (Abdelwahed *et al*., 2006; Sun and Yeo, 2012; Borhade *et al*., 2013). Baba *et al*. (2007) prepared 2-devinyl-2-(1-hexyloxyethyl)pyropheophorbide (HPPH) nanosuspensions without any stabilizer and reported formulation stability for more than 3 months. The self-stabilization of the nanosuspensions was attributed to a high zeta potential value (−40 mV) resulting from the deprotonation of the carboxylic end group of HPPH molecules. A similar self-stabilized nanosuspension was reported in another study in which amorphous all-trans retinoic acid nanoparticles were shown to be stable in an aqueous medium for up to 6 months. Epstein *et al*. (2007) prepared self-suspended alendronate nanosuspensions by combining the negatively charged alendromnic acid with gallium (Ga) or gadolinium (Gd) under sonication as complex nanoparticles. The alendronate-Ga nanosuspension was shown to be stable for more than 3 months, while the alendronate-Gd nanosuspension was stable for only 3 days. These stability profiles correlated well with their zeta potential values (33 mV for Ga complex vs. 21 mV for Gd complex).

3.3.1.2 Sedimentation or creaming

Drug particles can either settle down or cream up in the formulation medium depending on their density relative to the medium. The sedimentation rate is described by Stokes' law, which indicates the important role of particle size, medium viscosity, and density difference between medium and dispersed phases in determining the sedimentation rate. Decreasing particle size is the most common strategy used to reduce particle settling (Nutan and Reddy, 2010; Wu *et al*., 2011). Matching drug particle density with medium or increasing medium viscosity are the other widely used approaches to alleviate sedimentation problems (Lee *et al*., 2010; Chapman and Sparks, 2013). Although sedimentation is one of the key issues for colloidal suspension, the reported studies examining sedimentation issues in aqueous-based nanosuspensions are very scarce. This could be due to the following: (1) surfactants are generally used in most of the nanosuspensions to inhibit particle agglomeration in the medium, which alleviates the sedimentation issues, and (2) the small nanosized particles significantly reduce the sedimentation rate. In addition, many of the aqueous nanosuspensions are transformed to dry solid form by spray drying or freeze drying to circumvent the long-term sedimentation issue (Nkansah *et al*., 2013). Unfortunately, this solidification process cannot be applied to nonaqueous nanosuspensions where sedimentation/creaming is commonly present (Gulati and Gupta, 2011).

3.3.1.3 Change of crystalline state

Crystalline state is one of the most important parameters affecting drug stability, solubility, dissolution, and efficacy. The main issue with crystalline state change is the transformation between amorphous and crystalline state. The high-energy top-down manufacturing techniques tend to create partially amorphous nanosuspensions, and some bottom-up techniques can create completely amorphous particles. The high-energy amorphous particles are unstable and inclined to convert to a low-energy crystalline state over time (Kumavat *et al*., 2013). This conversion occurs depending on different parameters, such as temperature, dispersion medium, stabilizers, and the presence of crystalline particles and nanosuspensions via antisolvent precipitation under sonication (Lindfors *et al*., 2007). They demonstrated that amorphous nanoparticles were highly unstable in the presence of small amounts of crystalline particles. This was attributed to saturation solubility differences between amorphous and crystalline nanoparticles that initiated a similar diffusion process to Ostwald ripening, leading to a rapid conversion of amorphous nanoparticles to a crystalline state. Although most of amorphous particles have been shown to be unstable, a few amorphous nanosuspensions have been demonstrated to be stable over a certain period of time. Amorphous hydrocortisone nanosuspensions, produced through a bottom-up nanoprecipitation technique using microfluidic reactors, were found to remain stable after 3 months of storage at room temperature (Ali *et al*., 2009). Amorphous all-trans retinoic acid nanosuspensions, prepared by an antisolvent precipitation technique, were also shown to be stable over 6 months of storage at 4°C (Zhang *et al*., 2006).

The manufacturing process might also induce some other type of crystalline transformation. Lai *et al*. (2009) prepared the diclofenac acid (DCF) nanosuspensions by HPH with two different crystalline forms (DCF1 and DCF2). 5 w/w% Pluronic F68 was used as a stabilizer. X-ray diffraction analysis showed that these two crystalline forms belonged to the same polymorph with differences in molecular conformation and crystal size. It was demonstrated that the HPH process caused the partial transformation of DCF2 to DCF1 while no effect on DCF1 was observed. The change in the crystalline structure was attributed to the solubilization of DCF2 during the HPH process and its subsequent recrystallization as the DCF1 form.

3.3.2 Stability issues with the solidification process

When stable nanosuspensions are unattainable, the solid dosage form is the ultimate solution. The most common solidification processes are freeze drying and spray drying (Rabinow, 2004; Stenger *et al*., 2005; Kesisoglou *et al*., 2007; Goodwin *et al*., 2013). Since most solidified nanoparticle dry powders are usually reconstituted back into nanosuspensions during administration, drug nanocrystal growth or agglomeration during the drying process needs to be prevented in order to maintain the nanosizing features such as rapid dissolution following the reconstitution. Adding matrix formers, such as mannitol, sucrose, and cellulose, into nanosuspensions prior to drying is the common approach to overcome the stability issues during the solidification process (Yadollahi *et al*., 2015).

3.3.2.1 Chemical stability

Since drug nanocrystals are usually dispersed in nanosuspensions with a limited solubility, the possibility of chemical reactions is not as substantial as that in solution-based formulations. Consequently, the chemical stability of nanosuspensions is generally superior to that of solutions. Paclitaxel serves as a good example. Chemical stability is drug specific. Each molecule has its particular functional groups and reaction mechanism that affect the stability. For example, chemical functionalities, such as ester and amides, are susceptible to hydrolytic degradation, while amino groups may undergo oxidative degradation (Garad *et al*., 2010; Baldwin and Kiick, 2013). Although the chemical stability of nanosuspensions is usually not a major concern, extra attention should be paid to drug molecules with solubility greater than 1 mg/mL or with low concentrations in suspension (Garad *et al*., 2010). The common strategy to enhance the chemical stability is to transform the nanosuspensions into dry solid dosage form which is much more stable than nanosuspensions (Williams *et al*., 2013) or to increase the concentration of the nanosuspensions (Garad *et al*., 2010).

3.3.3 Stability issues related to biomolecule loading

Large biomolecules discussed in this review are mainly referring to therapeutic protein and peptide. The molecular structure of protein/peptide is distinctly different and more complicated compared to small molecules. The structures of large molecules are generally differentiated into four structures, i.e., primary, secondary, tertiary, and quaternary structures. These different structures refer to the sequence of the different amino acids, or regions where the chains are organized into regular local structures by hydrogen bonding such as alpha helix and beta sheet, the mechanisms on how the protein/peptide chain folds into a three-dimensional conformation and the composition of multiple protein/peptide molecule assembly, respectively (Torchilin, 2005; Rogueda, 2005; Murthy, 2007). The intact molecular structure of protein/peptide is essential to maintain its therapeutic efficacy (Bummer, 2007; Koppenol, 2007). Common stability issues associated with protein/peptide include deamidation, oxidation, acylation, unfolding, aggregation, and adsorption to surfaces. These stability issues are affected by temperature, solution pH, buffer ion, salt concentration, protein concentration, and added surfactants, with solution formulations being more susceptible to the influence from these factors than the suspension formulations. Although suspension formulations or solid states of protein/peptide have enhanced stability due to their reduced molecular mobility, other stability issues may arise during particle formation or the formulation process. For example, irreversible denaturation and aggregation upon reconstitution were often observed for dehydrated protein through freeze drying or spray drying (Blanco and Alonso, 1998; Quellec *et al*., 1998). To prevent this, supplementary excipients such as bulking agents or surfactants are usually introduced during lyophilization. The vulnerable structure of the protein/peptide creates challenges for formulation development. Instead of using "naked" protein, the common strategy to prevent protein/peptide denaturation is to encapsulate the biomolecules with a carrier such as liposome, solid lipid nanoparticle or polymeric materials (Wu *et al*., 2011).

In addition to improving the stability, protein/peptide encapsulation can enhance bioavailability and provide sustained therapeutic release (Blanco and Alonso, 1998; Quellec *et al.*, 1998; Hildebrand and Tack, 2000; Waeckerle-Men *et al.*, 2006). There has been plenty of work reporting on encapsulated protein/peptide nanoparticles but very scarce studies on pure protein/peptide nanoparticles. Gomez *et al.* (1998) produced bovine zinc insulin nanoparticles using an electrospray drying technique and reported retained biological activities of the particles. By using HPH, Maschke *et al.* (2006) attempted to micronize insulin in the medium of Myglyol 812. The stability and bioactivity of the insulin were maintained in spite of the harsh HPH process conditions. Merisko-Liversidge *et al.* (2004) also noticed retained stability and bioactivity of zinc insulin nanosuspensions that were produced through a wet milling process in the presence of Pluronic F68 and sodium deoxycholate. Nyambura *et al.* (2009a) utilized a bottom-up technique (combination of emulsification and freeze drying) to generate insulin nanoparticles (80 w/w% insulin with 20 wt% lactose). The particles were then dispersed into hydrofluoroalkane (HFA134a) to produce an metered dose inhalers (MDI) formulation. The molecular integrity of insulin formulation, measured by high performance liquid chromatography, size exclusion chromatography, circular dichroism and fluorescence spectroscopy, indicated that native structures (primary, secondary, and tertiary) were retained after particle formation and the formulation process. The presence of surfactant (lecithin) and lyoprotectant (lactose) was believed to be responsible for preservation of the insulin structures. In their follow-up work (Nyambura *et al.*, 2009b), they applied a similar approach to produce composite nanoparticles of lysozyme and lactose for MDI formulations. The retained biological activity of lysozyme was enhanced by increasing lactose concentration in the particles and reached a maximum (99% retained activity) with 20 w/w% lactose. Nanoprecipitation coupled with freeze drying was used as well in this work to produce spherical nanoparticles containing 80 w/w% lysozyme with fully preserved bioactivity. It was demonstrated that the bioactivity of lysozyme nanoparticles remained unchanged when in contact with HFA (Deutzmann, 2004). The suspension must be physically stable (no appreciable settling) for a sufficient time, chemically stable over the required time (shelf life), possess a viscosity that allows it to be used for its intended purpose, be easily reconstituted (redispersible) upon shaking, easy to manufacture and be acceptable in use to the patient, caregiver, or other user. This chapter deals in-depth, with the role and selection of commonly used excipients in developing stable pharmaceutical suspension dosage forms.

3.4 Sterility assessment of nanoparticles

The sterility of nanoparticles is an important part of nanosafety. Sterility testing guidelines have been addressed in various worldwide pharmacopeias, e.g., European Pharmacopoeia, Korean Pharmacopoeia, United States Pharmacopeia, and Japanese Pharmacopoeia, including section 21 of the code of Federal Regulation (CFR), International Conference on Harmonisation, and Food and Drug Administration points to consider documents. These documents provide a brief summary of the test methods and sample requirements used for the most common type of pharmaceutical products. In addition,

it might be recommended that nanobased products should pass sterility tests that are outlined under good manufacturing practice (GMP) guidelines.

The sterility evaluation of nanoparticles and nanosuspensions should be evaluated before starting *in vitro*/*in vivo* tests. Therefore this chapter describes the important tests for examining nanoparticle sterility such as the detection of endotoxins and bacteria/yeast/mold.

3.4.1 Endotoxins

At an early stage of nanobased formulation development, endotoxins can be less important; however, as the formulation progresses to the stage of preclinical assessment, where one begins to assess immunotoxicity and *in vivo* outcomes, sterility and endotoxin contamination become very important. An endotoxin, or lipopolysaccharide (LPS), is a component of the cell wall of gram-negative bacteria and is omnipresent (Fadeel *et al*., 2012). High levels of endotoxin in a nanoformulation can cause immune stimulatory reactions and can mask the true biocompatibility of the formulation and complicates the interpretation of toxic response in toxicological tests (Crist *et al*., 2013). The US Pharmacopeia has established limits on the amount of endotoxin allowed in intravenously administered clinical formulations as 5 Endotoxin Units (EU)/kg body weight/h, and for those delivered intrathecally, the limit is 0.2 EU/kg/h.

A new study shows that gold nanoparticles with a diameter of 7 nm synthesized in a conventional laboratory atmosphere activate human antigen-presenting dendritic cells (DCs) to induce the proliferation of peripheral blood mononuclear cells, when particles were mixed with either allergenic or autologous DCs. This effect was observed due to endotoxin contamination of the nanoparticles. However, when particles were produced under controlled conditions, endotoxin contamination was eliminated and the activation of DCs did not take place (Vallhov *et al*., 2006). The quantitative detection of endotoxin produced by gram-negative bacteria in nanoparticle preparation is possible using an end-point Limulus Amebocyte Lysate (LAL) assay (Sahu and Casciano, 2009). Gram-negative bacterial endotoxins catalyze the activation of proenzyme in the LAL. The activated enzyme then catalyzes the splitting of *p*-nitroaniline from the colorless substrate. The released *p*-nitroaniline was measured using a UV−Vis spectrophotometer at 405 nm after the reaction was terminated using a stop reagent. Using the standard curve the concentration of endotoxins in the samples can be estimated, as concentration is directly proportional to the absorbance (Thassu *et al*., 2007). It is recommended to use an endotoxin-free environment during particle synthesis, and the nanoparticles should be sterilized with gamma radiation before using them for any possible medical applications.

Lipopolysaccharide (LPS) contamination can be avoided if the good laboratory practice (GLP) is followed or the particles are synthesized in a GLP-certified laboratory. However, LPS level should always be determined in the preparation of nanoparticles if they are to be tested on living cells or animals. Furthermore, LPS contamination should be avoided in nanoparticles used for other applications, since there is a risk of an unwanted immune response if particles enter the body via various routes (Magalhães *et al*., 2007).

3.4.2 Bacteria/yeast/mold

A contamination test for the microbes should be carried out to ascertain the quality and safety of manufactured nanoparticles and nanomaterials that will be used in the production of nanobased medicine. All biological products manufactured under GMP conditions require that sterility testing be performed under GMP guidelines. There are two common approaches to assay the sterility: direct inoculation (immersion test) and membrane filtration. The pharmacopeias and 21CFR610.12 recommend using two media for both the direct inoculation and membrane filtration methods (Sahu and Casciano, 2009). In the case of nanoparticles, the direct inoculation method may be preferred since there are currently no filters small enough for nanoparticles. In general in both test methods the test articles or membrane is incubated for 14 days in the test media. However, protocols can be altered according to the characteristics and properties of the nanomaterials.

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Nanoparticles for DNA delivery

4

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4.1 Introduction

Gene therapy has been considered as a potential candidate for the treatment or prevention of diseases associated with defective gene expression (Engelhardt and Wilson, 1992; Maguire *et al*., 2008; Morgan *et al*., 2006; Ott *et al*., 2006; Parkman and Gelfand, 1991). This approach involves insertion of a therapeutic gene inside the cells followed by expression and production of deficient protein. These therapeutic genes are comprised of nucleic acids, antisense oligonucleotides, or small interfering RNAs (siRNAs) which enable replacement of a damaged gene, or expression inhibition of undesired genes (Hughes *et al*., 2001; Kaestner *et al*., 2015; Pouton and Seymour, 2001; Shuey *et al*., 2002). These materials are large in size, vulnerable against enzymatic degradation and anionic in nature. Such features make their delivery very challenging. Therefore carriers are required to effect their entry into the cells/tissues. Some encouraging *in vitro* results have been published; however, it has not been clinically successful due to several constraints in their delivery (Ferber, 2001). Researchers have been making sincere efforts in understanding the biological barriers during their delivery course for efficient gene expression *in vivo* (Bally *et al*., 1999; Nishikawa and Huang, 2001; Rolland and Sullivan, 2003). Currently, developing a safe and efficient vector is the primary focus of gene delivery research which can cross all the biological barriers *in vivo*. Broadly, gene delivery has been divided into two categories, viz., viral and nonviral vector-mediated delivery. Although high transfection efficiency has been achieved from viral vectors rather than nonviral ones, some obvious problems associated with such vectors have limited their *in vivo* applications (Baum *et al*., 2006; Bessis *et al*., 2004; Mingozzi and High, 2011; Thomas *et al*., 2003; Zhang *et al*., 2012). Besides, these viral vectors have also exerted several other limitations such as restricted cell targeting, limited gene-carrying capacity and higher cost for large-scale production (Marshall, 1999). While their counterparts, nonviral vectors, are safe, can carry a wide range of genetic material, are robust and are also amenable to large-scale production, still these vectors display a lower level of efficiency as compared to viral vectors (Amiji, 2004; Ledley, 1995; Lollo *et al*., 2000).

Nanotechnology has revolutionized the entire areas of biological and medical sciences (Reza, 2006). It has covered a very broad range of applications from energy generation to industrial productions (Raliya *et al*., 2013). To deal with this wide spectrum, nanotechnology has been subdivided, and the application of the nanotechnology in the field of biomedical sciences has been termed more specifically as nanobiotechnology (Sapsford *et al*., 2013). At present, nanobiotechnology refers to therapeutic

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nanomaterials which are comprised of organic and inorganic particles with a size range of ~10−200 nm. Due to their smaller size, these nanomaterials are easily taken up by the cells, avoid reticuloendothelial (RES) clearance and minimize undesirable side effects (Webster, 2011). Besides, these materials are also amenable to multifunctionalization and can be decorated with specific ligands to promote site specificity toward certain cells and tissues (Cheng *et al*., 2015; Sapsford *et al*., 2013; Webster, 2011). At present, these nanomaterials are not only finding their use as delivery agents but also act as promising materials for the treatment of various diseases, viz., cancers, inherited and infectious diseases and other complex neurodenerative disorders. More recently, these are being used in photodynamic therapy and imaging techniques (Lucky *et al*., 2015; Shin *et al*., 2013). Similarly, in drug (conventional and nucleic acid-based drugs) delivery applications, the use of nanomaterials has shown very encouraging results *in vitro* and *in vivo* (Dizaj *et al*., 2014; Panyam and Labhasetwar, 2003; Torchilin, 2008). In order to deliver nucleic acids inside the cells, a suitable carrier is required which can effectively bind nucleic acids, carry it to the desired destination, provide protection toward various endo- and exonucleases and release it in the cytoplasm or in the nucleus for transcription. These are the major steps involved in gene delivery to a particular cell or tissue. Over the years, researchers have been conducting numerous experiments to develop an efficient and safe delivery vector to deliver nucleic acids *in vitro* and *in vivo*. Among the various classes of delivery agents, cationic polymers constitute a special class of materials which offers several distinct advantages such as stability, reproducibility, large-scale production, ease of their assembly, low cytotoxicity, etc. A number of cationic polymers have been employed for this purpose, the account of which can be found out from various reviews and monographs. As the present chapter describes the nanoparticle-mediated delivery of DNA, it is beyond the scope of this chapter to include a discussion on all these polymers that have been employed for such applications (Levine *et al*., 2013; Lin *et al*., 2015; Park and Yeo, 2014; Vasir and Labhasetwar, 2006; Yi *et al*., 2015; Yin *et al*., 2014). Specifically, this chapter outlines the application of nanoparticles of the most often and widely used polymers, polyethylenimines (Boussif *et al*., 1995; Lungwitz *et al*., 2005; Patnaik and Gupta, 2013), which are commercially available in two variants, viz., branched and linear (Fig. 4.1), with a wide range of molecular weight and generally known as off-the-shelf cationic polymers, to effect gene delivery *in vitro* and *in vivo*.

Generally, branched polyethylenimine polymers of different molecular weight are prepared from acid-catalyzed polymerization of aziridine monomers in aqueous or alcoholic solutions controlling the temperature and initiator concentration. The process results in the generation of random branched polymers. Likewise, linear polyethylenimines are synthesized by cationic ring-opening polymerization of ethyl-2-oxazolines to generate poly (2-ethyl-2-oxazoline), which on acid- or base-catalyzed partial hydrolysis produces linear polyethylenimines. By varying the conditions in each of the processes, the degree of branching and molecular weight can be controlled. In these polymers, every third atom is nitrogen, which can be protonated. Basically, such high-charged polymers are ideal for nucleic acid condensation and delivery *in vitro* and *in vivo*. However, sometimes, a high charge poses problems leading to imparting

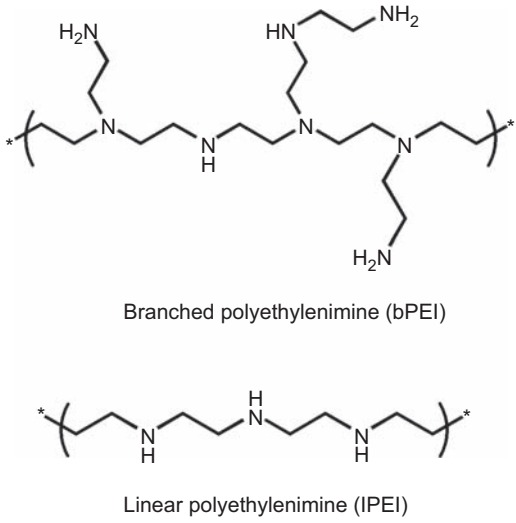

Figure 4.1 Chemical structures of branched and linear polyethylenimines.

toxicity to the cells or tissues. In general, there are some parameters, which greatly affect the efficacy of polyethylenimine-based vectors, viz., molecular weight, degree of branching, positive charge density, buffering capacity, cross-linking, etc. These parameters directly influence the DNA-binding ability of the polymers, size, and magnitude of charge on the resulting complexes (Campeau *et al*., 2001; Fischer *et al*., 1999; Godbey *et al*., 1999, 2000; Goula *et al*., 1998; Morimoto *et al*., 2003; Wightman *et al*., 2001).

Low-to-high molecular weight branched polyethylenimines (PEIs) have been used in gene transfection studies. High molecular weight PEIs exhibit high transfection efficiency due to the formation of small-sized complexes on interaction with negatively-charged DNA, which are more efficiently taken up by the cells. However, at the same time, the presence of high positive charge density imparts high toxicity to cells. In case of branched PEI, primary: secondary: tertiary amines are present in a 1:2:1 ratio, wherein the primary amines are mainly responsible for DNA condensation, and also a source of toxicity and secondary and tertiary amines offer buffering capacity over a wide range of pH that facilitates the endosomal escape of the complexes from the endosomes. This property is also known as the "proton sponge effect," which is crucial for PEIs for exhibiting higher transfection efficacy. Proton sponge effect helps PEI/pDNA complexes endosomal escape, as an increase in the pH of the endosome leads to a high protonation of PEI which results in osmotic swelling, leading to the rupturing of the endosome and the release of the complex in cytosol. In order to address the toxicity concerns (due to the high density of primary amines in high molecular weight PEIs), researchers have attempted to exploit both high and low molecular weight PEIs (Cho, 2012; Jin *et al*., 2014). Low molecular weight (LMW) PEIs are nontoxic but display poor gene transfer capabilities even at higher w/w or N/P ratios. These low molecular weight polymers show inefficient condensation of

nucleic acids and intracellular uptake, which are responsible for poor transfection efficacy. Low and high molecular weight linear polyethylenimines, on the other hand, are nontoxic but exhibit low transfection due to lower stability of the pDNA complexes, which may result in premature release of the bound pDNA followed by chewing up by the enzymes in cellular milieu. Gene delivery efficacy of PEI was reported for the first time in 1995 (Boussif *et al*., 1995); since then, there have been numerous studies published discussing its applications in gene delivery *in vitro* and *in vivo*. Different molecular weights of PEIs that have been investigated in gene delivery range from 430 to 800,000 Da, with 25 kDa PEI showing the highest transfection efficiency *in vitro*. The toxicity exhibited by PEIs is mainly due to aggregation of polymers at the cell surface while low molecular weight PEIs are less toxic due to decreased surface charge (Morimoto *et al*., 2003). Further, on interaction with pDNA, PEI/DNA complexes display an incapability to interact with a negatively charged cell membrane (Godbey *et al*., 1999; Thomas *et al*., 2003), hence low cellular uptake which may lead to poor transfection efficacy (Godbey *et al*., 2000; Goula *et al*., 1998; Wightman *et al*., 2001). The transfection efficiency of branched PEIs increases with an increase in molecular weight, but cytotoxicity has also been found to increase concurrently (Godbey *et al*., 1999). In order to address the cytotoxicity issue as well as to improve the transfection efficiency, different strategies have been developed. These include substituting or linking high molecular weight branched PEIs with polysaccharides, hydrophilic polymers such as PEG, disulfide linkers, lipid moieties, etc. (Cho, 2012; Jin *et al*., 2014). These moieties have been used either for grafting or cross-linking. The polysaccharides include chondroitin sulfate, hyaluronic acid, gellan gum, chitosan, dextran, etc. (Goyal *et al*., 2011; Pathak *et al*., 2009; Raemdonck *et al*., 2013; Tripathi *et al*., 2011, 2012a,b, 2013a). Similar results were obtained with cross-linked PEI nanoparticles; cross-linking can be covalent and ionic type. Cross-linking low molecular weight PEIs has proved to be an alternative to synthesize high molecular weight polymers, which also help in maintaining low cytotoxicity and high transfection efficiency. Some of the linkers used for this purpose are butane-1,4−diol bis glycidyl ether (BDG), PEG-bis (*p*-nitrophenylcarbonate), piperazine-*N*, *N*′-dibutyric acid, PEG-bis-succinimidyl succinate, glutaraldehyde, PEG-bis(aminoethylphosphate) and polyglutamic acid (Fig. 4.2). In this chapter, various studies, performed with preformed nanoparticles of PEIs, have been discussed with their ultimate effect on transfection efficiency and cytotoxicity. Conventionally, mixing cationic polymer with pDNA produces a heterogeneous population of particles (higher polydispersity), where large-sized nanoparticles are more susceptible to RES clearance and cellular membranes may also act as barriers to their smooth passage inside the cells.

4.2 Low molecular weight linear polyethylenimine

As previously discussed, one of the main limitations of linear polyethylenimines (lPEIs) is their inefficient interaction with nucleic acids, which results in their low degree of transfection efficacy. Their transfection efficiency has been improved in several ways by designing and synthesizing degradable and nondegradable derivatives, which

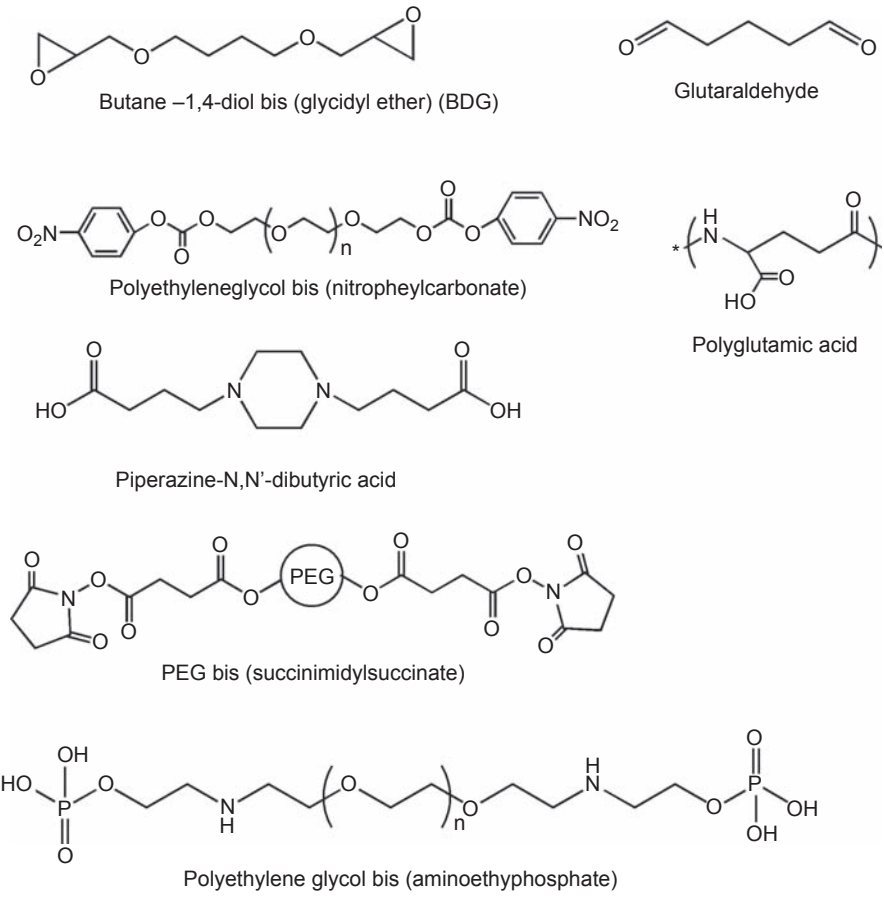

Figure 4.2 Chemical structures of cross-linkers.

will be discussed here. In order to enhance the transfection efficacy of the linear PEI (800 Da) with minimal cytotoxicity, Forrest *et al*. (2003) developed degradable ester derivatives of linear PEI using 1,3-butanediol diacrylate and 1,6-hexanediol diacrylate as cross-linkers (Fig. 4.3). These degradable cross-linked PEI nanoparticles were synthesized by Michael addition reaction between low MW linear PEI and diacrylates. The main goal to synthesize these degradable nanoparticles (NPs) was to increase the overall molecular weight maintaining minimal toxicity. The results showed an increase in the molecular weight of the cross-linked NPs (1400−3000 Da) wherein a number of PEI chains reacted to form a network, which efficiently condensed plasmid DNA to nanosized complexes. These complexes were efficiently taken up and displayed significantly reduced cytotoxicity due to degradable linkages in the NPs. The cross-linked PEI NPs also showed high transfection efficiency almost 2−16 folds higher than branched polyethylenimine (bPEI 25 kDa)/pDNA complex in different cell lines. The strategy emphasized the fact that high transfection efficiency could

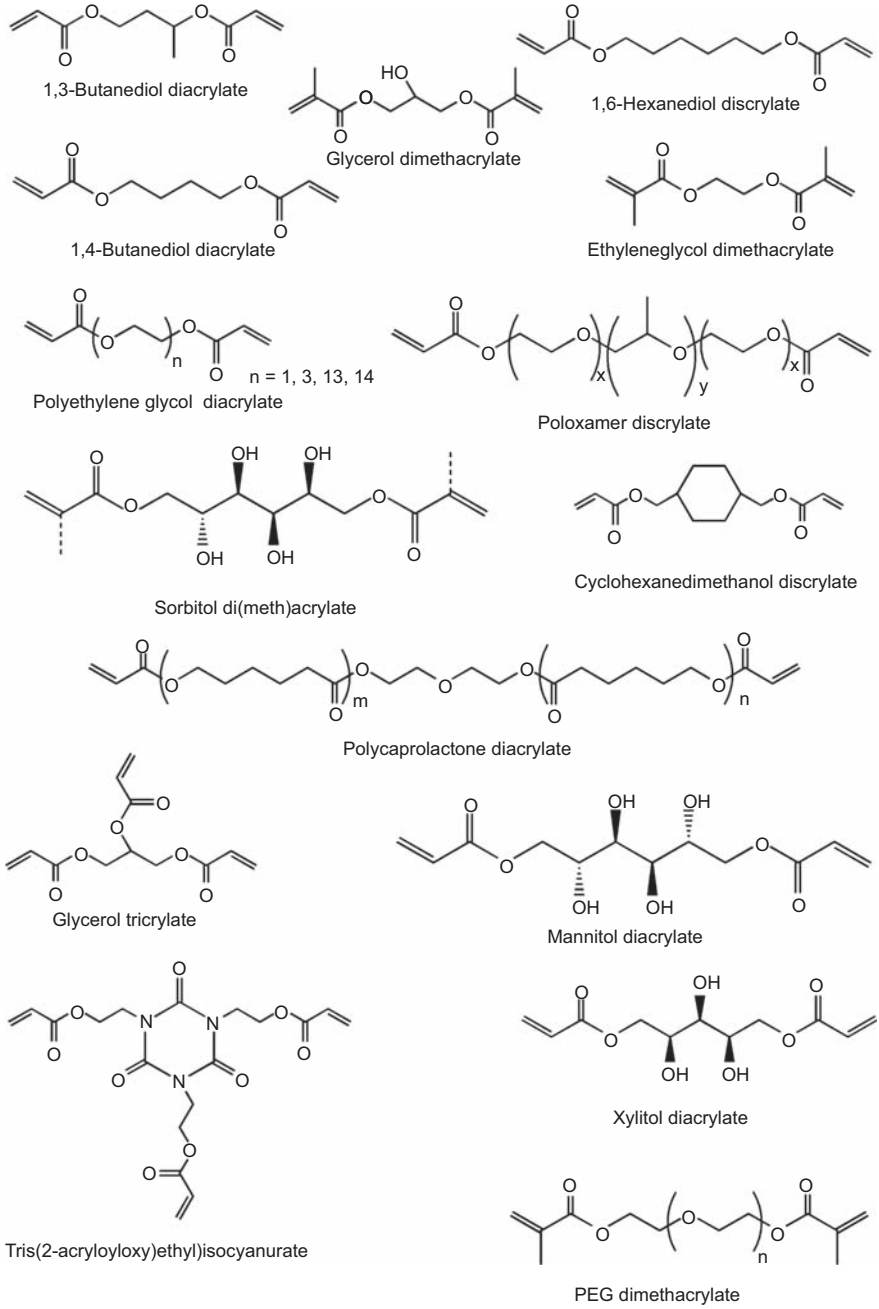

Figure 4.3 Structures of cross-linkers based on Michael addition reactions.

be due to strong binding of DNA with the nanoparticles, which provided enzymatic resistance against nucleases. Besides, degradation of the linkages weakened the binding, and easy unpackaging led to the higher release of DNA for transgene expression (Forrest *et al*., 2003). The half-life of the synthesized cross-linked degradable PEI NPs was ∼4 h due to rapid hydrolysis of ester linkages at physiological pH. In another attempt, degradable linear PEI NPs have been synthesized using Michael addition reaction of low MW lPEI (423 Da) with polyethylene glycol diacrylate of different MW (285, 575, and 700 Da) (Fig. 4.3; Park *et al*., 2005). Depending upon the reaction conditions, various cross-linked polymeric nanoparticles with a molecular weight range from 8000 to 12,900 Da were obtained. The incorporation of PEG significantly affected the transfection efficiency of the lPEI NPs in various cell lines due to its shielding effect in the copolymers. The shielding effect of PEG increases with an increase in MW of PEG. These cross-linked polymeric NPs showed high degradation at physiological pH. The NPs exhibited no cytotoxicity, and the transfection efficiency of these NPs was found to be dependent on PEG molecular weight and quite higher compared to bPEI (25 kDa)/pDNA complex. Moreover, these NPs were also tested for *in vivo* delivery of DNA in mice using two routes of administration, viz., aerosol and intravenous (i.v.). The aerosol route of administration was found to be more efficient in terms of transfection efficiency in all organs as compared to the i.v. route, even in the lungs, due to ease of access toward pulmonary tissues (Park *et al*., 2008). Further, to develop an accelerated gene transfer system, Islam and his co-workers cross-linked low molecular weight lPEI (423 Da) with sorbitol dimethacrylate (Fig. 4.3), an osmotically active transporter, to synthesize poly (amino ester) (PAE) (Islam *et al*., 2011). The electrostatic complexes of polymer/DNA showed excellent stability in the presence of serum and postlyophilization, which could be attributed to the presence of polyhydroxyls in the backbone of polymeric NPs. The presence of polyhydroxyl groups also leads to reduction in the surface charge of polyplexes, which results in high cell viability even at higher N/P ratios. The PAE exhibited higher transfection efficiency than the PEI 25 kDa/pDNA complex due to the synergistic effect of buffering capacity provided by PEI and osmotic active property by the polysorbitol chain. The same polymer showed therapeutic RNA interference *in vitro* by delivering osteopontin siRNA; osteopontin plays a crucial role in tumor angiogenesis (Islam *et al*., 2012). These studies demonstrated polysorbitol cross-linked lPEI NPs; an osmotic active property, as well as buffering capacity, could deliver DNA and siRNA far better than PEI 25 kDa/DNA complex via caveolin-1 and cyclooxygenase-2-mediated caveolae-dependent endocytosis.

Bioresponsive cross-linkers have also been used to cross-link PEIs to mimic their high molecular weight PEIs. These linkers, under a cellular environment, undergo cleavage to low molecular weight polymers which are nontoxic to cells or tissues. Several disulfide cross-linkers have been designed, synthesized, and used to cross-link PEIs electrostatically or covalently. In one of such reports, two series of bioreducible cross-linked linear PEI NPs of molecular weight <4.6 Da were generated using LMW lPEI and dithiobispropionic acid or cystine as cross-linkers (Fig. 4.4; Breunig *et al*., 2007). These cross-linked polymeric NPs were evaluated for transfection efficacy and cytotoxicity on seven different cell lines and the results were compared

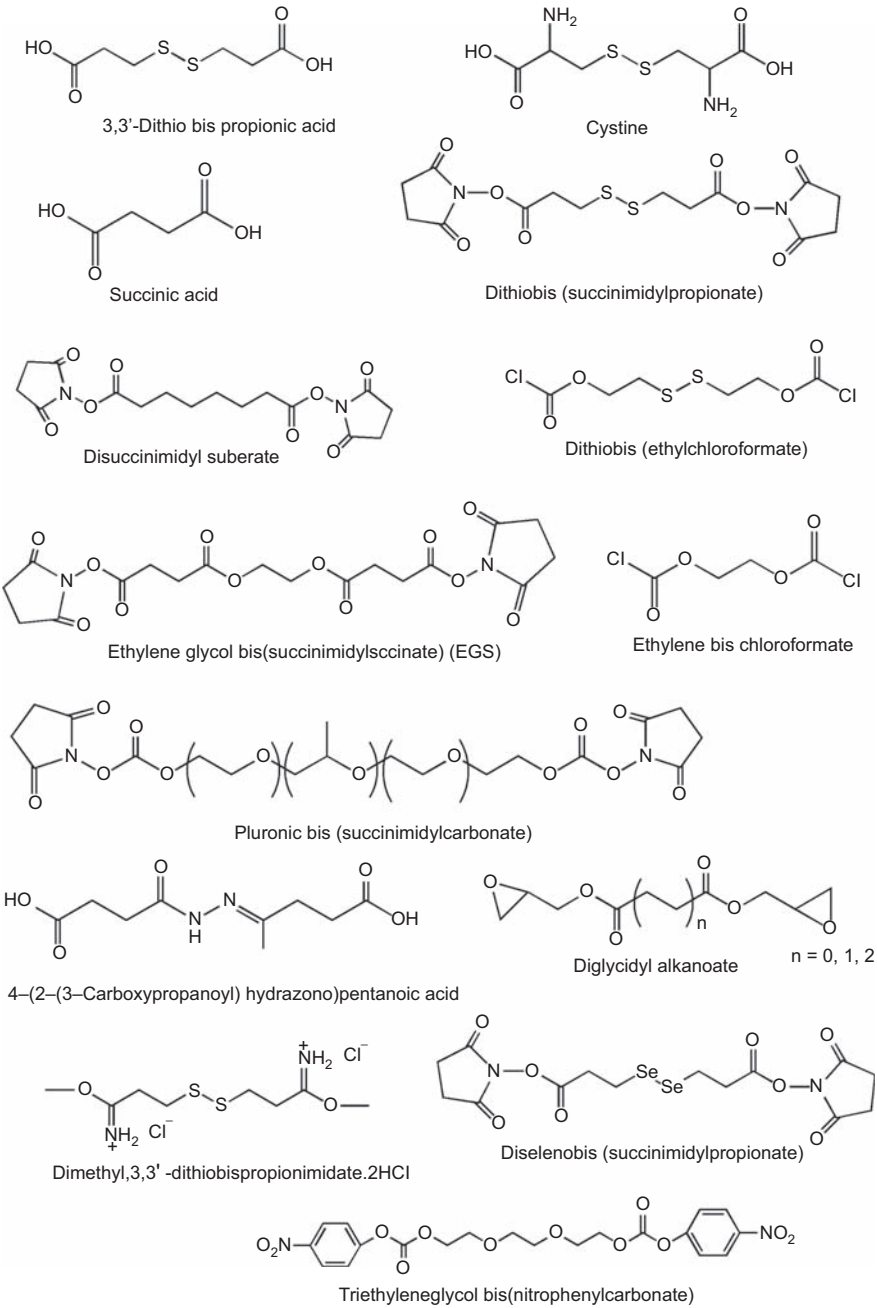

Figure 4.4 Some bioresponsive linkers for cross-linking polyethylenimines.

with seven different commercial transfection reagents of different origins. Around 1400 individual experiments were conducted and results showed $69.62 \pm 4.18\%$ transfection efficiency in HEK293 cells with up to $98.69 \pm 4.79\%$ cell viability. The same group of researchers illustrated the use of the reductive principle in combination with a low molecular weight PEI to be an ideal strategy for the delivery of biomolecules.

Certainly, cross-linked linear PEIs have shown the capability to maintain the transfection efficiency comparable to branched PEI (25 kDa) with minimum cytotoxicity. Hydrolytically degradable or disulfide cross-linkers have resulted in high transfection efficiency and cell viability. Bonner and co-workers synthesized a series of cross-linked lPEI polymers of a 2.5 kDa lPEI using either disulfide or nondegradable cross-linkers (Fig. 4.4), compared their efficiency and tried to evaluate the mechanism for exhibiting high transfection efficiency and cell viability (Bonner *et al*., 2013). Both the systems with degradable and nondegradable linkers exhibited similar results compared to commercial standards in terms of high transfection efficiency and minimum cytotoxicity. However, the incorporation of uncomplexed polymer critically played a significant role in enhancing the transfection efficiency of these polymers through efficient endosomal escape without any signs of cell membrane damage. Several studies demonstrated the effect of disulfide moiety on achieving high transfection efficiency and low cytotoxicity; however, this group illustrated that disulfide-mediated unpackaging may not be as important as conventionally thought for some PEI systems.

4.3 High molecular weight linear polyethylenimine

Bioreducible linear PEIs have also been synthesized and evaluated for their transfection efficiency and cytotoxicity. Lee *et al*. (2007) modified high molecular weight lPEI at its two ends and oxidized to form linear poly (ethylenimine disulfide) (lPEIS). A series of these polymers have been synthesized by varying the amine density. They found that as the amine density in lPEIS increased, transfection efficiency also increased. DNA complexes of L-PEIS-6 and -8 showed comparable transfection efficiency with that of bPEI (25 kDa)/pDNA complex; however, no cytotoxicity was observed due to the degradation of lPEIS in the reductive intracellular conditions.

Goyal *et al*. (2012) have synthesized a series of nondegradable nanoparticles of high molecular weight linear PEI (25 kDa) by varying the amounts of a cross-linker, butane-1,4-diol bis (glycidyl ether) (BDG, Fig. 4.1). These spherical nanoparticles (LPNs) were obtained in size ranging from $\sim$109 to 235 nm with a surface charge of $\sim$ +38 to +16 mV. Among all the LPN polyplexes, LPN-5/DNA complex exhibited $\sim$14.7-fold higher transfection efficiency than lPEI (25 kDa) and other commercial transfection reagents. Deviation from LPN-5 formulation on either side resulted in a decrease in the transfection efficiency. The series of nanoparticles synthesized were found to be nontoxic. Further, *in vivo* administration in Balb/c mice of LPN-5/Luc DNA complex particles showed the highest transfection of the reporter gene in the spleen.

4.4 Low molecular weight branched polyethylenimine

Branched PEIs have shown merits over their linear counterparts as they efficiently condense and provide protection to condensed nucleic acids due to their high amine density in the ratio of 1:2:1 of primary, secondary, and tertiary amines. In order to optimize the transfection efficiency of low molecular weight branched PEIs without compromising on cytotoxicity, cross-linking with bifunctional reagents with degradable linkages such as disulfide, ester, amide, carbamate, hydrazone, etc. (Figs. 4.3 and 4.4) has proved to be the most viable option (Mintzer and Simanek, 2009). Subsequent to cross-linking, the molecular weight of the polymers increases; however, cytotoxicity remains negligible due to degradation of the linkages under physiological conditions, which generates nontoxic products. Hence, these resulting polymers demonstrate nucleic acid condensing ability almost equivalent to high molecular weight branched PEI (25 kDa). Consequently, all these factors improve the transfection efficiency of the vectors exhibiting minimal toxicity. Feng *et al.* (2014) designed and synthesized a reusable disulfide carbamate linker to prepare cross-linked PEIs (PEI-SS-CLs) for the purpose of efficient gene delivery (Fig. 4.4). The highlighting features of their strategy were that on cleavage of disulfide linkages, intramolecular cyclization led to the removal of carbamate bonds. *In vitro* transfection assay revealed that the projected vector efficiently condensed DNA, carried it into the cells and displayed higher transgene expression than bPEI (25 kDa) and Lipofectamine/DNA complexes without showing significant toxicity. They found that another factor responsible for these results was the slow release of DNA from the cross-linked PEI. The cleavage of S−S bonds regulated the release of DNA, which could be attributed to these observations.

Li *et al.* (2011) synthesized diglycidyl esters of adipic acid, succinic acid, and oxalic acid and used them to cross-link branched PEI (600 Da) to high molecular weight analogs (Fig. 4.4). Gel permeation chromatography (GPC) revealed molecular weights of adipic acid-PEI, succinic acid-PEI and oxalic acid-PEI around 6861, 16,015 and 35,281 Da, respectively. Further, their degradation pattern was also followed by GPC. These cross-linked PEIs exhibited good DNA binding ability and buffering capacity and were found to be less toxic. DNA release was regulated by the biodegradability of the modified polymeric nanostructures. All the polymer complexes showed higher transfection efficiency than bPEI (25 kDa)/DNA complex. On comparison, diglycidyl succinate cross-linked bPEI polymers showed the highest transfection efficiency in various mammalian cells. Hydroxyl groups generated upon the reaction of PEI with glycidyl esters also improved the solubility of cross-linked polymers.

Disulfide linkage has drawn immense attention as it has a characteristic property of releasing DNA into the cytosol in the presence of GSH (glutathione) and addresses the issue of cytotoxicity as well. Generally, two strategies have been followed to introduce disulfide linkages into polymeric structures: one of them utilizes prethiolation of the polymers, which needs incorporation of the thiol group on PEI polymers and subsequent oxidation of thiolated PEIs (PEI-SH) to form cross-linked PEI (PEI-SS-PEI) polymers. The other method involves cross-linking with disulfide-containing bifunctional reagents which react with the polymers to yield the polymeric framework

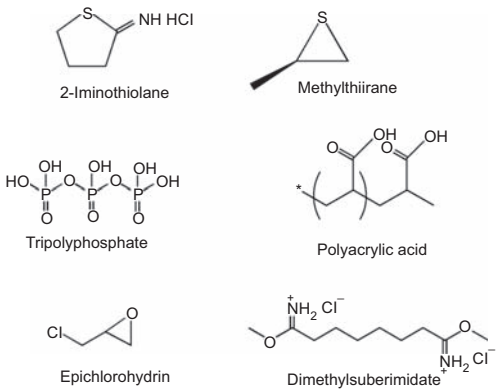

Figure 4.5 Reagents for direct and indirect cross-linking of polyethylenimines.

consisting of disulfide linkages. Peng *et al.* (2008) followed the first method and synthesized disulfide cross-linked branched PEI nanoparticles in two steps via the ring-opening reaction of PEI (800 Da) with methylthiirane to produce thiolated polyethylenimine (PEI-SHX) followed by oxidation to form a series of cross-linked PEIs (PEI-SSx) (Fig. 4.5). Due to comparable nucleophilicity of amine and thiol groups, suitable conditions were established for ring-opening reactions so that free thiol groups did not react with methylthiirane. The PEI-SSx series showed lower cytotoxicity and higher transfection efficiency than bPEI (25 kDa)/pDNA complex. The best formulation (PEI-$SS_{2.6}$) showed 10 times higher luciferase expression in mammalian cells with minimal toxicity compared to branched PEI (25 kDa), and even in the presence of serum, the transfection efficiency did not decrease. Subsequently, they investigated the influence of disulfide density and molecular weight in disulfide cross-linked PEI NPs in detail (Peng *et al.*, 2008, 2009). Among the three cross-linked PEIs (800, 1800, and 25,000 Da) composed of disulfide linkages, cross-linked branched PEI (800 Da) showed the highest transfection efficiency. Moreover, the PEIs with too low or too high degree of thiolation led to ineffective condensation of DNA, resulting in poor transfection efficiency (Peng *et al.*, 2009).

2-Iminothiolane provides a better option among other thiolating reagents, such as dithiobis(succinimidyl propionate) (DSP) and *N*,*N*′-cystamine bisacrylamide, since it maintains the total number of positive charges (Fig. 4.5), their condensing ability and buffering capacity by converting the amine groups into amidine groups (Kang *et al.*, 2011).

In the very first report on the introduction of disulfide linkage, low molecular weight bPEI (800 Da) was cross-linked with 3,3′-DSP and 3,3′-dithiobis propionimidate (DTBP) (Fig. 4.4; Gosselin *et al.*, 2001). Both the reagents reacted with primary amines of bPEI but generated different linkages. DSP cross-linking occurred via amide bond formation while DTBP reacted with bPEI via amidine linkage. Charge density was reduced partially in the first method while the latter one did not block the charge; rather it converted primary amines to amidine functions. These reactions yielded high molecular weight cross-linked PEI polymeric nanoparticles capable of binding DNA effectively while on reduction with dithiothreitol, and DNA binding ability of the

degraded polymers vanished. This phenomenon produced easy unpackaging of DNA complexes in intracellular compartments for nuclear uptake and transcription. An increase in effective molecular weight induced sufficiently high transfection efficiency; it was not more than bPEI (25 kDa), but these offered advantages in terms of reduced toxicity of the high molecular weight polymeric nanoparticles as well as after degradation, the products were found to be nontoxic and did not show nonspecific interactions with the endogenous DNA. Moreover, these low molecular weight adducts, postdegradation, could be cleared more easily than bPEI (25 kDa) due to enhanced diffusion and easier filtration through glomerular capillaries. Since then, several researchers have synthesized disulfide-containing degradable high molecular weight polymers and demonstrated their advantages over unmodified PEIs and commercial transfection reagents such as Lipofectamine, Superfect, Transfect, etc. Cross-linking of low molecular weight bPEI with disulfide linkage has also been achieved by utilizing click chemistry between azide-functionalized PEI (1800 Da) and disulfide containing cross-linker, bis(propargyl carbamate) ethyl disulfide (Jiang *et al*., 2011; Liu *et al*., 2010). These reducible polymeric nanostructures have been shown to exhibit superior transfection efficiency with much lower cytotoxicity compared to branched PEI (25 kDa). In another variation, biodegradable disulfide-containing PEI−PEG cross-linked NPs were synthesized by using click chemistry employing azide-functionalized tetraethylene glycol (TEG) and alkyne containing PEI (2000 Da), resulting in enhanced transfection efficiency and low cytotoxicity (Zhao *et al*., 2011).

From these studies, it has been postulated that bioreducible cross-linked polymeric nanoparticles have been shown as efficient and safe gene delivery vehicles because of their stability in an extracellular medium and rapid degradation in a reductive intracellular environment which facilitates release of the bound DNA for nuclear localization. Moreover, these have been shown to possess low cytotoxicity, and even after degradation, the biproducts are nontoxic and do not interact nonspecifically with other biomolecules or tissues. Based on these findings, Cheng *et al*. (2012) and Yue *et al*. (2014) designed alternative reduction-sensitive diselenide linkage and cross-linked low molecular weight oligoethylenimine (800 Da) with diselenobis (succinimidyl propionate) (Fig. 4.4) to obtain diselenide cross-linked high molecular weight polymeric nanoparticles. For comparison purposes, PEI (800 Da) was cross-linked with the disulfide-containing bifunctional reagent, DSP. Reduction kinetics revealed that diselenide linkage was more stable than S−S linkage. The rest of the properties of diselenide cross-linked PEI were almost similar to disulfide-linked bPEI. Cytotoxicity of both the series of nanoparticles was similar, but transfection efficiency of diselenide cross-linked PEI was comparable or higher than S−S linked bPEI and bPEI (25 kDa).

Other studies involving relatively more stable linkages than disulfides/diselenides are carbamates, amides, esters, and hydrazones. Several cross-linkers have been designed and used to cross-link low molecular weight PEIs into their high molecular weight analogs to address various limitations of nondegradable bPEI (25 kDa) in gene delivery. Here, both hydrophobic and hydrophilic bifunctional reagents have been utilized. The insertion of hydrophilic segments greatly improves the *in vivo* viability of these vectors, where long-circulating complexes are needed to target various tissues. In one of such studies, Wang *et al*. (2012a) cross-linked PEI

(800 Da) with ethylene bischloroformate to obtain low molecular weight PEI nanoparticles (PEI-Et, 2895 Da) (Fig. 4.4). These particles showed their ability to condense DNA and the resulting complexes at various w/w ratios and exhibited higher transfection efficiency than bPEI (25 kDa) and Lipofectamine complexes in various cell lines. Besides, PEI-Et NPs displayed cytotoxicity much lower to bPEI (25 kDa).

Similarly, Sun *et al.* (2012) designed a specific strategy to target macrophage mannose receptors (MR) using mannose decorated vectors. They cross-linked two low molecular weight PEIs (2000 and 600 Da) with triethyleneglycol bis (*p*-nitophenylcarbonate) (Fig. 4.4) to form PEI-TEG polymeric NPs which were further reacted with different amounts of mannopyranosyl isothiocyanate to obtain mannosylated PEI−TEG for the purpose of improving specificity for dendritic cells. On dendritic cells, there is an abundant expression of MR. These mannose receptors have been shown to select ligands carrying terminal sugars such as mannose and *N*-octyl glucosamine. Therefore, a number of mannosylated delivery vectors have been designed and synthesized to improve the target-specific delivery of drugs and genes by targeting its receptors on antigen-present cells (APCs). Based on this concept, DNA vaccines have been developed. Man−PEI−TEG−PEI−Man polymers with mannose substitution of 35%, 32.8%, and 24.4% still displayed sufficient affinity to bind DNA and carried it inside the cells. Cell viability was found to be above 80%. In DC 2.4 cell line, these particles with 32.8% substitution of mannose showed the highest transfection efficiency.

Polyester-cross-linked PEIs have also been synthesized using low molecular weight PEIs and 1,4-butanediol diacrylate or ethylene glycol dimethacrylate (Fig. 4.3), and subsequently, their cytotoxicity and gene transfer ability were assessed on melanoma B16F10 and other cells (Dong *et al.*, 2006). *In vivo* efficiency was determined by direct injection into mice and estimated the gene expression in animal muscles. These cross-linked PEIs mediated significantly higher transfection efficiency in various cell lines without exhibiting toxicity. Moreover, in the presence of serum, these complexes showed almost similar efficacy. *In vivo* results revealed the highest degree of gene expression in mouse leg muscles as observed under fluorescence microscopy (Dong *et al.*, 2007).

Another set of degradable hyperbranched poly(ester amines) (PEAs) was prepared by Kim *et al.* (2007) using low MW poloxamer (2500 Da) diacrylate and branched PEIs (600, 1200, and 1800 Da) (Fig. 4.3). Michael addition reaction yielded these polyamines in significant yields, which showed good DNA binding ability and further, evaluated for their cytotoxicity and gene delivery efficacy. The poly(ester amines) showed higher transfection efficiency in three different cell lines (A549, HEK293T, and HepG2) and much lower cytotoxicity. A higher percentage of poloxamer content in the modified polymers displayed low serum dependency. Besides, these polymers also showed degradability in a controlled fashion. Arote *et al.* (2007) employed hydrophobic polycaprolactone diacrylate as a cross-linker (Fig. 4.3) and reacted with branched PEIs (600, 1200, and 1800 Da) to synthesize degradable branched PEI nanoparticles with a half-life of 4.5−5 days. The synthesized polymeric NPs exhibited around 15−20 folds higher transfection efficacy than branched PEI (25 kDa). Later, the same group synthesized degradable branched PEI polymers with hyperosmotic characteristics by using hydrophilic glycerol dimethacrylate as a cross-linker and

branched PEI (1200 Da) (Fig. 4.3; Arote *et al*., 2008). The polymer resulted in much higher transfection efficiency *in vitro* due to the synergistic effect of buffering capacity by PEI and hyperosmotic property by polyglycerol. Further, glycerol triacrylate was also used for the same purpose (Fig. 4.3) and reacted with branched PEI (1200 kDa). The results were similar to earlier findings (Arote *et al*., 2009).

Yu *et al*. (2009) synthesized degradable branched PEIs using PEG dimethylacrylate (550 Da) as a cross-linker and branched PEIs (600, 1200, and 1800 Da) (Fig. 4.3). The half-life of the polymeric structures was longer at a lower pH (5.6), compared to a higher pH (7.4), and showed equivalent transfection efficiency to PEI (25 kDa) in 293 T, HeLa, and HepG2 cells. Transfection efficiency increased with an increase in MW of the starting PEI due to an increase in buffering capacity. In an exciting investigation, Luu *et al*. (2012) synthesized two sets of degradable PEI NPs using PEI (1200 Da) polymer and sorbitol diacrylate or sorbitol dimethacrylate as a cross-linker (Fig. 4.3). Results showed higher cytotoxicity was governed in degradable branched PEIs using sorbitol dimethacrylate as a cross-linker rather than sorbitol diacrylate. The results suggested that hydrophobic methyl groups in cross-linked polymer increased the cellular toxicity. The polysorbitol-consisting polymers exhibited higher transfection efficacy than PEI (25 kDa) through selective caveolae endocytosis pathway. To generate stimuli-responsive uptake polymers, osmotic mannitol diacrylate was reacted with PEI (1200 Da) to synthesize polymannitol-based gene transporter (Fig. 4.3), which exhibited higher transfection efficiency with minimal cytotoxicity than PEI (25 kDa) (Park *et al*., 2012), as the cross-linked polymer consisted of hyperosmotic property due to the polymannitol backbone and characteristic proton sponge effect. The caveolae-mediated endocytosis pathway of the polyplexes avoided lysosomal fusion and protected genes from degradation. Another similar attempt was made in order to synthesize degradable PEI using osmotic xylitol diacrylate and branched PEI (1200 Da) (Fig. 4.3; Lee *et al*., 2014). The polyxylitol-based polymers exhibited superior osmotic effect which led to enhanced endosomal escape via osmolysis, thus showing higher transfection efficiency and low cytotoxicity. The higher osmolytic characteristic of polyxylitol-based polymeric NPs enabled the gene to avoid lysosomal degradation and transferred more genes into cytosol and nucleus to express.

Unlike the viral method wherein the vector itself integrates into the host genome and exhibits long-term gene expression, clinical applications involving cationic polymer/DNA complexes require repeated administration to generate a long-term response. Hence, the toxicity issue needs greater attention. Therefore, degradable derivatives are finding a greater role in gene delivery compared to their high molecular weight gold standard (bPEI 25 kDa) (Ahn *et al*., 2002; Akinc *et al*., 2003; Lim *et al*., 1999, 2000, 2002; Nimesh and Chandra, 2011; Peterson *et al*., 2002; Shen, 2013; Thomas *et al*., 2005; Wang *et al*., 2001). There are a few reports wherein an increase in transfection efficiency was reported. In most of the systems, the transfection efficiency was either comparable or lower than the bPEI (25 kDa). Also, there was an increase in cytotoxicity at high w/w ratios. Likewise, there are examples where the PEI derivatives have worked very efficiently *in vitro* but their performance dropped miserably in the presence of serum or *in vivo*. Therefore, a subtle balance is required while

designing and synthesizing a vector for efficient gene delivery *in vitro* and *in vivo*. Working on this strategy, Thomas *et al*. (2005) further cross-linked low molecular weight branched PEI (2000 Da) with linear PEI (423 Da) using different cross-linkers and found that transfection efficiency of the degradable PEI NPs, synthesized with a different combination of PEIs and cross-linkers, enhanced up to 550 folds *in vitro* and 80 folds *in vivo* compared to native polymers without increasing their cytotoxicity. Two cross-linkers used in this study were ethylene glycol bis(succinimidyl-succinate) and disuccinimidyl suberate (Fig. 4.4). Subsequently, the same research group employed a combinatorial library approach and identified highly effective degradable PEI derivatives capable of delivering DNA *in vitro* and *in vivo* (Thomas *et al*., 2007).

In an interesting study involving poly (ester amines), Kloeckner *et al*. (2006) synthesized two biodegradable cross-linked polymer derivatives using oligoethylenimine and hexanediol diacrylate at two different temperatures (20 and 60°C). They observed that temperature strongly influenced molecular weight as well as ester/amide ratio. At low temperatures, the ester-based cross-linked polymer showed the molecular weight of 8700 Da, while at high temperatures, amide-based polymers showed 26.6 kDa, which could be due to conversion of ester linkages to amide linkages through inter- and intramolecular ester aminolysis at a higher temperature. Both the polymers exhibited high transfection efficiency in two cell lines with minimal toxicity. Of these two polymers, the latter one showed slow degradation kinetics while the ester-based polymer showed fast degradation under physiological conditions. Moreover, the low-temperature cross-linked polymer showed less cytotoxicity than the higher temperature-based one. In another investigation, Wang *et al*. (2012b) demonstrated that the interaction of low molecular weight PEIs (800, 1200, and 2000 Da) with tris[2-(acryloyloxy) ethyl] isocyanurate generated high molecular weight hyperbranched poly(ester amines) via Michael addition reaction capable of binding and condensing genetic materials and delivering them to their specific targets (Fig. 4.3). On *in vitro* evaluation, these poly(ester amines) displayed lower cytotoxicity in various cell lines. Among the poly(ester amines), the series prepared from bPEI (2000 Da) was found to be the best in terms of transfection efficiency. It exhibited five to eight folds higher transfection efficiency than bPEI (25 kDa) in dystrophic mdx mice through intramuscular administration. No muscle damage was observed with the modified polymer, suggesting the potential of these vectors as a safe and efficient delivery system for *in vitro* and *in vivo* applications.

Several researchers have also cross-linked low molecular weight PEIs with nonionic amphiphiles to form amphiphilic block polymers of high molecular weight and used them as nonviral gene delivery vectors. They have investigated the role of polymeric nanostructures with their biological performance to deliver a gene of interest *in vitro* and *in vivo*. In one of the studies, Fan *et al*. (2012) synthesized a series of polymeric structures by interacting low molecular weight PEI (2000 Da) with nonionic amphiphilic surfactant, pluronic bis (succinimidyl carbonate) (Fig. 4.4), consisting of both hydrophilic and hydrophobic segments, ethylene oxide (EO) and propylene oxide (PO) blocks, respectively. These structures were evaluated for their cellular uptake, gene transfer ability, cytotoxicity and localization in the cells. Pluronics are

well known to impart an interesting property to the polymers: higher hydrophilic–hydrophobic balance helps in homogeneous distribution of the complexes in the cytoplasm, while low balance facilitates accumulation of the complexes in the nucleus. Besides, these surfactants enhance cell interactions and DNA transport across cell membranes. The ethylene oxide segments decrease nonspecific interactions and improve dispersibility whereas propylene oxide segments improve interactions with the lipidic constituents present in the cell membranes. Hence, incorporation of such moieties in the cationic polymers serves two purposes, viz., decreases overall cytotoxicity and improves uptake of DNA complexes. By varying the ratio of EO/PO, one can regulate delivery of the nucleic acids in the cytoplasm or nucleus.

Fang *et al.* (2014) also synthesized a new series of degradable PEIs by using a hydrazone- based cross-linker. To enhance the transfection efficiency of low molecular weight PEI (1800 Da) without affecting its cytotoxicity, PEI was cross-linked with an acid labile hydrazone cross-linker (Fig. 4.4), which converted it into high molecular weight polymeric NPs. These NPs were further tethered with varying amounts of betaine, *N*,*N*-dimethyl(acrylamidopropyl)ammonium propane sulphonate. These modified analogs exhibited much lower cytotoxicity compared to bPEI (25 kDa) but displayed high transfection efficiency, which was comparable or higher than PEI (25 kDa)/pDNA complex in the presence and absence of serum (10%).

4.5 High molecular weight branched polyethylenimine

Transfection efficiency of PEIs is molecular weight dependent, i.e., high molecular weight PEIs exhibit high transfection efficiency while low molecular weight ones inefficiently trap DNA and are poorly taken up and, therefore, display poor transfection efficiency. Contrary to this, high molecular weight polymers show low cell viability while low molecular weight polymers are almost nontoxic. Toxicity in high molecular weight polymers is mainly due to high cationic charge density by which they interact with the negatively charged cell membranes and causes morphological damages followed by cell death. Many reports are there to subside their toxicity level by grafting or cross-linking using peptides, polyethylene glycols, polysaccharides, antibodies, imidazolyl, acylation, etc. These have been well documented, and here, some of the recent reports have been discussed.

The ionic or ionotropic gelation method is one of the methods that have been used for cross-linking polyelectrolytes in the presence of a counter ion. The method has been finding numerous applications in the design and synthesis of drug delivery systems. Here, it has been used to alleviate the charge-associated toxicity of the high molecular weight branched PEI (25 kDa). Huang *et al.* used tripolyphosphate (TPP) to prepare anion-enriched nanoparticles of PEI–TPP (Fig. 4.5). These nanoparticles showed a dramatic improvement in the cell viability, which enhanced up to ~90%. The transfection efficiency exhibited by these NPs was 1.11–4.20 folds higher than that with the native bPEI at all N/P ratios tested. These NPs not only protected the

condensed DNA against nucleases but also showed efficient uptake within 1 h of exposure. These nanoparticle/pDNA complexes transfected cells in a specific manner, which was found to be in the order HEK293>HepG2>HeLa>MCF-7 (Huang *et al*., 2014). bPEI-polyacrylic nanocomposites (ionic and covalently linked) have also been shown to deliver plasmid DNA efficiently inside the mammalian cells with minimal toxicity (Fig. 4.5; Tripathi *et al*., 2016). In this study, they compared the gene-carrying capability of both types of nanocomposites and found that covalently-linked nanocomposites performed much better than their ionic counterparts, which established that type of bonding significantly affected the performance of the nanomaterials.

Reduction-sensitive nanoparticles of high molecular weight PEI have also been synthesized (Bansal *et al*., 2015) using electrostatic interactions. bPEI (25 kDa) was cross-linked with 3,3′-dithiobis (propionic acid), and by varying the amounts of the cross-linker, a series of cross-linked bPEI nanoparticles has been synthesized. The cross-linking did not alter the DNA binding and condensing property of the NPs. These complexes have been efficiently taken up by the cells through endocytosis and showed high transfection efficiency in mammalian cells without exerting toxicity to the cells. The decrease in cytotoxicity could be attributed to diminished charge density on the NP formulations.

In an interesting study, high molecular weight linear and branched PEIs have been cross-linked in a two-step process using epichlorohydrin (Fig. 4.5; Goyal *et al*., 2014). In the first step, lPEI was reacted with epichlorohydrin via epoxide-ring opening to generate lPEI-chlorohydrin, which in the second step, reacted with bPEI in the presence of sodium hydroxide. In situ generation of epoxide ring was followed by nucleophilic attack by the primary/secondary amines of bPEI onto the epoxide ring. These stepwise reactions led to the formation of a series of copolymers, which showed excellent transfection efficiency and low cytotoxicity. *In vivo* delivery of DNA in Balb/c mice resulted in the highest gene expression in the spleen.

In another variant, high molecular weight bPEI was cross-linked with dimethylsuberimidate (Fig. 4.5) and synthesized a series of bPEI NPs by varying the amounts of the cross-linker (Tripathi *et al*., 2013b). The highlighting features of the strategy were that the cross-linker specifically reacted with primary amines of bPEI, which are considered as the main source of toxicity, cross-linking introduced hydrophobicity in the NPs, and facilitated interaction of the NPs with the lipophilic cell membrane. Reaction of primary amines with amidate esters did not block charge on the NPs; rather, these were converted into amidines, i.e., charge density remained unaltered. These cross-linked bPEI NPs exhibited significantly higher transfection efficiency in various cell lines with minimal cytotoxicity compared to native bPEI (25 kDa).

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Nanoparticles for siRNA-mediated gene silencing

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5.1 Introduction

RNA interference (RNAi) or posttranscriptional gene silencing has been emerging as a potent and effective strategy for silencing the expression of a specific gene. The small interfering ribonucleic acid (siRNA) has a great therapeutic potential to treat various types of diseases as it blocks the translational level rather than disturbing the sequence of DNA, thus reducing the chances of gene mutation or insertional mutagenesis. Despite several advantages, the siRNA gene silencing technique encounters many issues regarding the specificity, stability, off targeting, interferon induction and immune stimulation, and lastly, the efficient delivery of therapeutic siRNA to the target cell. These can be overcome by precise selection of the siRNA carrier that facilitates the successful silencing of an endogenous and exogenous gene through different *in vivo* and *in vitro* delivery systems. A number of viral and nonviral vectors have been used as delivery systems in the past for gene therapy. The use of viral vectors is limited due to their immunogenic and inflammatory properties and limited DNA packaging capacity.

There are various nonviral or synthetic vectors that have been extensively used for the siRNA delivery including cationic lipids and liposomes, cationic polymers (linear, branched, dendrimers, polysaccharides), cell penetrating peptides, and inorganic nanoparticles (Pack *et al*., 2005; Thomas and Klibanov, 2003). Among these, polymeric nanoparticles prove to be a promising candidate due to their unique properties such as biodegradability, biocompatibility, cost-effectiveness, low immunogenicity, and reduced cytotoxicity, in addition to ease of modifications, high transfection efficiency, and the safe delivery of genetic materials. A number of polymer-based nanoparticles have been investigated to enhance the target specificity of the vector and to increase its transfection efficiency.

The topic summarizes the structural and biochemical behaviour of different nanoparticles under physiological conditions, which decide the cellular uptake efficiency of siRNA. In order to improve the transfection efficiency, several factors play a key role, like the concentration of polymers, polymer molecular weight (MW), pH, N/P ratio, surface charge, and modifications. Further, strategies in the delivery of siRNA regarding different nanoparticles and their modifications are exploited for both the *in vivo* and *in vitro* delivery.

The physiochemical properties of different polymers vary accordingly, depending upon the structure and chemistry of the polymer. Numerous polymers have been

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employed for the formation of nanoparticles and for the delivery of oligonucleotides. Some of them include chitosan, polyethylenimine (PEI), poly-L-lysine (PLL), dendrimers, cyclodextrins, and poly(lactic-co-glycolic) acid, which are discussed respectively in the subsequent sections.

5.2 Chitosan

Chitosan belongs to the aminoglucopyran family, comprised of randomly arranged *N*-acetylglucosamine and glucosamine residues. The biopolymer has emerged as a promising candidate because of its versatile biological activity, biocompatibility, and biodegradability, as well as low toxicity (Abbasi *et al*., 2010; Rinaudo, 2006). Numerous efforts have been made to explore the unique properties and to utilize the full potential of this versatile biodegradable polymer, for the proper functionalization and derivatization of the polymer. Chitin, a modified polysaccharide, is found as a basic component in the cell walls of fungi, insects, and in the exoskeletons of crustaceans. It is found in almost all the parts of the world and contributes to be the second most abundant natural biopolymer next to cellulose. Chitin is a linear chain of cationic heteropolymer, composed of randomly arranged *N*-acetylglucosamine and glucosamine residues covalently bonded with β-1, 4-linkage. Chitosan is prepared by *N*-deacetylation of chitin using an alkaline substance. The chitosan of varying MW ranging from 5×10^4 Da and 2×10^6 Da can be obtained according to the degree of deacetylation (DDA) between 40% and 98% under controlled derivatization of chitin (Hejazi and Amiji, 2003). The DDA and degree of polymerization (DP) are the key factors that decide the biological application of chitosan as well as the MW of the polymer. On heating, this polymer degrades before melting, due to the presence of reactive hydroxyl and amino groups in the chitosan. This makes it less crystalline than chitin, and thus this polymer has no melting point. Moreover, chitosan is easily soluble in dilute acidic solutions below pH 6.0. Due to the presence of primary amino groups with a pKa value of 6.3, chitosan can be considered as a strong base. As this polymer possesses amino groups, the pH determines the charged state and properties of chitosan (Yi *et al*., 2005). As the pH is decreased, chitosan amino groups get protonated and become positively charged, thus making it a water-soluble cationic polyelectrolyte. Similarly, at a pH above 6, the amino groups become deprotonated; the charge on the polymer decreases and this makes it insoluble. This alteration between solubility−insolubility occurs at its pKa value between pH 6 and 6.5. Chitosan can be easily degraded either by lysozymes or by chitinases that are synthesized by the normal flora in the human intestine or present in the blood under physiological environment (Aiba, 1992; Escott and Adams, 1995; Zhang and Neau, 2002). These properties of the polymer can be exploited for drug delivery in pharmaceutical research and industry (van der Lubben *et al*., 2001). Studies have been reported for using chitosan as a safer alternative for siRNA delivery among other nonviral vectors.

5.2.1 Molecular weight and concentration of chitosan

The MW of chitosan has an important role in determining the physicochemical properties such as size, zeta potential, structure, complex stability, and *in vitro* gene

silencing of chitosan/siRNA nanoparticles. Stability of the nanoparticles is necessary for the protection of extracellular siRNA; however, the release of siRNA from the complex nanoparticle is also essential for silencing the expression of the targeted gene. Thus, equilibrium between both protection and dissociation of siRNA is required for the delivery of siRNA-mediated nanoparticles.

The MW of chitosan influences the particle size of the chitosan/siRNA complex. Katas and Alpar (2006) concluded that the lower the MW of chitosan (110 kDa), the smaller the size of nanoparticles. Liu *et al*. (2007) showed that the formulated chitosan/siRNA nanoparticles were in the MW range 8.9−173 kDa. In H1299 human lung carcinoma cells, it has been observed that the concentration of chitosan molecules (MW 64.8−170 kDa) must be around 5−10 times the length of the siRNA (MW of 13.36 kDa) to form a chitosan/siRNA stable complex, resulting in an efficient silencing of the gene. On the other hand, low MW (10 kDa) chitosan molecules are unable to condense the siRNA properly into stable nanoparticles, resulting in the formation of large aggregates and hence negligible knockdown. Additionally, Ji *et al*. (2009) investigated that higher MW chitosan (190−310 kDa) produced very small nanoparticles of 148 nm size with a low polydispersity index (PDI). The FHL2 gene expression was reduced by 70% in human colorectal cancer Lovo cells when FHL2 gene-specific siRNA complexed chitosan was introduced in the cells. Therefore, MW of chitosan could be dictating its siRNA-mediated knockdown efficiency.

The concentration of chitosan is a key factor in determining the properties of the complexes. It has been observed that with the increase in chitosan concentration from 25 to 300 μg/mL in distilled water, the mean particle size of obtained chitosan-siRNA nanoparticles was increased (Katas and Alpar, 2006). Furthermore, at a constant siRNA concentration, the zeta potential of the chitosan/siRNA nanocomplexes increased with the increase in the concentration of chitosan molecules. Thus, it can be concluded that MW and concentration of the chitosan acts as an important parameter for the efficient knockdown of the gene.

5.2.2 Degree of deacetylation of the polymer

The positive charge density of chitosan relies on the DDA under acidic conditions. The DDA value denotes the percentage of deacetylated primary amino groups along the polymeric chain, which in turn decides the positive charge density of chitosan. The chitosan with high DDA value (commonly above 80%) will have more positive charge, which further allows better siRNA binding capacity (Liu *et al*., 2007). On the other hand, it has been shown that in H1299 human lung carcinoma cells, chitosan with low DDA value exhibits low charge density. This results in low interaction of chitosan with siRNA, and the nanoparticles formed are unstable, further lowering the knockdown efficiency (Liu *et al*., 2007).

5.2.3 N/P ratio of the polyplex

The N/P ratio for chitosan/siRNA nanoparticles can be described as the molar ratio of amino groups (N) of chitosan to phosphate groups (P) of siRNA. Howard *et al*. (2006) investigated the effect of N/P ratio on the size of chitosan/siRNA nanoparticles by

observing that at lower N/P ratio, particles of larger size were obtained. Similarly, in regard to the effect on size, it has been studied that at low concentrations (250 μg/mL) of chitosan and N/P ratio of 71, the measured size of the formed nanoparticles was 181.6 nm but increased to 223.6 nm at N/P ratio of 6. Additionally, Liu *et al*. studied the effect of N/P ratio of chitosan170 kDa (with 84% DDA) nanoparticles on the gene silencing efficiency in H1299 human lung carcinoma cells. It was found that the enhanced green fluorescent protein (EGFP) expression silencing was increased at N/P ratios (50 and 150) compared to low N/P ratio (2 and 10) formulations and maximum level of EGFP silencing (80%) was observed at N/P 150 formed nanoparticles (Liu *et al*., 2007). At high N/P ratios, incomplete chitosan participates in the nanoparticle formation, although some of the fraction of chitosan gets loosely associated with the formulated nanoparticle in order to increase its stability and enhance gene silencing efficacy. Furthermore, it has been shown that on removal of the excess of chitosan before transfection, no cellular knockdown was observed, indicating the need of stabilized nanoparticles for the cellular uptake and gene silencing.

5.2.4 Salt form of chitosan

The physicochemical properties of the chitosan-siRNA nanoparticles are also controlled by chitosan salt form. Chitosan glutamate (G213, G113) has higher MW than that of chitosan hydrochloride (Cl213, Cl113), and further, it has been observed that chitosan glutamate produced smaller chitosan-siRNA nanoparticles than chitosan hydrochloride (Katas and Alpar, 2006). Studies indicated that when the weight ratio of chitosan nanoparticles to siRNA approached 100:1, it resulted in the complete binding of siRNA with chitosan except for low MW chitosan hydrochloride, Cl113. Moreover, siRNA adsorbed onto chitosan glutamate (83% ± 0.9% for G213% and 90% ± 0.3% for G113) showed higher siRNA loading efficacy in comparison to chitosan hydrochloride (72% ± 1.1% for Cl213% and 59% ± 0.8% for Cl113) (Katas and Alpar, 2006). In addition, in CHO K1 cells, the highest gene silencing expression was observed by chitosan glutamate, G213 (470 kDa) at 24 h posttransfection either by simple complexation (51% gene knockdown) or ionic gelation (82% and 63% gene knockdown for siRNA entrapment and adsorption, respectively) in comparison to chitosan hydrochloride.

5.2.5 pH of the system

Studies reported that there was no significant difference in the particle size of chitosan-siRNA nanoparticles was observed when combining chitosan with siRNA either in acetate buffer (0.1 M, pH, 4.5) or in distilled water, even though the pH of the system influences the charge of chitosan (Katas and Alpar, 2006). On the other hand, a study revealed that the strength of the interaction between siRNA and chitosan was pH dependent, and as pH varied from 4.1 to 6.1, 7.4, and 9.5, the interactions also decreased, indicating definite multimodal interaction forces at acidic pH. No significant interaction forces were observed at high pH between siRNA and chitosan molecules (Xu *et al*., 2007). In cell culture, the impact of pH on the siRNA gene silencing activity has not yet been reported, and extensive research and studies are required to

further explain it. The effect of pH on chemical properties and gene delivery efficiency of chitosan-DNA nanoparticles has been determined (Nimesh *et al*., 2010). The low MW chitosan (10 kDa)-formed nanoparticles, when suspended in double distilled water (pH 6.1), resulted in uniform hydrodynamic size of 243 ± 12 nm, which further increased to 911 ± 39.6 nm and 1213 ± 84 nm when placed in phosphate buffer saline (PBS) of pH 6.5 and pH 7.1, respectively. Rhodamine-labelled chitosan nanoparticles incubated with cells for 24 h reported the formation of large aggregates at pH 7.4, whereas rhodamine B isothiocyanate-chitosan fluorescence at pH 6.5 showed even distribution of nanoparticles on the cells (Fig. 5.1). It showed that the transfection efficiency (measured as % of cells expressing EGFP) was 26.3% at pH 6.5 and reduced to 9.2% and 0.2% at a considerably higher pH of 7.1 and 7.4, respectively (Nimesh *et al*., 2010). Moreover, in HEK 293 cell lines, the uptake of formulated nanoparticles was detected by flow cytometry and revealed the dependence on pH, with the maximum uptake taking place at pH 6.5 medium.

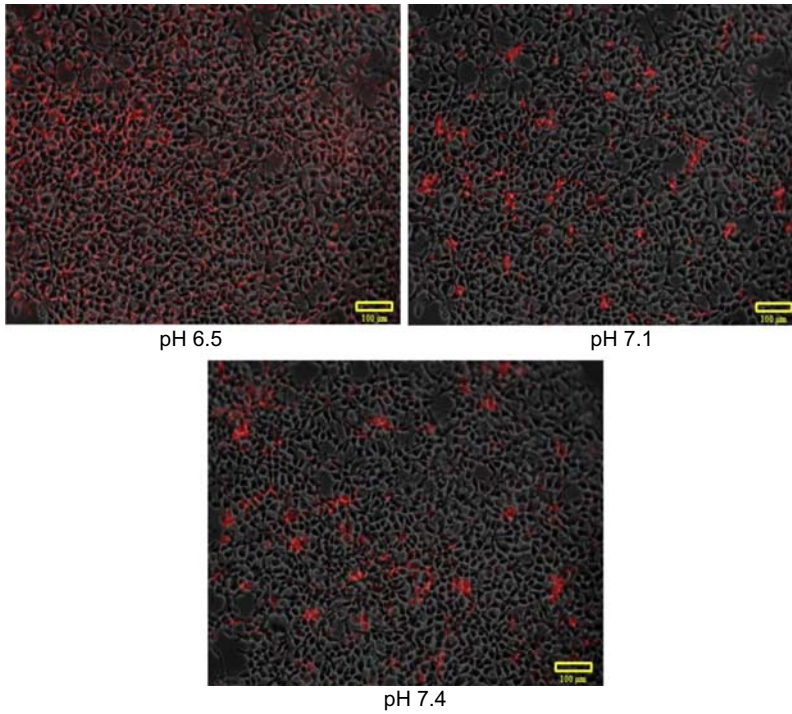

Figure 5.1 Fluorescence microscope images of HEK 293 cells transfected at different pH. Cells were exposed to rhodamine labelled chitosan/DNA complexes, and 24 h posttransfection media was exchanged with phosphate buffer saline and cells with internalized or adsorbed complexes visualized under fluorescent microscope at 10× magnification. Large aggregates present on the cell surface at pH 7.4 while complexes were more uniformly distributed at lower pH 6.5. Reproduced with permission from Nimesh S, Thibault MM, Lavertu M, Buschmann MD. Enhanced gene delivery mediated by low molecular weight chitosan/DNA complexes: effect of pH and serum. Mol Biotechnol 2010;46(2):182−96. http://dx.doi.org/10.1007/s12033-010-9286-1.

5.2.6 Presence of serum

The stability of siRNA complexed nanoparticles is necessary against digestion by nucleases to attain maximal knockdown activity in cells. Katas and Alpar (2006) examined the stability of the formulated nanoparticles in the presence of serum by incubating free and chitosan-complexed siRNA in 5% fetal bovine serum at 37°C, respectively. It was found that siRNA was stable and intact until the initial duration of 30 min, and it was completely degraded after 48 h. However, it was observed that chitosan-tripolyphosphate nanoparticle-entrapped siRNA started to degrade after 24 h and completely degraded after 72 h of incubation. The experiment also revealed that chitosan-siRNA nanoparticles considerably protected siRNA from digestion by the nuclease when 50% serum was added along with chitosan-siRNA nanoparticles. On the contrary, the unformulated siRNA and the siRNA recovered from chitosan-siRNA nanoparticles were intact and protected from degradation up to 7 h and completely degraded after 48 h of incubation in the presence of 50% serum.

5.3 Polyethylenimine

PEI, a cationic polymer, is one of the most widely used polymers for gene delivery due to its high transfection efficiency. Behr *et al.* used PEI for the first time for oligonucleotide delivery, and presently, it has been modified and derivatized to enhance its physicochemical and biological properties (Boussif *et al.*, 1995; Neu *et al.*, 2005). The high positive charge density of PEI, due to the presence of amino groups (a positive charge per 43 Da, which is the MW of a monomeric unit), results in the production of highly condensed nanoparticles. Structurally, PEI can be in either linear or branched form depending upon the arrangement of repeating units of ethylenimine in the polymer. BPEI (branched PEI) is prepared by the acid-catalysed polymerization reaction of aziridine, whereas LPEI (linear PEI) is synthesized through the ring-opening polymerization of 2-ethyl-2-oxazoline followed by hydrolysis (Brissault *et al.*, 2003; Jones *et al.*, 1944).

The vector used for the oligonucleotide delivery should maintain a suitable balance between the lipophilicity and hydrophilicity so that the vector can easily cross the plasma membrane barrier of the cells, further reducing the cytotoxicity in the cells. The net cationic charge density of the polymer must be sufficiently positive to form a stable polymer-siRNA nanoparticle and results in developing the proton sponge effect to assist the endosomal release of polyplexes. Oskuee *et al.* (2010) investigated the structural influence of various alkyl modifications on the biophysical properties and bioactivity of the formulation when alkylcarboxylate residues were grafted onto PEI (25 kDa), thus intending to enhance the efficiency of siRNA delivery through PEI-based carrier systems. Numerous studies have concluded that at a low degree of carboxylation (<20%), the buffering capacity results in an efficient endosomal escape of the polyplex. With the increase in the hydrophobic alkyl chain length, an enhanced stability of the polymer-siRNA complex has been observed (Philipp *et al.*, 2009). It has been shown that an association of additional negative charges on the PEI 25 kDa lowers the interaction potential of PEI with the cell surface and thus reduces

the toxicity of PEI. However, it was found that carboxyalkylation of PEI significantly improved the siRNA-mediated luciferase gene knockdown.

Some additional modifications were done on PEI to circumvent the problem of instability and low transfection efficiency. Polyethylene glycol (PEG) (2 kDa)-PEI (25 kDa) nanoparticles were prepared as a nonviral carrier of siRNA, targeting the CD44v6 in gastric carcinoma cells (Wu *et al*., 2010). Physicochemical studies dictate that the complexes formed below 10 N/P ratios were not suitable for cellular transfection because PEG-PEI/siRNA were unable to form a complex of appropriate size and potential. However, it has been observed that complete complexation of siRNA occurs at N/P ratios $\geq$10. The transfection efficiency of PEG-PEI/siRNA nanocomplexes was found to increase at higher N/P ratios, attaining the maximal transfection efficiency at N/P 15. Moreover, at N/P 30, no considerable increase in the transfection efficiency appeared. This could occur because of over condensation of siRNA at high N/P values, which subsequently results in the poor release of nucleic acids in the targeted cell. For the proper delivery of siRNA, Veiseh *et al*. (2010) synthesized magnetic nanoparticles comprising iron oxide (Fe_2O_3) bound with a cationic copolymer of chitosan-grafted PEG and PEI. Targeted peptides such as chlorotoxin were covalently bound with the nanoparticles to facilitate the site-specific delivery of the oligonucleotide. This modified magnetic vector was observed to deliver siRNA to the targeted brain tumour cells via receptor-mediated endocytosis, specifically silencing the expression of green fluorescent protein (GFP) in C6/GFP + glioma cells. In addition, Creusat *et al*. (2010) studied the mechanism of PEI-mediated siRNA delivery. Results indicate that the proton sponge effect plays a crucial role in the transfection efficiency of tyrosine-PEI conjugate, as it also controls the endosomal rupture and release of siRNA through an optimal pH-sensitive degradation of the PEI self-aggregates. In one of our studies, we combined the properties of cationic PEI polymer with polysaccharide alginate by synthesizing an ionic complex of BPEI (750 kDa) with varying concentration of alginic acid to obtain a series of nanocomposites (Patnaik *et al*., 2006). It was found that when the concentration of alginic acid was increased, subsequently the zeta potential and size of the nanocomposites decreased. It was shown that the zeta potential of PEI (22 mV) was decreased in the nanocomposite, as alginate masked the surface charge of PEI. The maximum transfection efficiency on various cell types was observed with the formulated PEI-alginate (6.26%) at w/w ratio 20:1, which was determined to be 2−16 times higher as compared to the native PEI. Besides, these PEI-alginate (6.26%) nanocomposites showed considerable suppression of 80% of GFP expression by efficiently delivering the targeted siRNAs into mammalian cells (Fig. 5.2).

Additionally, we formulated PEI nanoparticles by acylating PEI with propionic anhydride and further conjugating it with PEG-bis(phosphate) (Nimesh and Chandra, 2009). The size of the nanoparticles, as measured by dynamic light scattering (DLS) studies, was $\sim$110 nm, and atomic force microscopy (AFM) analysis showed spherical and condensed nanoparticles with an average size of 100 nm. The qualitative study for gene silencing was performed by visualizing under an inverted microscope after 48 h (Fig. 5.3a). *In vitro* siRNA delivery studies with the modified nanoparticles showed inhibition of GFP gene expression up to 85%, which was nearly comparable with the results for Lipofectin-mediated siRNA delivery (81% inhibition) (Fig. 5.3b).

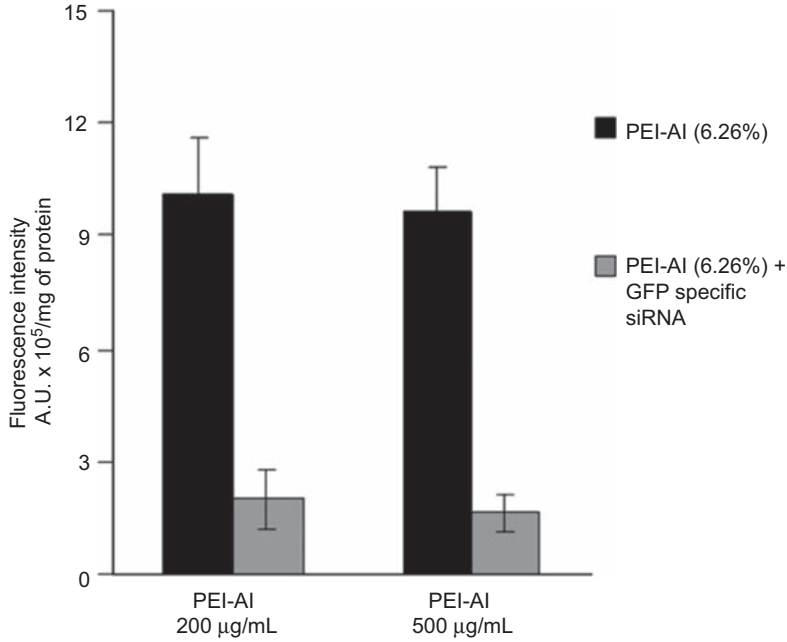

Figure 5.2 Polyethylenimine (PEI)-alginate (6.26%) as an effective carrier of small interfering ribonucleic acid (siRNA). PEI-alginate (6.26%) was tested for its ability to deliver green fluorescent protein (GFP)-specific siRNA into COS-1 cells [CV-1 (Simian) in origin carrying SV40 genetic material]. The expression of GFP in cell lysates was reduced by 80%, as monitored by measuring fluorescence on spectrofluorometer. Reproduced with permission from Patnaik S, Aggarwal A, Nimesh S, Goel A, Ganguli M, Saini N, Gupta KC. PEI-alginate nanocomposites as efficient in vitro gene transfection agents. J Control Rel 2006;114(3): 398−409. http://dx.doi.org/10.1016/j.jconrel.2006.06.025.

Kim *et al.* (2007) prepared water-soluble lipid polymer (WSLP) by attaching the cationic group of low MW BPEI (1.8 kDa) with cholesterol chloroformate (a hydrophobic lipid anchor) to reduce the cytotoxicity and increase the transfection efficiency in the cells. The complementary complex with siRNA was developed to silence human vascular endothelial growth factor (VEGF) expression and was found to form nanosized complexes (∼100 nm) and further protected siRNAs from enzymatic degradation. When WSLP/siRNA complexes were introduced in human prostate cancer (PC-3) cells derived from human prostate adenocarcinoma, a considerable reduction of VEGF production by 40% was observed in comparison to the unmodified BPEI. Additionally, it was shown that WSLP/siRNA complexes decrease 55% of the tumour volume in 21 days and 65% of the tumour in 28 days when compared with controls. These results illustrate the use of WSLP as a nonviral vector for siRNA delivery and in antiangiogenic tumour therapy.

Biswal *et al.* (2010) designed folate receptor (FR)-modified nanoparticles for the delivery of dihydrofolate reductase (DHFR) siRNA to block the expression of the DHFR gene in FR-positive KB cells, resulting in a target-specific treatment of tumours. A folate-PEG-PEI (25 kDa) (FOL-PEG-PEI) complex was formed and further conjugated with the DHFR siRNA sequence. This complex was characterized by particle size

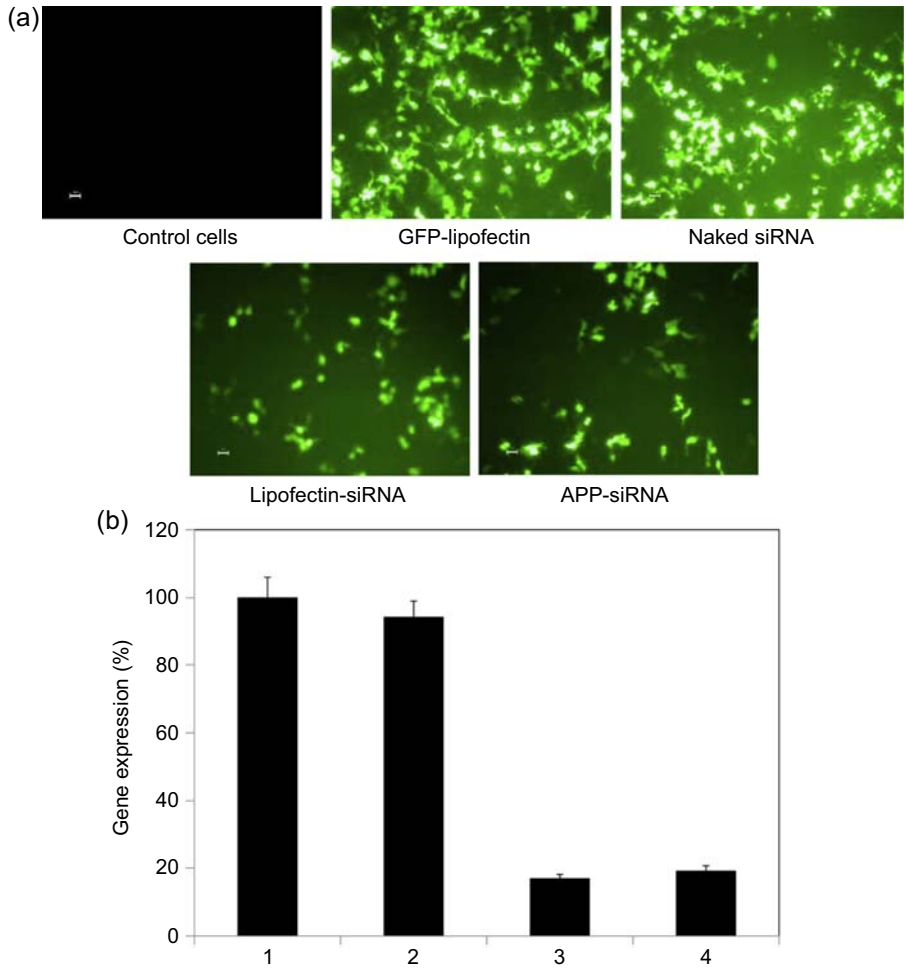

Figure 5.3 Comparison of gene silencing efficiency of various siRNA formulations after 48 h. (a) The GFP expression was observed under fluorescent microscope at 10× magnification. (b) The level of GFP expression was estimated by quantitation of green fluorescence after 48 h. The data was recorded at optimal inhibition efficiency for acetylated-PEI-PEG-bis-P (APP) nanoparticles, i.e., APP nanoparticles: siRNA at 30:1 ratio.*GFP*, green fluorescent protein; *PEG*, polyethylene glycol; *PEI*, polyethylenimine; *siRNA*, small interfering ribonucleic acid. Reproduced with permission from Nimesh and Chandra (2009).

analyser, gel retardation, and DNase protection assay. The complex was transfected to FRs overexpressing human epidermal carcinoma (KB) and FR-negative human lung carcinoma (A549) cells. The transfection studies were analysed through fluorescence microscopy and real time polymerase chain reaction and it observed that the complex released the siRNA and silenced the DHFR gene in KB cells, though it remained unaffected in the case of control A549 cells. Thus FOL-PEG-PEI complex-mediated siDHFR delivery inhibits the DHFR expression in FR-positive cells, resulting in the target-specific delivery to the tumour cells. The site-specific delivery was further

affirmed by transfection of pSUPER-siDHFR through lipofectamine in both KB and A549 cells, and the reduction in expression of the DHFR gene was observed. Another approach for the confirmation can be through blocking the receptors using free folic acid. This strategy can be applied for the study of a wide range of FR-targeted drug delivery and gene silencing therapeutics by siRNA expression pDNA.

Galactose and pullulan are among the several targeting moieties that have been employed in liver targeted gene delivery because of their potential to bind with asialoglycoprotein receptor. Pullulan is a polysaccharide biopolymer comprised of three α-1, 4-linked glucose polymers (maltotriose) and covalently connected with different α-1,6-glucosidic linkages. Kang *et al.* (2010) utilized pullulan with PEI for liver-specific targeting. Moreover, they designed a delivery system of pullulan-PEI/siRNA nanocomplexes for delivery in mice by either hydrodynamics or nonhydrodynamic-based injections through the tail-vein method. It has been found that during systemic injection of PEI-pullulan/siRNA complex, an increased fluorescence level in the liver was observed compared to the PEI/fluorescein-labelled siRNA complex, which raised the level of fluorescence in the lung. Besides, PEI/siRNA complexes of high N/P ratio contributed to a high mortality rate of mice, but pullulan-conjugated PEI greatly reduced the death of mice through systemic injection. Hence, PEI-pullulan polymeric complex facilitates an efficient siRNA delivery with low cytotoxicity into the liver.

Another strategy included the development of a stable nanocarrier siRNA system by using biocompatible/biodegradable glycol chitosan polymer (GC) and PEI with hydrophobic 5β-cholanic acid that were amalgamated to form stable, self-assembled GC-PEI nanoparticles (GC-PEI) (Huh *et al.*, 2010). The particle size of the freshly prepared GC-PEI nanoparticles were 350 nm with a zeta potential or positive-charged surface (ζ potential = 23.8), which is sufficient to condense the negatively charged red fluorescent protein (RFP)-siRNAs, designed for blocking RFP expression. The siRNA-condensed nanoparticles (siRNA-GC-PEI) exhibited the particle size of 250 nm at 1:5 weight ratio of siRNA to GC-PEI nanoparticles. In B16F10 tumour cells (RFP/B16F10), it was observed that the uptake profile of siRNA-GC-PEI nanoparticles were time-dependent and showed a rapid uptake within 1 h. Furthermore, the endocytosed siRNA-GC-PEI nanoparticles resulted in the silencing of gene expression. Hence, the formulated siRNA-GC-PEI nanoparticles showed a substantial decrease in the RFP gene expression level of RFP/B16F10-bearing mice, because of their higher tumour-targeting ability. These results indicated the application of GC-PEI nanoparticles as an efficient nonviral siRNA delivery system for cancer therapy.

On the other hand, reducible polymerized siRNA showed a possible solution for the low efficiency of siRNA delivery. The modified RFP siRNAs were polymerized in both the sense and antisense strands at their 5′ ends. Lee *et al.* developed polymerized siRNA (poly-siRNA) comprised of 30% oligomeric siRNA (50−300 bps) and 66% polymeric siRNA (above ∼300 bps) as fractions, and it was further reduced by reducing the solution through disulfide bond cleavage. It has been observed that the nanosized complexes with low MW PEI (1800 Da) and poly-siRNA were more condensed than siRNA (mono-siRNA) because of a strong electrostatic interaction based on the higher charge density of poly-siRNA. The compact and stable poly-siRNA/PEI complexes were protected from the degradation of siRNA from a polyanion competitor and

RNases present in serum. In addition, it was also found that in murine melanoma cells (B16F10), the uptake efficiency of poly-siRNA/PEI complexes was high, resulting about 80% inhibition in RFP gene expression than the untreated cells. These results conclude that strong polyanionic and reducible poly-siRNA can be employed as a potential therapeutic approach for human diseases (Lee *et al*., 2010).

Additionally, siRNAs conjugated linear jetPEI/*in vivo* jetPEI were used as a possible treatment for other pathologies as well. In an investigation on the treatment of guinea pigs against a lethal Ebola virus infection, the intraperitoneal injection of PEI/siRNA complex resulted in the targeting of the polymerase (L) gene of the Zaire species of EBOV and also considerable reduction in plasma viraemia levels (Geisbert *et al*., 2006). On intraperitoneal administration of PEI/siRNA polyplex, a decrease in the levels of hypoxia inducible factor 1α and plasminogen activator inhibitor 1 was observed (Segura *et al*., 2007). Ge *et al*. utilized PEI-complexed siRNAs targeted to the conserved regions of influenza virus genes for the treatment and protection from lethal influenza infections in the mouse. Moreover, a reduction in the virus production in lungs of infected mice was observed on introduction of the complexes before or after initiation of the virus infection (Ge *et al*., 2004). With their intravenous application, the lungs were considered as one of the primary organs of PEI/siRNA complex delivery (Ge *et al*., 2004). Similarly, under physiological conditions, a certain degree of aggregation of the PEI/siRNA polyplex may be suitable for efficient cell transfection in the lung. Besides, intravenous injection was also used for targeting IL-13. In one of the studies, it was determined that PEI/siRNA-assisted IL-13 silencing could also prevent the stimulation of allergen-induced airway dysfunction (Lively *et al*., 2008). Another study suggested that systemic delivery of PEI complexes, which was based on complete deacetylation of PEI, led to elevated levels of siRNA delivery to the lung and reduced the toxicity as well. This was shown by the low levels of expression of luciferase as a model gene or of the influenza viral nucleocapsid protein gene with a subsequent 94% reduction of virus titres in the lungs of influenza-infected animals (Thomas *et al*., 2005). With an objective of reducing the toxicity and increasing bioactivity, Werth *et al*. (2006) studied the purification of a low molecular weight (LMW) PEI (PEI-F25-LMW) through size exclusion chromatography, from the commercially available 25 kDa PEI. Complexes based on PEI-F25-LMW can be stored, lyophilized, or frozen, thus overcoming the requirement to synthesize complexes freshly and allowing the preparation of standardized aliquots (Hobel *et al*., 2008; Werth *et al*., 2006).

5.4 Poly-L-lysine

PLL is a cationic polypeptide composed of an amino acid lysine as a repeating unit and is one of the first polymers commonly studied for nonviral gene delivery. The DP of lysine can be in the range of 90−450, which decides the degree of biodegradability of the formed polypeptide chain, a property required for the *in vivo* analysis. Moreover, it has been shown that the DP directly corresponds to the toxic effects on the cultured cells, i.e., the longer the polypeptide chain, the more the cytotoxicity (Martin and Rice, 2007; Plank *et al*., 1999). The reason behind the toxicity is the slow degradability of the polymer; PLL of high MW are degraded slowly and are toxic to cultured cells (Putnam *et al*., 2001; Symonds *et al*., 2005). However, PLL have been widely used in

different forms such as the dendrimer and copolymer for siRNA delivery. Inoue *et al.* (2008) investigated the potential of dendritic PLL, KG6 (sixth generation dendritic PLL with 128 amine groups on its surface) as an efficient carrier for siRNA delivery. It was found to be highly efficient to deliver fluorescein-labelled oligonucleotide into cells, although a large amount of the fluorescence was observed from the endosomal compartment, indicating that the complex failed to move out of the endosomal compartment after endocytic uptake. To circumvent this problem, KG6 was modified with Endo-Porter (KG6/EP), which showed fluorescence from the entire cytosol. KG6 also displays considerable uptake efficiency (>90%), and is the most efficient carrier in comparison to HiPerFect, DoFect-GT1, lipofectamine 2000, EP, and KG6/EP. Moreover, Endo-Porter, a weak-base amphiphilic peptide when combined with KG6, showed effective knockdown of GAPDH along with low cytotoxicity. Additionally, in rat hepatoma H4IIEC3 cells, a reduction in the levels of glucose production was observed due to the knockdown of PEPCK, a rate-limiting enzyme for gluconeogenesis. Organic cation transporter 1 (OCT1), which is responsible for the metformin action, was subsequently knocked down and resulted in lowering the activity of metformin to inhibit gluconeogenesis in H4IIEC3 cells. Thereafter, KG6 was used as a nonviral vector to deliver ApoB-specific siRNA *in vivo* for the treatment of hypercholesterolaemia (Watanabe *et al.*, 2009). At a cation/anion ratio of 8.0 in 5% of dextrose, the particle size of the obtained KG6/siRNA complex as measured through DLS was 168 ± 9.9 nm. In order to determine the *in vivo* ability of KG6 to deliver siRNA designed to silence ApoB expression, the formulated complex was injected in C57BL/6 mice intravenously. It has been found that si-ApoBI complex treated mice showed 22% reduction in ApoB levels comparison with those in the nontreated group, at all of the doses evaluated. si-ApoBII complex indicates a considerable reduction of approximately 50% in the mRNA levels at 2.5 mg/kg dose. Eventually, progress in hypercholesterolaemia was observed when ApoE-deficient ($ApoE^{-/-}$) mice were treated with the KG6 siRNA complex. The injection of si-ApoBI complex led to the decrease in the levels of very low density lipoprotein and low density lipoprotein cholesterol for up to 96 h, and it was determined that the ApoB mRNA levels were also reduced but only in the si-ApoBI treated group (reduction in ApoB mRNA levels: $24.6 \pm 2.6\%$ vs. $7.6 \pm 9.4\%$, respectively). These results conclude that KG6 could be employed as an efficient carrier for the systemic delivery of siRNA and can be used in lowering the cholesterol levels in patients with hypercholesterolaemia. Matsumoto *et al.* (2009) synthesized polyion complex (PIC) micelles with a disulfide cross-linked core through the assembly of siRNA iminothiolane-formulated PEG-block-PLL [PEG-b-(PLL-IM)] and at a certain mixing ratio. The size of obtained PIC micelles was ~60 nm with a narrow size distribution. At physiological conditions, the structure of micelles was maintained, but under reducing environment, the structure got disrupted due to the cleavage of disulfide cross-links and thus facilitates the release of siRNA in the intracellular environment. However, it has been observed that the environment-sensitive PIC micelles showed 100 times higher siRNA transfection efficacy in comparison to the unstable noncross-linked PICs synthesized from PEG-b-PLL. These results indicated the potential of cross-linked PIC micelles for siRNA delivery into the target cells.

In order to avoid the possibility of polyplex dissociation in the extracellular environment, a pH- and redox-sensitive polymer was covalently linked with siRNA (Meyer *et al*., 2009). The conjugated siRNA composed of PLL (MW = 32 kDa, DP = 153) for RNA binding and polycation protection, PEG for solubilizing and shielding the polymer, the lytic peptide melittin masked with dimethylmaleic anhydride (DMMAn), gets cleaved at endosomal pH, where the siRNA is attached through a reducible disulfide bond at the 5′-end of the sense strand. The PEG-PLL-DMMAn-Mel-siRNA obtained was in the range of 80−300 nm, based on the handling methods (such as freeze thawing, dilution). In the presence of the polyanion heparin, the formed siRNA conjugate remained stable where the comparable siRNA complex disassembles. The release of siRNA could be triggered in the presence of a reducing agent such as glutathione along with heparin. In Neuro2α-eGFPLuc-cells, an efficient silencing of the gene was observed at ≥25 nM siRNA complex and elevated levels of *in vitro* biocompatibility (absence of cytotoxicity or haemolytic activity at neutral pH) was also found. Around 90% reduction in the level of luciferase expression was observed when PEG-PLL-DMMAn-Mel/luciferase siRNA polyplex (complexed at w/w ratio 2) was used. The conjugate with covalently attached GL3 luciferase siRNA showed 80% knockdown of luciferase at lower siRNA doses (0.125 and 0.25 μg) and around 90% knockdown at higher siRNA amounts (≥0.5 μg).

Lipid-modified polymers could be used as efficient gene carriers because of their potential to condense substantially large plasmid DNAs (>1000 base pairs) and protect them from intracellular degradation (Abbasi *et al*., 2008). The substitution of lipid on positively charged polymers significantly increased the uptake of plasmid DNA across cellular membranes. In regard to these studies, Abbasi *et al*. (2010) suggested the stearic acid-modified PLL (PLL-StA) to deliver siRNA, which downregulates the P-gp expression in a tumour cell model. P-gp is a membrane protein that efflux the foreign substances out of the cell. When the levels of P-gp are high, it results in multidrug resistance (MDR) in breast and ovarian cancer cells. It has been observed that at low siRNA carrier ratios (1:3 and 1:1), PLL-StA carriers protect the siRNA from degradation. The results showed an increased delivery of siRNA through modified PLL into MDR1 cells. The PLL-StA mediated siRNA delivery presented an increase of almost 2.5-fold in DOX-positive MDR1 cells. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cytotoxicity assay showed an increase of ∼35% and ∼25% in the DOX-treated cells and PTX-treated cells, respectively. However, the efficient siRNA delivery through nonviral carriers lowered the level of P-gp expression on the cell surface and resulted in an increased efficiency of chemotherapeutic agents *in vitro*.

PEG could be used as a promising candidate in therapeutic applications due to its biocompatibility, solubility, enzymatic tolerance, and minimum nonspecific interaction with blood proteins and cells. PEG could either be incorporated through grafting or as a copolymer with the cationic polymers. Shimizu *et al*. (2010) designed a PEG-PLL-based delivery system to deliver siRNAs to the targeted glomeruli. The particle size of the siRNA/nanocarrier complex as measured by the DLS was approximately 10−20 nm, which would facilitate the movement across the fenestrated endothelium to access the mesangium. Intraperitoneal injection of fluorescence-labelled siRNA/

nanocarrier complexes allowed the siRNAs to remain stable during the blood circulation for a prolonged time. It has been observed in a mouse model of glomerulonephritis that on repeated intraperitoneal administration of MAPK1 siRNA/nanocarrier complex, a considerable decrease in the glomerular MAPK1 mRNA and protein expression level was found, resulting in an improved kidney function, reduced proteinuria, and ameliorated glomerular sclerosis. Moreover, this therapy suppressed the expression of the profibrotic markers TGF-β1, plasminogen activator inhibitor-1, and fibronectin. Therefore, with the introduction of siRNA/PEG-PLL nanocarriers, intraglomerular genes were subsequently suppressed.

In one of the studies, Sato *et al*. (2007) synthesized and analysed a series of cationic comb-type copolymers (CCCs) exhibiting a polycationic backbone (less than 30 wt%) and abundant water-soluble side chains (more than 70 wt%) as a carrier for siRNA delivery with prolonged blood circulation time. Considerably, it has been shown that CCC with higher water-soluble side chains (10 wt% PLL and 90 wt% PEG) possess stronger interaction with siRNA in comparison to the CCC with the lower side chain content (30 wt% PLL and 70 wt% PEG), indicating that a highly dense PEG brush provides interpolyelectrolyte complex formation between the PLL polycationic backbone and siRNA. It was found that during *in vitro* studies for 24 h, the siRNA/CCC complex was protected from degradation in 90% plasma. The CCC with the higher side chain content enhanced the circulation time of siRNA by 100-folds, when studied in the mouse bloodstream. Although, when the mouse was treated with CCC and siRNA separately at 20 min intervals, blood circulation time of postinjected siRNA was subsequently increased. These results indicate the specificity of CCC toward siRNA through ionic interactions than other anionic substances present in the bloodstream. Afterwards, the same group synthesized a new series of PEG-grafted PLL (PLL-g-PEG) with different lengths of PEG (PEG 2, 5, and 10 kDa and PLL 28 and 40 kDa), to determine the *in vivo* masking effects of PEG on positively charged PLL (Kano *et al*., 2011). Among the series, 40K10P37 (40 kDa of PLL, 10 kDa of PEG, 37 mol% grafting) with MW of 10^6 as measured by MALLS, showed accumulation in tumours at around 8% of the injected dose/gram of tissue. Moreover, a modified PLL-g-PEG, when mixed with murine sera, protected the siRNA from degradation, indicating the association of siRNA with the formulated PLL-g-PEG in sera. These results shows that PLL-grafted PEG improves its blood circulation time and tumoural accumulation without losing the potential to associate with siRNA.

5.5 Dendrimers

Dendrimers derived from a Greek word “Dendron” (meaning tree, and “meros” means part) are basically multivalent branched well-defined structures. The molecules were first reported by Vögtle *et al*. as “cascade molecules”, and in another study, Tomalia *et al*. (1985) explained these molecules as dendrimers (Buhleier *et al*., 1978). The structure of this macromolecule is globular and in nanometre range. Typically, these are distinguished into three different domains: (1) a central core that comprises either a single or a group of atoms that has at least two chemical functionalities that can

facilitate linking of branches; (2) branches arising from the core that possess repeat units where the repetition could be in a geometric progression that arranges in a concentric layers; and (3) terminal functional groups at the periphery that determine its complexation or drug entrapment capability. In general, the large number of terminal groups favours its interaction with other macromolecules. However, the dendrimers have the advantageous properties of high solubility, reactivity, and binding properties.

Thus, it is evident that dendrimers carry various beneficial properties such as chemical homogeneity, high ligand, and functionality density, which allows them to be a beneficial carrier for the therapeutics by interior encapsulation, surface adsorption, or chemical conjugation. Polycationic dendrimers such as polyamidoamine (PAMAM) and polypropylenimine (PPI) have been the widely investigated vector for efficient drug or gene delivery, including its role in siRNA delivery.

5.5.1 Poly(amidoamine) dendrimers

Polycationic PAMAM dendrimers consist of primary amine groups on the surface and tertiary amine groups inside. The primary amine group plays an important role in nucleic acid binding and nanoparticle formation, whereas the secondary amine exhibits proton sponge effect in endosomes, enhancing the release of nucleic acids in cytoplasm. A comparative study between the delivery of siRNA alone and the delivery of antisense/siRNA using PAMAM dendrimer conjugated to a peptide showed higher efficiency of delivery using PAMAM G5. The siRNA delivered using PAMAM G5/Tat modified PAMAM G5 dendrimer against P-glycoprotein expression in viable NIH 3T3 MDR cells was evaluated using immunofluorescence and flow cytometry, and the results were compared to the effects of antisense and siRNA delivered by lipofectamine 2000. From the results, it was discerned that dendrimers were comparably more effective when compared to the results obtained from lipofectamine 2000. On the contrary, it was observed that cationic lipids were more effective than the dendrimers in reducing the expression of P-glycoprotein (Kang *et al*., 2005). Furthermore, conjugation of PAMAM dendrimers reduced delivery efficiency.

In a study, Zhou *et al*. (2006) developed polycationic PAMAM dendrimers for the purpose of intracellular siRNA delivery. In this process, triethanolamine was used as the core of the dendrimers where the branching started at 10 successive bonds from the central nitrogen, giving rise to a packed branching unit; compared to lower generation dendrimers, higher generation dendrimers were capable in delivering siRNA and inducing gene silencing in a mammalian cell culture system. The higher the generation number, the stronger the interactions of dendrimers with siRNA. The formation of siRNA complex at neutral pH and internalization at higher N/P ratio resulted in greater gene silencing efficiency due to G7-mediated buffering of the endosomal cavity. The N/P ratio was kept at 10−20 with siRNA concentration of 100 nM. However, the toxicity profile increased with an increase in the concentration of siRNA concentration. Further, these PAMAM dendrimers with a flexible triethanolamine core are used in delivering Hsp27 siRNA effectively into prostate cancer (PC-3) cells

(Liu *et al*., 2009). These resulted in the gene silencing of heat shock protein 27 that can act as therapeutic target prostate cancer (Liu *et al*., 2009).

The potential role of dendrimers for siRNA delivery was evaluated using PAMAM dendrimer (G3) in combination with α-cyclodextrin (α-CDE) to silence luciferase gene and compare results with the commercially available transfection reagents (Tsutsumi *et al*., 2007). It was observed that the ternary complexes of pGL3/siGL3/α-CDE exhibited RNAi effects with minimal cytotoxicity. Most importantly, it was observed that α-CDE conferred interaction with both pDNA and siRNA and protected it from serum degradation. A combination of α-CDE with fluorescent-labelled siRNA led to even distribution in cytoplasm compared to the commercially available transfection reagent that mainly retained the siRNA in both the nucleus and cytoplasm in NIH3T3 cells (Tsutsumi *et al*., 2007). Consequently, the siRNA/CDE complex led to significant siRNA delivery in transiently as well as stably expressing luciferase gene cell lines. Also, Shen *et al*. (2007) studied various properties of dendrimer-RNA complexes using AFM. The study revealed that fabrication of a stable nanoparticle largely depends on the size of the RNA molecule, dendrimer generation, and N/P ratio. Thus, it was concluded that larger RNA molecules, higher generations of dendrimers, and higher N/P ratios lead to the development of uniformly stable dendrimer-RNA complexes (Shen *et al*., 2007).

In a study by Patil *et al*. (2008), PAMAM-NH_2 dendrimers was converted into PAMAM-NHAc dendrimers in order to overcome the cytotoxicity issue. In the process of derivatization the surface amine groups were modified to the acetyl group, whereas the internal tertiary nitrogen groups were quaternized. Further, the physicochemical properties and siRNA delivery efficacy of the native, modified dendrimers as well as hydroxyl-terminated QPAMAM-OH dendrimer were done. From AFM studies, it was reported that QPAMAM-OH and QPAMAM-NHAc formed stable, well-condensed spherical nanoparticles, while PAMAM-NH_2 formed nanofibers (Patil *et al*., 2008). Thus from the surface modification of surface amine groups, the cytotoxicity of dendrimers QPAMAM-NHAc decreased significantly. From the confocal microscopy studies on A2780 human ovarian cancer cells, it was revealed that PAMAM-NH_2-siRNA and QPAMAM-OH-siRNA complexes were not taken up by the cells, even after 80 min of incubation time. While QPAMAM-NHAc-siRNA complexes were rapidly internalized by the cells with uniform distribution in the cytoplasm and nuclei (Patil *et al*., 2008). Thus the studies revealed that the amide groups on the surface of the dendrimer play a crucial role in uptake by facilitating the hydrogen bond with the cell membrane to enhance the cellular uptake.

Further, to overcome the cytotoxicity issue, the primary amines of PAMAM-NH_2 are acetylated using acetic anhydride (Waite *et al*., 2009). It was found that dendrimers with 60% acetylation of primary amines resulted in formation of ∼200 nm complexes with siRNA. Moreover, with the increase in the degree of acetylation the cytotoxicity of the polymer decreased gradually in U87 cells, along with the increase in the dissociation of dendrimer-siRNA complexes. However, in addition to this, acetylation of dendrimers reduced the buffering capacity of the dendrimers, thereby resulting in a reduction in the delivery of siRNA (Waite *et al*., 2009). In addition, confocal microscopy proved the escape of payload from endosomes to be a major barrier in the siRNA

delivery system. It was later concluded that some fraction of modification (20%) of PAMAM can maintain the delivery efficiency and gene silencing efficiency, as higher neutralization reduces gene silencing efficiency vectors. To further address the issues of cytotoxicity, the primary amines of PAMAM-NH_2 were acetylated to different extents using acetic anhydride (Waite *et al*., 2009). Dendrimers with up to 60% of primary amines acetylated resulted in the formation of ~200 nm complexes with siRNA. With the increase in amine acetylation polymer, cytotoxicity reduced in U87 cells, along with enhanced dissociation of dendrimer-siRNA complexes. Acetylation of dendrimers reduced the cellular delivery of siRNA, which correlated with a reduction in the buffering capacity of dendrimers upon amine acetylation (Waite *et al*., 2009). Confocal microscopy suggested that escape from endosomes is a major barrier to siRNA delivery in this system. It was concluded that a modest fraction (~20%) of primary amines of PAMAM can be modified while maintaining the siRNA delivery efficiency of unmodified PAMAM, but higher degrees of amine neutralization reduced the gene silencing efficiency of PAMAM-siRNA delivery vectors.

In one of the studies, it has been observed that on the attachment of a synthetic analogue of luteinizing hormone-releasing hormone (LHRH) onto the QPAMAM-OH dendrimer, there was an increase in the efficiency of siRNA delivery (Patil *et al*., 2009). Additionally, the complex produced from QPAMAM-OH and QPAMAM-OH-LHRH dendrimers revealed a significant decrease in the size of the complex with the increase in charge ratio. AFM studies confirm the formation of spherical complexes at N/P ratio 3. QPAMAM-OH and QPAMAM-OH-LHRH dendrimers with a neutral charge on the surface showed more than 90% cell viability even at high concentrations of 12.5 μM (Patil *et al*., 2009). Furthermore, the uptake studies revealed the uniform distribution of dendrimers and dendrimer-siRNA complexes in the cytoplasm and nucleus of A2780 cells. The degree of quaternization, to a small extent, also effects the internalization of siRNA and the gene knockdown efficiency. It has been determined that dendrimers with a lower degree of quaternization (20−30%) delivered siRNA effectively in comparison to those with a higher degree (70−85%). In A2780 cells, the introduction of nontargeted QPAMAM-OH and targeted QPAMAM-OH-LHRH dendrimers showed a considerable decrease in the levels of *BCL2* gene expression with the targeted dendrimer (Patil *et al*., 2009).

Perez *et al*. (2009) explored the use of "not flexible" ethylendiamine (EDA) core PAMAM dendrimers of different sizes to form siRNA condense complexes and demonstrated the impact of ionic strength of media on biochemical properties such as the size, relative binding affinity, and zeta potential of complexes, and its interaction with the cell membrane for cellular uptake analysis and knockdown activity. Markedly, the size of the formed complexes was relatively larger in the presence of NaCl in comparison to the complexes in the absence of it (several μm−800 nm, 0 zeta potential, vs. 30−130 nm, +25 mV zeta potential, respectively). Besides, the uptake efficiency and the silencing effect of the EGFP gene expression were also dependent on the size of the complex. Complexes synthesized in the presence of NaCl in the medium generated an intrinsic effect on phagocytic (J-774-EGFP) cells, while it was inactive on nonphagocytic cells (T98G-EGFP). Although maximum suppression of the EGFP gene was observed at a noncytotoxic concentration of 50 nM siRNA with

the complexes formed in the NaCl-absent medium. Strikingly, siRNA-G7 complexes showed the maximum silencing of EGFP expression of about 35% in T98G-EGFP and 45% in J-774-EGFP cells, respectively (Perez *et al*., 2009).

Moreover, to characterize the complexes formed by siRNA and G7 PAMAM dendrimers, the combinational approach, using the structural and calorimetric strategies along with the molecular dynamics simulations, have been employed (Jensen *et al*., 2010). Complexes of 150 nm were evaluated by DLS, and it was determined that at a higher N/P ratio, the size of the complex decreases. Small-angle X-ray scattering (SAXS) showed no change in the structural behaviour of the complex, and the size remains the same on siRNA binding (Jensen *et al*., 2010). The obtained values of ΔH in binding and size were comparable with molecular dynamics simulations.

Additionally, the mechanism of self-assembly between siRNA and various generations of PAMAM dendrimers was explained, and the obtained complexes were characterized (Jensen *et al*., 2011). It has been studied that G4 and G7 dendrimers exhibit equivalent efficiencies for dendriplex formation, while G1 dendrimers do not. Although, it has been concluded that at a high concentration of dendrimers, the particle size decreases, along with an increase in polydispersity of the complex through DLS and nanoparticle tracking analyser. The nanoparticle tracking analysis suggested that electrostatic interactions between the complex maintains an equilibrium between different sized complex aggregates, in which the centre of mass is dependent on the dendrimer-siRNA ratio. Initially, with the increase in generations, the binding became increasingly exothermic, and the values were determined by molecular dynamics simulations (Jensen *et al*., 2011). The maximum entropic penalty was shown by flexible G1 and further by rigid G7, hence making the G4 most appropriate for complex formation, displaying a considerable charge for siRNA interaction.

5.5.2 Poly(propylenimine) dendrimers

There have been few studies on PPI dendrimers as a potential siRNA delivery system. siRNA have limited stability and low internalization. To overcome this issue, PPI dendrimers were introduced that include layer by layer modification caging of siRNA-PPI (generation 5) dendriplex followed by coating of PEG (Taratula *et al*., 2009). In order to achieve targeted uptake by the cells a synthetic analogue of LHRH peptide was conjugated to the PEG polymer. These attributes were supportive in contributing serum resistance, increased stability, tumour-specific targeting, and enhanced permeation of the particles into the cells retaining the gene silencing capacity (Taratula *et al*., 2009). Further, it was observed that higher generations (G4 and G5) of PPI dendrimers were comparatively more effective in forming a complex with siRNA, compared to the lower toxicity, nanoparticle formation, and intracellular internalization of G5 and G4 dendrimers, ultimately leading to specific gene silencing. The formulated PPI-siRNA dendrimer complexes exhibited marked knockdown of targeted mRNA expression in A549 human lung cancer cells.

In another approach, Chen *et al*. (2010) used low generation (G3) PPI dendrimers to package and deliver siRNA efficiently. The AuNPs were used to package siRNA with low generation dendrimers into discrete nanoparticles. But most notably, the AuNPs

could be selectively removed without affecting the integrity of siRNA complexes. These G3 PPI, along with AuNPs, could be internalized into the cancer cells and efficiently silence their target mRNA. This approach was found to be superior to the higher generation dendrimers (Chen *et al*., 2010).

5.5.3 Carbosilane dendrimers

Carbosilane dendrimers possess an interior carbon-silicon bond that starts to dissolve at a slow rate when dissolved in water. This ultimately results in the slow release of its exterior branches and their payload within 4−24 h. Therefore, in order to provide stability, transfection of siRNA, and the release of siRNA inside the cells, carbosilane dendrimers (CBS) were introduced (Weber *et al*., 2008). Initial studies were done to determine their stability and cytotoxicity. The CBS bind to the siRNA via electrostatic interaction and showed resistance to RNase. Also, cytotoxicity studies with peripheral blood mononuclear cells (PBMC) and the lymphocytic cell line SupT1 showed a maximum safe dendrimer concentration of 25 μg/mL. Flow cytometry and confocal microscopy was also performed with fluorochrome-labelled siRNA, either naked or complexed with CBS, that showed higher transfection efficiency. From the studies, it was revealed that dendriplexes with N/P ratio of two showed the highest transfection efficiency with a low level of cytotoxicity, including in hard-to-transfect HIV-infected PBMC. These CBS-siRNA dendriplexes potentially silenced GAPDH expression and reduced HIV replication in SupT1 and PBMC. Thus, from these studies, feasibility of the dendrimers could be revealed for their use in RNAi therapeutics and an alternate therapy in HIV infection.

5.6 Cyclodextrins

Generally, cyclodextrins (CDs) are (α-1,4)-linked oligosaccharides of α-D-glucopyranose with a central hydrophobic core and hydrophilic outer surface. The CDs are not cylindrical in shape due to the absence of free rotation about the bonds and are toroidal or cone shaped. In this structural framework, primary hydroxyl groups are present at the narrow side of the toroidal structure, whereas secondary hydroxyl groups are located at the wider region. The most available form of CDs are α-CD, β-CD, and γ-CD that comprise six, seven, and eight glucopyranose units, respectively. Davis *et al*. introduced linear cationic polymers containing β-CD in the polymer backbone for the purpose of gene delivery (Gonzalez *et al*., 1999). Generally, modification of CDs with cationic polymers exhibited lower toxicity and better gene delivery. During the process of a gene delivery system using cyclodextrin polymers (CDPs) the polyplexes can be further modified by inclusion complex formation due to the presence of large amounts of CD moieties (Davis and Brewster, 2004; Pack *et al*., 2005). Different cationic polymers, including linear and branched PEI, as well as PAMAM dendrimers with grafted CDs, were used to determine their role in gene delivery. Thus, upon grafting CD moieties to the CDP, there was a significant reduction in the cytotoxicity. Similarly, in another study, Uekama *et al*. investigated the role of

CD grafting onto PAMAM dendrimers that showed enhanced transfection efficiency and intracellular trafficking of the pDNA (Arima *et al*., 2001; Kihara *et al*., 2002; Wada *et al*., 2005).

The cyclodextrins with short polycations can confer low cytotoxicity and enable the ease of assembly with other components such as targeting ligands for delivering siRNA. Upon binding CDP with siRNA, a complex of 50 nm diameter is formed, in which the imidazole rings aids in endosomal release and intracellular trafficking of the encapsulated nucleic acids (Davis *et al*., 2004). Thus, from most of the studies, it has been revealed that CDP can effectively protect siRNA from nuclease degradation and can bypass the need for chemical modification of the nucleic acid. Further, the colloidal stability of the particles in biological fluids can be improved by decoration of the surface with PEG via inclusion complex formation for interaction with the cell surface receptors. The complete formulation can be prepared by mixing the components for self-assembly.

The nonvirally delivered siRNA also showed efficacy in *in vivo* studies. For this, Hu-Lieskovan *et al*. (2005) used a mouse model possessing metastatic EFT in NOD/scid mice that constitutively expresses luciferase. Further, targeted delivery of siRNA against EWS-FLI1 was used to control metastatic tumour growth. A complex containing modified CDP was used to deliver siRNA. The CDP was first mixed with adamantine-PEG5000 (AD-PEG) with AD: β-CD at 1:1 (mol/mol) ratio. Further, polyplexes with transferrin modification of AD-PEG were used. Similarly, imidazole-terminated CDP with siRNA was delivered against the sequence EWS-FLI1 breakpoint in TC71. This significantly reduced EWS-FL11 protein levels by 50%. The siGL3-containing polyplexes strongly reduced the luciferase signal for more than 90% after 2−3 days of injection. However, the luciferase downregulation was transient. A three-consecutive daily injection of siEFBP2 showed a decrease in the tumour signal lasting 2−3 days. Moreover, the EWS-FLI1 expression in the tumours significantly decreased with two consecutive siEFBP2 formulations that showed a 60% downregulation of EWS-FLI1 RNA level in comparison to siRNA-treated tumours. In addition, no abnormalities were observed in the interleukin-12 and IFN-A levels as well as in the kidney, liver, pathology, or the blood counts tested for long-term low pressure and low volume tail-vein administration. Thus this study strongly provides evidence for its safety and efficacy as an siRNA delivery system.

In another similar type of study, Bartlett and Davis (2007) investigated the role of CDP in the delivery of nucleic acids. The polycation-nucleic acid complexes were 60−150 nm with a zeta potential ranging from 10 to 30 mV, and MW from $\sim 7 \times 10^7$ to 1×10^9 g/mol. For the purpose of cell-specific targeting, inclusion complexes were formed between AD-containing molecules and the β-CD molecules that ultimately facilitate modular attachment of PEG (AD-PEG) conjugates for steric stabilization and targeting ligands (AD-PEG-transferrin). Consequently, it was found that a 70-nm particle can possess ∼10,000 CDP polymer chains, ∼2000 siRNA molecules, ∼4000 AD-PEG5000 molecules, and ∼100 AD-PEG5000-Tf molecules. Thus from the study, it is evident that a high amount of payload or siRNA targeting toward a ligand could be achieved. As mentioned in previous literature, the particles protect the payload from nucleases and do not aggregate at physiological salt

concentration; they also reduced erythrocyte aggregation and complement fixation. Furthermore, from the uptake experiments, it was revealed that transferrin-targeted particles displayed enhanced uptake due to avidity effects. Moreover, the efficacy of pDNA/siRNA could be demonstrated by either delivering the luciferase-expressing gene or luciferase-targeting siRNA. Cells showed that CDP-imidazole particles carrying the plasmid and siRNA against luciferase conferred luciferase activity that was ~50% lower than cells that received CDP-imidazole particles with either the plasmid alone or the plasmid plus a control siRNA.

In a study, Davis *et al*., conducted a phase I trial where the patients with melanoma were treated with siRNA therapy that failed to respond to standard therapy. The siRNA used was directed against the M2 subunit of ribonucleotide reductase (RRM2). The nanoparticle doses were administered at different intervals: 1, 3, 8, and 10 of a 21-day cycle by a 30-min intravenous infusion. The nanoparticles were comprised of (1) a linear CDP, (2) a human transferrin protein targeting ligand on the exterior of the nanoparticle for interaction with the TFR on cancer cells, (3) a hydrophilic polymer PEG, and (4) and siRNA designed to reduce the expression of the RRM2 (Heidel *et al*., 2007a). The nanoparticle, i.e., CALAA-01, was found to be well tolerated in studies done with nonhuman primates. These nanoparticles (clinical version denoted as CALAA-01) have been shown to be well tolerated in multidosing studies in nonhuman primates (Heidel *et al*., 2007b). A biopsy sample from tumours of patients showed intracellular localization of nanoparticles that was found to be the same as that of the concentration of formulation administered. Further, it was observed that the messenger RNA (RRM2 and protein), most notably, the mRNA fragment from the patient's sample, denoted successful RNAi mechanism. Thus it was suggested that RNAi-mediated therapy can be successfully administered in humans for specific gene inhibition.

5.7 Poly(lactic-co-glycolic) acid

Among most of the available noncondensing vectors, poly(lactic-co-glycolic) acid (PLGA) has been considered to be one of the most potent candidates in the field of nucleic acid delivery. Earlier studies have shown that PLGA nanoparticles are efficiently taken up by the cells either specifically or nonspecifically through endocytic pathways (Panyam and Labhasetwar, 2003). It has been observed that the escape of nanoparticles from the lysosomal compartment depends on the property of surface charge reversal of PLGA nanoparticles in the acidic environment of lysosomes (Panyam and Labhasetwar, 2003; Panyam *et al*., 2002). Thereafter, the PLGA nanoparticles released in the cytoplasm have the ability to release their payload at a slow rate for more than 2 weeks (Panyam and Labhasetwar, 2004). Thus PLGA nanoparticles have gained tremendous attraction for gene silencing due to their beneficial properties such as efficient cellular uptake, rapid lysosomal escape, and sustained intracellular drug release. In addition, PLGA nanoparticles have proven to be highly biocompatible, nontoxic, and easy to synthesize as this degrades to physiologically amiable metabolites, i.e., lactic and glycolic acid. In general, PLGA are available in

different ranges of MW that are usually synthesized by ring-opening polymerization of cyclic dimers, i.e., lactide and glycolide, with the help of metal catalysts. This produces PLGA that are amorphous with glass transition temperature within 40−60°C.

5.7.1 Poly(lactic-co-glycolic)acid as small interfering ribonucleic acid carrier

The versatile nature of PLGA allows it to be tailored to produce various derivatives that can be efficiently used for delivery purposes. In accordance to this, the surface of PLGA has been modified by chitosan to obtain a positively charged surface for better adsorption of siRNA. However, it was observed that PLGA-chitosan nanoparticles formed were larger (400−1000 nm) in size and had higher PDI, which could be attributed to the MW and concentration of chitosan as well as type of PLGA. Thus on decreasing the MW of chitosan, the particle size of PLGA-chitosan decreased. Further, it was found that the siRNA binding capacity depended on several physical properties. For example, siRNA loading capacity was reported to increase with the use of a higher degree of "uncapped end groups". Also, the use of trehalose in the process of freeze drying reduces the chance of aggregation caused by freeze drying. In addition, a formulation could be successfully prepared to achieve sustained release of nucleic acids by modifying the degradation rate of PLGA (Katas *et al.*, 2009). Above all, it was shown that PLGA-chitosan formulations were nontoxic to the cells with more than 95% cell viability for the particles to siRNA weight ratio (100:1 to 500:1), by employing MTT assay.

The incorporation of chitosan has not only aided in increasing the binding efficiency and delivery of siRNA, but also has significantly increased bioavailability of PLGA nanospheres used in siRNA delivery (Tahara *et al.*, 2010). The siRNA-loaded nanospheres prepared by the solvent emulsion diffusion method had a size ∼300 nm with positive zeta potential, while the native PLGA nanospheres carried a negative charge. The siRNA-loaded PLGA nanospheres exhibited spherical, smooth, and homogenous surface distribution along with a high uptake of siRNA-loaded chitosan-modified PLGA nanospheres by the cells relative to native ones. Also, luciferase knockdown efficiency of modified PLGA was higher in A549 cells when compared to the native PLGA formulation and naked siRNA (Tahara *et al.*, 2010). The gene knockdown efficiency was comparable to that of uptake studies due to electrostatic interactions. In another study, it was found that with the use of 1% of polyvinyl alcohol (PVA) as an emulsifier, uniform and small-sized particles of chitosan-modified PLGA could be obtained (Yuan *et al.*, 2010). Further, it was observed that the size of PLGA nanoparticles increased from 204 to 543 nm with the increase in the concentration of chitosan in chitosan-PLGA nanoparticles. Evidently, in comparison to the native PLGA nanoparticle, *in vitro* gene silencing studies performed in HEK 293 cells showed maximal gene silencing (63.3 ± 5.6%) of GFP that was comparable to lipofectamine. In a study, Zeng *et al.* (2011) silenced the expression of hepatitis B virus (HBV) X and HBV S region for treating HBV infectious diseases by fabricating the chitosan-PLGA nanosphere in order to improve pDNA loading efficiency and cellular uptake. The formulation was prepared by a spontaneous emulsion diffusion method that obtained a particle size of ∼60 nm. Further, the formulation exhibited positive zeta potential while the native

PLGA nanoparticles possessed negative zeta potential that was in correlation to other studies using CS-PLGA NS. A significant reduction in the HBV-X mRNA and HBV-S mRNA levels were noticed with CS-PLGA NS in HepG2.2.15 cells that reduced to 0.39 ± 0.02-fold and 0.49 ± 0.01-fold compared with the control (Zeng *et al*., 2011). Consequently, the gene silencing efficiency of CS-PLGA NS was higher than native PLGA nanoparticles and the control.

In a similar line, the properties of PLGA could be improved by fabricating branched biodegradable polyesters by adding hydrophilic, positively charged amine groups to PVA that could be grafted with PLGA side chains (Nguyen *et al*., 2008). Small-sized siRNA-loaded nanoparticles were prepared using the solvent displacement method. The size of amine-modified-PVA-PLGA/siRNA nanoparticles were in the range of 150−200 nm with a zeta potential between +15 and +20 mV in PBS. The nanoparticles degradation started within 4 h in PBS. In order to determine the potential of nanoparticles as siRNA carriers, *in vitro* knockdown of the luciferase gene was deployed in a human lung epithelial cell line, H1299 luc. The nanoparticles were able to knock down 80−90% of gene expression with 5 pmol antiluc siRNA, even after nebulization.

Poloxamers have been employed in conjugation with PLGA for better delivery of plasmid DNA (Csaba *et al*., 2005, 2006). In one study, Luo *et al*. (2009) used siRNA-loaded PLGA: poloxamer nanoparticles against the sequence methyl-CpG binding domain protein 1 (MBD1) tested in pancreatic cancer BxPC-3 cells. The size of the nanoparticles with or without pDNA was in the range of 198−205 nm. Nanoparticles were able to release pDNA in two phases that were characterized by the rapid release (>30%) of DNA in Tris ethylenediaminetetraacetic acid buffer within first day, followed by slow release of the pDNA (>90%) in 11 days. It was revealed that MBD1 protein expression decreased within 2−5 days in the gene knockdown experiment. The number of apoptotic cells was higher in the transfected cells (24.19%) in comparison to control cells (4.79%).

To improve siRNA encapsulation in PLGA nanoparticles, a cationic polymer, PEI, was incorporated in the PLGA matrix (Patil and Panyam, 2009). This was formulated using the double emulsion-solvent evaporation method. Further, in the study, it was observed that various properties of PLGA-PEI (40 kDa) nanoparticles depended on the concentration of PEI. The hydrodynamic radius of PEI-PLGA nanoparticles increased effectively from 280 to 330 nm and 586 nm at PEI concentrations in the range of 0.5−500 μg/30 mg PLGA, respectively. In addition, the encapsulation of siRNA increased by two-fold in the presence of PEI and simultaneously also improved the siRNA release profile. The silencing effect of the siRNA-loaded PLGA-PEI nanoparticles was found to be effective in both the stably transfected cell line (EMT-6G/L) as well as in an inducible cell line (MDA-Kb2). Also, inhibition of luciferase expression was more effective with the formulated nanoparticles in comparison to the commercial transfecting agent DhamaFECT (Patil and Panyam, 2009). When MDA-Kb2 cells were stimulated by dexamethasone the luciferase expression reduced more than 70% that sustained for more than 3 days. The silencing efficiency of the nanoparticles was attributed to the effective cellular uptake of PEI in PLGA nanoparticles. The nanoparticles were stable in a serum-containing medium and conferred less cytotoxicity that further adds to its therapeutic potential.

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Nanoparticles for locked nucleic acid delivery

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6.1 Introduction

Antisense technology has gained considerable attention due to its applicability in the treatment and diagnosis of diseases. While a multitude of work has already been done, the main key objective is to find an efficient nucleic acid analogue for *in vivo* and *in vitro* delivery. Since 1995, intensified efforts have been dedicated toward the development of modified oligonucleotides with efficient therapeutic properties and minimal toxicity. Modified nucleotides are of three types, viz., analogues with unnatural bases, those with modified sugars (especially at 2′ position) and those with an altered phosphodiester backbone. Phosphorothioates (PS) have been one of the most widely used oligonucleotide analogues, which are categorized as first generation antisense (AS) molecules. The modification involves substitution of nonbridging oxygen in the phosphodiester linkage with a sulfur atom. This could cause some destabilization in the base pairing, but at the same time, it imparts resistance against nuclease digestion. The polyanionic backbone renders interaction with proteins that contribute in an increase in their half-life in biological fluids, with a concomitant role in cellular toxicity (Eckstein, 2000). The second generation oligonucleotide molecules mainly involve nucleotides with an alkyl modification at 2′ position of ribose, such as 2′-O-methyl and 2′-O-methoxy ethyl RNA. These are generally nontoxic and imbibe the property of binding with a target sequence with a greater affinity and meditative antisense effects (Sierakowska *et al*., 1996). The third generation of modified oligonucleotides comprised of morpholino oligonucleotides, N3′-P5′-phosphoroamides (NPs), 2′-deoxy, 2′-fluoro-β-D-arabino (FANA) nucleic acid, and peptide nucleic acids. Among these, NPs display high binding affinity and exhibit nuclease resistance toward a complementary RNA strand (Gryaznov and Chen, 1994). These NPs also exhibited their antisense effects by steric blockade of translation analogous to 2′-O-methyl RNA. Indeed, the NPs exhibited properties superior to PS in downregulating the expression of the c-myc gene (Skorski *et al*., 1997). Morpholino oligonucleotides (MF) have morpholino moieties and they do not activate RNase H, but block translation by preventing ribosome binding. MFs could circumvent unwanted interaction due to their uncharged backbone. On the other hand, FANA, a 2′ epimer of RNA, is known to induce RNase H cleavage of the bound RNA molecule (Damha *et al*., 1998). The peptide nucleic acids (PNAs) are another type of modified DNA analogue, which possesses a peptide backbone consisting of *N*-(2-aminoethyl)-glycine unit that make it uncharged and achiral in nature. In accordance with the uncharged backbone, these PNAs exhibit poor

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water solubility. However, they can form sequence specific bonds with RNA and DNA. In addition, they can form chemically stable structures and display higher thermal stability. Although all the approaches were implemented for attaining a better therapeutic agent, there still awaits the development of efficient methods for its delivery into the cells (Elayadi and Corey, 2001; Nielsen, 2000).

6.2 Locked nucleic acids: structure

Locked nucleic acids (LNA) have been observed to display high stability, low toxicity and good aqueous solubility in *in vivo* systems. LNA is a class of nucleic acid analogues that structurally resembles RNA molecules which follow Watson-Crick base pairing rules as well as form duplexes with complementary DNA and RNA with profoundly high thermostability and improved selectivity. This property of LNA is attributed to its locked conformation introduced by O2′, C4′ methylene linkage in the furanose ring of the ribose sugar (Fig 6.1). Although several structural analogues of LNA have been synthesized that exhibited high binding affinity and efficiency to RNA, β-D-LNAs have been known to be superior of all diastereomeric forms. In addition, α-L-LNA also exhibited high thermostability and contributed in establishing a molecule with remarkable binding affinity and specificity in forming unique nucleic acid mimics (Fluiter *et al*., 2005; Rajwanshi *et al*., 2000).

6.3 Locked nucleic acids: hybridization and conformation properties

Hybridization properties of various oligonucleotides (novel abasic LNA monomer, unmodified abasic DNA monomer and thymine LNA monomer) were investigated to prove enhanced stability of LNA monomers. It was elucidated that the thymine LNA monomer exhibited more duplex stability and thermal stability, which hitherto has been found to be +8 to +5°C for deoxy LNA and +11 to +5°C for ribose LNA. In contrast, undesirable results were obtained for abasic LNA monomers and

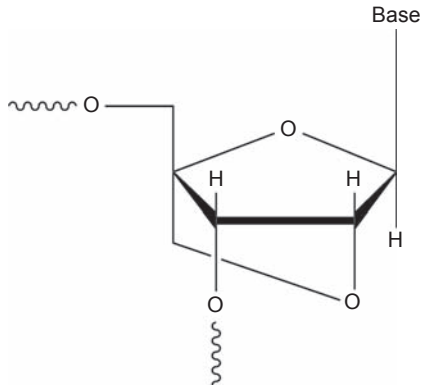

Figure 6.1 Chemical structure of locked nucleic acid.

abasic DNA monomers. The possible explanation for the duplex stability was its probable nucleobase stacking and oxymethylene bridge link between 2′ and 4′ carbon atoms of furanose ring. In addition, nucleobase is also believed to play a crucial role in maintaining the LNA stability. However, in particular, LNA-DNA and LNA-RNA hybrids showed an increasing A-like character with an increase in LNA content of the LNA strand (Kværnø and Wengel, 1999). In order to understand these remarkable structural properties, studies were conducted using NMR spectroscopy which determined the high resolution structures of LNA: RNA hybrids relative to the reference unmodified DNA: RNA hybrids. It was proposed that the progressive change of the duplex toward A-type duplex geometry is not solely due to the C3′-endo locked LNA nucleotides but rather due to the 2′-oxygen of LNA that alters the charge distribution in the minor groove, which ultimately propels the conformational shift of its 3′- neighbor, that shifts the equilibrium toward N-type conformation (Petersen *et al.*, 2002). Similarly, in another study, stability of LNA: DNA duplexes containing four LNAs were shown, in which it was observed that the stability properties of the LNA: DNA duplex increased with an increase in the number of modifications and that it saturated after a while with increasing modifications (Nielsen *et al.*, 2000). Recently, another stereoisomer analogue of LNA, viz., α-L-LNA, was used to investigate its thermal stability toward duplex formation. It was observed that high affinity binding comparable to that of LNA was seen for fully modified α-L-LNA toward DNA. However, both mixmer α-L-LNA and completely modified α-L-LNA exhibited considerable duplex formation with RNA. α-L-LNA base pairing is strong corresponding to LNA:LNA binding affinity. Interestingly, it was also observed that these oligonucleotides also propelled RNase H activity when bound to RNA. Similar studies were also conducted with 9- and -10 mer α-L-LNA/DNA:DNA duplexes which adopted s-type sugar puckers and retained B-type helix features in contrast to LNA/DNA:DNA duplexes. The same α-L-LNA/DNA chimera displayed helical geometry intermediate between A- and B-type helixes which remained very similar to the native DNA:RNA helix. Therefore an α-L-LNA confers substantial elevation in duplex stability (Nielsen *et al.*, 2002, 2003).

6.4 Serum stability and nuclease resistance

LNA oligonucleotides are also known to have better potential for therapeutic applications, which implies that they should be resistant to the nucleases in the media or serum. In a study, LNA containing oligothymidylate stability was tested by treating the oligonucleotides with 3′-exonucleases (snake venom phosphodiesterase). A significant modification at the 3′ end of the oligonucleotide with three LNAs showed greater stability relative to the completely modified oligonucleotide. Also, α-L-LNA exhibited a considerable stability against both endo- and 3′- exonucleases (Frieden *et al.*, 2003b). When studying the effects of human serum on chimeric LNA/DNA oligonucleotides, it was found that the oligonucleotides exhibited a considerable increase in the half-life from 1.5 to 15 h in the presence of three LNAs at 3′ and 5′ ends, and the further addition of LNA did not show any significant changes in its stability. In addition, it was also seen that a stretch of seven or eight DNA monomers in chimeric LNA/DNA

oligonucleotides were sufficient to trigger RNase H cleavage of the target RNA. In another study, it was noticed that LNA/DNA mixmers were much more stable relative to LNA/DNA/LNA gapmers and phosphorothioates. In addition, it was also reported that the LNA-containing oligonucleotides displayed enhanced stability against nucleases in the rat brain as well as against *Escherichia coli* cell lysates (Wahlestedt *et al.*, 2000). LNA has also been used in designing decoy agents for transcription factor κB. This approach involved modification of 5′ and 3′ terminal bases of double-stranded oligonucleotides containing NF-κB consensus binding sequence that provided maximum resistance to DNase 1 endonuclease degradation. Whereas in the case of resistance toward exonuclease digestion, two LNA bases per strand modification were required as demonstrated in their particular experimental conditions (Crinelli *et al.*, 2002). Thus it becomes a prerequisite to develop modified oligonucleotides that are potently more resistant to nucleases. In this regard, LNAs are widely favored due to their high biological stability and low toxicity. On investigating its stability against S1 endonuclease, it was indicated that fully modified LNA and fully modified α-L-LNA imparted high stability against S1 endonuclease digestion, whereas the presence of DNA gap made it more prone to cleavage (Frieden *et al.*, 2003a).

Generally, polymerases also exhibit nuclease activity, to some extent. The effect of these polymerases was studied during primer extension with few LNA monomers near or at the 3′ end. LNA nucleotides significantly lowered the degradation by 3′−5′ proofreading exonuclease activity of Pfu and vent, while the activity of DNA polymerase and of Klenow fragment was not hindered. In opposition, it was observed that a single LNA nucleotide in the penultimate position supports complete resistance to all the nucleases tested in this study (Di Giusto and King, 2004).

6.5 Delivery and toxicity

To qualify as an efficient therapeutic tool, delivery becomes especially important for LNA, provided they enter through the cell membrane barrier and interact with the target intracellular site. Unlike PNAs and morpholinos, LNAs possess a charged backbone which allows lucid transfection of the oligonucleotides by routinely used conventional methods. Among the commercially available delivery vectors, namely FuGENE, lipofectin, lipofectamine 2000 and polyfect (Jepsen *et al.*, 2004; Obika *et al.*, 1998), lipofectamine 2000 proved to be the most efficient in the delivery of oligonucleotides such as PS, LNA/PS gapmers, or fully modified LNA oligonucleotides in breast cancer cell line (MCF-7). Phosphorothioate oligonucleotides were uniformly distributed, whereas LNAs were localized particularly in nucleoli, when delivered by lipofectamine. Studies conducted with other delivery agents, such as lipofectamine, effectin on HeLa cell lines and DU145E prostate cancer cells, suggested that LNA localized in the cytoplasm as well as the nucleus, whereas LNA got exclusively accumulated in CV-1 monkey kidney cells (Braasch *et al.*, 2002; Elayadi *et al.*, 2002). Toxicity evaluation studies suggested that LNA chimeras and fully modified LNA oligonucleotides were well tolerated up to an optimum dose (Arzumanov *et al.*, 2001; Fluiter *et al.*, 2003). Thus, in comparison to the toxic profile of phosphorothioates which resulted in damage

to the rat brain, LNAs were well tolerated as their DNA counterparts (Obika *et al.*, 1998). The low toxic profile of LNAs could be attributed to its property of high affinity and specificity. In addition, the LNA-based antisense oligonucleotides could be reduced to a shorter length which can mediate desired biological effect by decreasing the degree of binding to serum proteins (Arzumanov *et al.*, 2001; Fluiter *et al.*, 2003).

6.6 Therapeutic applications of locked nucleic acids

6.6.1 Locked nucleic acids as microRNA inhibitors

MicroRNAs (miRNAs) are short, noncoding RNAs that play a crucial role in the development, differentiation and metabolism by posttranscriptional regulation of gene expression by interaction with the 3′ untranslated region (UTR) of target mRNAs. These miRNAs are basically synthesized as long primary transcripts which are then further processed by RNase 3 (drosha and dicer) digestion into 21−23 mature miRNA nucleotides. miRNAs are present in a wide range of organisms ranging from nematodes to plants and humans, and its action of mechanism varies with the type of organism. Most of the miRNAs belonging to plant species form perfect complementarity with the target sites and propel RNA-induced silencing complex (RISC)-mediated mRNA cleavage, while most of the animal miRNAs form incomplete base pairing with their target sites at 3′ UTRs that ultimately leads to translational repression of the target genes (Bagga *et al.*, 2005; Bartel, 2004; Lim *et al.*, 2005; Nakahara and Carthew, 2004). miRNAs also exhibit certain other biological functions, such as apoptosis, fat metabolism, neuronal asymmetry and brain morphogenesis. However, it is now well documented that miRNA has a pivotal role in diseases, such as cancer, as noticed in most of the reproductive cancers where miRNA expression is deregulated. In addition, the role of miRNA was found to be positive in the case of esophagus, gastrointestinal, lung, and bladder cancer (Naeini and Ardekani, 2009). It has also been observed that the deregulated expression of miRNA-1 and miRNA-133 caused human heart failure (Care *et al.*, 2007; Ikeda *et al.*, 2007; Yang *et al.*, 2007). In addition, several inflammatory disorders were investigated for having being controlled by certain miRNA expression, as seen in rheumatoid arthritis and systemic lupus erythematosus (Dai *et al.*, 2007; Tili *et al.*, 2008). Therefore it has been a challenge to establish and understand miRNA function and its biological process, and it calls for robust and improved technologies for miRNA detection and functional studies.

LNA has been gaining attraction for its implication in designing probes specific for miRNA detection in northern blots. Further, it has also been reported that these miRNAs are tenfold more sensitive than the DNA probes. Apart from being an efficient tool for northern blot, it has also been used for in situ localization of miRNA in cells and tissues (Válóczi *et al.*, 2004; Wienholds *et al.*, 2005). The use of LNA-modified oligonucleotides for miRNA inhibition in cultured cells was first demonstrated by Ørom *et al.*, in which the efficacy and specificity LNA oligonucleotides were shown by well-characterized interaction between *Drosophila melanogaster bantum* miRNA and its target gene hid as a model (Ørom *et al.*, 2006). Thereafter, several studies were done to elucidate the functional properties of LNA oligonucleotides for their

application in therapeutics. In one of the studies, phosphorothioate-modified LNA-inhibitor-miR-221 displayed anti-multiple myeloma (MM) activity where it exerted strong antagonistic activity against miR-221 and caused upregulation of p27Kip1. LNA proved to be a capable agent against MM cells and suitable for clinical use. The simple systemic delivery of LNA-antimir-122 displayed an antagonizing effect on liver-expressed miR-122 in nonhuman primates. Investigations on African green monkeys revealed downregulation of miR-122 in the liver, without any signs of toxicities or other histopathological effects and further proved to be effective and long lasting. The same group also probed into the potential use of this miR-122 as an anti-hepatitis C virus agent, in which they found a significant decline in the miR-122 expression levels (Elmén *et al*., 2008; Lanford *et al*., 2010).

6.6.2 Locked nucleic acids in short interfering RNA stability

The targeted silencing of disease-associated genes has now become a robust method of choice for the manipulation of gene expression in mammalian cells (Dorsett and Tuschl, 2004). This gene silencing, in particular, is achieved by an RNA interference (RNAi) technique that counts in the potential use of short interfering RNA (siRNA). Typically, 21-bp siRNA forms perfect complementarity with the target RNA and triggers RNAi by sequence specific degradation of mRNA when bound to the RISC by its complementary siRNA strand (Meister and Tuschl, 2004; Sontheimer, 2005). It has been demonstrated that synthetic siRNA can stimulate the human innate system, resulting in the release of inflammatory cytokines and interferons (Bridge *et al*., 2003; Kim *et al*., 2004; Sledz *et al*., 2003). This can be abrogated by the application of chemical modification in siRNA. Among all the chemical modifications, LNA modifications have proved to be a potent nucleotide analogue that exhibits enhanced thermal and serum stability. One of the studies has depicted that LNA thermally stabilizes the siRNA without affecting its function (Braasch *et al*., 2003). Further, in a systematic study, it has been reported that the functional activity of siRNA depends on the location and number of LNA incorporated. Moreover, it was observed that on incorporation of LNA, the serum stability of siRNA was enhanced, which possibly favors successful *in vivo* application (Elmén *et al*., 2005; Fluiter *et al*., 2003). Further, in another study, the efficacy of LNA-modified siRNA was tested by designing end-modified siRNA and heavily modified siRNA against green fluorescent protein (GFP) *in vivo* and *in vitro*. On comparison with the unmodified siRNA, it was observed that the end-modified siRNA displayed satisfactory serum stability and silencing efficacy, while heavily modified siRNA showed comparable serum stability accompanied with lower knockdown efficiency *in vivo*. It was also observed that the end-modified LNA showed potential off-target effects. These results depict that end-modified siRNA holds promise for *in vivo* applications (Mook *et al*., 2007).

6.6.3 Locked nucleic acid antisense

An antisense oligonucleotide could be described as a short chain of synthetic deoxyribonucleotide that can form complementary base pairing with the target mRNA. The

RNA−DNA duplex formed acts as a substrate for cellular RNase H that ultimately degrades the mRNA, thus forming a blockade in the transfer of genetic information (Chan *et al*., 2006). In addition, these have also been used in studies related to the loss of gene function and as an approach for the treatment of disease linked to dysregulated gene expression. They have also been known to play a pivotal role in correcting splicing defects (Sazani and Kole, 2003). Upon hybridization, the RNA−DNA hybrid either triggers the activity of RNase H or the steric hindrance of ribosomal subunit binding. The RNase-H-dependent mechanism results in the degradation of the target mRNA and ultimately target protein knockout, whereas steric blocker oligonucleotides prevent splicing only when targeted to 5′ or AUG (start codon) initiation codon (Dias and Stein, 2002). Other mechanisms include modulation of mRNA maturation, splicing activation and 5′ cap formation (Crooke, 1999). From earlier studies it is evident that LNA oligonucleotides are known to have unprecedented high binding affinity, resulting in a stable, nontoxic, and potent antisense oligonucleotide that is capable of recruiting RNase H. These properties enable them to become a promising molecule for the development of oligonucleotide-based therapeutics for gene silencing (Fluiter *et al*., 2003; Wahlestedt *et al*., 2000).

In a study, various LNA oligonucleotides were synthesized that comprised of mismatch as well as fully complementary bases of different lengths to test the inhibition of telomerase, among which the fully complementary LNA−DNA one chimera inhibited telomerase with an IC_{50} value of 10 nM. In accordance with it, a fully substituted LNA oligomer also contributed in the inhibition of telomerase with an IC_{50} of 10 nM. Also, a short eight-base LNA was observed to possess 200-fold better inhibition capability than PNA molecules with no obvious toxicity to cells (Elayadi *et al*., 2002). Similarly, in another antisense study, LNA gapmers showed potent downregulation of endogenous proteins (cyclin-dependent kinase inhibitor p21 and estrogen receptor-α) in human breast cancer cell line MCF-7, in comparison to phosphorothioates oligonucleotides, whereas fully modified LNA antisense oligonucleotides or LNA mixmers were found to be inactive (Jepsen *et al*., 2004). Antisense LNA oligonucleotides have also been used in targeting the superoxide dismutase (*SOD1*) gene in the choroid plexus of rats, and gene expression was inhibited via albumin- antisense oligonucleotide (ASO) conjugates. Also, in a study done by Emmrich *et al*., it was shown that the LNA-antisense oligonucleotides selectively downregulated the truncated splice isoforms of p73 collectively called DNp73 that are known to act as oncogenes. Three different ASOs (ASO-115, 116, and 185/451) were prepared and targeted against the splice forms ΔEx2, ΔEx2/3, or ΔN′ for its selective suppression, and further it was observed that the strongest antisense effect was obtained after six hours and was involved in the selective knockdown of the target variants, resulting in no or low knockdown of the other p73 isoforms. This ASO has also displayed certain modulation in the isoform expression, resulting in the inhibition of tumor growth *in vivo* accompanied by induction of apoptotic Tap73 (Emmrich *et al*., 2009). In another study related to hepatocellular carcinoma (HCC), β-catenin suppression was performed using LNA antisense treatment after establishing *ctnnb-1* mutation, and it was observed that it resulted in abrogation of HCC after 10 treatments in tumor-bearing mice. Thus it

provides proof of the concept that β-catenin suppression in HCC could be of significant therapeutic benefit (Delgado *et al*., 2015). In another similar kind of study, it was demonstrated that modulation of the expression of *myc* by LNA oligonucleotide proved to be a promising agent for the treatment of various disorders which mainly involves hyperproliferative disorders such as cancer (43).

Antisense drugs have also been investigated in a variety of diseases, which includes hypercholesterolemia and cardiovascular diseases. The first evidence for the efficacy of LNA ASO against PCSK9, responsible for familial hypercholesterolemia and cardiovascular disease, was demonstrated by us, in which the LNA−ASO efficiently reduced the mRNA and protein levels of PCSK9 with a significant increase in low density lipoprotein receptor (LDLR) levels in HepG2, HuH7, and β-TC3 cell lines (Fig. 6.2; Gupta *et al*., 2010). On further investigation in mice, it was observed that the level of PCSK9 was decreased by 60% and lasted for more than 16 days. In

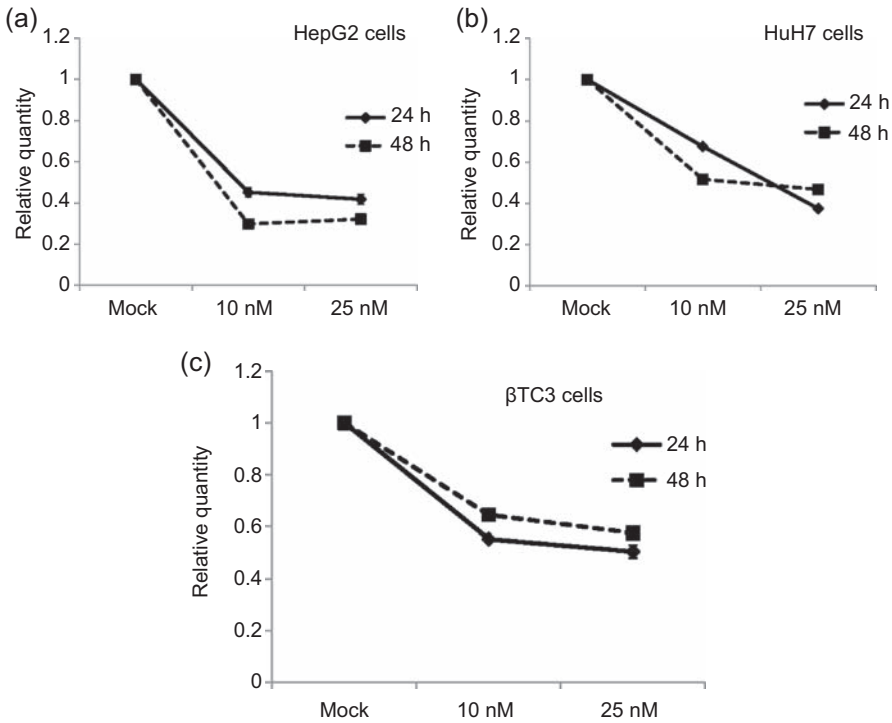

Figure 6.2 Intracellular reduction of targeted PCSK9 mRNA by locked nucleic acid antisense oligonucleotide. Two human hepatic cell lines, HepG2 and HuH7, and a mouse insulinoma b-TC3 cell line were transfected with locked nucleic acid antisense oligonucleotide at concentrations of 10 and 25 nM. Total RNA was extracted at two different time points, 24 and 48 h posttransfection, and quantitative polymerase chain reaction analysis was performed using specific primers. The levels of PCSK9 mRNA were normalized to S14 mRNA for (a) HepG2 and (b) HuH7 cells and (c) with S16 mRNA for b-TC3 cells.
Reproduced from Gupta *et al*. A locked nucleic acid antisense oligonucleotide silences PCSK9 and enhances LDLR expression *in vitro* and *in vivo*. PLoS One 2010;5.

addition, the LDLR levels were upregulated by 2.5−3 folds for eight days and two folds for a period of 16 days. Finally, upon measuring the liver alanine aminotransferase, it was further confirmed that LNA−ASO did not cause any toxicity (Gupta *et al*., 2010). Similarly, in another study, reduction in low density lipoprotein cholesterol (LDLC) was displayed upon targeting PCSK9 in nonhuman primates with a loading dose of 20 mg/kg and four weekly maintenance doses of 5 mg/kg. This resulted in the reduction of PCSK9 level by 85% and 50% reduction in LDLC levels with no observed effects on toxicological parameters. Therefore with this pharmacological evidence, it can be indicated that LNA−ASO targeting PCSK9 could be used as a viable therapeutic strategy (Lindholm *et al*., 2012).

6.6.4 Locked nucleic acid-modified DNAzymes

DNAzymes are the DNA molecules that possess catalytic action and are capable of cleaving almost any RNA substrate, efficiently and specifically under physiological conditions. The 10-23 DNA enzymes could be applied for the manipulation and inactivation of the RNA targets similar to that of antisense oligodeoxynucleotides. DNAzymes possess the property of both recognition as well as cleavage of the targets and operate with catalytic turnover (Santoro and Joyce, 1997). Due to their high flexibility and excellent catalytic efficiency, these DNAzymes contribute their role in several therapeutic applications, including the treatment of cancer, by targeting vascular endothelial growth factor receptor 2 as well as targeting promyelocytic leukemia gene/retinoic acid receptor a fusion gene, and also in brain disorders such as Alzheimer's disease, Huntington disease, Parkinson's disease, and in muscular disorders. However, DNAzymes are also prone to nucleolytic degradation in physiological conditions, which calls for the modification of the DNAzymes using LNA that ultimately increases its melting temperature and enhances its binding affinity. Therefore these LNA-modified DNAzymes prove to be more stable than the unmodified DNAzymes (Kurreck, 2003; Vester *et al*., 2002). Similarly, in another study, it was shown that the incorporation of two α-LNA monomers per binding arm increased the overall cleavage reaction time 50 times faster than usual, which also depended on the length of the binding arms and number of modified LNA monomers (Vester *et al*., 2006). LNA-modified DNAzymes are also used as an antiviral agent by targeting the 5′ noncoding region to block reverse transcription and dimerization of the HIV-1 RNA template, resulting in the suppression of HIV replication in the cell culture system.

6.6.5 Locked nucleic acid in diagnostics

In accordance with the studies mentioned in previous sections, it is evident that LNA with higher binding affinity and sequence specificity could also have applications in the field of diagnostics. It can be utilized as a probe for different hybridization assays including single nucleotide polymorphism (SNP) genotyping and so forth. SNPs are usually the DNA sequence variations taking place when a single nucleotide in the genome differs between members of a species. These SNPs can be seen at the coding or noncoding regions of the genome that may have regulatory effects. Alterations in

the coding region by these SNPs can cause several genetic disorders or other ailments. Therefore a better approach could be a well-designed LNA technology with high discrimination power to replace the time-consuming conventional methods for SNP screening. LNA, with its remarkable inherent property, could make it an ideal tool for hybridization-based genotyping assay (Jacobsen *et al*., 2002; Ørum *et al*., 1999). One of the studies employed the use of allele-specific polymerase chain reaction (PCR) (AS-PCR) for SNP genotyping, in which incorporation of LNA at the 3′ position of allele-specific PCR primers significantly increased its specificity and reliability. The results were further confirmed by applying this method on plasmid and human genomic DNA that exhibited a profound discriminating ability even under broad PCR conditions (Latorra *et al*., 2003). Similarly, this approach was also implemented to determine the accurate and sensitive quantification of the methylation level of cytosine within a complex genome by employing methylation specific real-time PCR with SYBR green (dye). The presence of LNA at the 3′ end of the primers exhibited an efficient discrimination power between the methylation states of cytosine which presented its sensitivity at a level as low as 1% of the overall population (Thomassin *et al*., 2004). This remarkable property of the LNA probes has also been put to good use in enzyme linked immunosorbent assay-like assays where the LNA capture probes detect SNPs in PCR amplicons from human genomic DNA, for example, factor V laden mutation, apolipoprotein B (apo B) R3500Q mutation, a two-mutation apolipoprotein-E, and so forth (Jacobsen *et al*., 2002; Ørum *et al*., 1999). In addition to solid phase SNP genotyping, LNA probes have also been widely used in homogenous (liquid) hybridization for SNP genotyping. On conjugating the LNA probe to fluorescent dye such as rhodamine/hexachlorofluores, the fluorescence polarization value showed a significant increase upon hybridization of the probe to the target molecule. Similarly, a homogenous SNP genotyping assay was also used to screen co-segregating variants of toll-like receptor-4 (Grannemann *et al*., 2005; Simeonov and Nikiforov, 2002). LNA therefore has shown its remarkable application in the field of diagnostics, outperforming the work of DNA probes.

6.7 Nanoparticle-mediated delivery of locked nucleic acid and locked nucleic acid-modified oligonucleotides

6.7.1 Gold nanoparticles

The aforementioned facts clearly depicts the potential of LNA in diagnostics and therapeutics due to its capability to successfully penetrate into the cells and interact with the target site. The charged backbone of LNA allows appropriate delivery into the cells. The conventional methods involve both carrier and noncarrier systems for efficient delivery. It was observed that noncarrier systems such as scrape loading and electroporation damaged cells (for example, MCF-7 cell line), while the carrier systems such as FuGENE 6, lipofectin, lipofectamine 2000, and polyfect proved to be an efficient delivery system. Although these chemical transfection methods have

been dominating in gene and drug delivery research, the advent of nanoparticles has been gaining considerable interest as these are easy to prepare, less immunogenic, and less cytotoxic. For example, among a multitude of research conducted on LNA, Dwight S. *et al*. were the first to report preparation and application of LNA nanoparticles (NP) conjugates (LNPs). To date, studies have mainly described the implication of deoxyribonucleotides as a functional element in the preparation of nucleic acid-NP conjugates. However, it was revealed that LNAs are ideal for engineering LNPs as they are commercially available, have suitable binding properties, could display stability in aqueous media, and have highly tunable and enhanced recognition properties. Therefore the preparation, characterization, and the applications of LNA-NP conjugates were studied. Those NP conjugates that were densely loaded with LNA produced remarkably stable duplexes compared with nucleic acids. The LNPs were synthesized using thiol-terminated oligonucleotides in a solution possessing 13 d 1 nm Au NPs. Four LNA modifications were incorporated in the ends of a recognition sequence for higher affinity. The loading efficiency of LNPs were comparable with that of DNA-modified NP analogues (DNPs) which were used for comparison purposes to highlight the novel properties of LNA conjugates. The number of attached LNA oligonucleotides was determined by fluorescence methods, which revealed that LNPs were functionalized with 205 ± 18 LNA nucleotides. This had further enhanced its stability in various media, including deionized water, highly concentrated solutions, and biological solutions such as serum containing cell culture media. Fluorescence-based melting experiments were utilized to determine the binding properties of LNPs, where the fluorescein labeled sequence was made to hybridize with the NP conjugates so that the fluorescence got quenched when in close proximity with the Au NP surface. LNP-complementary oligonucleotide complexes showed melting transition (T_m) at $84 \pm 18°C$, which denotes its property of enhanced stable complex formation. LNPs were also evaluated *in vitro* to test their ability to downregulate the expression of the gene survivin (a member of inhibitors of apoptosis gene family) in lung carcinoma cell line A549. The cellular entry of LNPs did not require any additional transfection agents. With western blot analysis, it was observed that LNPs significantly decreased the expression of survivin protein levels ($50 \pm 8\%$), while DNPs displayed a $31 \pm 4\%$ decrease in survivin levels relative to the controls. In addition to the reduction in protein levels, changes in the cell morphology of the culture cells were observed for those treated particularly with antisurvivin LNPs, which was clearly depicted by dark and detaching cells, whereas such effect was not seen in cells treated with "nonsense" LNPs or antisurvivin DNPs. Therefore these NPs functionalized with LNA oligonucleotide could contribute in a variety of diagnostic and therapeutic applications (Seferos *et al*., 2007). In its extension, Prigodich *et al*. developed an ideal theranostic agent (oligonucleotide gold nanoconjugate) that is capable of regulating and detecting the intracellular gene expression. This sort of study was possible earlier only by either using antisense nucleotides or molecular beacons which played their role in regulating and detecting mRNA, respectively. In the process of this development of theranostic agents, nanoconjugates containing fluorophore-labeled "flare" oligonucleotides were designed to dissociate upon target binding. The nanoconjugate recognition sequence possessed

DNA−LNA chimera, where these LNA bases served to increase mRNA binding affinity, thereby increasing its detection and regulation efficiency. Nanoconjugates were prepared using citrate-capped gold nanoparticles and thiol-terminated antisense oligonucleotides with a recognition sequence such as DNA−LNA chimera targeting against survivin, which is a well-established gene for cancer treatment and diagnosis. After synthesizing the nanoconjugates, various parameters were monitored which involved its response to the targets, rate of flare release, and specificity. It was observed that in a solution (phosphate buffer saline) containing synthetic target sequence, the nanoconjugates exhibited a 3.7-fold increase in fluorescence signal when compared to control nanoconjugates. The rate of flare release was also monitored by adding an excess of the target to the solution, where the fluorescence increased rapidly. Further, these nanoconjugates were subjected to experiments to characterize specificity, where they were challenged with a series of targets containing four, three, two, or one mismatches in which it was found that those carrying more than one mismatch displayed low fluorescence. Targets with one mismatched base pair still showed a considerable fluorescence response. However, the signal was easily distinguishable from the fully complementary target, which demonstrated that these novel nanoflares could be implemented in the detection of targets even with single base pair mismatch. Indeed, an *in vitro* study with HeLa cells depicted 1.7 ± 0.1 times increase in fluorescence in cells treated with high concentrations of survivin nanoconjugates when compared with control nanoconjugates. Therefore these nanoconjugates hold a promising step toward the development of mRNA-directed theranostics with the combined advantage of gene therapy (Prigodich *et al.*, 2009). Similarly, Riahi *et al.* demonstrated the ability of gold-nanorod-LNA (GNR) complex for characterizing the spatiotemporal gene expression in viable cells and tissues along with induction of photothermal ablation of single cells. They characterized the GNR−LNA complexes for intracellular detection in human breast adenocarcinoma cells and mice tissues. The spatial cellular heat shock responses were examined by measuring the intensity distributions of the HSP70 probes in human cells and mice lung tissues. It was revealed that with longer irradiation (15−30 min), the cell response was upregulated in 100−300 μm away from the laser beam and was downregulated in the zone of the laser. It was later confirmed that the temperature elevation at other regions due to the heat source did not induce the release of probes from the GNRs, and similar types of gene expression profiles were seen in MCF-7 cell cultures, which confirms that the temperature elevation caused at different distances from the heat source does not induce the nonspecific release of probes from GNRs present inside the cells. Further, on evaluation of the cells exposed to prolonged irradiation, it was observed that, with short duration of laser exposure (one minute or less), cell ablation was seen (25 μm). With an increase in the duration, the region of apoptotic cells expanded. In addition, it was observed that the HSP70 expression increased with both irradiation time and temperature in a nonlinear manner. Therefore this data illustrates the utility of studying spatiotemporal dynamics of the cells and tissues during photothermal operation, using plasmonic nanostructures (Riahi *et al.*, 2014). In another study, Dong H *et al.* demonstrated the synthesis of a simple nanocarrier of polyethylenimine-grafted graphene nanoribbon (PEI-g-GNR) with loaded locked nucleic acid modified molecular beacon (LNA-m-MB) probes. The GNR was prepared by longitudinally unzipping the

carbon nanotubes and treatment with strong acids followed by sonication to attain the surface of carboxylic acid groups for the graft of PEI via electrostatic assembly. Herein, the PEI-g-GNR appeared to protect LNA-m-MB from nuclease digestion or single-strand binding-protein interaction. It showed better transfection properties with low cytotoxicity and apoptosis under optimum conditions. With the remarkable affinity and binding properties of LNA, a method for the successful detection of mRNA was developed using HeLa cells. These results further suggested that PEI-g-GNR could act as a potent gene delivery vehicle for in situ detection of gene and clinical applications (Dong *et al.*, 2011). In tumor cells, oncogenic miRNAs, also known as oncomirs, are reported to be linked to the process which directly determines cancer initiation, progression, and response to therapy. Therefore a nanocarrier is a prerequisite for the delivery of oligonucleotide antagonists into the cell to target miRNAs.

6.7.2 Lipid and polymer-based delivery vectors

In this regard, Pedro costa *et al.* developed a lipid-based nanocarrier for the targeted delivery to glioblastoma (GBM), where LNA-modified ASOs or siRNAs were encapsulated into the lipid formulation with covalently coupled chlorotoxin (CTX), a peptide known to bind selectively to the glioma cells. This resulted in the formation of stable nucleic acid-coupled nanoparticles (SNALPS) that displayed excellent features for both *in vitro* and *in vivo* applications with a small size and neutral surface charge. The prepared CTX-coupled SNALPS were subjected to physicochemical characterization before implementing it to *in vitro* and *in vivo* studies. Upon evaluation of cellular association and cellular internalization of SNALPS, it was observed that extensive association was established within four hours after exposure of U87 cells to 1 μmol/L of oligonucleotides encapsulated in targeted SNALPS when compared to nontargeted liposome-encapsulating oligonucleotides. Further, it was demonstrated that cellular association of the CTX-coupled SNALPS was mediated by specific interaction with the cellular receptors. In addition, it was proved that CTX-labeled SNALPS specifically targeted the cancer cells which were determined by a decrease in the extent of association with nonmalignant human embryonic kidney cell line, HEK293T, when compared with that determined in U87 cell lines. Confocal microscopy data revealed that rhodamine-labeled CTX-coupled liposomes encapsulating fluorescein amidite-labeled anti-miR-21 oligonucleotides exhibited intensive red (lipid) and moderate green (oligonucleotide) fluorescence inside the cell cytoplasm. Moreover, this also resulted in nanoparticle-mediated miR-21 silencing in U87 human GBM and GL261 mouse glioma cells. This further showed an increase in levels of tumor suppressors, PTEN and PDCD4, and activation of caspase 3/7. A significant decrease in mRNA levels of survivin were also observed in cells exposed to CTX-coupled liposomes encapsulating antisurvivin siRNAs, which clearly provided evidence that targeted nanoparticles can efficiently deliver LNA-modified ASOs as well as siRNAs to GBM/glioma cells (McKenzie *et al.*, 2007).

New nanotools were also generated using fluorescent semiconductor nanocrystal quantum dots (QDs), in which lipid oligonucleotide (ONs) conjugates (LONs) targeting microRNAs were used to functionalize the QDs. The oligonucleotide-based nanoplatform was established by implementing three steps. At first, LONs were prepared

using two different approaches which involved 1, 3-dipolar cycloaddition click and phosphoramidite reactions. For this kind of study, two ON sequences were studied. The first model possessed DNA to avoid self-complementary strands, and the second sequence possessed 23-mer LNA/DNA sequence antisense oligonucleotide directed against the microRNA miR-21. In the second step, QDs possessing a hydrophobic shell (trioctyl phosphine oxide) were used in encapsulating and solubilizing in water by a layer of amphiphiles. This approach leads to the formation of micelle and preserved optical properties. Natural phospholipids (DOPC), as well as synthetic phospholipids such as nucleolipids (PUPC and PUOH), were used for encapsulation, and it was observed that the latter leads to a more compact layer formation relative to the former. The hydrodynamic size of the selected QDs was roughly around 6.5 nm with a fluorescence emission peak at 620 nm with 80% quantum yield. Finally the LONs were conjugated on the surface of the QD by hydrophobic moieties in which 20 LON molecules were found to be grafted on each QD after subjecting it to various concentrations of LONs. Intracellular hybridization studies revealed that LON-QD was successfully taken up by the cells. Therefore, taken together, the results highlighted the robust properties of QDs that combined cell internalization, endosomal escape, and hybridization with the target ON (Aime *et al.*, 2013).

Although many approaches have been constantly exploited for the delivery of nucleic acids which mainly consist of secondary carriers and complex multicomponent charge-neutralizing formulations, Anthony M. Rush *et al.* demonstrated the efficient delivery of an LNA-polymer conjugate that assembles into spherical micellar nanoparticles, namely LNA-polymer amphiphile (LPA) that is capable of displaying nucleic acids at the surface and further contribute in forming a stable and nontoxic delivery agent. These LPA NPs were prepared using solid phase coupling of a carboxylic acid-terminated norbornyl polymer with an amine-modified LNA oligonucleotide on controlled pore glass beads. LPA nanoparticle size was around 20 nm in diameter. On examining the efficiency of cellular uptake and intracellular mRNA interaction, it was observed that the antisense fluorescein-labeled LPA (AS-FL-LPA) nanoparticles showed a tenfold increase in fluorescence in the treated HeLa cells relative to those treated with single-stranded fluorescein labeled LNA analogue.

A similar trend of observation was observed for the uptake of Cy-5 labeled LPA which were designed to interrogate the influence of incorporated dye on LPA NPs uptake in HeLa cells. In addition, based on confocal fluorescence microscopy, it was observed that LPA NPs were distributed in most of the cells within two hours after incubation. Furthermore, the utility of the LPA NPs role in regulation and propagation of genetic information was demonstrated by treating HeLa cells with antisense (AS-FL-LPA) against the survivin gene, which ultimately resulted in the downregulation of the target mRNA levels. In five differently originated cell lines, uptake times for LPA NPs were studied. It was observed that cell lines showed remarkable rapid uptake time of 10 min for LPA nanoparticles after exposure. Maximum uptake in four cancerous cell lines was attained after 30 and 60 min of incubation. Furthermore, it was observed that LPA nanoparticle uptake by HeLa cells was dependent on cholesterol, as shown by the decrease in LPA nanoparticle association when treated with methyl-β-cyclodextrin (Rush *et al.*, 2014).

6.7.2.1 Delivery of locked nucleic acid -aptamers

LNA has also been known to play a crucial role in the development of chimeric and stable aptamers, which are single-stranded DNA or RNA that are able to bind their target with high affinity and selectivity. Therefore, by integrating the advantages of nanomedicine with the cell-targeting ability of aptamers, aptamer-functionalized NPs could be designed for biomedical applications. In general, the aptamers devoid of unmodified nucleotide bases are more susceptible to nuclease-mediated degradation which could be overcome by LNA incorporation in aptamers to increase its stability, biodistribution, and targeting efficiency facilitating accurate targeting (Keefe *et al*., 2010). Such a work was done by Jagat *et al*., where LNA aptamer (antinucleolin and EpCAM) complexes were loaded in an iron-saturated bovine lactoferrin (Fe-blf)-coated dopamine modified surface of superparamagnetic iron oxide (Fe_3O_4) NPs, which was successful in delivering the specific aptamers in a co-culture model of cancer cells and normal cells (Kanwar *et al*., 2011). In another study, Kislay Roy *et al*. demonstrated the anticancer activity of the alginate-coated chitosan NPs encapsulating dominant negative survivin (SR9) with LNA aptamers targeting EpCAM and nucleolin (called nanobullets) *in vitro* (2-D and 3-D cell culture) and *in vivo* (colon cancer mouse xenograft model). The aptamers were incorporated with LNA to enhance the stability, and it was observed that mucoadhesive nanobullets exhibited six-fold higher internalization in cancer cells relative to noncancerous cells, which suggested its tumor-specific uptake. The nanobullets were accumulated at both the periphery and the core of tumor spheroids and displayed 2.26-fold ($p \leq .05$) reduction in 24 h and 4.95-fold reduction ($p \leq .001$) in the spheroid size after 72 h. In addition, it was also seen that there was a four-fold decrease in tumor size in those subjects fed with nanobullets, and it showed a significantly high apoptotic ($p \leq .0005$) and necrotic index in the tumor cell population ($p \leq .005$) relative to void NPs (Roy *et al*., 2015).

6.7.2.2 Delivery of locked nucleic acid–short interfering RNA

The siRNAs form a potent therapeutic tool in the treatment of various diseases. Since it imparts its role in the inhibition of protein synthesis, its therapeutic effect is also supposed to last from days to weeks. This long-lasting therapy has gathered the attention of researchers as it would not only bring down the expense of medical treatment but also help with patient compliance. It has been estimated that the efficacy of siRNA therapeutics would remain at a picomolar level and that it has an added advantage of reducing adverse drug reactions. Finally, the large manufacture of therapeutic siRNA is considerably straightforward and efficient. But the biggest problem with siRNA-based medicines is its delivery into the cells (Perkel, 2009). To be utilized as a therapeutic, siRNA is required to be stable in the environment where it is exposed, such as during its manufacture, storage, and delivery. Unfortunately, as discussed earlier, these siRNA are prone to RNases which have the capability to rapidly degrade the siRNA provided its unstable condition. To overcome these issues, two approaches could be applied which includes (1) the use of siRNA encapsulating vectors that

provide serum stability as well as RNase inhibition that could degrade siRNA and (2) small chemical modifications of siRNA in order to inhibit RNase interaction sites. And in practice, the challenge of increased stability could be achieved by applying both of the approaches at the same time (Yazbeck *et al*., 2002). Gao *et al*. (2009) has compared the effect of chemical modifications, nanoparticle formulations on siRNA stability, and biodistribution in mice. In this regard, experiments were performed to determine the effects of chemical modifications and pharmacokinetics of nanoparticle-based siRNA vectors in blood clearance and biodistribution. A different range of siRNAs (siLNA-light, sisiRNA, siRNA-thio, and siRNA-cholesterol) were used. Different detection strategies were also incorporated for siRNA analysis, such as scintillation count, direct gel electrophoresis, and northern blot technique. On investigating siRNA integrity, it was observed that LNA modification significantly reduced its susceptibility to nuclease degradation and significantly prolonged the blood circulation time for siRNA-chol. In addition, it was also observed that the unmodified siRNA displayed degradation in blood within a minute after intravenous administration, whereas almost all of the chemically modified siRNA were detected in blood and organs, 30 min postinjection. The majority of siRNA duplexes displayed a six- to eight-fold higher radioactivity in the kidneys than in any other organs, and it was demonstrated that siRNA circulatory half-life could be extended by conjugation with cholesterol. In dose-dependent experiments, a gradual increase in a siLNA-light dose did not contribute in blood clearance or biodistribution pattern but increased the relative excretion ratio. Although chemically modified siRNA accumulated greatly in the kidneys, the strongest intensity was observed in the lungs. On delivering these siRNAs with different cationic polymers, it was found that the Jet-PEI/siRNA formulation showed rapid clearance <5 min after injection; however, this facilitated equal distribution of siRNAs throughout the organs. On the other hand, chitosan-based NPs delivery of siRNA displayed higher circulatory half-life given its higher cationic-charged surface that could interact with the serum proteins. These NPs contributed in siRNA stability and displayed pronounced accumulation at 24 h in the kidneys after intravenous administration. Results obtained from liposome formulations were not as pronounced as Jet-PEI or chitosan formulations (Gao *et al*., 2009). Later, Gao *et al*. (2014) used nonwater-soluble chitosan polyplex nanoparticles to deliver siRNA in proximal tubule epithelial cells (PTECs). Various studies were done to determine accumulation of chemically modified siRNA/chitosan in kidneys, its mode of uptake and crucial parameters for the optimal delivery *in vivo*. In addition, the knock down of water channel protein aquaporin (AQP1) was done to account it as a proof-of-principle for utilizing this novel strategy for the purpose of targeting specific genes in PTECs. Chitosan formulated with different chemically modified siRNA were used to study its effect on biodistribution in mice. All the chemically modified siRNA were accumulated in kidneys 24 h post injection, whereas unmodified siRNA could not be detected. Therefore LNA-modified siRNA was used for *in vivo* experiments. Furthermore, on investigating the contributing parameters for successful delivery into the targets, it was noticed that accumulation of the modified siRNA in the kidneys depended on the molecular weight of chitosan and the endocytotic uptake of this formulated chitosan/siRNA in PTECs which were

mediated by megalin. Among different formulations, it was observed that NPs prepared with chitosan A (40 kDa) with high amine to phosphate (N/P) ratio (N/P = 60) led to significant accumulation in the cortex of kidneys. Further, it was found that chitosan/Cy3-siRNA uptake in PTECs was mediated by megalin, which was confirmed by immunohistochemical staining and confocal microscopy. To facilitate monitoring of the dynamic changes in siRNA blood clearance, urine secretion, and kidney accumulation Cy5 dye were implicated as an siRNA tracer. It was observed that the chitosan/siRNA specifically accumulated and resided in the kidneys for at least 48 h postinjection. The specificity and effectiveness were further evaluated by silencing the AQP1 gene which is predominantly expressed in PTECs. Therefore it was confirmed that the chitosan/siRNA could be delivered in PTECs for specific knock down of gene expression (Gao *et al.*, 2014). In another study, particular features of siRNA-AuNPs were determined, which are critical for stability in serum and recognition by dicer (Patel *et al.*, 2011). In this experiment, anti-eGFP dicer substrate RNAs of 25−27 base pairs immobilized on 13 nm gold nanoparticles were examined using fluorescent recovery assay to investigate its stability and dicer activation. Initially they used two siRNA-AuNP substrates, where one possessed two base pair overhangs on 3′ terminus of the antisense strand and one possessed a blunt end. It was observed that dicer preferred 3′ overhangs on siRNA-NPs and that the catalysis rate was twice as blunt as A-U substrates, respectively. To overcome this issue, the terminal two bases of overhangs were modified using LNA, which increased the stability of this substrate against nonspecific degradation modestly from 1.16×10^{-2} nm/min for the unmodified substrate to 7.37×10^{-3} nm/min for substrates with LNA and phosphorothioate modifications. However, it was observed that dicer recognition and processing significantly decreased upon LNA addition. Later, the orientation of the duplex was reversed so that the more stable end was in the distal end of the NPs, which mediated interaction with the serum proteins, improving its stability due to the serum degradation rate decreasing to 4.9×10^{-4} nm/min in comparison to 7.37×10^{-3} nm/min for blunt end A-U substrates. The designed siRNA-AuNPs were further subjected to a cell culture system (C166-GFP cells) to check its ability to knock down eGFP. The results obtained showed a 15-fold increase in serum stability for siRNA structures with blunt ends of high thermal stability and low duplex breathing. However, this increase in serum stability also enhanced the cellular uptake by 300% and thus led to greater gene knockdown. Therefore in a nutshell, it was demonstrated that with the implementation of modifications in nucleic acid structural features such as distal 3′ overhangs, the dicer processing, and serum stability of siRNA-AuNPs could vary dramatically (Patel *et al.*, 2011).

6.8 Conclusion and future prospects

To explore and understand the subtle enigma of life filled with a wide array of human health diseases, researchers have been investing billions of dollars to investigate the complexity of health disorders. Gene therapy has been an approach for various

therapeutic applications, as nucleic acids have proved to be a promising tool in therapeutics. In order to improve the stability and specificity of nucleic acid, several nucleic acid analogues have been developed for their application in contemporary molecular biology. LNA is one nucleic acid analogue that has proved to be an efficient tool due to its high stability in biological fluids, lack of toxicity, and improved hybridization behavior. LNA oligonucleotides, along with other oligonucleotides such as LNA, LNA/DNA chimera, and LNA/RNA chimera, are considered to be compatible with different oligonucleotides. This has also generated avenues for diagnostic applications due to its heightened ability to discriminate between mismatches in nucleic acids. Despite its ability to be a strong therapeutic agent, its efficiency gets restricted due to its limited cellular uptake, poor endosomal escape, and improper biodistribution in the cells. These shortcomings can be circumvented by the incorporation of nanotechnology that could be achieved by the use of nanoparticle-mediated delivery of LNA. Although there has been a limited number of studies related to nanoparticle-mediated delivery of LNA in the scientific scenario, most of the researchers have come up with effective results using quantum dots, gold, lipid, and polymer nanoparticles for the convenient delivery of LNA-modified oligonucleotides such as siRNA, aptamers or LNA-DNA, and LNA/RNA chimera, etc.

Thus nanoparticle-mediated delivery of LNA and LNA-modified oligonucleotides is known to make a greater impact on every nook and corner of biotechnology and medicine. However, a thorough study and understanding is required for this to occur at a wider scale.

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Nanoparticles for ribozymes delivery

7

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7.1 Introduction

In the initial era, RNA was regarded as one of the entities of the central dogma which was translated into a functional unit called protein. The change in the pristine perspective occurred after Kruger *et al.* (1982) acquainted the world with their unique discovery of RNA with the enzymatic properties. These RNA-based nucleic acid enzymes were capable of conducting catalytic activities like protein enzymes without undergoing any structural change and thus were called ribozymes. These ribozymes catalyzed chemical reactions without the presence of the protein component (Schubert and Kurreck, 2004). Naturally occurring ribozymes are seven in number, all of which carry out the same reactions, i.e., either splicing or ligating the phosphodiester backbone of RNA using trans-esterification or hydrolysis of the phosphate group (Doherty and Doudna, 2001).

On the basis of size and reaction mechanisms there are two kinds of catalytic RNA: small and large ribozymes. Small ribozymes are comprised of hammerhead, hairpin, hepatitis delta ribozymes, and varkud satellite (VS) RNA as well as artificially selected nucleic acids, whereas large ribozymes include self-splicing group I and group II introns and the RNA part of RNase P. With the exception of ribonuclease P and ribosomes, all the other naturally occurring ribozymes catalyze intramolecular reactions while the intermolecular reactions can be carried out by artificially made ribozymes. The synthetic ribozymes are produced by exploiting the properties of catalytic centers of the already existing natural ribozymes. The main function of all these reactions is to inactivate or silence the other RNA molecule while the other processes in which they play their role are RNA splicing, the processing of RNA, and the formation of peptide bonds during translation (Nissen *et al.*, 2000). These RNA catalytic functions show high sequence specificity between the RNA molecule and the target molecule. All the genetic information for recognizing, binding, and cleavage of the substrate molecule lies in the ribozymes. Due to this hidden genetic information and sequence specificity, ribozymes can be exploited in different therapeutic areas. The use of ribozymes as therapeutic agent has already begun in areas such as in the treatment of viral diseases and diseases such as cancer, diabetes, and rheumatoid arthritis (Puerta-Fernandez *et al.*, 2003). The rate of success in using ribozymes to fight diseases depends upon various factors such as its efficiency, stability of both the ribozymes as well as its target molecule, localization of target RNA with its complementary ribozyme, etc. These factors have been improved by either using small ribozymes (examples mentioned earlier)

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or by using vectors for these ribozymes. Though each individual factor has a scope for improvement by using different methods, the unique properties of RNA led researchers inquisitive of exploiting the properties of RNA using nanotechnology, which led to the advent of RNA nanotechnology.

Nanotechnology concerns itself with the creation of structures at the nanoscale by employing any one of the approaches, i.e., the top-down or the bottom-up approach. Due to the defined structures of the macromolecules, such as nucleic acids and proteins at the nanoscale level, these macromolecules can further be exploited to formulate nanostructures. The utilization of nanotechnology in the field of DNA and proteins has been extensively studied by researchers for a long time, but the RNA nanoparticles were first constructed in the year 1998 using multiple re-engineered natural RNA molecules (Guo *et al*., 1998). The choice for RNA as one of the candidates in nanotechnology by researchers has been increasing day by day. It can be attributed to its diverse functions governed by its versatile structure. Similar to DNA, RNA can be easily manipulated and mimic proteins in terms of flexibility in their structures and functions. Different properties such as noncanonical base pairing, tertiary structure formation, base stacking, presence of single stranded stem loops for inter/intramolecular interactions make it a significant moiety for constructing a large number of nanostructures (Guo *et al*., 2012). RNA molecules can also function as aptamers, short interfering RNAs (siRNA), riboswitches, ribozymes, miRNA, etc. These varied functional forms of RNA further cater to gene silencing and regulation, cell recognition and binding, intracellular control, etc. In order to use these RNA modules successfully for clinical trials, safe, efficient, specific, and nonpathogenic delivery has to be ensured. Thus RNA nanotechnology has given all different forms of RNA a platform to hone their properties in such a manner that they could be used in the field of nanomedicine and therapeutics.

RNA nanoparticles have been observed to possess greater thermostability than their counterpart, i.e., DNA. Also, RNA serves as a better gene/drug delivery carrier due to the ease with which it penetrates the cell membrane. RNA nanoparticles manage their own survival inside the endosome that has a characteristic acidic environment and then get distributed in the entire area of the cellular compartment. Contrary to thermostable property, the chemical instability of RNA makes it prone to breakdown by the active enzymes such as RNases. However, RNA nanotechnology can be utilized to produce nanoparticles that are resistant to the RNase attack, thus rendering it to be highly stable and garnishing it with greater potential to be utilized in the field of nanomedicine. The present chapter highlights some of nanotechnology-based aspects for the delivery of ribozymes.

7.2 Types of ribozymes

Ribozymes can be divided into two broad categories: large and small ribozymes. The differences between these two can be understood in terms of their reaction mechanism and size. The former group is comprised of ribozymes possessing 3000 nucleotides and generates reaction products with a free 3′-hydroxyl and 5′-phosphate group. Small

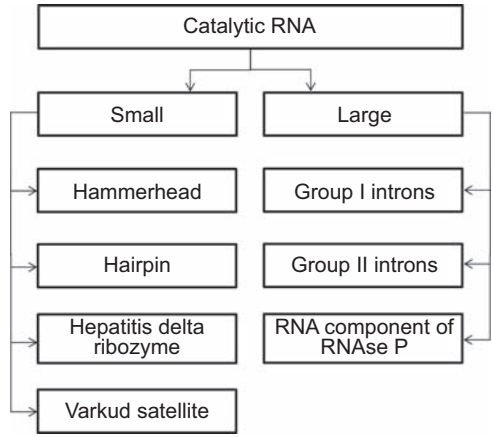

Figure 7.1 Different kinds of catalytic RNA.

ribozymes vary in the size range of 30−150 bases, and their mechanism of action generates products with a 2′-3′-cyclic phosphate and a 5′-hydroxyl group. Examples of large ribozymes include group I and II introns and the RNA component of RNase P (Fig. 7.1). The early 1980s marked the discovery of group I introns. It is the first catalytic RNA found in the pre-rRNA of ciliate *Tetrahymena thermophilia* along with its presence in rRNA, mRNA, and tRNA of several organisms and organelles such as the nuclei of protozoa, the mitochondria of fungi, the chloroplasts of algae as well as bacteria and their phages (Schubert and Kurreck, 2004). However, they are absent in higher eukaryotes, for example, in vertebrates (Abera *et al*., 2012).

7.2.1 Group I introns

Group I introns are involved in catalyzing their own excision (self-splicing), which requires guanosine factor and divalent cations, either Mg^{2+} or Mn^{2+}(Abera *et al*., 2012). Self-splicing occurs via a trans-esterification reaction, which is a two-step process. An already existing catalytically active site for the trans-esterification reaction can also be re-engineered to catalyze reactions. One of the applications of these group I introns was the treatment of sickle cell anemic patients in which a mutated part of the β-globin RNA was replaced with γ-globin-3′- exon. As a result the γ-globin was found to be resistant to the pathological polymerization of hemoglobin (Schubert and Kurreck, 2004). In order to correct abnormal β-globin RNA, scientists have also developed an expression cassette for transferring the ribozyme coding gene into mammalian cells (Lan *et al*., 1998).

7.2.2 Group II introns

Although very little information is known about the group II introns, the unique features allow its use at the genetic level. They differ from group I introns in terms of their distribution and self-splicing reaction mechanism. Less distributed than group I

introns, they are present in bacteria and organelle genes of eukaryotic cells. Self-splicing reaction carried out by these multidomain RNA metalloenzymes resemble that carried out by spliceosomes. This class possesses the property of retrohoming, in which a subgroup of this class of ribozymes is able to insert itself into an intronless allele on the DNA level by reverse splicing and reverse transcription.

7.2.3 Ribonuclease P

RNase P is a ribonucleoprotein complex that consists of RNA (375 ntd long) and a small polypeptide. The catalytic center of this complex lies in the RNA part, and it cleaves precursor tRNA and other RNA into its mature form, which is required for cellular metabolism. The RNA part alone expressing the catalytic activity *in vitro* was the first example of an RNA-based catalyst acting in trans on multiple substrates (Guerrier-Takada *et al*., 1983). However *in vivo* conditions require even the protein component to exhibit the full enzymatic activity.

The catalytic property of RNase P can be harnessed to cleave the particular target mRNA by either exogenously introducing External Guide Sequence (EGS) into cells or expressing it intracellularly. These EGS are oligonucleotides that are designed to be complementary to the mRNA of our choice. The hybridization of EGS with the target region resembles the top portion of the natural substrate of RNase P (Guerrier-Takada and Altman, 2000). Scientists have successfully employed this technology against the viral genes *in vitro* to interfere with the replication of herpes, hepatitis, human immunodeficiency, influenza, and cytomegalovirus (Dunn and Liu, 2004). A different approach could be understood in terms of covalently linking a guide sequence to the RNA component of *Escherichia coli* RNase P, referred to as the M1 ribozyme, to yield M1GSRNA, a sequence-specific ribozyme (Liu and Altman, 1995). These were used to cleave various viral and oncogenic targets in cell culture. RNase P can be considered to be the only true naturally occurring *trans*-cleaving RNA enzyme known to date.

7.2.4 Hammerhead ribozyme

It is one of the smallest ribozymes that was found in plant virus satellite RNAs, viroids, and transcripts of a nuclear satellite DNA of newts. It has been utilized in several applications, and different forms of this ribozymes have been developed for cleaving the target RNA in trans. The hammerhead ribozyme consists of three helical sections connected via a three-way helical junction. Several changes in the structure of hammerhead ribozymes have led to its utilization in *in vitro* and *in vivo* experiments and further are being used for clinical trials.

7.2.5 Hairpin ribozyme

Hairpin ribozymes exhibit similarity in their reaction to the other small ribozymes. Using rolling circle replication, they cleave the concatameric precursor molecules into mature satellite RNA. Apart from the cleavage reaction, they are also involved

in the RNA ligation depending on the availability of favorable reaction conditions (Hegg and Fedor, 1995). Of three different hairpin ribozymes that have been found, the best characterized hairpin ribozyme is the satellite RNA associated with tobacco ring spot virus (Hampel and Tritz, 1989). The naturally occurring hairpin ribozyme structurally consists of the catalytic domain which is a four helix junction containing approximately 50 nucleotides for initiating the metal ion-dependent cleavage reactions in trans. Scientists have utilized hairpin ribozymes to target the RNA of HIV-1 in cell culture experiments. Ribozymes that were expressed in the cell were found to be resistant toward HIV-1. Successful evidence became an impetus for researchers to conduct the phase one clinical trial for the hairpin ribozyme-based strategy to work against the HIV-1 virus.

7.2.6 Hepatitis delta virus

The other two small catalytic ribozymes are Hepatitis delta virus (HDV) ribozyme (Shih and Been, 2002) and varkud satellite ribozyme (Lilley, 2004) that have limited therapeutic use. HDV is the satellite virus of hepatitis B virus, a major human pathogen, and HDV is one of the sources of the ribozyme (Wu *et al*., 1989). Though there is a difference in the sequence of the genomic and the antigenomic strand, they exhibit similar secondary structure. Crystalline structure reveals five helical regions organized by pseudo-knot structures.

7.2.7 Varkud satellite ribozymes

The varkud satellite (VS) ribozyme is transcribed from the plasmid of Neurospora. It is 154 nucleotides long and is the only ribozyme whose crystalline structure is not known.

7.3 Ribozymes and their limitations

It is evident in the studies that a large portion of the human genome codes for the noncoding RNA (Lander *et al*., 2001) such as microRNA (miRNA), short interfering RNA (siRNA), snRNA, etc. Although noncoding, this class of RNA has shown their significance in a number of research areas such as gene expression (Novoa *et al*., 2012), gene regulation (Bagga *et al*., 2005; Keene, 2010), catalytic reaction in the cellular compartments (Kaida *et al*., 2010), etc. As these noncoding RNA undergo some anomalies in their structure or functions, it results in the malfunctioning of the cellular system in the form of diseases such as cancer, heart failure, etc. A few examples where the aforementioned noncoding RNA has been found to be associated with cancer are as follows: presence of SNORD33, SNORD66, and SNORD76 (snoRNAs) in the form of biomarkers in nonsmall cell lung cancer (Liao *et al*., 2010); microRNAs (miRNAs) role as oncogenes or tumor suppressors (Boll *et al*., 2013; Fabian *et al*., 2010; Krutzfeldt *et al*., 2005). The involvement of noncoding RNAs in the diseases has led the scientists to exploit RNA properties for the

treatment of the diseases. Thus these different forms of RNA, such as siRNA, miRNA, RNA aptamers, riboswitches, and ribozymes, have been in use as therapeutics. Many scientists now have cited the literature for the use of various forms of RNA such as siRNA, RNA aptamer, and miRNA as measures to guard against diseases. However, the present chapter will precisely focus on ribozymes as a treatment strategy.

A new set of tools to study the gene expression system can be studied in terms of ribozymes. As described earlier, ribozymes are catalytic RNA which possess sequence specificity in terms of recognition and catalyzing the cleavage reactions of the target molecule. One of the versatile ribozymes are hammerhead ribozymes, which are small ribozymes containing two motifs. One is a catalytic core and the other one is a hybridizing helix (Kashani-Sabet, 2002). The catalytic core carries out the cleavage reaction while the hybridizing helix not only helps in flanking the catalytic core but also binds to the target molecule. Although there has been an increasing interest in the ribozyme-based technology due to its intrinsic catalytic activity, the loopholes in terms of its design and delivery also hold importance and cannot be neglected. Another aspect is its *in vivo* efficiency, which has been found very low in order to produce a desirable biological effect. In order to reach effective solutions, we will first need to understand the concerns related to the design and delivery of ribozymes.

7.4 Selection of the target gene

The three main points for the selection of the target gene are to be considered prior to designing a ribozyme against it:

1. stability of the target molecule
2. sequence
3. accessibility to the ribozyme for the target molecule

The target molecule holds great importance in terms of producing the desired phenotype. Thus the higher the stability of the target molecule, the higher will be the inhibition time to stem its expression by the ribozyme (Kashani-Sabet, 2002). Thus it is required that the ribozymes express at a higher rate in order to achieve the sustained level of inhibition. Another feature is the sequence of the target gene which varies with each target gene. Thus it becomes imperative to test every individual target gene sequence as the ribozyme identifies three sites, namely, the 5′ end of mRNA, the 3′ untranslated region, and the sequence in between them (Kashani-Sabet, 2002). The accessibility of the ribozyme to interact with the sites of target molecules has also been one of the challenges faced by the scientists. This is because long RNA molecules fold to form secondary and tertiary structures in a highly ordered form, which makes it difficult for the ribozyme to interact with the target RNA. Along with this, the binding of protein with RNA also precludes the ribozyme to make any base pairing with its substrate. It has been studied that nearly 90% of the cleavage sites have shown inaccessibility to molecules such as ribozymes, DNAzymes, and oligodeoxynucleotides which serve as antisense-based molecules

(Kurreck *et al*., 2002). Therefore an effective solution can be the designing and thereafter testing of several ribozymes which will be complementary to the RNA of interest (Kashani-Sabet, 2002).

7.5 Stability of ribozymes

The second challenge in designing the ribozyme deals with the sensitivity of the ribozymes toward the serum nucleases and their interaction with the plasma proteins. In other terms, an effective approach toward making a ribozyme more stable requires certain alterations. The stability of ribozymes in turn is not dependent on the sequence of a nucleotide but rather on the chemistry of the oligomers' nucleotide backbone (Kashani-Sabet, 2002; Schubert and Kurreck, 2004). A ribozyme delivered in its RNA form is prone to degradation by RNases. To combat these hindrances, researchers have developed several alternatives and have studied their effects. Chemical modification includes several base analogues such as, 5 IU or 5 BrU (Shu *et al*., 2014), have been developed which were found to be resistant to the RNases. Another modification includes the introduction of the sulfur atom, resulting in the formation of phosphorothioate molecules (Eckstein, 2000; Kashani-Sabet, 2002). Although phosphorothioate molecules were able to achieve success, they brought along several side effects and toxicity due to nonspecific binding with the proteins such as heparin-binding proteins and growth factor receptors (Guvakova *et al*., 1995; Schubert and Kurreck, 2004). Galbraith *et al*. (1994) has reported that when the phosphorothioate oligonucleotide was incorporated in the monkey, it adversely affected the cardiovascular system. To overcome this issue, modification at 2′OH of the ribose sugar with 2′-O-methyl and 2′-methoxyethy was suggested, and it was found to be better than the previous one in terms of toxicity, bioavailability, and affinity toward the target molecule (Wang *et al*., 2003).

Although we have addressed the two major issues and their solutions, a bigger challenge appears in terms of the internalization of the ribozymes through some channels. The modes of internalization of ribozymes have been further discussed in detail.

7.6 Delivery of ribozymes

One of the challenges of ribozymes lies in their delivery to the desired subcellular compartment of the targeted cell. Scientists have conducted experiments which emphasized the importance of the co-localization of the ribozyme and target RNA. Separate viral vectors were chosen for the expression of the lacZ gene, its RNA and the ribozymes targeted against it. Co-packaging of the ribozymes and the target RNA in the virions resulted in the cleavage of genomic RNA, while the cytoplasmic LacZ mRNA remained untouched (Sullenger and Cech, 1993).

There are certain factors which need to be understood while studying the delivery of ribozymes in the human body. For the ribozymes to work in the cells of the human

body, it is important to deliver them in the individual cells to interact with their target molecules. The delivery of the ribozymes can be in two forms, namely, the exogenous form and the endogenous form. The exogenous form involves the uptake of the pre-synthesized ribozymes by the target cell which then get released from the endosomal compartment and degrade their target RNA. This form of entry has the limitation of the presence of negative charge on ribozymes and their high molecular weight (Kashani-Sabet, 2002). Moreover, stability remains an issue as discussed earlier. However, to maintain stability, chemical modifications in the form of introducing base analogues or thio alterations had been in favor earlier, but due to its toxicity and cost-related factors this has become impasse. The advent of vector systems as the carrier of the ribozymes was thus in large part to overcome such issues. There are two kinds of vector systems: virus based and nonviral vectors. The polyanionic nature of ribozymes makes the movement of the ribozymes difficult across the hydrophobic cell membrane, and hence investigators had to make use of the latter vector system, i.e., the nonviral vectors. Examples are liposomes, polymers, and polypeptides. A few scientists coupled ribozymes with the positively charged liposomes to ensure the smooth entry via the lipophilic cell membrane. Liposomes are the first delivery systems to be used which proved successful (Puri *et al*., 2009). Lower efficiency, thermodynamic instability, and limited reproducibility (Douglas *et al*., 1987; Moon and Giddings, 1993) of these vector systems has led the scientists to use the viral vectors as their choice of vector system in spite of its cost effectiveness, relatively nontoxic nature and ease of synthesis (Douglas *et al*., 1987; Moon and Giddings, 1993). Researchers have been working to improve the drug delivery by liposomes by either changing the phospholipid structure or by incorporating polyethylene glycol for its stability. Saupe *et al.* (2005) and Wissing and Muller (2002) have developed more biocompatible Solid lipid nanoparticles (SLN), demonstrating many advantages.

Another vehicle to administer the ribozymes is the viral vectors such as adenovirus, herpesvirus, retrovirus, lentivirus, etc., which are derived from the detoxified virus. All the vector systems mentioned earlier have great potential as gene delivery vehicles. These offer better efficiency, which not only delivers a stable form of ribozymes but also helps in the constitutive synthesis of the same by the cell. This could thus help in overcoming the deadlock of the appropriate number of ribozymes needed to reach the target cell. Homogenous character and a well-defined structure of viral vectors offer advantages to fabricate nanoparticles. However, these systems might induce immunogenic response, and their production and safety remains an issue. Other factors that hold a key role in ribozyme delivery are:

The endogenous system, i.e., the vector system, highlights a better means of infiltration across the cell membrane, but whether it provides the best means as a ribozyme-carrying vehicle still remains a question.

Thus a novel field that has been gaining attention is RNA nanotechnology. Scientists around the world have developed and incorporated various modules of RNA in the nanoscale devices with the vision of using them for the betterment of society. The versatility of its structure and the diversity and flexibility in its function, just like proteins, has made RNA a choice for the scientist to exploit at the nanoscale level (Guo, 2005). The study related to RNA, its modules and its use in nanotechnology

is still in a very nascent stage. RNA nanotechnology has a number of advantages over the traditional vector systems. These are the following:

1. The size of the particles at the nanoscale enhances permeability and retention effects (Guo, 2010; Guo *et al*., 2012).
2. The unique shape of RNA nanoparticles permits passive targeting through tumors and also appropriate biodistribution (Haque *et al*., 2012; Shu *et al*., 2011).
3. RNA nanoparticles are hydrophilic in nature which shields the ribozyme from degradation and confirms the crossing across the membrane. Also, these particles do not aggregate under normal physiological conditions (Shu *et al*., 2014).
4. In comparison to the vector systems or other heterogeneous nanoparticles, RNA nanoparticles confer less immunogenicity and almost no side effects (Haque *et al*., 2012; Shu *et al*., 2011).
5. In the case of chronic diseases, repeated treatment by these particles can be a possibility, as these induce a minimum production of antibodies (Shu *et al*., 2014).
6. RNA nanoparticles are multivalent in nature. Thus various molecules, including targeting molecules, therapeutic molecules, and imaging molecules, can all be conjugated in a single nanoconstruct without the need for cross-linking. This helps in enhanced and synergistic effects (Shu *et al*., 2011).
7. For any system to work inside the body, toxicity is a major concern. RNA nanoparticles offered a great advantage for nontoxicity as studied in mice (Abdelmawla *et al*., 2011); moreover, it called for favorable pharmacokinetic and pharmacodynamic profiles *in vivo* (Shu *et al*., 2014).
8. Targeted delivery, retention time, and dose requirement has been the important concerns for the researchers. Enhanced permeability and retention effect and active targeting by conjugation with specific ligands of the particular disease such as cancer can provide a solution (Shu *et al*., 2011). Abdelmawla and coworkers revealed that when thermodynamically and chemically stable RNA nanoparticles were systemically injected in mice, there was very little accumulation in vital organs or tissues, and particles displayed a strong and specific binding to the tumor.

There are different kinds of nanodelivery systems based on their physicochemical properties and the material other than RNA nanoparticles, which have also been used for the treatment of diseases like cancer and viral infections such as DNA nanoparticles (Andersen *et al*., 2009), inorganic nanoparticles (Pericleous *et al*., 2012), polymer-based nanoparticles (Lu *et al*., 2011), lipid-based nanoparticles (Puri *et al*., 2009), and viral nanoparticles (Lehto *et al*., 2011). The various advantages associated with RNA nanoparticles led them to be the preferred choice over all the other delivery systems.

Details about lipid-based nanoparticles and viral nanoparticles have already been discussed before. In the polymeric-based nanoparticles, both the lipophilic and lipophobic drugs can be incorporated. These nanoparticles can be synthesized from both the natural and artificial polymers and have a stable structure which can encapsulate a drug with better efficiency (Lu *et al*., 2011; Vasey *et al*., 1999). These nanoparticles can be easily modified so that targeted and multifunctional drug delivery can be achieved. In spite of these comforts of using polymeric nanoparticles as delivery systems, the formation of nonuniform-sized nanoparticles, nonbiodegradability, toxicity, and the cost display its limitations. Inorganic nanoparticles face similar challenges and thus cannot be used for clinical applications; however, high photothermal conversion rate makes them good candidates for photothermal therapy. Its usage can be found in medical imaging due to its brightness and photostability (Shukla *et al*., 2005).

DNA nanoparticles, on the other hand, confer their application in the medical field in the form of entrapment of the enzyme by a DNA nanostructure to be released in the cellular compartment for the induction of the apoptosis (Andersen *et al*., 2009; Zadegan *et al*., 2012). The simple structure of DNA makes its use limited as nanoparticles in clinical applications compared to RNA nanoparticles.

7.7 Applications of RNA nanoparticles emphasizing ribozymes

The advent of ribozymes to be used as one of the candidates in medical applications started when Haseloff and Gerlach displayed the catalytic activity of the hammerhead ribozyme (Haseloff and Gerlach, 1988). It was a milestone in terms of using a ribozyme as a tool to manipulate the expression of the gene. It was after this that hammerhead ribozymes were used against HIV infection (Sarver *et al*., 1990). The role of ribozymes did not remain limited to the viral infections but also achieved success against human cancer targeting c-fos oncogene, which was found to be resistant to several chemotherapeutic drugs, one of them being cisplatin (Scanlon *et al*., 1991). The role of the antioncogene ribozyme could also be seen in targeting H-ras and HER-2/neu in the therapy of bladder and breast cancer (Suzuki *et al*., 2000). In addition to this, an *in vivo* plasmid-based ribozyme study was done by Kashani-Sabet *et al*. (2002) to identify the pro-metastatic function of the NF-κβ gene in melanoma. Scientists have even found its role in dermatologic diseases (Kashani-Sabet, 2002). Thus the versatile nature of ribozymes has made them a potential tool which could be exploited by entrapping in nanosystems.

The inability of conventional therapies (radiation, surgery, or chemotherapy) to distinguish malignant cells from normal cells for cancer treatment has led researchers to find a better treatment regimen which could be involved in specific targeted delivery with zero toxicity and no side effects. The quest for the search came to an end by the discovery of RNA nanoparticles as therapeutics to cancerous cells by targeted delivery. Due to the multivalent nature of RNA nanoparticles, various therapeutics, such as siRNA, miRNA, ribozymes, detection molecules like radionucleolide, fluorophore, and targeting molecules like aptamers, can all be combined under one roof, i.e., all in a single nanoparticle. Scientists have done a tremendous task in this particular field by investigating one such RNA called pRNA, which has utilized siRNA, miRNA, ribozymes, and aptamers for gene silencing and targeted delivery to cancer receptors. As the focus of the present chapter is to study the delivery of ribozymes by nanoparticles, hence the scope will be limited to the use of pRNA in the delivery of ribozymes.

pRNA is an important component of the phi29 DNA packaging motor. It is 117 nucleotide-long molecule which has two essential functional domains, namely, (i) the 5′/3′ helical domain and (ii) the central domain or the interlocking domain or intermolecular interaction domain (Guo *et al*., 2005). The folding of these two domains is independent of each other. The helical domain is a double-stranded RNA region with an open 5′/3′ end. Studies regarding helical domain suggested that there would

be no change in the structure and function of pRNA even if the primary sequence of any nucleotide of this region is modified, provided the pRNA remains double stranded (Zhang *et al*., 1994). Also, the addition of any variable sequences to the 5′/3′ end of the helical domain does not have any effect on its folding and structure (Shu *et al*., 2009). siRNA, a double-stranded molecule (Brummelkamp *et al*., 2002), can be replaced with the helical region of pRNA, producing pRNA with the same intermolecular interactions as before. Khaled *et al*. (2005) and Guo *et al*. (2005) constructed chimeric pRNA consisting of siRNA which was found to inhibit gene expression. Thus pRNA, due to its unique characteristics, is able to circumvent two major concerns: degradation by exonucleases and improper folding in the cell. Hence it has become a suitable vector system for scientists to deliver therapeutic RNA.

The central domain of the pRNA consists of two interlocking loops, i.e., the right-handed loop (RH loop) and left-handed loop (LH loop). These loops are engineered to form dimers, trimers, or hexamers (Guo *et al*., 1998; Shu *et al*., 2007). Dimers of pRNA serve as a monomeric unit for hexamer ring formation using intermolecular interaction (Chen *et al*., 2000). Each loop is composed of four nucleotide sequences which form complementary base pairs with the sequences in the other loops (Chen *et al*., 1999). These interactions with the other loops are called "hand in hand" interactions and are responsible for creating multimers (Shu *et al*., 2014). These two loops, i.e., RH loop and LH loop, are joined by a connector known as the three-way junction (3WJ) motif. Shu *et al*. showed that three pieces of small RNA oligomers of high affinity were used to form the 3WJ motif of pRNA. The formation of this complex confers stability and was found to be resistant to denaturation even in the presence of a strong reducing agent such as 8M urea. Each of the three RNA oligomers carrying siRNA, or a receptor-binding aptamer or a ribozyme, when incubated resulted in the formation of trivalent RNA nanoparticles which can be used a therapeutic agent. pRNA 3WJ was found to be the most stable of several 3WJ motifs that were retrieved from varied biological systems (Shu *et al*., 2011).

Shu *et al*. has described the bottom-up approach to design pRNA nanoparticles that provided an advantage of incorporating all the modules into the nanoparticles keeping the folding or functioning of both the core scaffold and the individual modules intact. RNA functional modules of the RNA nanoparticles are comprised of nicks which aids in the *in vivo* stability of the RNA nanoparticles. This approach also helps in acquiring the homogenous nature of the particles along with its high efficiency.

For the production of thermodynamically stable nanoparticles, three different methods were used, the first of which was the extension of loop sequences which were further analyzed by the online RNA folding program M-fold (Zuker, 2003). Another was the introduction of the palindromic sequence at the 3′ side of the RNA building block which was able to form foot-to-foot self-dimers, trimers, tetramers, and so on, which were revealed by atomic force microscopy (AFM) and native page (Shu *et al*., 2013). To construct the large macromole RNA nanoparticles, researchers used the 3WJ core which was further assembled using three RNA oligos mixed in equal molar ratio without any metal ions. The use of even two 3WJ cores was deployed to construct arm-on-arm branched hexavalent RNA nanoparticles. Misfolding of the RNA nanoparticle complex was significantly reduced using the aforementioned three

strategies. Several assays were performed that revealed the correct folding and functionalities of each incorporated module independently within the nanoparticles. One of the assays was the use of the Hepatitis B virus (HBV) ribozymes. They exhibited the cleavage of 135-nt HBV RNA genome substrate into two fragments (60 nt and 75 nt) after it was incorporated into the tetrameric pRNA nanoparticle, which was an indication of the ribozyme retaining correct folding and function after its incorporation into the pRNA nanoparticles.

Due to the unique properties of the pRNA-based nanoparticle, such as its uniform distribution of nanometer sized particles, *in vivo* stability and its strong natural folding, it has provided a meaningful platform to scientists in the field of RNA nanotechnology. The properties of pRNA-based nanoparticles such as monomer pRNA, pRNA 3WJ, and pRNA-X moiety can be exploited at the clinical arena.

To test the feasibility of its properties, chimeric pRNA dimers were designed. One of the units of the dimer is connected to the receptor-binding RNA aptamer or folate for cell recognition, while the other was connected to siRNA, ribozyme, or chemical group. The chimeric dimer was then tested for the silencing of genes such as green fluorescent protein (GFP), luciferase, survivin, and other pro/anti-apoptotic members of the bcl-2 family by intracellular delivery to several cancer cells (Guo *et al*., 2005).

pRNA/ribozyme (survivin) is a chimeric pRNA that harbors a hammerhead ribozyme against survivin. Survivin finds its presence in the cancer cells and is found absent in the normal cells. In cancer cells it is found to inhibit apoptosis, and hence suppression of survivin leads to the induction of apoptosis in the cancer cells (Grossman *et al*., 2001; Pennati *et al*., 2003). Thus it was chosen as the target by pRNA/ribozyme.

Several human cancer cell lines were chosen for transfection with pRNA/ribozyme (survivin), and it was found that pRNA/ribozyme (survivin) was able to inhibit the apoptosis as more than 80% of the cells were found to be nonviable after 24 h (Guo *et al*., 2005).

The application of pRNA/ribozyme to downregulate the gene can also be found in viral diseases. Hammerhead ribozymes were incorporated in pRNA to form a chimeric pRNA/ribozyme that could cleave HBV polyA signal. pRNA 5′/3′ was chosen where the ribozymes could be connected, and the ribozymes were comprised of two arms for targeting the HBV while it is being escorted by the pRNA. It was observed in *in vitro* experiments that ribozymes were completely able to cleave the polyA signal of HBV mRNA. Northern blot and e-antigen assay were employed to observe the effect of inhibition of HBV replication by chimeric pRNA/ribozyme. Increased inhibition was found by the chimeric moiety rather than the ribozyme alone (Hoeprich *et al*., 2003).

7.8 Conclusion and future prospects

A fate of the cell or eradication of a disease will depend on efficiency and how effectively a ribozyme has been able to perform its functions and which in turn will depend

upon many factors as already discussed earlier. As the pros and cons of various ways to mark the entry of ribozymes inside the cell has been discussed thoroughly, it has become an important event to optimally design the ribozyme in order to achieve the maximum efficiency. Thus nanotechnology has provided a platform to synthesize the carrier/vehicle for the ribozyme and deliver it at the nanoscale level. RNA nanotechnology has a great potential to be used in the biomedical field, such as at target gene/drug delivery for treating diseases, and serves as a promising candidate at the therapeutic level.

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Nanoparticles for DNAzymes delivery

8

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8.1 Introduction

It has long been observed that various debilitating diseases have been prevalent in society for a very long time. Fatal diseases could be exemplified as cancer, atherosclerosis, Huntington's, Alzheimer's, Parkinson's, etc. Several different kinds of pathways that led to such infectious diseases were targeted in order to manage the critical conditions. However, the root cause of any chronic disease boils down to the abnormality at the genetic level. With the development in science and technology, traditional measures of treatment are at an impasse. Thus to keep the pace with newer technologies, scientists have developed deep into genetic accounts with an aim to discover newer possibilities in the era of drug discovery. Contemporary notions by the scientists in the field of molecular genetics have revealed new tools for selective gene silencing. With the progress in the field of research, the invention of catalytic nucleic acids provides an opportunity to work at the genetic level. These catalytic nucleic acids are complementary to a unique molecular target by manifesting its sequence-specific binding properties. Such a property holds an advantage in medicine and chemical biology. Vitravene (fomivirsen), manufactured by Isis Pharmaceuticals, got an approval by the Food and Drug Administration in 1998 for the treatment of cytomegalovirus retinitis in immunocompromised patients, such as those with AIDS (Marwick, 1998). Yet another drug, Genasense (Oblimersen), was manufactured with an intent to treat patients suffering from relapsed or refractory chronic lymphocytic leukemia. This was manufactured by Genta, Inc., and it was prescribed to be given in combination with fludarabine and cyclophosphamide. Bcl-2, an oncogene, was selected as a target for Genasense.

The ability of nucleic acids as biological catalysts has become well established. Formerly, biochemical dogma dictated that proteins performed all biological chemical reactions, and nucleic acids merely carried the genetic information that encoded the proteins. In particular, significant advances in genomics have led to a substantial shift away from conventional perceptions and dogma to focus on intricate molecular and cellular pathways regulated by an array of key genes. This change has come with the discovery of certain RNA strands that were capable of catalyzing ligation reactions, analogous to that of RNA-dependent RNA polymerase. It is at this interface that nucleic acid molecules emerged as a potent force in further characterizing important molecular pathways and in defining themselves as a sustainable therapeutic class of agents. The ability to selectively attenuate the expression of specifically targeted genes represents an appealing method of therapy and a means of dissecting molecular function.

Advances in Nanomedicine for the Delivery of Therapeutic Nucleic Acids
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The use of antisense oligodeoxynucleotides (ODNs) is an approach to study cellular and viral gene functions and to block gene expression in a therapeutic context. Antisense therapy mainly involves delivery into cells of small DNA oligonucleotides (7−30 nt in length), complementary to target RNA. These oligonucleotides specifically hybridize with the target RNA within the cell and interfere with the function of RNA by blocking RNA transport, splicing, or translation.

Catalytic nucleic acids that have been in use are small interfering RNA (siRNA), ribozymes (Rz), oligonucleotides, microRNAs, DNAzymes (Dz), etc. The therapeutic approaches involving the use of oligonucleotides attracted researchers worldwide and led to the discovery of drugs targeting diseases as could be understood by the examples mentioned earlier.

However, the present chapter highlights DNAzymes as a tool to modulate the outcomes of a disease by regulating the expression of the genes. A holistic study of deoxyribozymes, which includes their properties and advantages, their delivery into the cellular compartments, etc., would help to comprehend the subject in a better manner.

8.2 DNAzymes

Conventionally, DNA molecules were thought to be the messengers of genetic information from one generation to another; however, they were found to be associated with material science (Seeman, 2003).

DNAzymes, which are also known as DNA enzymes or deoxyribozymes, were invented by Professor Ronald R. Breaker and Gerald F. Joyce in 1994. The first discovered DNAzyme, which was used to cleave RNA, was cationic (Pb^{+2}) dependent (Breaker and Joyce, 1994). Deoxyribozymes could be understood as single-stranded(ss) molecules that are not naturally present but are synthesized by an *in vitro* selection procedure (Dass *et al*., 2008a). These single-stranded deoxyribonucleic acid molecules aid in cleaving mRNA by exhibiting high sequence specificity and substrate recognition along with an additional advantage of being highly stable molecules (Akhtar *et al*., 2000). Although similar in functionality with the ribozymes, DNAzymes differ in structure due to the absence of a 2′-hydroxyl group, which plays a central figure in the functionality of Rz. Thus, the absence of 2′-hydroxyl made the discovery of DNAzymes an unexpected event (Kumar *et al*., 2013). These deoxyribozymes consist of a catalytic core at the central position, which is flanked by two arms on both sides. These two arms, arm I and arm II, are complementary to the RNA molecules of interest and bind them according to the Watson-Crick base pairing (Kumar *et al*., 2013). The target molecule, i.e., the mRNA, is being cleaved between an unpaired purine and a paired pyrimidine through a de-esterification reaction (Dass *et al*., 2008a). According to Santoro and Joyce, DNAzymes have an enormously high catalytic efficiency (k cat/K m) of $10^9\ M^{-1} \times min^{-1}$ under multiple turnover conditions. In spite of having a small size, they outnumber other known nucleic acids with respect to their catalytic efficiency (Santoro and Joyce, 1997). The catalytic efficiency was also found to be metal ion dependent as studied by the researchers. Apart from the catalytic efficiency, stability holds a major importance. Studies done by different scientists reveal that DNAzymes exhibit a high level of stability as compared to ribozymes. Stability of the molecules

is brought about by DNA motifs (Kumar *et al*., 2013). Several functions that are carried out by DNAzymes are RNA ligation (Silverman, 2009), carbon−carbon bond formation (Chandra and Silverman, 2008), and the hydrolytic cleavage of DNA (Kumar *et al*., 2013). Of all these functions, the best characterized function is found to be cleavage of RNA molecules. As mentioned earlier, since DNAzymes have to be synthesized and are not naturally present, two variants of DNAzymes were identified, namely 10−23 DNAzymes and 8−17 DNAzymes. These two variants were found to cleave target mRNA in a RNase independent manner in the presence of a divalent cation. Presence of an unpaired purine and a paired pyrimidine site in a large number in the secondary structure of the target mRNA renders its cleavage in an easy mode (Lu *et al*., 2005).

8.3 The 10−23 DNAzymes

It is the most widely known Dz, named as the 23rd clone which was identified following 10 cycles of selective amplification. This DNA interacts with its RNA targets using canonical base-pairing interactions between the DNA and RNA and depends on the binding of a divalent cation (such as magnesium) for activity (Santoro and Joyce, 1998).

The structure of 10−23 Dz (Fig. 8.1) is made up of a catalytically active core of 15 near-invariant nucleotides, flanked by substrate binding arms that can be changed to a base pair with the sequence of the desired target RNA. 10−23 Dz cleaves the RNA sequence at a phosphodiester bond between a purine and a pyrimidine residue (5′AU 3′ most efficiently cleaved). Two products are obtained after the cleavage of mRNA: one with 2′3′ cyclicphosphate and another with 5′OH at the end. A single nucleotide change in the catalytic motif diminishes its sequence-specific cleavage activity; **G14C** completely abolishes the catalytic cleavage activity. These Dzs have a high substrate specificity and are able to discriminate the target RNA sequences that differ by a single nucleotide.

It has been seen in few instances that the rate at which deoxyribozyme catalyzes a cleavage reaction can also be improved by asymmetric arm length truncation in the binding domains. Essentially, stability of the DNA−RNA heteroduplex becomes the

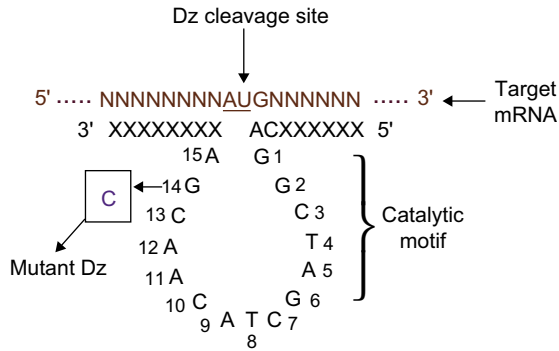

Figure 8.1 Secondary structure of 10−23 DNAzymes.

necessity for the DNAzyme catalytic efficiency (Khachigian, 2000). Catalytic efficiency depends on the stability of the DNA−RNA heteroduplex. Thus the catalytic efficiency may increase to a maximum level with an increase in the stability of the duplex above a threshold level while it may decrease with a decline in the stability of the duplex below the threshold. It could be due to any change incorporated in the form of either a mismatch or truncation in the binding domain (Kumar *et al.*, 2013). Kumar and coworkers reported the catalytic domain of 10−23 DNAzyme to be highly generalizable with respect to the substrate sequence as compared to the 8-17 motifs. Thus it is the most extensively used enzyme.

8.4 The 8−17 DNAzymes

This class of Dzs can cleave an RNA sequence at a phosphodiester bond that is located between an A and G residue in the presence of a divalent cation such as magnesium, lead, and zinc, for which the order is $Pb^{2+} \gg Zn^{2+} \gg Mg^{2+}$. The catalytic domain consists of 13 nucleotides with a four-nucleotide loop adjacent to the cleavage site and a stem loop region that resembles the "stem loop II" region of the hammerhead Rz. The stem loop region in 8−17 Dz is essential for catalysis (Breaker and Joyce, 1994). This Dz has a special requirement for an "rG-dT wobble" pair located immediately downstream from the target site (Fig. 8.2). Substitution with a Watson-Crick pair at this position eliminates catalytic activity.

8.5 Mechanism of DNAzymes

The catalytic mechanism of RNA cleaving is thought to be similar to that of hammerhead Rz (Santoro and Joyce, 1997). The putative mechanism involves the divalent metal ion associated deprotonation of the 2′-hydroxyl group adjacent to the cleavage

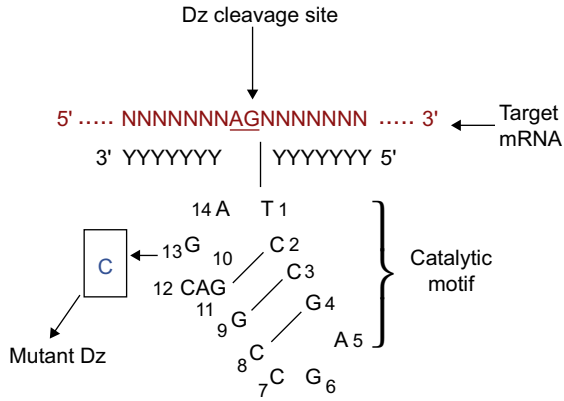

Figure 8.2 Secondary structure of 8−17 DNAzymes.

site. This mechanism is favored as it explains the divalent metal ion dependence of many small nucleic acid enzymes as well as the effect that pH has on their reactions. Also, Dzs with recognition domains of a particular length are more sensitive to mismatches compared with RNA enzymes with guide sequences of the same length, which may allow the avoidance of off-target effects resulting from the cleavage of nontarget RNAs (Herschlag, 1991).

8.6 Advantages of DNAzymes

For *in vitro* applications, DNA is inherently more stable both chemically and biologically than RNA and protein; and this stability can be further enhanced via chemical modifications. Such modifications can be readily incorporated. Because of relatively higher stability, Dzs also have potential advantages for *in vivo* therapeutic applications via mRNA degradation. The cost of synthesis of Dzs is much less in comparison to that of Rzs, and are easier to be synthesized.

8.7 Stability of DNAzymes

As mentioned before, DNAzymes are found to be more stable than the ribozymes. However, Kumar *et al*. reveal the need of modification in DNAzymes due to the inefficient uptake of DNAzymes by the cells (Kumar *et al*., 2013). In spite of being the efficient drug candidate, these are prone to degradation by nucleases and thus need to undergo modification to be further utilized for *in vivo* applications.

Modifications initially included the use of two phosphorothioate (PTO) moieties or two 2′-O-methyl-substituted residues at both the 5′ and 3′ ends. This resulted in the resistance toward the exo- or endonucleases present in the serum. Subsequent studies by the researchers toward stability led them to infer the disadvantages incurred by the PTO and thus propelled the discovery of new modifications. Therefore 3′-3′inverted thymidine modifications were introduced, which overcame the limitations brought about by the PTOs such as toxicity (Wahlestedt *et al*., 2000), immunological side effects (Fluiter *et al*., 2003), and nonspecific interactions with cellular membrane proteins (Rockwell *et al*., 1997). Additionally, PTOs rendered more negative charge to DNAzymes and decreased affinity to bind the target mRNA, thereby decreasing its catalytic potency (Dass *et al*., 2002). Burgin (2001) also mentioned that PTOs inhibited topoisomerase activity in the dividing cells, which could result in malignancies. The 3′-3′ inversion modification, on the other hand, worked favorably with DNAzymes by enhancing both their stability and catalytic activity by up to 20-fold compared to the unmodified form (Dass *et al*., 2002). Also, it was reported by Dass *et al*. (2002) that the half-life of a DNAzyme was extended from 70 min to 21 h in human serum. Another peculiar modulation could be understood in terms of circozymes. These circozymes are circular DNAzymes, which are formed by cloning in some suitable vector or ligating it to an oligonucleotide sequence, thus rendering

stability against nucleases (Seifert *et al*., 2006). The circozymes formed were found to be more stable than the linear counterparts that exhibited stability for only 24 h (Kumar *et al*., 2013).

With the unremitting efforts in the field of science and technology, every now and then new and unique discoveries are made. Hence modifications to DNAzymes further gave way to the introduction of locked nucleic acid (LNA). Improvement in the rate of catalytic efficiency and sensitivity toward the target molecule was achieved with help of LNA (Fahmy and Khachigian, 2004). Locked nucleic acid consists of a 2O-4C methylene bridge that locks in a C3′-endo conformation (Braasch and Corey, 2001). LNA not only strengthened thermal stability but also conferred resistance against 3′-exonucleolytic degradation. Furthermore, as it was similar in structure to nucleic acids, LNA aided in an increase in the solubility (Tan *et al*., 2009).

With an aim in the enhancement of stability, a novel invention is the use of hairpin DNAzymes. It involves the use of substrate binding ends where stem-loop hairpins are added. Compared to the earlier alterations that rendered stability for less than two days, hairpin DNAzymes were found to be resistant to nucleases for up to three days after transfection. It is also proved to be a better tool for gene silencing than the nonhairpin DNAzymes that have the same catalytic domain (Abdelgany *et al*., 2007). No cytotoxic or nonspecific effects have been registered using hairpin DNAzymes.

8.8 Application of DNAzymes

Many biological and chemical reactions have found their catalysis applications in Dzs. The list includes RNA cleavage reactions (Geyer and Sen, 1997), RNA ligations (Purtha *et al*., 2005), DNA phosphorylation (Wang and Sen, 2001), DNA ligation (Sreedhara *et al*., 2004), DNA adenylation (Li *et al*., 2000), oxidative DNA cleavage (Carmi and Breaker, 2001), DNA depurination (Claudia Höbartner, 2007), and many more. Dzs have been used in many *in vivo* applications against a number of diseases. A brief summary of these applications is shown in Table 8.1.

Not only the biological and chemical applications but various physical applications of Dzs have been demonstrated. They are now being used extensively in the fields of nanotechnology, logic and gates applications, and as sensors. Their usage for amplified biosensing was accomplished by designing aptamer-Dz conjugates that combine recognition units and amplify readout units as in integrated biosensing materials (Willner *et al*., 2008).

8.9 DNAzyme delivery via metallic and polymeric nanoparticles

The most challenging task in developing these technologies as various classes of drugs is that this area requires substantial development efforts along with the uphill battle of winning targeted cellular delivery. This momentous work can be channelized with a multidisciplinary approach that involves a greater understanding of each step of the

Table 8.1 A summary of catalysis applications in DNAzymes

Gene	Model	Applications	References
c-Jun	Rabbits, rats, mice	Restenosis, neovascularization, inflammation, tumor growth	Fahmy *et al.* (2006)
Transforming growth factor-β1	Rats	Glomerulonephriti	Isaka *et al.* (2004)
Xylosyltransferase-1	Mice	Spinal regeneration	Grimpe and Silver (2004)
PAI-1	Rats	Myocardial infarction	Xiang *et al.* (2004)
Tumor necrosis factor-α	Rats	Congestive cardiac failure	Iversen *et al.* (2001)

process, including a knowledge of enhanced DNAzyme pharmacodynamics. Several varying researches, including gene vector design, biomolecular and chemical engineering, drug delivery, pharmaceutics, membrane biophysics, and nanotechnology are meant to come under one umbrella to present tangible solutions to increase the efficacy of DNAzymes.

There are certain key points to ponder while dealing with the efficacy of DNAzymes. (1) The gene of interest should be specifically targeted without hampering the normal physiological processes. (2) The target gene should be very well associated with the respective disease process (noncompensable). To achieve this, a suitable delivery system should circumvent the issue of nonspecific (off-target) effects. Delivery vehicles have drawn the attention of the researchers in order to offer a stable complex that could also provide protection from degradation. Therefore greater attention has been given to developing efficient delivery vehicles that can deliver DNAzymes to the target cells.

With the emergence of nanotechnology, few obstacles could be resolved such as biological permeability restrictions, poor pharmacokinetic properties, appropriate trafficking in cells, entry into the nucleus, and locating their target mRNA. Moreover, along with the management of toxicity, it helps to reduce the amount of drugs to enter in the cellular compartment by keeping a checkpoint on the dosage of DNAzymes.

To examine the application of DNAzymes in conjunction with a variety of delivery systems, researchers exploited a myriad of polymeric systems. However, taking into account the discrepancies with each and every macromolecule that was used, scientists were compelled to select a more novel branch, i.e., the use of nanoparticles. Thus Tack *et al.* (2008) started working on DNAzyme delivery via gold nanoparticles. Gold nanoparticles were employed for the delivery of DNAzymes into HT29 human colon carcinoma cells to target the c-Jun gene. The method for preparing the nanoparticles exploited the reducing property of sodium citrate, which reduced tetrachloroauric

[III] acid. DNAzymes were encapsulated in 10 nm gold nanoparticles and were internalized into the cellular compartment with the help of targetors (molecules which aid in directing delivery systems to target). Targets chosen ranged from polylysine (PL) or polyethylenimine (PEI) to transferrin (Tf) while polyvinyl pyrrolidone (PVP) was added as a co-stabilizer. The outcome of the experiments was that inferred PEI-based nanoparticles were more efficient in terms of cell transfection with DNAzymes and more toxic to HT29 cells as compared to PL-based nanoparticles. Transfection was found to be more efficient at low pH, low DNAzyme concentrations, and in the absence of the PVP stabilizer. It was reported that 56% of the DNAzymes have been delivered while 36% of the cells were alive. The experimental setup, however, failed to validate the delivery of DNAzymes into the cells and its activity to cleave the target mRNA. Thus the problem was readdressed by Dass *et al*.

DNAzymes made to target against c-Jun were encapsulated into chitosan nanoparticles and were tested for activity against osteosarcoma (Dass *et al*., 2008b). The results suggested downregulation of the gene. Yet another use of chitosan could be viewed in osteosarcoma SaOS-2 cells (Zimmermann *et al*., 2006). Chitosan is a biocompatible, biodegradable polysaccharide, cheaper than other vehicles such as liposomes or synthetic particles that involve biohazardous procedures. A DNAzyme called Dz13 was encapsulated using a simple complex coacervation method to yield Dz13−chitosan nanoparticles with a median diameter of 350 nm, a high positive surface charge, and high encapsulation efficiency. These particles were not only found to be stable for a month at room temperature but also efficient at intracellular delivery. Moreover, no loss in activity was reported and *in vivo* analysis showed that there were no toxic side effects to the nearby tissues, thus rendering them as safe entities.

Another study in the field of cancer using nanotechnology was carried out by Chen *et al*. (2013) where they worked with nanohydroxyapatite particles (nHAP) as nonviral vectors. Arginine-modified hydroxyapatite nanoparticles (Arg-nHAP) were designed and subjected to assess the absorption efficiency of Arg-nHAP and DZ1 *in vitro*. DZ1 is the DNAzyme which was designed to curb the expression of the target protein known as the Epstein−Barr virus-encoded latent membrane protein 1 (LMP1). It was evident by the results obtained that the nanoparticles proved to be an efficient delivery vehicles for the cells, as the efficiency was near 100%. It was also demonstrated that the uptake of the Arg-nHAP/DZ1 complex by the cells was an energy-dependent endocytosis pathway. To validate the results, confocal microscopy was used, which confirmed nuclear localization and effective intracellular delivery. A decrease in the expression of LMP1 in nasopharyngeal carcinoma cells was also observed. *In vivo* results of a tumor xenograft model of mice also demonstrated a decrease in the expression of LMP1, suggesting the presence of the complex tumor tissue. Thus it confirms Arg-nHAP may be an efficient vector for nucleic acid-based drugs with potential clinical application.

Zhu *et al*. (2004) also worked with pEGFP-N1 loaded nHAP. It was reported that nHAPs are able to deliver DNA into gastric cancer cells and showed zero cytotoxicity. Further modifications in nHAP included the addition of protamine to improve the gene transfection efficiency (Tan *et al*., 2007). The use of nHAP with the NT-3 gene was

studied by Sun *et al.* (2008) The NT-3 gene was delivered into cochlear neurons of guinea pigs both *in vitro* and *in vivo* using nHAP with polyethylenimine modification. It also ensured low toxicity with greater transfection efficiency (Wu *et al.*, 2012). Another aspect that was taken into account by Yan-Zhong *et al.* (2011), was the surface charge of nHAP. One of the 20 amino acids, i.e., arginine, was used to alter the surface charge of nHAP to improve adsorption capacity in human epithelial cells. All of the aforementioned studies on nHAP reveal it to be an efficient and, at the same time, safe gene vector that possesses the potential to be used in clinical applications.

One of the potential vectors for the nonviral gene delivery, studied by Roy *et al.* (2003), was calcium phosphate nanoparticles. The hydroxyapatite [$Ca_{10}(OH)_2(PO4)_6$,HA], which is a ceramic compound, exhibits several advantages such as absorbability and a high binding affinity with a variety of molecules, low cytotoxicity, nonimmunogenicity, lack of oncogenicity, excellent biocompatibility, and relatively high loading capacity and transfection efficiency (Chen *et al.*, 2011; Loo *et al.*, 2010).

With the variety of options of polymers available, Xing *et al.* (2015) employed the use of polyethylenimine derivative *N*-acetyl-L-leucine-polyethylenimine (*N*-Ac-L-Leu-PEI) as a nanoparticle. (*N*-Ac-L-Leu-PEI) was used as a delivery vehicle for DNAzymes to target aurora kinase A using the PC-3 cell as a model. Aurora kinase A is a kinase enzyme that plays a central role in centrosome maturation and spindle assembly (Vader and Lens, 2008). It was found to be overexpressed in prostate cancer (Qu *et al.*, 2008). Though several other strategies have been used to inhibit the gene expression, Xing *et al.* employed DNAzymes as a potential tool to combat the disease by harnessing the property of nonviral carriers, especially cationic polymers, as a delivery system. These cationic polymers exhibit advantages such as flexible designability, high gene-loading capacity, low immunogenicity and low production cost (El-Aneed, 2004; Tian *et al.*, 2013). Due to these advantages, these have been commonly used as a gene delivery system. Branched polyethylenimine has a molecular weight of 25 kDa, and the presence of greater positive charge and high proton buffering capacity aids in the development of resistivity toward nucleases (Patnaik and Gupta, 2013). Thus the uptake by the cells becomes an effortless affair. However, it has also been studied that a cationic polymer such as PEI25K could also render harmful effects to the cell membrane due to excessive positive charge and thus indulge in nonspecific interaction with the proteins that are negatively charged. This leads to toxic effects and serum instability (Zhao *et al.*, 2012). Thus these damaging effects demand the need for modifications in such polymers in the form of hydrophobic groups (Tian *et al.*, 2012). As described by the scientists and earlier in this chapter, these alterations are done in order to improve the transfection efficiency and simultaneously reduce the cytotoxic effects. Thus in one of the studies done by Li *et al.*, PEI25K was modified using hydrophobic *N*-Ac-L-Leu, forming a complex *N*-acetyl-L-leucine-polyethylenimine (*N*-Ac-L-Leu-PEI). The complex was found to be biocompatible and was used to deliver the p53 gene to mitigate the proliferation of tumor cells (Li *et al.*, 2015).

A similar complex was used by Xing *et al.* as a carrier for the delivery of a DNAzyme targeting aurora kinase A. Flow cytometry and confocal laser scanning microscopy were employed to confirm the presence of DNAzymes in the cell and to assess the intracellular distribution of the DNAzyme. It was observed that cells could internalize the nanoparticle

complex in an energy-dependent manner. However, the process of internalization differed from the previous study as it used the clathrin-mediated pathway rather than the endosomal approach. Also the concentration of DNAzyme in the cytoplasm was found to be high. As the aim of the study was to inhibit aurora kinase A, its level was observed to be downregulated. Inhibition in the proliferation of cells was also demonstrated, which could possibly be due to the activation of apoptosis and cell cycle arrest. Wound healing and Transwell migration assay were employed to evaluate if the transfection by DNAzymes is able to hamper cell migration. All the results demonstrated that *N*-Ac-L-Leu-PEI could successfully mediate the DNAzyme delivery and downregulate the expression level of aurora kinase A, triggering a significant inhibitory effect of excessive proliferation and migration of tumor cells (Xing *et al.*, 2015).

In addition to the modification of the polymers by adding unique groups, variations in terms of adding specific ligands have also gained the attention of scientists. These ligands are chosen in accordance with their respective receptors. Studies regarding ligand-conjugated nanoparticles have been reported by researchers both at the *in vivo* and *in vitro* level.

Bellocq *et al.* (2003) reported gene delivery using a transferrin-modified cyclodextrin polymer-based system in K562 leukemia cells. Tf is a glycoprotein that is iron bounded and is one of the most widely studied ligands for tumor targeting (Singh, 1999). It is a ligand for the transferrin receptors, whose expression is elevated in cancerous cells. Transferrin-containing iron recognizes and binds to the transferring receptors present on cell surfaces. After the entry of transferrin into the acidic compartment via endocytosis, there is a dip in the level of pH. Due to the drop in pH, iron dissociates from transferring and consequently gets recycled to the cell surface and gets released (Qian *et al.*, 2002). Due to the need of iron, the levels of transferrin receptor (Tf-R) were found to be upregulated in rapidly dividing cells (Thorstensen and Romslo, 1993). Thus the transferrin receptor (Tf-R) is selected as a tumor-targeting ligand for drug delivery systems. In the study conducted by Bellocq *et al.*, nanoparticles were synthesized using transferrin conjugation to target the cancer cells (K562 leukemia cells) that overexpress the transferrin receptor. The nanoparticle was formed by the condensation of a cyclodextrin polycation with nucleic acid. A cyclodextrin-based polymeric delivery vehicle was chosen, keeping in view the systemic delivery of nucleic acids. The formulation of the delivery vehicle included four parts: the nucleic acid of interest, a polycation for condensation of the nucleic acid, adamantane polyethylene glycol (AD-PEG) for particle stabilization, and AD-PEG-Tf for tumor targeting. Cyclodextrinpolycation is a linear cyclodextrin-based polycation whose ends are tailored with imidazoles. It is reported that it was used as the nucleic acid condensing agent (Bellocq *et al.*, 2003). Optimization of the parent cyclodextrin was done in relation to their structure for efficient *in vitro* transfection using adherent cell line PC-3 and suspension cell line K562 leukemia cells. The role of imidazoles was to enhance the efficiency of transfection and as buffers in endosomes (Davis *et al.*, 2004). The results of this study concluded the transferrin-modified nanoparticles to be safe and effective tools for the systemic delivery of nucleic acid therapeutics for metastatic cancer applications.

A similar study by Pun *et al.* (2004) was undertaken at an *in vivo* level that employed transferrin as a tumor-targeting ligand. Linear β cyclodextrin-based

polymers were conjugated with DNAzymes to form nanoparticle-sized "polyplexes." The size of these polyplexes came out to be 50 nm as assessed by dynamic laser scattering. These polyplexes were found to be modified by two molecules, one of them being transferrin. Transferrin as a modifier was used with an aim to augment targeting to those tumor cells that express transferrin receptors. Another form of modification exploited the surface property of adamantane and β cyclodextrin substructure for the formation of inclusion complexes. Adamantane is a crystalline chemical compound which is colorless and has a camphor-like odor. Adamantane was conjugated with polyethylene glycol (PEG), which was then further conjugated with polyplexes. The formation of inclusion bodies by the action of adamantane on β cyclodextrin of the polyplexes resulted in the formation of a sterically stable layer of PEG. The DNAzymes to be incorporated within these polyplexes were fluorescently labeled and were administered to nude tumor-bearing mice. With the help of a fluorescence imaging system, biodistribution and clearance kinetics of DNAzymes could be monitored. In this study, four methods of administration were studied: subcutaneous injection, intravenous bolus, intraperitoneal bolus, and infusion. Results demonstrated that the highest fluorescent signal for a DNAzyme was observed for intravenous and intraperitoneal bolus at the tumor site. Uptake by the tumor cell was observed only for intravenous bolus, while transferrin targeting was required for the delivery into cells (Pun *et al*., 2004).

8.10 Conclusion and future prospects

Challenges faced by scientists in the field of medical biotechnology have been numerous. Drug discovery and drug delivery are not only indispensable but also unavoidable chapters of this field. Playing with the genes has led to the discovery of antisense oligonucleotides (ODNs), siRNA, miRNA, ribozymes, and DNAzymes that work at the genetic level by either silencing genes or knocking out the genes to combat diseases such as cancer, cardiovascular diseases, etc. DNAzymes have outnumbered their counterparts due to their advantages over traditional methods to alleviate the diseased conditions. This has imparted new hope for the targeted gene therapies; however, high demands that have to be met still lie ahead of scientists. This is in the form of delivery of the drugs that are continuously being synthesized by the pharmaceutical companies every year and released into the market. Researchers need to catch the drug delivery systems in order to use them at a clinical level. This requires meticulous planning and simultaneous observation of the loopholes that restrain researchers from introducing the drug delivery vehicles into the market. Although chitosan and PEG-based systems have been proven to be efficient, greater efforts would be required for safety, pharmacokinetics, and the pharmacodynamics front of the drug candidate that will have to be delivered in the body. Though the discovery of DNAzymes along with siRNA or ribozymes has created an easy and an uncomplicated way to reach up to a particular disease, the success of the same exclusively lies in the invention of smooth and approachable delivery vehicles and their prudent execution.

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Pharmacokinetics and biodistribution of the nanoparticles

9

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9.1 Introduction

9.1.1 Nanotechnology

Nanotechnology is a rapidly emerging area of medical research which focuses on the development of materials in the size range of 5−200 nm (Faraji and Wipf, 2009). The global market for nanomedicine is growing day by day. The Organization of Cooperation and Development estimates that by the end of 2016, the worldwide nanotechnology-related market will reach up to US $1 trillion (Shono *et al.,* 2012). The application of nanotechnology in drug delivery is widely accepted, and numerous formulations have now been approved by various federal agencies (Raza *et al.,* 2014). There are varieties of biological molecules possessing a low therapeutic index and posing challenges for formulation scientists. In an endeavor to overcome these problems, nanotechnology-based nanosized formulations are regarded as the better and safer alternatives over conventional formulations (Onyuksel *et al.,* 2005; De Villiers *et al.,* 2008; Farokhzad and Langer, 2009). Nanotechnology-based approaches not only increase the surface area of drugs, but also modulate the physiochemical properties of the active pharmaceutical ingredients. The overall effect is reflected in enhanced efficacy, better dose management, and also reduced dose (Katare *et al.,* 2010). These drug delivery carriers generally employ novel materials and approaches, which might result in better outcomes. However, the pharmacokinetic profile of all these nanocarriers is a function of size and composition and cannot be generalized, due to the diverse nature of materials employed for these nanoconstructs. The case with the toxicological profile of nanoparticulate carriers is similar, as nanotechnological treatments not only alter the dimensions, but also transform the drugs, resulting in unpredictable outcomes (Elsaesser and Howard, 2012).

9.1.2 Nanoparticles in modern drug delivery

In 1905, Nobel laureate Sir Paul Ehlrich envisioned the drug molecules as "magic bullets" to hit the specific target sites with an aim to attain absolute efficacy and safety. This objective, however, could not be achieved but directed the way to an alternate approach. This approach turned the concept of "magic bullets" to "magic guns,"

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i.e., drug delivery systems. These nanocarrier system-based delivery of molecules attempts to deliver the drugs to specific receptor sites, without affecting the normal tissues and organs of the body (Katare *et al.,* 2010; Raza *et al.,* 2014). These carriers are colloidal, solid, and rounded particles which have dimensions below 100 nm and are fabricated from natural, semi-synthetic, or synthetic polymers (Kumar *et al.,* 2016). Nanoparticles have been successfully employed for cancer therapeutics, antimicrobial actions, vaccine delivery, gene delivery, and site-specific targeting of a variety of drugs (Kaur and Saini, 2000, 2008; Kakkar *et al.,* 2011; Raza *et al.,* 2013, 2013a, 2013b, 2013c). Many anticancer drugs like paclitaxel, docetaxel, doxorubicin, carboplatin, and etoposide have been successfully delivered by means of nanoparticulate carriers (Parveen *et al.,* 2012). The smaller size (in nm) range helps in the easy penetration of nanoparticles in various cells, especially cancer cells, due to the enhanced permeability and retention effect (Petros and DeSimone, 2010). Varieties of nanoparticles are employed for the delivery of various drugs. The examples include liposomes, niosomes, solid lipid nanoparticles (SLNs), polymeric nanoparticles, nanolipidic carriers (NLCs), dendrimers, resealed erythrocytes, pharmacosomes, phytosomes, transfersomes, ethosomes, invasomes, carbon nanotubes, graphenes, and C_{60}-fullerenes (Mishra *et al.,* 2010). Nanoparticles composed of phospholipids, biologically derived lipids, natural polymers, and strategically designed biodegradable polymers like poly lactic-co-glycolic acid (PLGA) are regarded as biocompatible and devoid of untoward effects. On the contrary, nanoparticles composed of inorganic materials like metallic nanoparticles and carbon-based drug delivery systems are regarded as toxic to the biological systems. Even systematically designed dendrimers are also not regarded as safe. However, a few systematic studies have been reported which discuss the biological safety of such carriers (Raza *et al.,* 2015). Pictorial representation of a few selected nanocarriers is given in Fig. 9.1.

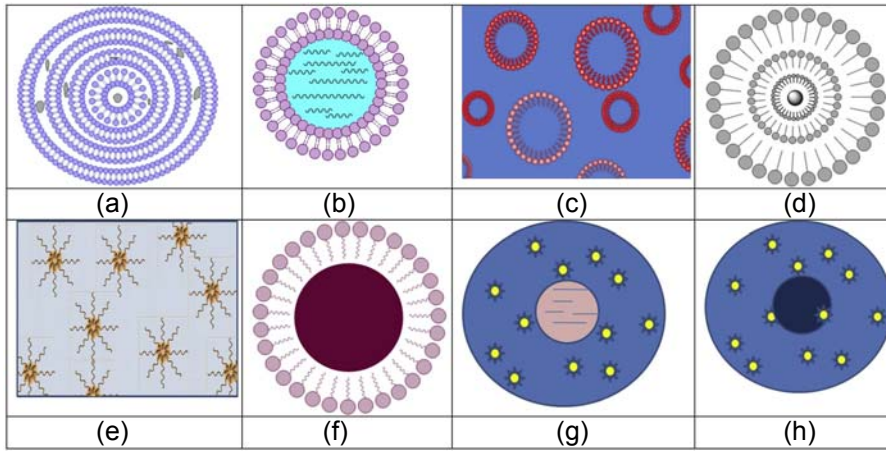

Figure 9.1 Picture of various nanocarriers: (a) liposomes; (b) ethosomes; (c) nanoemulsions; (d) niosomes; (e) organogels; (f) lipospheres; (g) nanolipidic carriers; and (h) solid lipid nanoparticles.

9.1.3 FDA approved nanoparticles

Due to the advantageous features of nanotechnology-based products, over 500 drug products based on this approach have already been catalogued globally, and the number is escalating in an exponential manner. Numerous nanoparticles have now been marketed and approved by various regulatory agencies. A list of a few US-FDA approved nanoparticulate systems has been presented as Table 9.1 (Gu *et al.*, 2007; Bawa, 2008; Barenholz, 2012). The systems not only belong to the anticancer category but cover a diverse domain of anti-HIV drugs, antimicrobials, vaccines, lipid modifiers, hormones, and drugs for multiple sclerosis. In medicine, the use of nanoparticles is increasing, but in the cosmetic industry, its applications are being frequently explored.

9.1.4 Basic pharmacokinetic considerations

The term pharmacokinetics was first introduced by F.H. Dost in 1953. It consists of two words: "*Pharmakon*" and "*Kinetics*." *Pharmakon* is a Greek word, which means "drugs and poisons," and *Kinetics* is defined as "change of one or more variables as a function of time" (Wagner, 1981; Gladtke, 1988). Pharmacokinetics can be understood as the science dealing with what the body does to a drug. It is the study of absorption, distribution, biotransformation, and elimination of drugs in men and animals (Benet and Zia-Amirhosseini, 1995). It finds its application in dose adjustment, *in vitro—in vivo* correction, bioavailability/bioequivalency determination, toxicity and studies, and also for the study of drug interaction. Torsten Teorell is known as the "father of pharmacokinetics" due to his valuable contribution in the area of pharmacokinetics (Paalzow, 1995).

Commonly employed pharmacokinetic approaches

The primary objective of pharmacokinetic modeling is to identify key properties of a drug *in vivo*, which allow the characterization and prediction of the course of time of a drug under studied physiological and pathological conditions (intensity and duration) (Breimer and Danhof, 1997). Pharmacokinetic models may be considered either empirical or explicative. Empirical models are purely mathematical descriptions of the course of time of a drug concentration in a sample of biological tissue or fluid. Explicative models incorporate physiological hypotheses about pharmacokinetic behavior; the classical compartmental models have been considered as explicative since they require compartments for absorption, distribution, and elimination (Balant and Gex-Fabry, 1990; Colburn, 1988). Commonly employed modeling approaches in pharmacokinetics will be briefed in the subsequent section.

Compartmental approach

This approach is one of the most frequently exploited methodologies in pharmacokinetic studies. A compartment is not a real physiologic or anatomic region but is considered as a tissue or group of tissues that have similar blood flow and drug affinity.

Table 9.1 List of FDA approved nanotechnology-based drug products

Product name	Drug nanoparticle system	Delivery route	Company name	FDA approved indication	FDA approval date
Diprivan	Propofol liposomes	Intravenous	Zeneca Pharma	Anesthetic	October, 1989
Adagen	PEGylated adenosine deaminase	Intravenous	Enzon	Enzyme replacement therapy for patients with severe immunodeficiency disease	March, 1990
Abelcet	Amphotericin B phospholipid complex	Intravenous	Enzon	Invasive fungal infection in patients who are intolerant of conventional Amphotericin B therapy	November, 1995
Doxil Caelyx (outside the US)	PEGylated doxorubicin HCl liposomes	Intravenous	Ortho Biotech Schering-Plough	Metastatic ovarian cancer and AIDS-related Kaposi sarcoma	November, 1995
DaunoXome	Encapsulated daunorubicin citrate liposomes	Intravenous	Gilead Sciences	Advanced HIV-related Kaposi sarcoma	April, 1996
Amphotec	Colloidal suspension of lipid-based Amphotericin B	Subcutaneous	Sequus	Invasive aspergillosis patients who are refractory to conventional Amphotericin B	November, 1996
Copaxone	Glatiramer acetate (copolymer L-glutamic acid, L-alanine, L-tyrosine, and L-lysine)	Subcutaneous	TEVA	Relapsing remitting multiple sclerosis	December, 1996
AmBisome	Amphotericin B liposomes	Intravenous	Gilead Sciences	Fungal infection	August, 1997

DepoCyt	Sustained release cytarbin liposomes	Intravenous	Skye Pharma Enzon	Lymphomatous meningitis	April, 1999
Estrasorb	Estradiol Hemihydrate miceller nanoparticle	Transdermal	Novamax	Reduction of vasomotor symptoms, such as hot flushes and night sweats, in menopausal women	October, 2003
Macugen	PEGylated anti-VEGF aptamer	Intravitreal	OSI Pharmaceuticals Pfizer	Neovascular age-related macular degeneration	December, 2004
Abraxane	Paclitaxel bound albumin nanoparticle	Intravenous	Abraxis Bioscience Astra Zeneca	Metastatic breast cancer patients who have failed the combination therapy	January, 2005
Renagel	Cross-linked poly(allylamine) resin (sevelamer hydrochloride)	Oral tablets	Genzyme	Control of serum phosphorous in patients with chronic kidney disease on dialysis	May, 2005
Triglide	Nanocrystalline fenofibrate	Oral tablets	Skye Pharma First Horizon	Lipid disorder; markedly reduces elevated plasma concentration of triglycerides, LDL, and total cholesterol and raises abnormally low level of HDL	May, 2005
Elestrin	Estradiol gel (0.06%) incorporated in calcium phosphate nanoparticles	Transdermal	BioSante	Treatment of moderate to severe hot flashes in menopausal women	December, 2006

VEGF, vascular endothelial growth factor.

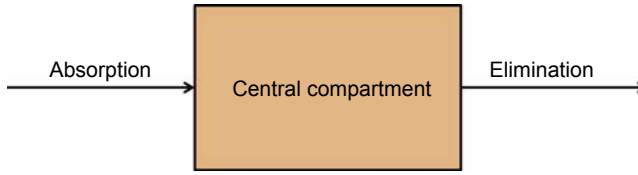

Figure 9.2 Pictorial representation of a one-compartment open body model with absorption.

Within each compartment, the drug is considered to be uniformly distributed. Mixing of the drug within a compartment is rapid and homogeneous and is considered to be "well stirred," so that the drug concentration represents an average concentration, and each drug molecule has an equal probability of leaving the compartment. Rate constants are used to represent the overall rate processes of drug entry into and exit from the compartment (Shargel *et al.,* 2007). This approach depicts the body as an entity, which is divided into one or more compartments. From the site of administration, the drug enters the central compartment (process of absorption), from where it is exchanged with the peripheral compartments (distribution) and also irreversibly removed from the central compartment (metabolism and elimination). Fig. 9.2 shows the schematic representation of a classical one-compartment open body model.

Noncompartmental approach

Noncompartmental pharmacokinetic modeling is less structured than classical modeling methodologies (Veng-Pedersen, 2001). Noncompartmental methods develop methods of estimating pharmacokinetic parameters that do not require the tedious and somewhat subjective method of nonlinear regression. Such methods are more readily automated, so that minimal decision-making and intervention are required from the user. For example, it is easier to automate the estimation of area under the curve (AUC), area under the first moment curve (AUMC), and λ_z [and, consequently, Cl, V_z, mean residence time (MRT), Vss, and F] than to automate the identification and parameter estimation process of compartmental modeling, though attempts have been made, for example, AUTOAN (Gillespie, 1991).

Pharmacokinetic formulae at a glance

Various empirical formulae are employed to determine a variety of pharmacokinetic parameters along with the course of time of a drug in a biological system. A compilation of the same has been presented in Table 9.2. The abbreviations employed should be read as follows: C, plasma drug concentration; C_0, plasma drug concentration a $t = 0$; C*, the last studied concentration; K, first order elimination rate constant; K_0, zero order administration rate constant; K_a, first order absorption rate constant; F and f_t, fraction drug absorbed; V, volume of distribution; C_t, plasma drug concentration at a given moment of time; $X_{u,t}$, unchanged drug excreted at time t; $X_{u,\infty}$, unchanged drug excreted at ∞ time; and dX_u/dt, rate of drug elimination (Gillespie, 1991; Veng-Pedersen, 2001; Shargel *et al.,* 2007).

Table 9.2 Various equations employed to determine various pharmacokinetic parameters

S. No.	Pharmacokinetic model/parameter	Formula
Compartmental approach		
1.	1 CBM (IV bolus)	$C = C_0 e^{-Kt}$
2.	1 CBM (IV infusion)	$C = \frac{k_0}{kv}[1 - e^{-kt}]$
3.	1 CBM (oral)	$C = \frac{k_a F X_0}{(k_a - k)v}[e^{-kt} - e^{-k_a t}]$
4.	1 CBM (oral Wagner–Nelson method)	$1 - f_t = 1 - \frac{C_t + k[AUC]_0^t}{k[AUC]_0^\infty}$
5.	1 CBM (oral modified Wagner–Nelson method)	$f_t = \frac{C_t + k[AUC]_0^t}{k[AUC]_0^{t*}}$
6.	1 CBM (preoral urinary treatment)	$f_t = \frac{(dX_u/dt) + k(X_u)_t}{k[X_u]_\infty}$
Noncompartmental approach		
7.	Area under curve	$[AUC]_0^t = \frac{1}{2}[C_1 + C_2][t_2 - t_1]$
8.	Area under curve	$[AUC]_0^\infty = [AUC]_0^t + \frac{C^*}{k}$
9.	Area under first moment curve	$[AUMC]_0^t = \frac{1}{2}[C_1 t_1 + C_2 t_2][t_2 - t_1]$
10.	Area under first moment curve	$[AUMC]_0^\infty = [AUMC]_0^t + \frac{C^* t^*}{k} + \frac{C^*}{k_2}$
11.	Mean residence time (MRT) (for instantaneous route)	$MRT = \frac{[AUMC]_0^\infty}{[AUC]_0^\infty}$
12.	Mean transit time	$MTT = MAT + MRT$
13.	Mean absorption time (for noninstantaneous route)	$MAT = [MRT]_{\mathrm{ORAL}} - [MRT]_{IV}$ OR $MAT = MTT - MRT$
14.	Mean residence number	$MRN = MRT/MTT$

9.2 Pharmacokinetics of nanoparticles

Nanoparticles have been exclusively explored for their advantages since 2005, especially in the cosmetics, beverage, and pharmaceutical industry. To understand the behavior and dose regimens of nanoparticles loaded with drug(s), it is compulsory to measure the drug concentration in the different organs. It is also a mandate for the determination of various pharmacokinetic parameters like C_{max}, AUC, Cl, $t_{1/2}$, and MRT. Pharmacokinetics of nanoparticle-loaded drug(s) is generally concerned with four components: absorption, biodistribution, metabolism, and elimination (Owens and Peppas, 2006; Li and Huang, 2008).

9.2.1 Absorption of nanoparticles

Absorption is the process by which a drug enters the bloodstream from the site of administration. The absorption of nanoparticles has been studied from various routes like oral, dermal, pulmonary, and nasal (Li and Huang, 2008), and this is discussed in the subsequent section.

Oral adsorption

Nanoparticles possess two possibilities after administration in the gastro intestinal tract (GIT): firstly, they get absorbed into the system, and secondly, they get cleared through the feces or other routes (Lai *et al.,* 2009). Two major barriers have been reported for the absorption of nanoparticulate-encapsulated drugs, viz., the epithelium and the mucous of GIT. The mucous layer acts as a barrier for nanoparticles and renews itself continuously. Various scientific studies report that nanoparticles with a size between 50 nm and 200 μm are absorbed via Peyer's patches in the small intestine (Des Rieux *et al.,* 2006). Nanoparticles are also reported to be absorbed by means of intestinal enterocytes (Kohli and Alpar, 2004). In general, nanoparticles get bioadhered with gastrointestinal mucosa and enhance the chances of drug absorption (Desai *et al.,* 2012). Nanoparticles like spontaneous emulsifying systems (SES) promote lymphatic absorption and are well suited for drugs which undergo extensive first-pass metabolism (Dixit and Nagarsenker, 2008). Analogous to SES, SLNs, and NLCs also inherit the property of absorption from the lymphatic system, circumventing the pre-systemic metabolism. Despite this, SLNs/NLCs are also believed to be superior to SES for the sustenance of therapeutic levels of drugs (Müller *et al.,* 2008; Desai *et al.,* 2012). Strategic fabrication of polymeric nanoparticles can fetch the desired benefits. For instance, nanoparticles composed of acid-soluble Eudragit E100 polymer possess the capability of enhancing the absorption of the drug from gastric mucosa, even in substantially higher amounts than that from cyclodexrin complexes (Khachane and Nagarsenker, 2011).

Percutaneous absorption

Nanoparticle interaction with the skin has now been established and well studied (Raza *et al.,* 2013a). Nanoparticles are absorbed in the skin by means of the lymphatic system and lymph nodes (Li *et al.,* 2010). A diagrammatic representation of nanoparticle absorption via the skin has been shown in Fig. 9.3. Nanoparticles comprised of

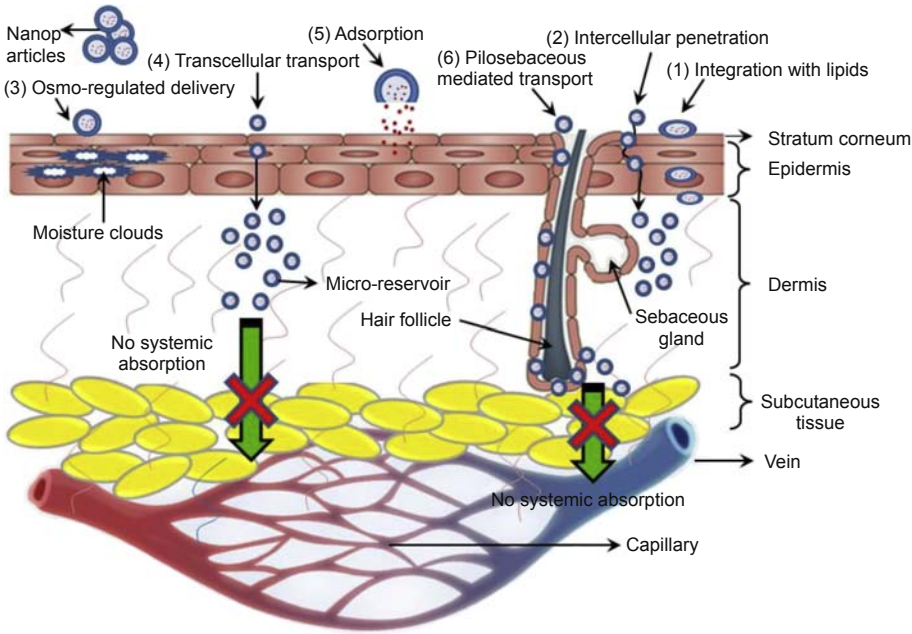

Figure 9.3 Pictorial representation of nanoparticle absorption from the skin.
Adopted from Raza K, Kumar M, Kumar P, Malik R, Sharma G, Kaur M, Katare O. Topical delivery of aceclofenac: challenges and promises of novel drug delivery systems. Biomed Res Int 2014;2014, 406731 11 pages.

biocompatible components like phospholipids and PLGA are known to integrate with the lipids of skin, whereas nanoparticles, especially liposomes with a size smaller than 600 nm, are believed to penetrate the skin, as such (Schramlova *et al.,* 1996; Katare *et al.,* 2010). Lipid nanoparticles like SLNs and NLCs with a particle size smaller than 200 nm form a monolayer on the skin and prevent the loss of moisture from the skin surface. This results in the loosening of packing of corneocytes and facilitates deeper drug penetration (Wissing and Müller, 2003; Desai *et al.,* 2010). Polystyrene nanoparticles with the size of approximately 20 nm have been reported to accumulate in deeper follicular regions, where particles up to 200 nm have been reported to show time-dependent follicular penetration (Alvarez-Román *et al.,* 2004). A group of scientists believe that intact PLGA nanoparticles cannot cross *stratum corneum*, whereas (polylactic acid) PLA-based nanoparticles employ hair follicles and sebaceous glands for percutaneous penetration (Stracke *et al.,* 2006; Luengo *et al.,* 2006; Redhead *et al.,* 2001). Magnetic nanoparticles <10 nm can passively penetrate the skin layers up to the *stratum granulosum* (Baroli *et al.,* 2007).

Pulmonary absorption

Nanoparticles inhaled through the lungs in this type of exposure generally face two competitive processes, viz., nonabsorptive clearance and absorption (Yang *et al.,* 2008). The larger surface area of alveoli is advantageous for nanoparticle absorption,

which may follow endocytosis. The nanoparticles absorbed from alveoli have easy access to the blood and lymph (Oberdörster *et al.,* 2005; Li *et al.,* 2010). However, the nanoparticles retained to the upper respiratory tract (tracheobronchial regions) have the probability to be removed by the mucocilliary movements. Particles with a size ranging between 1 μm and 5 μm are deposited in the bronchioles and smaller airways, whereas particles smaller than 500 nm are deposited in alveoli. As a general rule, particles in the size range of 5−10 μm have access to primary bronchi, 1−5 μm reach secondary bronchi, 1−3 μm are sediment in bronchioles, 500 nm−1 μm can reach alveoli, and smaller than 500 nm are exhaled in the expired air (Zhang and Monteiro-Riviere, 2008).

Nasal absorption

Various animal studies have reported that nanoparticles deposited in the olfactory region have easy access to the central nervous system (Oberdörster *et al.,* 2005). This route is frequently employed to cross blood−brain barrier (BBB). However, the data available is generally of animal origin, and the significant difference in the physiochemical and biological nature of animal and human nasal mucosa demands appropriate human studies.

Injections

Various injectable routes, such as intraperitonaeal, intramuscular, intradermal, and subcutaneous routes, are employed for the delivery of nanoparticles. Absorption from these sites is a prerequisite for execution of drug response. As depicted in Fig. 9.4, various mechanisms operate, which results in the absorption of nanoparticles. The role of regional lymph nodes, macrophages, and dendritic cells has been reported for the absorption of nanoparticles from these sites (Wilczewska *et al.,* 2012).

9.2.2 Biodistribution of nanoparticles

Numerous studies advocate the successful biodistribution of nanoparticles to various organs and tissues after administration from various routes. Physicochemical,

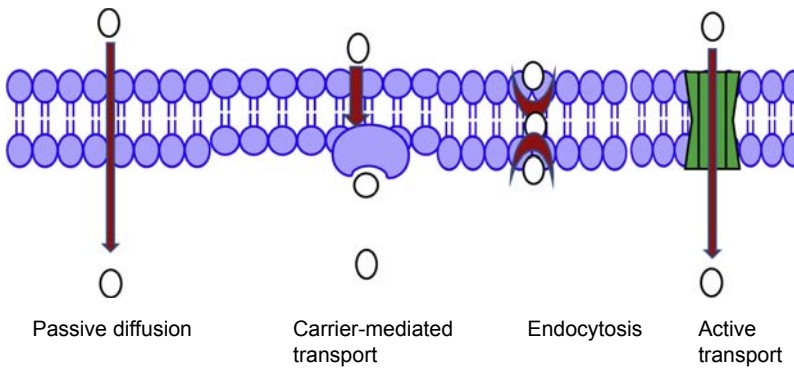

Figure 9.4 Pictorial representation of various mechanisms of drug absorption.

micromeritic, electric, and surface properties of nanoparticles, along with the interactions with the biological components, decide the distribution pattern from the site of administration (Owens and Peppas, 2006; Almeida *et al.,* 2011). The following section will discuss the generalized biodistribution patterns based on certain parameters.

Composition

In an interesting study, researchers employed radiolabeling to trace the pattern of biodistribution of nanoparticles of different compositions and formulations (Douglas *et al.,* 1986). Mesoporous silica nanoparticles were found to offer a higher affinity toward the lungs, while the polymeric nanoparticles were found to be more prone to liver metabolism. The clearance pattern was entirely different for all the selected systems, indicating the dependence on the interaction and solubility. Hence the make, size, and interaction of the nanoparticles have significant impact on the biodistribution (Sa *et al.,* 2012).

Size and morphology

For better and prolonged biodistribution, the lower limit of nanoparticle size is believed to be 5.5 nm, i.e., the cut-off size for renal filtration (Ernsting *et al.,* 2013). However, the renal filtration cut-off size is believed to be 10 nm by some researchers (Choi *et al.,* 2011). To bypass liver hepatocytes, the size of the nanoparticle should be below 50 nm, as the larger particles are rapidly taken by the liver and spleen, resulting in shorter blood circulation time (Yuan *et al.,* 1995; Ernsting *et al.,* 2013; Almeida *et al.,* 2011). However, the smaller size is not always desired, as they possess higher circulation time, but rarely retained at the desired site. In a study, the researchers employed liposomes of varied sizes and observed that the better retention in the desired site after i.v. injection was offered by the population in the size range of 100−200 nm. Liposomes below 50 nm and above 300 nm were not able to have access to the desired site (Moghimi *et al.,* 2001). Similar findings of optimal size range have also been reported by Hrkach *et al.,* where the research group investigated polymeric nanoparticles in the range of 28−224 nm. The lead formulation selected by the group possessed the particle size of 100 nm, which is the generally accepted diameter for better retention and lower clearance (Li and Huang, 2008; Hohnholt and Dringen, 2013; Ernsting *et al.,* 2013). In general, size range decides the protein adsorption on the nanoparticles and ultimately the clearance by the mononuclear phagocytic system (MPS). The larger the particle size, the higher the protein adsorption; however, PEGylation can also reduce protein adsorption onto the nanoparticles (Alexis *et al.,* 2008; Ernsting *et al.,* 2013; Fang *et al.,* 2006).

Morphology also exhibits its impact on the biodistribution of nanoparticles. In general, any deviation from the spherical shape enhances the circulation time of the nanoparticles (Ernsting *et al.,* 2013). In a study, the researchers employed filamentous micelles (filomicelles) and PEGylated stealth vesicles and reported that the filomicelles were circulated up to a week, while PEGylated spherical vesicles were cleared in 2 days (Ernsting *et al.,* 2013; Geng *et al.,* 2007).

Surface charge

Scientists have explored the relationship between the isoelectric point (pI) of proteins and the zeta potential of nanoparticles for the adsorption phenomenon. Positively charged nanoparticles generally adsorb proteins with $pI < 5.5$, while proteins with $pI > 5.5$ get adsorbed to negatively charged nanoparticles (Gessner *et al.,* 2002, 2003; Ernsting *et al.,* 2013).

Coating effect

Nanoparticles are generally coated with polymers like polyethylene glycol (PEG), polyethylene oxide, dextran, polysorbates, and starch and small molecules like citrate (Alexis *et al.,* 2008; Zhang *et al.,* 2009; Almeida *et al.,* 2011; Ernsting *et al.,* 2013). Scientific studies reveal that the coating of such materials generally enhances the biodistribution (Hsu *et al.,* 2014). PEG and polysorbate 80 are the widely employed coating materials used to enhance the circulation time of nanoparticles (Gaucher *et al.,* 2009). It has been reported that apolipoprotein E substantially binds to the polysorbate 80 of the coated nanoparticles and is involved in the transport of polysorbate-coated nanocarriers. Additionally, polysorbate 80 is a Pgp inhibitor, and its coating on nanocarriers loaded with drugs like doxorubicin is beneficial (Alexis *et al.,* 2008; Bartlett *et al.,* 2007). PEG of mass range 10 kDa is believed to be a better coating material, out of a variety of PEGs, due to lower protein adsorption tendencies (Ernsting *et al.,* 2013).

9.2.3 Metabolism of nanoparticles

Drug metabolism mainly occurs in the liver (Remmer, 1970). Nanoparticle composition and surface properties decide the metabolism pattern of the nanoparticles. The majority of drugs have to be transformed into polar metabolites before excretion using cytochrome P450 and other metabolic pathways (Watkins, 1992; Klotz, 2009).

Biodegradable nanoparticles composed of lactic acid and glycolic acid (PLGA) are relatively easily metabolized, and the degraded products are used in biological cycles like the Krebs cycle (Mahmoudi *et al.,* 2011). Other nanoparticles like silver, gold, iron oxide, quantum dots, carbon, and silica are quite stable and difficult to be metabolized in the whole system; they persist in the human body for long time periods. One scientific study reported that quantum dot nanoparticles reside in the human body for 2 years (Li *et al.,* 2010). Iron oxide nanoparticles have been reported to be metabolized by the astrocytes in the brain (Hohnholt and Dringen, 2013).

9.2.4 Elimination of nanoparticles

Orally administered drugs/nanoparticles are first metabolized and then eliminated from the body either via urine or feces (Sadauskas *et al.,* 2009). However, renal excretion is the major route of elimination for most of the exogenous materials. Renal excretion is a complex process comprised of glomerular filtration and tubular secretion, but excluding tubular reabsorption, as depicted in Fig. 9.5 (Choi *et al.,* 2007). Tubular secretion is mainly studied as two types: organic acid transport and organic base

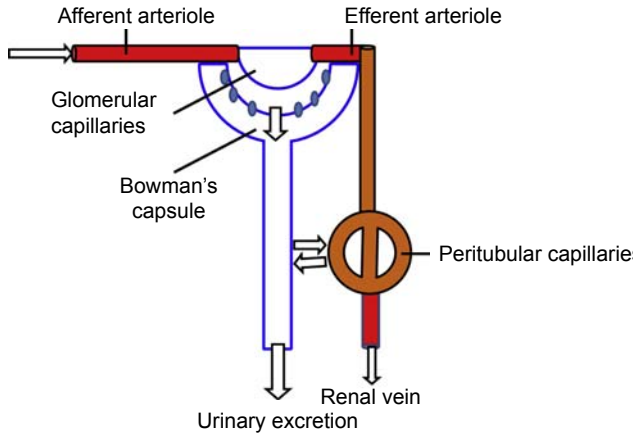

Figure 9.5 Diagrammatic representation of the urinary excretion process.

transport. Penicillin and salicylates are secreted by an organic acid transporter. Uric acid is also absorbed through this transporter. Organic thiazides, quinine, and procainamide are secreted by organic base transporters. Both of these transport mechanisms are bidirectional in nature (Shargel *et al.,* 2007).

There are various factors affecting the clearance, such as physiochemical properties of the drug/nanoparticle, its distribution and binding characteristics, plasma concentration, urine pH, biological factors, diseased state, drug interactions, and blood flow to the kidneys. Particle size, lipid solubility, and pk_a are the main physiochemical parameters affecting renal excretion. Small particles (Ø $<$ 5.5 nm) are easily filtered from the glomerulus and hence are easily excreted. If a drug/nanoparticle is not bound to a plasma protein, then it is easily excreted. A linear relationship between the rate of excretion and plasma drug concentration of a particular nanoparticle/drug generally holds well (Alexis *et al.,* 2008).

9.2.5 Factors affecting pharmacokinetics

Numerous factors are responsible for the alteration in the pharmacokinetic behavior of the molecules/nanoparticles (Shargel *et al.,* 2007). A few have been summarized in the following section.

Size

The nanoparticle with a size range lower than 10 nm (5.5 nm, more precisely) can get easily filtered through renal filtration or through extravasation, while larger nanoparticles may have a higher tendency to be cleared by cells of the MPS (Shargel *et al.,* 2007; Swami *et al.,* 2012). Nanoparticles with a diameter of approximately 100 nm have a potential for prolonged blood circulation and a relatively low rate of MPS uptake (Li and Huang, 2008). Liu *et al.* (1992) injected the radioisotope-labeled liposomes intravenously into the mice, examined the recovered dose in those tissues

4 h later, and reported the biodistribution of liposomes of different sizes (30−400 nm) in the blood, liver, spleen, and tumor. After investigation, it was found that approximately 60% of the injected liposomes between 100 and 200 nm were detected in the blood. While the nanoparticles with a size above 250 nm or below 50 nm constituted only 20% of the plasma population. The liver uptake of liposomes with approximately 100 nm was observed to be 20% for particles above 250 nm. Particles below 50 nm were found to accumulate more in the liver. The size of these particles were smaller than the pore size of liver fenestrae (100 nm), hence it was concluded that they got easy access through the endothelial walls, resulting in an enhanced liver uptake. For spleen uptake, liposomes less than 100 nm in size show minimal spleen uptake, whereas an increase in particle size (400 nm) resulted in an increase in the rate of spleen uptake (40−50% after 4 h of injection). The tumor uptake of liposomes of approximately 100 nm was four folds higher compared to liposomes greater than 300 nm or less than 50 nm (Alexis *et al.,* 2008; Ernsting *et al.,* 2013).

Shape

Particle shape is also a major parameter that can impact circulation time, intravascular transport, binding, and accumulation of nanoparticles at the desired site (Alexis *et al.,* 2008; Ernsting *et al.,* 2013). Champion and Mitragotri (2006) investigated the interaction of diversely shaped microsized polystyrene particles with *macrophages*. They defined all things with the help of Ω, a dimensionless shape-dependent parameter related to the length-normalized curvature. After investigation, it was found that the particles with $\Omega \leq 45$ degrees (ellipsoid or sphere) were internalized successfully via actin-cup and ring formation, with phagocytosis velocity inversely correlated to Ω (up to 45 degrees); on the other side, when $\Omega > 45$ degrees (ellipsoid), cell spreading was observed, but internalization was not reported (Champion and Mitragotri, 2009).

Surface charge

Surface charge on nanoparticles is generally defined in the terms of zeta potential. For nanoparticles, it has been successfully correlated with pharmacokinetics and MPS uptake (Alexis *et al.,* 2008; Swami *et al.,* 2012; He *et al.,* 2010; Xiao *et al.,* 2011). It has been reported that the negatively charged nanoparticles ($\xi_0 \leq 10$ mV) show higher MPS uptake and positively charged ($\xi_0 > 10$ mV) generate a higher immune response. Neutrally charged particles ($\xi \pm 10$ mV) have been linked with the lowest MPS uptake and prolonged circulation time (Ernsting *et al.,* 2013). In a study, it was found that the nanoparticles exhibiting $\xi_0 \leq 40$ mV offered >90% clearance in 10 min, while neutral particles ($\xi_0 \pm 10$ mV) exhibited <10% clearance in 10 min (Levchenko *et al.,* 2002).

Coating and surface engineering effects

Surface coating and surface modification are pharmacokinetic influencing parameters for nanoparticles. To prolong the circulation time and reduce opsonization, hydrophilic coating is done (Alexis *et al.,* 2008; Ernsting *et al.,* 2013). PEG polymers are FDA

approved and possess low toxicity and immunoneutrality; hence they are being frequently employed for this purpose. Sadzuka *et al.* (1998) demonstrated that the PEGylated liposome-loaded drug offered three-fold reduction in MPS uptake, six-fold higher area under the curve, and three-fold enhanced tumor uptake. The surface modification of nanoparticles with poloxamine 908 (a tetrafunctional ethylenediamine block copolymer) and poloxamer are also believed to enhance the circulation time and reduce the instances of MPS uptake (Moghimi *et al.,* 2012). Redhead *et al.* (2001) demonstrated that the nanoparticles with a surface modification by virtue of poloxamer 407 and poloxamine 908 offered reduced Kupffer cell uptake (Liu *et al.,* 2008).

9.3 Generalized pharmacokinetic profile of various nanoparticles

9.3.1 Metallic nanoparticles

Metallic nanoparticles are being used in the delivery of biological materials, vaccines, and drugs in medical sciences (Lin *et al.,* 2015). Various metals and metallic salts like silver, titanium dioxide, zinc oxide, iron, and gold have been extensively explored for the formation of metallic nanoparticles. Pharmacokinetic assessments of metallic nanoparticles are essential for their safety and biomedical applications. Particle size and their type, surface coating, surface charge, route of administration, protein binding, animal species, and doses are the various factors on which the pharmacokinetics of metallic nanoparticles depend. These nanoparticles have a shorter plasma half-life in rats and mice vis-à-vis rabbits and monkeys. Dermal, inhalation, and oral absorption of these carriers is generally less, but these issues can be resolved using surface tailoring and other appropriate coatings. Metallic nanoparticles have the tendency of significant distribution throughout the body and are reported to remain for months. They get accumulated in the spleen, liver, and lymph nodes. This accumulation is due to the nonspecific uptake by MPS. Metallic nanoparticles with a size <100 nm can easily cross the BBBs, and these can be coated employing neuropeptides. Biliary and renal excretion are very low due to the accumulation in tissues, but renal excretion can be enhanced by altering the surface and coatings (Weissleder *et al.,* 1989). Cho *et al.* employed gold coated with PEG 500 to formulate nanoparticles and reported higher blood circulation time. These nanoparticles remained deposited in the liver and spleen for more than 7 days. The group also reported acute inflammation and apoptosis in the liver (Cho *et al.,* 2009). In another study, Cho *et al.,* used silver and PEG to formulate the silver nanoparticles and reported nanoparticles with a size above 100 nm get easily cleared within 24 h while smaller particles with a size <20 nm were retained for weeks. They also reported that silver nanoparticles (7 nm, 14 nm, and 100 nm) accumulated in the liver and spleen for 6 months and were eliminated via urine and bile (Cho *et al.,* 2010). Balasubramanian *et al.,* also studied the biodistribution of silver nanoparticles and observed that silver nanoparticles were accumulated in the liver and spleen during the entire frame of the study, for at least 2 months. However, the group reported nil accumulation of silver nanoparticles in the brain (Balasubramanian *et al.,* 2010).

9.3.2 Cationic and anionic nanoparticles

Varieties of charged nanoparticles are employed in drug delivery, and many times, charge is also induced by the use of chemicals like dicetyl palmitate or stearyl amine (Jeon *et al.,* 2013; Bilensoy, 2010). Bexiga *et al.* (2011) employed polystyrene to formulate the cationic nanoparticles, which resulted in increased cell permeability of dyes and apoptosis. Lu *et al.* employed PEG-PLA and cationic bovine serum albumin to develop cationic nanoparticles and reported higher tissue uptake by the liver and spleen. They also reported enhanced BBB permeability and decreased bioavailability of 6-coumarin by cationic nanoparticles (Lu *et al.,* 2007). Chertok *et al.* (2009) employed heparin and PEG to formulate the cationic magnetic nanoparticles and reported 11-fold enhanced bioavailability. Anionic nanoparticles have now been established for biomedical applications like detection, diagnosis, and therapy (De *et al.,* 2008). Out of cationic or anionic nanoparticles, cationic nanoparticles are generally preferred for better electrostatic adhesion onto the negatively charged mucin (Aljayyoussi *et al.,* 2012). However, the advantages and disadvantages of the charged nanoparticles should be weighed, as they are reported to induce hemolysis and tissue necrosis (Goodman *et al.,* 2004).

9.3.3 Functionalized/tailored nanoparticles

The tailoring of the nanoparticles is a tool to achieve better drug delivery and desired pharmacokinetics (Hans and Lowman, 2002; Han *et al.,* 2007). Cheng *et al.* employed PEG and polyacrylic acid to formulate the coated functionalized nanoparticles and reported the enhanced blood circulation half-life. The developed systems remained accumulated in the liver and spleen (Cheng *et al.,* 2011). Guo *et al.* (2011) used PEG and PLGA to formulate the functionalized nanoparticles and reported prolonged accumulation and enhanced pharmacokinetics. In general, the tailoring of nanoparticles is performed to get benefits like desired drug loading, bypassing of MPS, tissue targeting, and prolonged circulation (Radad *et al.,* 2012).

9.3.4 Targeted/tagged nanoparticles

Nanoparticle-targeted delivery is a disease-specific and target-oriented technology to deliver the drug efficiently to the desired site (Olivier, 2005). Milane *et al.* (2011) employed PLGA, PEG, estimated glomerular filtration rate (EGFR) peptide, and n poly (ε-caprolactone) to formulate targeted lonidamine/paclitaxel nanoparticles and reported superior pharmacokinetic profile vis-à-vis the marketed formulations. Löw *et al.* (2011) employed human serum albumin and EGFR peptide to formulate the cetuximab nanoparticles and reported the intracellular accumulation of targeted nanoparticles. Various organic molecules like folic acid, nicotinamide, and estrogen are employed to tag the nanoparticles for facilitated transport to the desired site (Torchilin, 2000).

9.4 Challenges and future prospects

A modification in the size of the drug carrier offers various benefits, but also poses challenges, especially when human health is a concern. Conventional pharmacokinetic treatment of drug, loaded onto nanocarriers, cannot serve the purpose, as the nanomaterial itself has been introduced in the biological system. There is a need of holistic research which will take all the factors in consideration. The nanocarriers available are of varied varieties, starting from biocompatible PLGA and phospholipids to complex inorganic nanoparticles. Therefore there is a need for systematic research, not only at the preclinical level, but at clinical levels too, as the species difference in pharmacokinetics is well understood. However, various nanocarrier-based nanomedicines have emerged in the market after rigorous scrutiny from stringent federal agencies. These products are better and safer than the erstwhile conventional counterparts, supporting the hypothesis of nanomedicine. It provides a ray of hope by which the present therapies can be transformed from good to best. Still, various challenges like cost factor, limited targeting, and drug release are nightmares for nanoparticle-based therapies. Various drug delivery groups are working on these challenges, and we expect further fine tuning of the processes and the materials. The knowledge on pharmacokinetics of nanoparticles is dispersed and generally concerns the drug, not the nanoparticles as a whole. There is an immense need for pharmacokinetic studies which not only focus on the drug, but also take into consideration the material of the nanoparticle. This will result in a better understanding of the fate of nanoparticles in general and the fate of nanomedicine specifically.

9.5 Conclusions

Various physiochemical parameters like composition, shape, size, and surface charge are the major factors affecting the pharmacokinetics and biodistribution of nanoparticles. The range of nanoparticles is quite large, hence generalization for one category may not fit into the other. However, the effect of surface charge, shape, and size on the pharmacokinetics can be generalized for all the varieties of nanoparticles. The research in the domain of pharmacokinetics of nanoparticles in various preclinical models is seemingly increasing, witnessing a positive indication for the better understanding of what the body does to nanoparticles. Still, there is a scope for further research, especially for the nanoparticles composed of relatively toxic materials and inorganic nanoparticles.

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Nanotoxicology: evaluation of toxicity potential of nanoparticles

10

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10.1 Introduction

Scientific progress and technical innovations are key factors not only to secure the present level of prosperity and raise the competitiveness of national economies, but also for further breakthrough in medicine and for sustainable development. Unintended side effects of technology, such as hazards of new technologies for the natural environment and human health or for our common social life, can influence the balance considerably. The alarming increase in CFCs, the ozone hole, long unrecognized carcinogenic effects of asbestos, and historical catastrophes like Bhopal tragedy or Cherbonyl are well-known examples of environmental toxicity (Durodié, 2002).

Today, the advent of nanotechnology has created much hype and hope. The large surface area to volume ratio of nanoparticles leads to an alteration in biological activity compared to the parent bulk materials. Since 1995, the use of nanoparticles in experimental and clinical fields has risen exponentially due to their wide range of biomedical applications, for example, in drug delivery, imaging, and cell tracking (Cheng *et al*., 2011; Huang *et al*., 2011; Pan *et al*., 2011; Thanh NTK, 2011). Nanoparticles can mimic or alter biological processes (e.g., infection, tissue engineering, de novo synthesis, etc.), and a wide spectrum of nanoscale technologies is beginning to change the scientific landscape in terms of disease diagnosis, treatment, and prevention. The use of nanoparticles as carrier systems for drugs, particularly chemotherapeutic drugs, is gaining in popularity due to the ability to specifically target cancer cells, enhance efficacy, and reduce systemic toxicity. These technological innovations, referred to as nanomedicines by the National Institutes of Health, have the potential to turn molecular discoveries arising from genomics and proteomics into widespread benefits for patients.

The nanomedicinal devices include, but are not limited to, functionalized carbon nanotubes (CNTs), nanomachines (e.g., constructed from interchangeable DNA parts and DNA scaffolds), nanofibers, self-assembling polymeric nanoconstructs, nanomembranes, nanosized silicon chips, biosensors, and laboratory diagnostics. According to data collected by the National Nanotechnology Initiative, the quantity of manufactured nanoscale material is growing significantly every year. The Business Communications Company had projected a $10 billion global demand for nanoscale materials, tools, and devices by 2010 (http://www.nano.gov/). This large increase in demand and production could lead to the enormous exposure of humans and other organisms to nanomaterials/nanoparticles.

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Several nanoparticles are currently being used as a drug delivery system, namely, polymeric biodegradable nanoparticles, metallic nano, ceramic, polymeric micelles, dendrimers, liposomes, etc. These aforementioned nanoparticles also have the ability to act as nucleic acid carriers, for example, DNA-protein nanostructures. The generation of semisynthetic DNA-protein conjugates allows one to combine the unique properties of DNA with an almost unlimited variety of protein components, which have been tailored over billions of years of evolution. Consequently, these can perform highly specific functions, such as enhancing catalytic turnover, and ultimately can aid in other applications such as in energy conversion or translocation of other components. In a study, it was discerned that oligonucleotide—enzyme conjugates (DNA—SN conjugate, DNA-5′-thiolated oligonucleotides, and calf intestine alkaline phosphatase/HRP/beta galactosidase) enhanced kinetic rate constants for hybridization with double-stranded DNA targets. In general, DNA gold nanoparticle conjugates provide an attractive and applicable scaffold for the delivery of nucleic acids. Another simple but highly efficient method for delivering nucleic acids is employed using the magnetofection approach, which is basically done by applying the appropriate magnetic field to concentrate nucleic acids containing particles or DNA to the target cells. However, for cancer therapy, a high field, high gradient, and rare earth permanent magnet is placed above the solid tumor in order to retain administered magnetic nanoparticles with bound nucleic acids *in situ* until they internalize and transfect malignant cells.

So a thorough scientific validation is necessary for the risk assessment of nanomaterials, and screening assays are needed to assess their chemical and physical properties. In this context, nanoparticle toxicity refers to the ability of the particles to adversely affect the normal physiology as well as to directly interrupt the normal structure of organs and tissues of humans and animals. Potential hazards of nanoparticles can be investigated by the basic knowledge on particle toxicity and inhalation toxicity.

One important feature of nanotoxicology is that materials which are not harmful in their bulk form may be toxic on the nanoscale. However, nanoparticles are more reactive that are known to interact with the cells, various biological components such as proteins, thereby activating inflammatory and immunological responses (Oberdorster, 2010).

It is necessary to put the safety and risk evaluation of nanoparticles on a scientific basis. The nanotoxicological classification system (NCS) plays a pivotal role in deciding the toxicity of nanoparticles by their simple yet valuable classification system for cosmetics and pharmaceuticals. In addition, it also has implications for other consumer products (e.g., personal care, nutrition). Basically, it places the nanomaterials in four groups: group I possesses no or very little risk involved, group II and III cover medium risk, and group IV includes potential higher risk nanoparticles.

This classification acts as a guide for toxicological studies related to nanoparticles and focuses first on particles with the potential highest risk, for which the NCS can be used as selection tool.

There are several physiochemical parameters to determine toxicity such as particle size, shape, surface charge and chemistry, composition, degradability, biocompatibility, and subsequent nanoparticle stability. Further particle-related factors, the administered dose, route of administration, and extent of tissue distribution seem to

be important parameters in nanocytotoxicity. Another probable mechanism of nanotoxicity could be oxidative stress and proinflammatory gene activation leading to cytotoxic activity (Hsin *et al*., 2008; Arora *et al*., 2008; Samberg *et al*., 2010).

10.2 Size of nanoparticles

The size of the nanoparticles, an important toxicity-determining parameter, plays a crucial role in the accessibility of particles to cells. This could be attributed to the fact that nanoparticles of size >100−1000 nm have access to only a small number of cells, like macrophages, and are less harmful, whereas nanoparticles <100 nm can be internalized by any cell via endocytosis, that can ultimately pose more harm to the biological system. Besides, biodegradability also plays an important role to toxicity and related undesired effects, as nonbiodegradable particles can stay forever in the body, thus imparting higher risk.

Another important point is the biocompatibility (B) or nonbiocompatibility (NB) of the nanoparticles. A nonbiocompatible surface of a particle belonging to class I can also activate the immune system by opsonin adsorption. Therefore for a full picture, each class needs to be differentiated, resulting in a total of eight classes from I-B, I-NB to IV-B, and IV-NB. The classes can also be shown in the form of a traffic light system: green (I), yellow (II, III), and red (IV), for ease of understanding by politicians and the average consumer (Muller *et al*., 2011).

10.3 Dose-dependent toxicity

Typically, dose can be defined as an effective amount or quantity of substance reaching a biological system. The dose is directly related to exposure or the concentration of the substance in the relevant medium (air, food, water) multiplied by the duration of contact. In order to observe dose-related cellular or tissue toxicity, cell-based toxicity studies use increasing doses of the nanoparticle. Such dose−response correlations are the basis for determining safe limits of particle concentrations for *in vivo* administration. Experimentally, the low dose (10 mg/m^3) exposure to TiO_2 particles (20 nm diameter) resulted in a greater lung tumor incidence than the high dose (250 mg/m^3) exposure of 300 nm diameter particles. Moreover, it is the surface area and not the mass dose that is to be considered for assessing the negative health effects of nanoparticles (Oberdorster *et al*., 1994, 2005; Stoeger *et al*., 2006; Donaldson and Stone, 2003).

The health hazards related to nanoparticle toxicity rely on extrapolating *in vitro* concentration into *in vivo* scenarios to determine organ toxicity with predetermined doses and biochemical changes occurring inside living systems. Because of their shapes and surface areas, they can diffuse, aggregate/agglomerate, and sediment according to their size, density, and physical and chemical properties in solution and require separate *in vitro* assays from soluble chemicals. Therefore traditional *in vitro*

assays may misrepresent the response and cellular-uptake data for nanoparticles, making the test results less comparable across particle types than for soluble chemicals (Teeguarden *et al*., 2007).

10.4 Nondosage-dependent actions/route

Predicting the toxicity of a nanoparticle not only depends on the dose but the route which controls the biodistribution, accumulation, metabolism, and excretion of nanoparticles. Substances may enter the body via various routes such as oral ingestion, inhalation, dermal penetration, and intravascular injection and subsequently distribute to any organ system.

10.5 Surface area-dependent toxicity

Inflammatory effect is dependent on the surface area of nanoparticles, when chemical composition and crystal structure remain the same, suggesting a need for changes in definitions and regulations related to dose and exposure limits. Indeed, smaller nanoparticles have a higher surface area and particle number per unit mass compared to larger particles. The body will react differently to the same mass dose consisting of billions of nanoparticles compared to several microparticles. Larger surface area leads to increased reactivity, giving rise to an increased source of reactive oxygen species (ROS), as demonstrated by *in vitro* experiments (Donaldson and Stone, 2003; Roduner, 2006). Intratracheal instillation studies on mice with TiO_2 anatase showed that small nanoparticles (20 nm) induce a much greater inflammatory response than larger nanoparticles (250 nm) for the same mass dose. If instilled with the same surface area and dose, they generated similar toxicity, fitting the same curve (Oberdorster *et al*., 2005). The higher surface area of nanoparticles causes a dose-dependent increase in oxidation and DNA damage, much higher than larger particles with the same mass dose (Donaldson and Stone, 2003; Risom *et al*., 2005).

Extrapolating the research findings of mice to humans and environmental pollution, the critical surface area of nanoparticles becomes 30,000 cm^2 (Stoeger *et al*., 2006). In a busy urban area with nanoparticle concentrations of up to 10 $\mu g/m^3$ with a specific surface area of 110 m^2/g, deposition efficiency of 70%, the lung burden accumulates 150 cm^2/day. Deposition of particles in the lungs causes the surface threshold for significant inflammatory effects to be achieved in about half a year (Stoeger *et al*., 2006). However, patients already at high risk and suffering from respiratory or cardiovascular diseases may have a lower threshold. In addition, cardiovascular consequences may appear at a lower pollution threshold. Some contradictory results of surface area-related toxicity states that toxicity of smaller nanoparticles against larger nanoparticles of similar composition showed that they were capable of generating similar cytotoxicity or inflammatory reaction within the lung when two different forms of titanium dioxide, rutile and anatase, were used (Warheit *et al*., 2006; Gurr *et al*., 2005). Similar

composition does not necessarily imply similar chemistry and chemical bonds, for which the best example could be carbon, whose allotropes are graphite, diamond, carbon nanotubes, and fullerenes, each with distinct physical and biological characteristics.

10.6 Concentration-dependent toxicity

There are many contradictory results related to the toxic effects of nanoparticles at different concentrations. When comparing the results of different studies based on concentration, the differences in the aggregation properties of nanoparticles in air and water should be taken into account, which results in inherent discrepancies between inhalation studies and instillation or *in vitro* experiments. The aggregation may depend on surface charge, material type and size. It is well documented that aggregation of nanoparticles is essential in determining their toxicity, due to more effective macrophage clearance for larger particles compared to smaller ones, leading to the reduced toxicity of nanoparticle aggregates larger than 100−200 nm (Oberdorster *et al*., 2005; Takenaka *et al*., 2001). It has been established that a high concentration of nanoparticles would promote particle aggregation and therefore reduce toxic effects compared to lower concentrations (Gurr *et al*. 2005; Churg *et al*., 1998; Takenaka *et al*., 2001). Most aggregates are observed to be larger than the 100 nm threshold limit for many of the adverse health effects of small particles. Therefore experiments performed with low concentrations of nanoparticles may not be as toxic as higher concentrations of the same nanoparticles, because of their capability to form aggregates.

10.7 Particle chemistry and crystalline structure-dependent toxicity

Particle chemistry is critical in determining the toxicity of nanoparticles. Although there have been suggestions that size may be more important than chemical composition in deciding nanoparticle toxicity, one cannot generally extrapolate the results of studies showing a similar extent of inflammation for different nanoparticle chemistries (Risom *et al*., 2005). Both composition and chemistry of a nanoparticle play a critical role in determining toxic effect, as nanoparticles may vary in their chemical or crystalline structure. Let's take, for example, rutile and anatase of TiO_2. Rutile nanoparticles (200 nm) were found to induce oxidative DNA damage in the absence of light, but anatase nanoparticles of the same size could not (Gurr *et al*., 2005). Also, nanoparticles could change their crystal structure after interaction with water or liquids. This is clearly evident from previous reports, where it was presented that zinc sulphide (ZnS) nanoparticles (3 nm across containing around 700 atoms) rearrange their crystal structure in the presence of water and become more ordered, closer to the structure of a bulk piece of solid ZnS (Zhang *et al*., 2003). Nanoparticles often exhibit unexpected crystal structures due to surface effects. Gold nano- and microparticles can be prepared by evaporating gold on heating with an electron beam and allowing the vaporized atoms sufficient time and density to condensate into clusters before collection on a

substrate. Condensation dynamics dictate that gold under these conditions will form crystalline particles. The crystalline effects on condensation are clearly observed in the faceting and fine (nano) structure of the crystal faces. Incidentally interesting are the dendritic patterns on the faces where the condensation forms a classic diffusion-limited aggregation structure. These nanoparticles are similar to the engineered nanoparticles produced in many industrial processes; they are engineered or designed by developing unique recipes that yield materials with beneficial characteristics.

10.8 Aspect ratio-dependent toxicity

The toxicity is related to the aspect ratio; the higher the aspect ratio, the more toxic the particle is. The biopersistence of the long aspect ratio fibers leads to long-term carcinogenic effects. The toxicity of long aspect fibers is closely related to their biodurability. The biodurability of a fiber depends on its dissolution and mechanical properties (like breaking). More exactly, lung cancer was associated with the presence of asbestos fibers longer than 10 μm in the lungs, mesothelioma with fibers longer than 5 μm, and asbestosis with fibers longer than 2 μm. The minimum thickness of all these fibers is about 150 nm (Lippmann, 1990). Long fibers ($\geq$20 μm for humans) will not be effectively cleared from the respiratory tract due to the inability of macrophages to phagocytize them, as their diameter lies between 14 and 21 μm (Oberdorster, 2002; Hoet *et al*., 2004). Longer fibers that break perpendicular to their long axis become shorter and can be removed by macrophages. Asbestos fibers break longitudinally, resulting in more fibers with smaller diameter and this makes their removal more difficult. If the lung clearance is slow and these fibers stay in the lung for a long time, the probability of an adverse response increases. Fibers that are sufficiently soluble in lung fluid can disappear in a matter of months, while the insoluble fibers are likely to remain in the lungs indefinitely. Even short insoluble fibers that are efficiently phagocytized by alveolar macrophages may induce biochemical reactions (release of cytokines, ROS, and other mediators). Long aspect ratio-engineered nanoparticles, such as CNTs, are new materials of emerging technological relevance and have attracted a lot of attention due to their possible negative health effects as suggested by their morphological similarities with asbestos (Warheit *et al*., 2004; Lam *et al*., 2004; Maynard *et al*., 2004; Donaldson and Tran, 2004; Cherukuri *et al*., 2004; Muller *et al*., 2005; Monteiro-Riviere and Inman, 2006; Cui *et al*., 2005; Jia *et al*., 2005). However, there is no consensus in the characterization of CNT toxicity.

The diameter of CNTs ranging between 0.4 and 100 nm have varying lengths from several nm to cm. Due to their hydrophobicity and tendency to aggregate, they are harmful to living cells in culture (Monteiro-Riviere and Inman, 2006; Cui *et al*., 2005). For many applications, CNTs are oxidized to create hydroxyl and carboxyl groups, especially in their ends, which make them more readily dispersed in aqueous solutions. To conclude on carbon nanotube cytotoxicity, it can be stated that in general CNTs are very toxic, inducing cell death at sufficiently high doses of 400 μg/mL on human T cells, and 3.06 μg/cm^2 on alveolar macrophages (Jia *et al*., 2005; Bottini *et al*., 2006). Cell cultures with added single walled carbon nanotubes (SWCNTs) at

much lower doses of 3.8 μg/mL did not show cytotoxicity. However, various studies often contradict for dose-related inflammation or cell death. It was found that cells actively respond to SWCNTs by secreting proteins to aggregate and surround them. Also SWCNTs upregulate genes associated with apoptosis (Cherukuri *et al*., 2004). Long-aspect ratio particles (like SWCNTs) were reported to produce significant pulmonary toxicity compared to spherical particles (amorphous carbon black). Pharyngeal introduction of SWCNTs resulted in acute inflammation with the onset of progressive fibrosis and granulomas in rats. For the purpose of comparison, when equal doses of carbon black or silica nanoparticles were used, it did not induce granulomas or alveolar wall thickening and caused only a weak inflammation and limited damage (Maynard *et al*., 2004). The enhanced toxicity was attributed to physicochemical properties and fibrous nature. Carbon nanotubes are not eliminated from the lungs easily and their elimination rate is very slow. It was observed that 81% of the carbon nanotubes were found in the rat lungs after 60 days of exposure (Muller *et al*., 2005).

10.9 Surface coating and functions attributed

As nanoparticles are chemically very reactive the combined effects of inhalation, ingestion, or dermal application of nanoparticles with other nanoparticles, chemicals, and gases are largely unknown. A simple additive process could not explain the estimated risk of two or more pollutants. Particle surface plays a critical role in toxicity as it makes contact with cells and biological material. Surfactants can drastically change the physicochemical properties of nanoparticles, such as magnetic, electric and optical properties, and chemical reactivity, affecting their cytotoxicity (Oberdorster *et al*., 2005; Yin *et al*., 2005; Gupta and Gupta, 2005). Surface coatings can render noxious particles nontoxic while less harmful particles can be made highly toxic. The presence of oxygen, ozone, oxygen radicals, and transition metals on nanoparticle surfaces leads to the creation of ROS and the induction of inflammation (Donaldson and Stone, 2003; Risom *et al*., 2005; Sayes *et al*., 2004). For example, the specific cytotoxicity of silica is strongly associated with the occurrence of surface radicals and ROS (Hoet *et al*., 2004). Experiments performed on hamsters showed that the formation of blood clots is more prominent when the surface of polystyrene nanoparticles is animated (Nemmar *et al*., 2002). The interaction of diesel particles with ozone cause increased inflammation in the lungs of rats compared to diesel particles only (Risom *et al*., 2005). Nickel ferrite particles show different cytotoxicity with and without surface modification with oleic acid (Yin *et al*., 2005). The cytotoxicity of C_{60} molecules systematically correlates with their chemical functionality in human (skin and liver) carcinoma cells with cell death occurring due to lipid oxidation caused by the generation of oxygen radicals (Sayes *et al*., 2004). Spherical gold nanoparticles with various surface coatings are not toxic to human cells, despite the fact that they are internalized (Connor *et al*., 2005; Goodman *et al*., 2004). When coated appropriately, Quantum dots of CdSe can be rendered nontoxic (Derfus *et al*., 2004).

Above all, the interaction of nanoparticles with the biological system is crucial in determining its toxicity. Many toxicity studies, until now, have been done at much higher doses than is realistic, and they may exemplify Paracelsus's observation of "the dose makes the poison"; toxic substances are harmless in small doses and harmless substances are poisonous when overconsumed (Oberdorster, 2010). Quantifying real-life occupational exposures and emissions of nanoparticles into the environment is a challenge. Modeling studies that consider various release scenarios based on the life cycle of the nanomaterials and products that contain them have been presented, but to improve these models we require data on the industrial production of nanomaterials, the amounts released at different stages of the life cycle of the materials, and the form in which they are released (Gottschalk and Nowack, 2011). The chemical and physical properties of nanoparticles have a strong influence on the way in which they interact with biological components or the environment at large and also on the way they move, accumulate, and clear in the body. For example, nanoparticles acquire a "corona" of proteins when exposed to biological fluids, and this layer is thought to influence the way the cell perceives the nanoparticle (Lynch and Dawson, 2008). It has also been shown that certain nanoparticles can induce proteins to unfold, leading to an inflammatory response. Similarly, nanoparticles are coated with natural organic matter when they enter water, soil, or sediment environments and this layer influences their reactivity, bioavailability, and other transformations in the environment (Deng *et al*., 2011). These dynamic interactions add complexity to the challenge of determining the biological outcome of nanoparticles.

In studying the influence of the various properties of nanomaterials, the dose, the exposure route and time, and identifying the right model systems are expensive and time-consuming. High-throughput and computational approaches are on the horizon to rapidly screen and prioritize nanomaterials for toxicological tests and to develop causal relationships between material properties and biological behaviors (Fourches *et al*., 2011). Researchers have shown, for example, that the quantitative structure–activity relationship (a statistical model traditionally applied to chemicals) can predict the cytotoxicity of a small set of metal oxide nanoparticles (Puzyn *et al*., 2011); there are also opportunities for computational scientists to develop appropriate structural parameters for describing nanomaterials and to work with toxicologists to design new assays (Burello and Worth, 2011).

In order to gain sustainable development, the new technology should require a good balance between benefit and risk. Nanotoxicology is intended to address the toxicological activities of nanoparticles and their products to know whether and to what extent they may pose a threat to the biological system. It also deals with the quantitative assessment of the severity and frequency of nanotoxic effects in relation to the exposure of the organisms. The knowledge from the nanotoxicity study will be the base for designing safe nanomaterials and nanoproducts and also direct uses in the nanomedical sciences.

A number of studies on the effects of nanoparticles in *in vitro* and *in vivo* systems have been published. However, there is still a need for further studies that conclusively establish their safety/toxicity, due to the many experimental challenges and issues encountered when assessing the toxicity of nanomaterials. Most of the methods

used for toxicity assessment were designed and standardized with chemical toxicology in mind. However, nanoparticles display several unique physicochemical properties that can interfere with or pose challenges to classical toxicity assays. Recently, some new methods and modified versions of pre-existing methods have been developed for assessing the toxicity of nanomaterials. This chapter highlights some important methods employed in nanomaterial toxicology and to provide a critical analysis of the major issues/challenges faced in this emerging field.

10.10 Risk management of nanotechnology-specific aspects

Humans have been exposed to airborne particles, especially to nanoscale particles, during their total developmental period, but this exposure has increased enormously since 1916 due to anthropogenic sources.

Risk characterization in nanotechnology: the first step in managing risk is to identify potential risks and their causes (Fig. 10.1). Risk identification should consider all areas of a technology and both internal and external factors. Research is therefore needed with respect to environmental health and safety on the following topics:

- particle uptake into living organisms
- accumulation of nanoparticles in specific organs

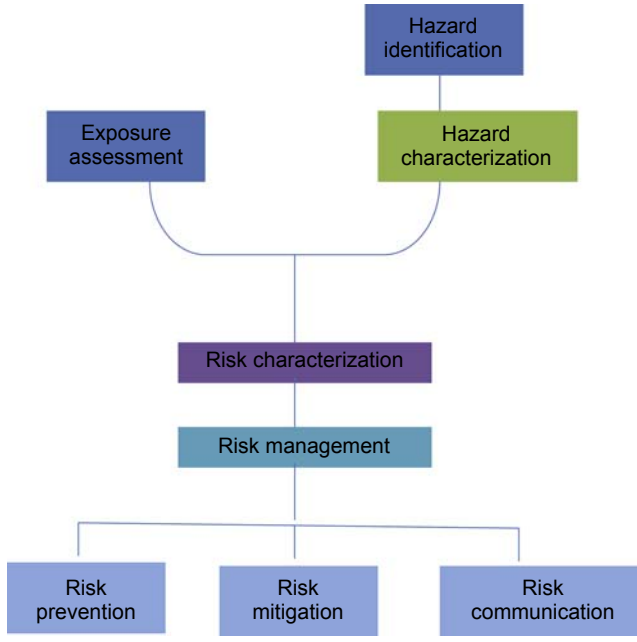

Figure 10.1 Risk assessment of nanoparticles.

- specific effects of nanoparticles in the respiratory system
- fate of nanomaterials in the environment (mobilization of heavy metals, binding of toxic substances)
- possible entry into the food chain: accumulation
- desorption/adsorption
- surprising effects

Rapid growth in the development of nanoparticles for uses in a variety of applications, including targeted drug delivery, cancer treatment, cosmetics, imaging, and as biological sensors, has led to questions about the potential toxicity of such particles to humans.

One of the most important points in assessing risks of nanotechnology is to distinguish between free nanomaterials, especially nanosized particles, and fixed nanoparticles, because of the tremendous differences in mobility.

Next, we have to discriminate between nanoparticles from technological processes and those that were unintentionally produced and released into the environment by several conventional technologies. Some engineered nanomaterials possibly exhibit critical characteristics when emitted into the environment or taken up by the organisms. The mechanistics of transition and potential dermal or systemic toxicity need to be evaluated. In the future, the penetrative capacity of certain nanoparticles could be exploited for transdermal drug delivery. Intravenous and oral nanoparticle administrations inherently have a more rapid systemic effect compared to transdermal administration and once within the circulation, most substances are subjected to first-pass metabolism within the liver, where they may accumulate or distribute via the vasculature to end organs including the brain. Despite its innate protection by the blood–brain barrier against external chemical insults, the potential for nanoparticulate matter to percolate through tight junctions renders the brain vulnerable to potential particle-mediated toxicity. Reliable data on nanoparticle toxicity is therefore necessary to avoid detrimental adverse effects.

The *in vitro* assays that are routinely practiced for the toxicity assessment of nanoparticles are cell viability assay, oxidative stress assay, and inflammatory assay.

Comet Assay: due to sensitivity of the method and the reactivity of many nanomaterials, the most common type of cell viability assay is comet assay. In the alkaline version of the comet assay, DNA strand breaks and alkali-labile sites are detected. In the process, oxidatively damaged DNA can be analyzed using the enzyme formamido pyrimidine glycosylase. The toxicity of manufactured nanoparticles has shown to cause DNA strand breaks or oxidative DNA lesions by comet assay. Finally, the potential use of the comet assay in human biomonitoring studies, which could provide valuable information for hazard identification of nanoparticles, is well documented.

Lipid peroxidation and imbalance in glutathione (GSH) level can act as a reliable parameter to study oxidative stress. Lipid peroxidation is the oxidative degradation of cell membranes initiated by the presence of ROS and is most commonly measured by assaying the presence of malondialdehyde or other thiobarbituric acid reactive substances. This assay has been used extensively to demonstrate the ability of a variety of nanomaterials to elicit lipid peroxidation in multiple cell types, such as fullerenes in

human dermal fibroblasts and human liver carcinoma (HepG2) cells (Sayes *et al*., 2004). The dose-dependent increase in DNA damage, lipid peroxidation, and protein carbonylation along with a significant decrease in activity of superoxide dismutase, catalase, total glutathione levels, and total antioxidant capacity indicated that the cells were under oxidative stress (Dubey *et al*., 2015).

Oxidative stress acts by alterations in superoxide dismutase or glutathione production. An increase or decrease in these responses can be interpreted as evidence for oxidative stress, as the cell either compensates for increased stress by upregulating the production of antioxidants, or the exhaustion of cellular stores of superoxide disumutase, or GSH by oxidation from reactive nitrogen species or ROS. GSH is an essential antioxidant that is oxidized during oxidative stress to form a GSH−GSH disulphide between two GSH molecules yielding oxidized glutathione (GSSG). The most quantitative assessment monitors the ratio of GSH and its disulphide oxidative product GSSG using high performance liquid chromatography, but chromatographic separation steps are time-consuming and allow for auto-oxidation, leading to overestimation in the amount of GSSG. For this reason, combined GSH and GSSG have been assayed instead, during the nanotoxicology studies to date, using 5, 5′-dithio-bis(2-nitrobenzoic acid) (DTNB) (Sharma *et al*., 2007). The total GSH concentration is determined by the colorimetric detection of 5-thio-2-nitrobenzoic acid after reaction of DTNB with GSH.

Inflammatory responses of nanoparticles can be detected through ELISA: enzyme-linked immunosorbent assay (ELISA) is a biochemical technique used mainly in immunology to detect the presence of an antibody or an antigen in a sample. In ELISA, an unknown amount of antigen is affixed to a surface, and then a specific antibody is applied over the surface so that it can bind to the antigen. This antibody is linked to an enzyme and in the final step a substance is added so that the enzyme can convert to some detectable signal, most commonly a color change in a chemical substrate. The most commonly tested human and murine inflammatory markers are the chemokine Interleukin-8 (IL-8), followed by TNF- α and IL-6 (Lequin, 2005).

10.11 Conclusion and future prospects

Nanoparticles are usually more toxic to some cell subpopulations than others, and toxicity often varies with cell cycle. Cells exposed to nanoparticles may undergo repairable oxidative stress and DNA damage or be induced into apoptosis. Exposure to nanoparticles may cause the cells to alter their proliferation or differentiation or their cell−cell signaling with neighboring cells in a tissue. Single-cell nanotoxicity assays and inclusion of various cell-damaging factors apart from cell death by necrosis therefore dictates the use of flow and scanning image cytometry approaches to measure nanotoxicity. Flow cytometry is fast and quantitative, provided that the cells can be prepared into a single-cell suspension for analysis. But when cells cannot be put into suspension without altering nanotoxicity results, or if morphology, attachment, and stain location are important, a scanning image cytometry approach must be used (Eustaquio and Leary, 2012).

Reverse transcription-polymerase chain reaction is a relatively simple and inexpensive technique to determine the expression level of target genes and is widely used in biomedical science research including nanotoxicology studies for semi-quantitative analysis (Mo *et al*., 2012). The omics technologies are particularly well suited to evaluate toxicity in both *in vitro* and *in vivo* systems and for mechanistic insight into nanotoxicity. Metabolomics, specifically, can rapidly screen for biomarkers related to predefined pathways or processes in biofluids and tissues, specifically oxidative stress. The application of both liquid chromatography/mass spectroscopy and nuclear magnetic resonance-based metabolomics approaches to study the potential toxicity of nanoparticles is gaining popularity (Schnackenberg *et al*., 2012).

Nanoparticles have certain unique characteristics which can be and have been exploited in many biomedical applications. However, these unique features are postulated to be the grounds for nanoparticle-induced biotoxicity which arises from the complex interplay between particle characteristics (e.g., size, shape, surface chemistry, and charge), administered dose, and host immunological integrity. More emphasis has been placed onto understanding the role of the route of particle administration as a potential source for toxicity. Current research focuses on elucidating the mechanism underlying nanoparticle toxicity which is postulated to range from inflammatory cell infiltration and cellular necrosis to ROS-induced apoptosis. Despite the wealth of toxicity studies available, the authors have identified several points of criticism which currently hinder the progression into clinical settings. Firstly, the application of the so-called "proof of principle" approach, where cell cultures or experimental animals are exposed to ultra-high nanoparticle concentrations to ensure cytotoxicity, leads to unrealistic results which cannot be extrapolated into the human scenario since diagnostic and therapeutic interventions usually only require the administration of minimal concentrations. Thus not only are we faced with scientifically unreliable data, such practices may, quite dangerously, cause unnecessary alarm in the public. Additionally, the authors have identified two further limitations of current toxicity studies: firstly, the chronicity of nanoparticle exposure in the case of therapeutic applications needs more thorough long-term evaluation. Secondly, different studies apply different particle formulations, leading to conflicting and unreliable results. Consequently and for the future, more emphasis should be placed on defining the dose of nanoparticles in relation to the route of administration. As mentioned at the beginning of this chapter, end-organ accumulation and distribution, as well as metabolism and excretion, are variable depending on the routes of administration, such that intravenous nanoparticle administration may have more implications with regards to systemic adverse effects than dermal application of nanoparticles. However, one must treat and compare toxicity results from different studies with caution, as current toxicity protocols lack uniformity with respect to nanoparticle formulations and application protocols. It has become apparent that a unifying protocol for the toxicological profiling of nanoparticles may be required in order to achieve reliable outcomes that have realistic implications for the human use of nanoparticles. In summary, current difficulties in evaluating nanoparticle toxicity originate in the inherent discrepancies found among toxicity study protocols, and it has become apparent that a unifying protocol for the toxicological profiling of nanoparticles may be required in order to achieve reliable

outcomes that have realistic implications for the human use of nanoparticles. Not only is there a pressing need for long-term studies, the future of nanotoxicology must also more heavily rely on realistic particle dosages and composition principles as well as differentiating more carefully between various routes of administrations if nanotechnology is to fully unfold its clinical potential.

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Regulatory aspects of nanoparticulate mediated nucleic acid delivery systems

11

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11.1 Introduction

Since 1996, nanomedicine has emerged as an effective tool for controlled drug/gene delivery to specific sites, enabling it to diagnose diseases at an early stage and monitor them. Several studies have shown that nanoparticles have the capability to produce some plausible risk to a patient's health and environment and can have cytotoxic or genotoxic effects as discussed in the previous chapter. So there arises a need for establishing more stringent and defined regulations regarding ethical, legal, and social impacts. This involves the rapid development of certain regulatory norms, enclosing different guidelines, rules, codes, approvals, and protocols for the standardization and accomplishment of extensive analysis of potential risks posed to human health, environment, and safety. From an institutional perspective, these policies can be executed by the government, nongovernment authorities, and self-regulatory agencies that create, limit, and constrain the law and conduct regulatory duties based upon clinical and nonclinical approaches (Hodge *et al*., 2010; Mühlebach *et al*., 2014; Twenty-seventh Report of the RCEP presented to the UK Parliament in November, 2008). The regulatory system should mandate an appropriate balance between the risks and the benefits posed by the technology. Many countries have already started revising their preexisting regulatory approaches, including new nanotechnology-based therapeutics. A significant amount of improvements and alterations are required before the policies are finally sanctioned.

This chapter outlines various issues and challenges of scientific and regulatory regimes related with nanoparticle-mediated drug delivery systems, the current regulatory approaches at the international level, and different strategies that can be adopted for better regulation. In turn, this will further facilitate to analyze the global governance gaps and solutions associated with the pharmaceutical nanoparticles.

11.2 Issues related with nanoparticle mediated nucleic acid delivery systems

The issues can be grouped under two categories: first, the potential risks posed to the environment, human and worker safety; second, the legal, ethical, and social conflicts dealing with control, management, and regulation, both nationally and internationally.

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11.2.1 Environment, health, and safety impacts

Limited information is available and not much is known about the impacts on health, environment, and safety issues pertaining to nanoparticle delivery systems. There has been an enormous gap between the development of nanoparticulate-based delivery systems and the evaluation of health, safety, and environmental risk data (Saner and Pelley, 2009). On the basis of expenditure, the expense used for the development of technology and for consumer application has outrun the investments for the environment, health, and safety research (Twenty-seventh Report of the RCEP presented to the UK Parliament in November, 2008). There has been no evidence reported regarding the harmful effects of nanomaterials on health and environment. Still, some specific properties, because of their small size, may result in undesirable interactions in humans at the cellular level and with the environment. Further, some secondary information needs to be attributed concerning the effect of pharmaceutical nanoparticles on health during oral, cutaneous, and inhalation uptake; transport in air, water, soil, and biosystems; their bioaccumulation; disposal and biodegradation; their interaction with different substances along with the environment; and their possible conversion into toxic compounds. In addition, proper strategies should be framed based upon risk assessment and risk management that deal with the production, application, and exposure to humans followed by the disposal of nanoparticles (International Risk Governance Council, 2007).

11.2.2 Ethical, legal, and social issues

Apart from the environmental, health, and safety (EHS) issues, potential risks posed to social, ethical, and legal implications should also be taken into consideration. This necessitates the framing of stringent guidelines and approaches toward Ethical, legal, and social issues (ELSI), which includes privacy issues, acceptance of the regulatory measures, regulation, and its control (Hullmann, 2008). One of the issues is concerned with a lack of communication between the developed and underdeveloped countries regarding the adoption of risk assessment and management procedures. These differences may impede the framing of standardized rules and protocols and in turn may complicate the policy coordination at an international level. Various attempts should be made for public participation in technology assessment and governance of the regulations (2007). Another concern is the knowledge gap between scientific and regulatory authorities, civil society, and the public, leading to the failure of proper estimation of the risk and management of the nanocarriers.

The enforcement of law-binding obligations at manufacturing sites encompasses worker protection, packaging regulation, and geographical distribution of the industry (Kelty, 2006). The management of intellectual property rights addresses the need for the interaction of the scientific community with the industry for the development of pharmaceutical nanoparticles. Also, the ownership of knowledge is in inquisition as to what should be/should not be patentable, depending upon the behaviour and characteristics of pharmaceutical nanoparticles. This in turn aids in maintaining the confidentiality of the manufacturers, ideally at an international level. In this concern,

Europe has already initiated the development of a "nanoscience and nanotechnology patent-monitoring system" under the European Union Commission Action Plan 2005–09 organized by the European Patent Office (EPO) (2005). The EPO is liable to allot a special tag to the patents to label nanotechnology products, so they can easily be identified (Keawchaoon and Yoksan, 2011). These promote knowledge and enhance the awareness level among society and the general public so that appropriate legislations can be established.

11.3 Current approaches for regulating pharmaceutical nanoparticles

Various commissions, regulatory policies, and guidelines have been issued by different countries in order to accomplish the appropriate measures and standards to approve the nanoproducts commercially. Several countries that contribute to the proper regulation of rules and further administration of general public policies include the United States (US), the United Kingdom (UK), the European Union (EU), Australia, and Canada. These play a major role in framing and establishing scientific and regulatory regimes concerning nanoparticle delivery systems.

11.3.1 The United States

For the innovation of research and discoveries in the area of nanoscience, the US government research and development initiated the formation of the National Nanotechnology Initiative (NNI) in January 2000, under the presidency of Bill Clinton, with an initial budget of US $422 million. NNI ensures that the investments are made in a coordinated and timely manner by regulating the budgetary and planning agencies. It acts as a centre for the channelization of federal funding related to nanotechnology (Roco, 2001; Saner and Pelley, 2009). With the increase in commercialization and advancement in technology, the federal investments raised to over US $1.5 billion for the president's 2015 budget under the provisions of 21st century Nanotechnology Research and Development Act of 2003 (2014). The NNI strategic plan describes the goals and investment policies through which the objectives can be achieved, and consequently, these programs are further revised according to the requirement for future nanotechnology-based innovations. The NNI is based upon four major goals, which are as follows:

1. worldwide promotion of nanotechnology research and development program;
2. propose an idea for creating new products based on these technologies that maximize economic and public benefits;
3. sustain scientific resources, skilled workers, and a productive infrastructure and tool set to be developed for the continuous advancement, extended innovation and discoveries in nanotechnology; and finally,
4. enable the pertinent development of nanotechnology.

Even though the US federal government has not executed any measure for the regulation and administration of nanoparticle-mediated nucleic acid delivery systems,

attempts have been made in this field by the director of the office of science and technology policy and chairperson of the council about environmental quality in 2007. It contributes to international harmonization among countries and further promotes economic regulatory efforts to gain advancement (Connaughton and Marburger, 2007).

On the other side, the National Nanotechnology Initiative Amendment Act of 2009 was passed by the US House of Representatives, which placed emphasis on increased funding regarding the environmental, health, and safety risks of nanotechnology. The Project on Emerging Nanotechnologies, established in 2005, is a US-based organization dedicated to address the social, public safety, and political aspects of nanotechnology. It provides online inventories about current research in health and environment implications, best available nanotechnology-based consumer products in the market, synthetic biological maps that show biological activity, agrifood nanotechnology research, development databases, medicinal developments, remediation maps, and many more (Contreras-Ruiz *et al*., 2011).

The two major US regulatory agencies that govern and administer the various schemes and policies related to nanomaterials are the US European Protection Agency (EPA) and the Food and Drug Administration (FDA). Some of the others include the Consumer Product Safety Commission, controlling consumer-related products and policy discussions, the Occupational Safety and Health Administration under the US Department of Labour, and the National Institute for Occupational Health and Safety, working on the risks and safe management practices at the workplace (2009).

11.3.1.1 The US Environmental Protection Agency

The Environmental Protection Agency's Science Policy Council established a cross-agency nanotechnology workshop in December 2004 for analysing plausible environmental applications and implications of nanotechnology and further channelizing these aspects to the stakeholders and the people. In February 2007, the group published a document, the US EPA nanotechnology white paper, illustrating the various environmental benefits of nanotechnology and opportunities and challenges related to the risk assessment of nanomaterial-based products. For the significant development of nanoscale materials, the EPA relies on a proactive approach. This program also considers the current challenges in this field (2007). The Nanoscale Materials Stewardship Program, a voluntary program, was launched officially by the US EPA in January 2008, addressing two subprograms, the basic program in which the companies were required to submit the information related to the material they produce and an in-depth program, which allows the companies to corporately work with the EPA in making regulatory decisions and to formulate such methods to generate this information. This program was formed to establish a scientific foundation for regulatory authorities to evaluate the submitted information regarding manufacturing, importing, processing, and usage of nanoscale materials (2009).

The EPA covers a wide range of policies and schemes for the regulation of nanomaterials. Some of the organization includes the Toxic Substances Control Act

for the regulation of chemical substances; the Federal Insecticide, Fungicide, and Rodenticide Act for the regulation of pesticides; the Clean Air Act for the hazardous air pollutants; the Clean Water Act for the regulation of substances toxic to aquatic life; the Safe Drinking Water Act that regulates hazardous contaminants in drinking water; the Comprehensive Environmental Response, Compensation, and Liability Act and the Resource Conservation and Recovery Act for the regulation of solid waste; and the Toxics Release Inventory Program, which is a publicly available database encompassing information on toxic chemical discharge and other waste management activities reported annually by manufacturing facilities in particular other sectors (2007; Saner and Pelley, 2009).

11.3.1.2 The US Food and Drug Administration

The FDA plays a significant role in protecting and promoting public health by ensuring the safety, efficacy, and security of humans and animals. The FDA is responsible for making decisions about products ranging from cosmetics to chemotherapeutic drugs to food packaging, thus regulating the manufacturing and marketing of nanotechnology-based products.

The agency created the FDA Nanotechnology Task Force Act in August 2006 to frame the regulatory approaches concerning the probable risk posed by nanomedical products, pharmaceutical drugs, food additives, and medical devices. The Food, Drug, and Cosmetic Act, under the authority of the FDA, addresses various policies and schemes for the use of conventional products based on nanotechnology. Currently, the FDA funds around US $1 trillion of products in a year for ensuring the safety and effectiveness safeguard to novel drugs, devices, nanomedical products, cosmetics, and food additives. Proper regulatory methods and guidelines should be framed for fostering innovations and promoting novel nanomedicine research (Harris, 2009). Several regulatory bodies are controlled by the FDA, some of which are the Centre for Drug Evaluation and Research; the Centre for Biologics Evaluation and Research, working for the safety, purity, and effectiveness of related products; the Centre for Devices and Radiological Health, which participates more in the regulation of pharmaceutical nanomaterials; the Centre for Food Safety and Applied Nutrition, concerned with the regulation of food, dietary supplements, and cosmetics; and the National Centre for Toxicological Research.

11.3.1.3 Regulatory developments by state and municipal organization

The US plays a significant role in developing legislation regarding nanotechnology, both at the state and municipal levels. Several laws and nanotechnology-related legislations have been issued by state legislatures created by the National Conference of State Legislatures. As per the list of July 2008, the rules enacted were focused on supporting educational innovations and discoveries through the funding of projects, creating study commissions and other promotional activities. However, no formal norms have been enacted toward the regulation of potential health, safety, and environmental impacts of nanoparticulate-based gene delivery systems.

The two main municipalities that have initiated efforts for the regulation of nanotechnology-based products are Berkley, California and Cambridge, Massachusetts. The Berkeley Municipal Code was modified in December 2006 through the introduction of new guidelines and measures concerning the health and safety of industrialized nanomaterials, involving the safe handling, monitoring, disposal, control release, and mitigation of the nanoproducts. The Cambridge City Council in January 2007 addressed various regulations for nanotechnology and further recommended appropriate amendments for Cambridge. However, the Cambridge Public Health Department and Cambridge Nanomaterial Advisory Committee concluded that there is a need to better understand the behaviour, characteristics, and properties of nanomaterials and their activities prior to the enactment of statutes (Saner and Pelley, 2009).

11.3.2 The United Kingdom

The UK Government initiated the "Better Regulation Taskforce" in 1997 by formulating five principles, which include proportionality, accountability, accuracy, transparency, and targeting to improve the quality of regulations (2000). The *Scientific Research: Innovation With Controls,* a report created by the Better Regulation Taskforce in January 2003, concluded that the government should foster public discussions concerning the risk posed by nanoparticulate material. The report was focused on environmental, ethical, and health safety, as well as political and social issues based on the nanoparticle-based delivery system, and also concluded the proper framing of guidelines and policies. The UK government started the planning of nanotechnology-based strategies with an initial investment of £45 million annually for the development of nanotechnology research during the period of 2003–06. The Better Regulation Commission in 2006 and subsequently the Risk and Regulation Advisory Council in January 2008 came into existence for establishing a proper regulatory framework in the field of nanotechnology.

The UK Royal Society and The Royal Academy of Engineering commissioned a report titled *Nanoscience and Nanotechnologies: Opportunities and Uncertainties*, underlining different obscurities and challenges encountered, and also emphasized the need to study the potential risk posed to human health by the use of nanoparticles (2004). This also addresses the issues regarding the need for public engagement and financial assistance for the human health-related research and environmental adversities related to nanoparticles. In 2007, the Council for Science and Technology released an independent review, *Nanosciences and Nanotechnologies: A Review of Government's Progress on its Policy Commitments*, concluding that several obligations have already been made, but the government research amendments particularly concerning toxicology, health, and the environmental implications of nanomaterials and long-term environmental fate should also be taken into consideration.

Subsequently, in November 2008, the Royal Commission on Environmental Pollution (RCEP) UK, released a report titled *Novel Materials in The Environment: the case of Nanotechnology*, highlighting observations with respect to toxicology,

functionality, adaptive governance, and the fate of nanomaterials. In response to the review by the Council for Science and Technology, in 2008 the Ministerial Group on Nanotechnologies was approved and governed by the Minister of State for Science and Innovation. The regulatory body constitutes various organizations, including the Department of Environment and Rural Affairs, the Department of Health, the Department of Work and Pensions, and the Department for Business Enterprise and Regulatory Reforms. This enables the proper interaction between the Ministerial Group and the stakeholders and the framing of policies and schemes by the government departments (Saner and Pelley, 2009; Twenty-seventh Report of the RCEP presented to the UK Parliament in November, 2008). The government was engaged in maintaining accessibility and unambiguity related to its activities and uncertainties in the field with an intention of developing a nano-oriented regulatory framework as per the requirement. The Ministerial Group, in January 2009, revised their commitments for the proper advancement in nanotechnology comprised of the involvement of various institutions, NGOs, and industries for framing the strategies, raising the awareness level concerning the products that are manufactured and brought to the market (2008; Saner and Pelley, 2009).

The UK Government has initiated several meetings for public interaction with stakeholders through the nanotechnologies stakeholder forum by the Department for Environment, Food, and Rural Affairs to maintain the transparency in research and innovations (Grabnar and Kristl, 2010). The departments that play a significant role in the development of nanotechnology in UK are the Food Standard Agency (FSA) for the regulation of food products, the Department of Health for the control of medicinal products, the Health and Safety Executive for worker safety, the Department of Business Enterprise and Regulatory Reform, and the Department of Innovations, Universities and Skills, which includes innovative research based on nanotechnology (2006; 2008; Frater *et al*., 2006).

11.3.3 The European Union

In 2000, the EU and its member states published a document titled *Communication from the Commission on the Precautionary Principle* that illustrates the framing of standard measures among the member states and the EU regarding risk assessment, evaluation, administration, and communication of risks in conditions lacking absolute scientific evaluation. Proportionality, integrity, consistency, examination of the advantages and expenditure, and evaluation of the scientific developments are the key features of the document (2000; Grobe *et al*., 2008). The EU and its member states discussed various approaches toward the risk analysis, concluding that the risk analysis is comprised of four components, namely, risk identification, assessment, management, and communication. As compared to other state jurisdictions, the EU and its member states assign each of the risk components to distinct professional groups. Nevertheless, this was determined to be a point of condemnation by the experts about the precautionary principle in response to public and political pressure (Calster, 2008; Saner and Pelley, 2009).

However, the European Commission officially released its plan for developing a regulatory framework for nano-oriented products through its communication *Towards a European Strategy for Nanotechnology* in May 2004. The document concluded to develop the guidelines for the use of nanotechnology-related products concerning their possible health and environmental impacts (2004). The strategies were analysed by the Competitiveness Council and the European Economic and Social Committee, and suggestions by the stakeholders were also considered on the commission's proposed strategy through an open survey by the Nanoforum and the European Commission in October 2004. The outcomes of the survey were compiled in a report titled the *Outcome of the Open Consultation on the European Strategy for Nanotechnology* in January 2005 (Malsch and Oud, 2004). Subsequently, the European Commission established an action plan titled *Nanosciences and Nanotechnologies: An Action Plan for Europe 2005–09*, highlighting the modifications relative to the risk assessment regarding the health of consumers and workers, research and innovation, effective interaction with the stakeholders and fostering the international collaborations in nanosciences and nanotechnologies (2005). This document further initiated the Scientific Committee on Emerging and Newly Identified Health Risks (SCENIHR) and was later modified in 2006 to execute suitable policies and schemes for the potential risks posed by the nanoparticles.

Moreover, in the list of numerous initiatives, it is worthy to mention a report in February 2008 on *Code of Conduct for Responsible Nanosciences and Nanotechnologies Research* with an aim to direct the member states in the establishment and reinforcement of research-related strategies. The code is based on fundamental principles such as significance, sustainability, preventative measures, inclusiveness, superiority, novelty, and liability in nanoscience (2009; Grobe *et al*., 2008).

There are various perspectives in which the nanotechnology approach differs in the EU compared to other state jurisdictions, such as the openness to public participation and consultation in formulating regulatory measures; its preventative measures keep previous experience with genetically modified organisms and food in view. Also, preexistence of scientific committees and regulatory bodies provide suggestions and warns about the potential risk posed by nanoparticles. The independent committees include the Scientific Committee on Consumer Products, which controls the risk associated with the insoluble nanoparticles in cosmetics and other consumer products. On the other hand, The Scientific Committee on Health and Environmental Risks, and the SCENIHR, deals with the policy and regulatory aspects concerning the issues related to ethical, legal, social, health, and environmental implications (Saner and Pelley, 2009).

11.3.3.1 Advancement in regulation in the European Union

In June 2008, the European Union organized a regulatory review regarding the rules and legislations, thereby ensuring a high level of protection to human health, safety, and the environment in the relevant fields of nanoscience and nanotechnologies. The EU released a report on the regulatory aspects of nanomaterials that addresses the existing regulatory framework related to the nanotechnology-based products and

any specific modifications that are required for maximizing the social benefits associated with it and minimizing the hazardous impacts (2008). The EU examines the legislative instruments based on safety, health, and the environmental aspects of nanomaterials, i.e., whether the instrument manages (1) chemicals, (2) worker protection, (3) products, or (4) environment protection (Saner and Pelley, 2009). The regulatory authorities that frame stringent rules and guidelines with relevance to nanotechnology and its related products are as follows:

- the European Chemicals Agency;
- the European Food Safety Authority (EFSA); and
- the European Medicines Evaluation Agency (EMEA).

The EU established the European Chemical Agency in June 2007 with an aim to regulate the chemical legislations and make sure that the chemicals are used safely in order to protect human health and the environment. Under this, various programs are involved, such as REACH in June 2007, responsible for the Registration, Evaluation, Authorization and Restriction of Chemical substances; CLP, addressing the Classification, Labelling, and Packaging regulation to aid workers and consumers become familiar with the symbols for using the chemicals in a safe way throughout the world. This will in turn assist the international trade of chemicals and the use in a standardized manner. The law on prior informed consent is another program that controls and sets some standardized protocols for the import and export of some hazardous chemicals so that countries have prior information about the chemicals (Liu *et al.*, 2011).

The General Food Law, under the Regulation (EC) No 178/2002, launched the EFSA, which underlines the basic fundamentals of general food safety requirements and forms the food law in the European Union (Saner and Pelley, 2009). Regarding the nano-oriented products in food, the EFSA has established novel food regulation for the foods that are produced from advanced technologies and are not consumed. This necessitates the premarket safety assessment and analysis of the product regarding biological hazards, chemical toxins, food consumption, and emerging risks related to the nanosciences and nanotechnologies (Nagpal *et al.*, 2010).

The EMEA is a decentralized scientific authority of the European Union that governs the regulatory challenges associated with the nanomedicines through the proper assessment and analysis for safety. Periodically, the authority releases the standardized scientific guidelines detailing the objective, notifications, legal issues, precautions, sustainability, and the modified rules based on the nanobased products.

11.3.4 Australia

The formation of the Australian Prime Minister's Science, Engineering, and Innovation Council in March 2005 assisted the government authorities to implement policies and schemes related to nanotechnology while maintaining the safety and health standards of the Australians. The following year, the Senate Community Affairs References Committee stated the potential risk emerging through nanoparticles that cause harm at the workplace. Further, Friends of the Earth, Australia confirmed this by naming the nanoparticles as the "new asbestos" and banning the commercial

production of them until strict regulations have been sanctioned concerning the protection of worker health (2005). In July 2005, the Department of Industry, Tourism, and Resources created a National Nanotechnology Strategy Taskforce that underlines the health, safety, and environmental issues and the related guidelines to be followed for the implementation of the strategies. Subsequently, the Australian Office of Nanotechnology was developed to address the plausible impacts and promote the innovation and research in the field of nanotechnology (Saner and Pelley, 2009).

The Australian government announced a report in July 2008 titled the *Australian Government Approach to the Responsible Management of Nanotechnology* that outlines the economic, social, and environmental challenges and frames various schemes and policies to gain the benefits of nanotechnology. The government highlighted the key goals for the proper regulation of nanomaterials as (1) the sustainability and safety of human health and environment, (2) promote public discussions, and (3) accomplish the social and economic benefits (2008).

Several regulatory agencies have been formed for the administration and management of guidelines and the laws related to nanotechnology are

- the National Industrial Chemicals Notification and Assessment Scheme: an authorized scheme responsible for chemical safety among the population as in food additives, medicines, pesticides, or any other industrial chemical;
- Food Standards Australia New Zealand: a government body that develops food standards with a goal to protect and support the health of the people for both Australia and New Zealand; and
- the Therapeutic Goods Administration: an administration that controls the import, export, and supply of the manufactured therapeutic goods through the premarket assessment, postmarket analysis, and implementations of the rules and standards in Australia (Millotti *et al*., 2011; Wang and Li, 2010; Zhu *et al*., 2010).

The Australian Safety and Compensation Council is a federal body that works in coordination with Occupational Health and Safety (OHS) to develop new policies, strategies, and programs through OHS concerning the health of the workers.

Many initiatives have been taken by the state and territorial governments in the area of nanotechnology, that includes the governments of Victoria and New South Wales. In February 2008, the *Victorian Nanotechnology Statement: Taking Leadership in Innovations in Technology* released national regulatory guidelines to protect the consumers, the workers, the environment, and society from the potential risk posed by nanoparticles. This statement also included the industry-based standardized rules for the proper management and development of industry design according to the set protocols in relevance to the development of nanotechnologies (2008; Saner and Pelley, 2009).

In addition to this, the Legislative Council Standing Committee on State Development in New South Wales established a report in October 2008, emphasizing the social, environmental, and health impacts and the development of stringent rules and laws regarding the labelling scheme of the engineered nanomaterials, further creating awareness among the public for the nanobased products (Saner and Pelley, 2009).

11.3.5 Canada

In 2005, The Prime Minister's Advisory Council on Science and Technology released a plethora of reports related to nanotechnology and their products. The initiation was carried out by The Office of the National Science Advisor, which presented the document *Towards a National Nanotechnology Strategy for Canada* at a workshop for nanotechnology and the environment in October 2005. In spite of such efforts, the Office of National Science Advisors failed to develop guidelines with respect to health, safety, and environmental issues circling the technology, and the office no longer exists.

The government of Canada published a paper in 2007 titled *Mobilizing Science & Technology to Canada's Advantage*, addressing the uncertainties, opportunities in nanotechnology and creating effective regulatory measures to protect the health of Canadians. In February 2008, another report on *Nanotechnology and Its Impact on Consumers* was proposed by the approval from the Industry Canada Office of Consumer Affairs with an aim to provide knowledge and awareness among the Canadian public about the nanotechnology-based products (Nielsen, 2007). In response to the appeal made by the Minister of Health, the Council of Canadian Academies in July 2008 released a report for the government of Canada titled *Small is Different: A Science Perspective on the Regulatory Challenges of the Nanoscale*. The document deals with the current regulatory strategies, risk assessment methods, and various regulatory issues, and it also ensures health and environmental safety. This outlines the work of an expert panel in the field of nanotechnology and will develop approaches for the proper research, innovation, risk analysis, and surveillance (2008).

In March 2007, a workshop on the *Discussion Paper on a Policy Framework for Nanotechnology* was organized by The Canadian Institute for Environmental Law and Policy, addressing the need for regulatory strategies in accordance with risk management and stakeholder participation. Later, this paper was revised and released in 2008, defining the policy into 12 major areas including goals, public awareness, research innovation, accountability and intellectual property rights, participation of stakeholders and investors, product labelling, and social and economic benefits, along with industrialization, scientific issues, training, security issues, research support, agencies, and authorities (Holtz, 2007, 2008). Canada is actively cooperating with various organizations such as OECD and ISO at an international level, aiming to establish standardized terminology, classification, measurement, techniques, and test methodologies. Canada also aids as an organizer for the ISO Nanotechnology Technical Committee (TC229), which is a Joint Working Group on Terminology and Nomenclature. Health Canada and the Environment Canada are the two active departments that operate the policies of nanotechnology regulation. Health Canada is in partnership with international counterparts, including the US FDA, that deals with health and safety issues and the development of regulatory approaches. This reduces the knowledge gap between the stakeholders and the government by setting up inventories, devices, systems, and structures for legally administering the policies and programs pertinent to nanomaterials (Azizi *et al.*, 2010; Saner and Pelley, 2009).

The Environment Canada and Health Canada mutually planned for the *Proposed Regulatory Framework for Nanomaterials* under the Canadian Environmental

Protection Act, which included a two-phase program for the enforcement of regulatory regimes and to address issues relative to nanomedicines (Saboktakin *et al.*, 2011).

11.3.5.1 The organization for economic cooperation and development

The OECD is an international economic authority responsible for framing strict rules and regimes and analysing the risk pertaining to the use of nanotechnology-based products. Among the jurisdictions that are working for the management of regulatory framework are the US, the UK, Australia, Canada, and the EU, which participate cooperatively with each other. The body is divided into two major groups: the Working Party on Manufactured Nanomaterials (WPMN) and the Working Party on Nanotechnology (WPN) organized by the OECD Chemical Committee and the OECD's Committee for Science and Technology Policy, respectively. The WPMN, established in 2006, placed emphasis on the assessment and testing methods for human health and environmental aspects by which standard safety protocols are developed for nanobased materials. On the other side, WPN, established in 2007, directs impending issues regarding science and technology to encourage research facilities and innovation in nanosciences and nanotechnologies. In addition, WPN runs parallel with the WPMN, the Working Party of Biotechnology, and the group of National Experts for Scientific and Technological Indicators. Several attempts have been made to ensure a persistent international approach for risk assessment and evaluation of nanomaterials. Many projects are currently going on, where each has a particular objective and time period. Some of them include the development of research strategies, an online OECD database, safe testing methods, development of guidelines, evaluation, and estimation of risk assessment, voluntary programs responsible for future development, and regulatory amendments related to nanomaterials. The WPMN works in association with the International Standards Organization (ISO) to develop protocols for nanomaterials, classification, nomenclature, test methodologies, health, safety, and environmental practices (Saner and Pelley, 2009).

11.3.6 Other industrialized and emerging countries

Several countries have investigated the potential risks pertaining to the use of nanotechnology-oriented products and are following the current regulatory strategies for maintaining human and environmental safety. Some of them have introduced voluntary programs and have framed rules and guidelines for the safe handling of nanomaterials. Many countries such as China, Korea, South Africa, Brazil, Russia, and India are taking the initiative to decrease the gap between developed and underdeveloped countries for the proper evaluation of risk and assessment of the nanocarriers. They are investing an increasing amount on public funding for general awareness and for promoting research and innovation in nanoscience and nanotechnology (Michelson, 2008).

In 2008, the Ministry of Economy, Trade, and Industry of Japan, took the initiative by approving a voluntary meeting regarding the risk assessment and management for manufactured nanomaterials. The National Institute of Occupational Safety and Health

Japan issued various research papers on safety and health impacts on exposure to nanoparticles in the workplace. In 2010, the Japan Industrial Standards Committee, an authority on nanotechnology standards in Japan, aided in the development of international standardized protocols in the field of nanotechnology.

The National Nanosafety Strategic Plan (2011−15) was proposed by the Republic of Korea for the development of strategies and policies related to nanomaterials. Additionally, risk management platform technology for nanoproducts is being set up with an aim to establish a certification system for nanobased products. Korea is also organizing various research programs on EHS and ELSI aspects.

In Taiwan, a strategic plan for the responsible development of nanotechnology, the Nanomark Certification system, is governed and managed by the Industrial Technology Research Institute with an intention to increase public awareness in nanoscience (Elvio Mantovani *et al*., 2011; Kumar, 2014).

In Thailand, the National Nanotechnology Centre was proposed by the government in 2011 to formulate the rules and regulatory regimes regarding social, environmental, and health safety implications. The certification and industrial standardization system was launched in late 2011 under the name NanoQ (Elvio Mantovani *et al*., 2011; Grayson *et al*., 2006; Kumar, 2014).

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Clinical studies and future prospects

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12.1 Introduction

Nanomedicine, an emerging interdisciplinary research field, is a widely investigated area often referred to have potential to improve strategies for the treatment of many diseases. Generally, nanoparticles from 10−100 nm are prepared, as those smaller than 10 nm are filtered by the kidneys, followed by excretion through urine. The major advantage of employing nanoparticles (NPs) has been its tailorability and ability to treat diseases with minimal side effects. This is mainly achieved by the targeted approach of drug delivery, which could be either passive or active by the NPs. Passive delivery of the drugs is attributed to the small size of the NPs and the enhanced permeability retention effect due to high vascularization and permeability of the solid tumours (Boylan *et al*., 2012; Harris *et al*., 2001; Ogris *et al*., 1999; van Vlerken *et al*., 2007). On the other hand, active targeting is achieved by employing NPs grafted with surface ligands that specifically bind to the cancer cells. Further, stable nanoparticles are synthesized and tailored for formulating nucleic acid-based therapies. These have recently emerged to be an exciting class of therapeutics for modulating and controlling the expression of the target protein in a cell. Various nucleic acid molecules have been investigated such as plasmid DNA, antisense olideoxyoligonucleotides, siRNA, and miRNA (Elsabahy *et al*., 2011; Gao *et al*., 2011; Guo *et al*., 2010). These molecules carry high negative charge, limiting its delivery into the cells as a therapeutic molecule. For efficient delivery, nanoparticulate delivery systems such as cationic lipids and polymers are generally used to carry and deliver the payload due to several advantages. Among most of the nanocarriers, cationic liposomes are considered to be one of the best NPs for delivering nucleic acids. As a result of the biodegradable and biocompatible nature of these NPs, it has been widely exploited in the delivery of siRNA molecules for most of the *in vivo* studies (Bhavsar *et al*., 2012; Xiong *et al*., 2011). In addition, polyplexes are also commonly deployed due to their ability to protect NA against intracellular and extracellular enzymatic degradation. These offer several potential benefits to medicine, which include early detection of several diseases, increased biocompatibility, biodegradability, along with imaging and therapeutic capabilities, making way for both imaging and treatment of the disease.

However, challenges are always accompanied with opportunities. Concerns regarding characterization, safety, and manufacturability with effective control methods are essential. In addition, NPs could be ineffective if the payloads are released during circulation itself or degraded inside the NPs. Thus this might lead to issues related to

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toxicity and nonspecificity. Therefore a complete knowledge and understanding of particle size, formulation, composition, its stability, reactivity, toxicity, biodegradability, and biocompatibility is required to bring the nanoparticles to the clinical level.

Despite all the aforementioned challenges, the first few generations of nanomedicines have already been brought into the market after gaining recognition from the clinical cancer community. For instance, a liposomal-based drug doxil was approved by the FDA in the mid 1990's due to its low cardiotoxicity relative to the free doxorubicin (Torchilin, 2005). Similarly, another drug, liposomal amphotericin B (Ambisome; Gilead), is an approved drug for antifungal infections (Adler-Moore and Proffitt, 2002).

12.2 Regulatory pathways in the process of clinical trials

Basically, drugs for human use involve the approval from the FDA through the Centre for Drug Evaluation and Research, which is a tedious and expensive process with low approval rates (DiMasi *et al*., 2003; Marchetti and Schellens, 2007). In general, the process of drug development and its approval can be divided into three main phases: the preclinical, clinical, and postmarketing phase. The preclinical phase involves the discovery of the drug and an investigation of its efficacy and toxicity. Thus during this process, animal testing is done to determine its safety and efficacy so that investigational new drug (IND) application could be filed with the FDA. Having obtained approval from IND, the drug enters into the clinical phase, where the drug is tested on humans. A clinical trial mainly involves the role of human volunteers in research with informed consent, basically involving three phases: phase I, phase II, and phase III. The drugs that are approved by new drug application (NDA) are thereafter marketed, and following that, FDA could further go for the postmarketing phase (phase IV). In general, the overall process of drug development takes about 10−15 years, costing nearly $1 billion (DiMasi, 2002; DiMasi *et al*., 2003; Kola and Landis, 2004; Marchetti and Schellens, 2007).

Prior to entry of the drug into the clinical phase, a thorough study in the preclinical phase is mandatory. This is probably achieved by different combinatorial approaches or in silico-based methods. In the case of nanoparticle-based therapeutics, the nanoparticles are required to be modified to achieve their desired function. There are already well-established platforms for the design of nanotherapeutics such as polymers, liposomes, metal oxides, dendrimers, and quantum dots (Brooks *et al*., 2007; FDA; Lee *et al*., 2007; NCL; Shacham *et al*., 2004; Sivanesan *et al*., 2005). For the purpose of human use, nanotherapeutics demand the use of proper resilient methods for their synthesis and characterization. Considering these issues, National Cancer Institute established Nanotechnology Characterization Laboratory. This basically aids in the development and standardization of protocols for the emerging nanotherapeutics (NCL). For the progress of the drug into the clinical phase, the IND application should possess the information regarding animal pharmacology, toxicity, manufacturing information, protocols, and investigator information (FDA). The following describes the clinical phases in detail:

Phase I: Studies are mainly conducted with 20−100 healthy volunteers with dose escalation in order to determine the safety and adverse effects of the drugs. In addition, pharmacokinetics,

as well as adverse effects of the drugs, are also evaluated. The study can take about 6 months to one and a half years to complete.

Phase II: Studies generally monitor the effects of the test drug on patients (100−300) and compare the results with the patients receiving a different treatment or a placebo along with the short-term adverse effects. The duration of the study is prolonged from 6 months to 2 years in order to thoroughly interrogate the drugs with a large number of people from different sites before it enters into phase III.

Phase III: The effectiveness of the drug is checked in a larger group of people (100−3000) with different drug dosage and drug combinations. Typically the study takes 1−10 years to complete. However, 10% of the studies get rejected at this phase.

Following phase III studies, the drug is subjected to NDA approval by the FDA, where the complete history of the drug analysis done in the preclinical and clinical phase is evaluated.

Phase IV: The drug is made available to the public after approval by the FDA, while researchers track its safety, risks, and benefits for its optimal use on a long-term basis (FDA).

12.3 Barriers in the progress of clinical trials

12.3.1 Decreased cellular bioavailability and nuclear uptake

The delivery of nucleic acids in biological fluids is usually accompanied with several physiological barriers affecting its bioavailability and biodistribution. Nucleic acids without any modifications further enhance their degradability by the extra- and intracellular enzymes, hence decreasing the therapeutic outcome. However, it has been observed that, despite employing nonviral vectors as gene carriers, satisfactory results are not procured. Although nonviral vectors such as nanoparticles have the capability to proficiently transfect the cells *in vitro* (rapidly dividing cells cultured in a monolayer), it does not necessarily transfect the cells in *in vivo* conditions. Also, it is seen that during transfection experiments, >95% of the cells take up the vector and only <50% of them are successful in expressing the transgene (Mukherjee *et al.*, 1997). Similarly, the delivery systems could seem to be stable in physiological conditions but cannot transfect cells *in vitro*. Moreover, the translocation of nucleic acids after transfection could be due to the loss of nuclear membranes during cell division. All these aforementioned factors mislead several companies, ultimately crippling nanotherapeutics to its clinical level.

Among most of the delivery systems, cationic liposomes and polymeric nanoparticles are the most broadly used nonviral vectors. The binding is promoted via electrostatic interactions between anionic groups of nucleic acids and cationic groups of the vector. Furthermore, due to their structural differences, there is a substantial difference in the interaction of plasmid DNA and short oligonucleotide sequences with the cationic vectors. From the studies, it is evident that ribo-oligonucleotides show altered binding properties with the cations relative to that of deoxyribose oligonucleotides. Apart from the mentioned properties, it is necessary to optimize formulations for the delivery of RNA and DNA for its efficient transfection and endosomal release (Schaffer *et al.*, 2000; Wolfert *et al.*, 1999; Zelphati and Szoka, 1996). Besides this, there are several other factors that affect the efficiency of the nanoparticles, which are discussed in the next section.

12.3.2 Serum stability

Albeit, several steps are taken to develop a suitable delivery system, cationic lipids still face many drawbacks when encountered by the serum proteins in the bloodstream. These proteins can potentially interact with the lipid, disrupting its integrity. Hence the lipid nanoparticles are liable to aggregate, inhibiting their uptake by the cell. Therefore to increase their stability, the lipid NPs could be PEGylated or could be combined with cholesterol. On the other hand, stability of the polyplexes depends on its structure and (amine to phosphate ratio) N/P ratio of the polyplexes. Neutral polyplexes form aggregates in a physiological condition, whereas cationic polyplexes exhibit time-dependent aggregate formation. Further, the adsorption of negatively charged proteins on the NPs could result in the formation of nanoparticles and clearance from the body by the reticuloendothelial system (Dash *et al*., 1999; Lai and van Zanten, 2001; Ogris *et al*., 1999).

12.3.3 Biodistribution of nucleic acids and its immunogenicity

Cationic formulations carrying nucleic acids tend to accumulate predominantly in the liver and lung after intravenous administration. These can also interact with the serum proteins and get cleared by the reticuloendothelial system. Many studies have shown that nucleic acid formulations accumulate mostly in the liver and lungs in a nonspecific manner, hence it cannot be concluded that nanoparticles are efficient enough upon delivery to the liver (Geng and Discher, 2005; Ishida *et al*., 2001; Patel, 1992; Semple *et al*., 1998). The uptake of nanoparticles by the macrophage phagocytic system could be hindered by increasing the circulation life of the formulation. A possible approach to overcome this problem was the incorporation of polyethylene glycol (PEG) to the cationic formulations. This substantially enhances the efficacy of the NPs by improving its circulation half-life, decreasing aggregation and its surface charge, which leads to accumulation in lungs (Li and Huang, 2008; Zhang *et al*., 2005). However, some cationic liposome formulations can promote cytokine release due to the presence of CpG motifs on the plasmid DNA, which is mainly identified by the toll-like receptors of the immune cells. In the case of developing siRNA formulations, the chemically synthesized small nucleic acids could be subjected to modifications that preferentially reduce their immunogenicity and improve nuclease resistance. Moreover, it was observed that PEGylated liposomes conferred immunogenicity by producing anti-PEG (immunoglobulin-M) Ig-M (Dow *et al*., 1999; Judge *et al*., 2006; Sakurai *et al*., 2007; Zhao *et al*., 2004).

12.4 Ethical concerns in clinical trials

The ethical aspects of the study are mainly governed and forwarded by the institutional review board (IRB). Before the approval of any research work, IRB committee is required to assess the risk minimization, outcome, benefits and safety associated with the research work. In addition, proper documentation of informed consent is well considered in the clinical study (Levine, 1988). Risk minimization is done by several methods, which can include literature survey or the scrutinizing of preclinical data.

Further, a definite field, nanotoxicology, is implemented to evaluate the effect of nanoparticle-based materials on living beings. The mode of administration of nanomaterials and different body stresses can invariantly affect humans in unpredictable ways. Also, studies on the effect of chronic exposure of the nanodrugs have not been performed to date (Service, 2004). Thus a lot of work is required to obtain the desired target. Besides, it is also observed that the results procured in preclinical data do not match with the data obtained at the clinical phase. Another limitation is that the preclinical research occurs only for a short duration and long-term effects of the drugs are rarely investigated. Further, another pitfall in the clinical trial pipeline is the management of risks and benefits of the drugs. For instance, if the risk is more than minimal in the study, the benefits are needed to outweigh the risk to meet the legal and ethical demands (Wendler *et al*., 2005). Above all, the final issue is the informed consent to human subjects regarding risks, benefits, costs, and beneficial nature of the study, as many people have misconceptions about therapy and research (Emanuel *et al*., 2000). Thus researchers are required to eliminate the misconception of the patients in a lucid manner without overestimating or underestimating the risks involved in the study.

12.5 Nanotherapeutics in clinical trials

12.5.1 Polymeric nanoparticles

There is an increasing momentum in the pace of gene delivery using polymeric nanoparticles. A number of polymeric formulations have already made their way into the clinical trials. From the previous chapters, it is clearly understood that several improved cationic polymeric vectors such as polyethylenimine (PEI), (poly-L-lysine) PLL, chitosan, and (polyamidoamine) PAMAM have been developed. Several strategies such as PEGylation, combination, and multifunctional modifications have been done to overcome the barriers in successful gene delivery. For instance, the translation of polymeric vectors for gene delivery has been one of the recalcitrant tasks in therapeutics. Among many available polymers, PEI is considered to be one of the most preferred polymers for the purpose of gene delivery due to its suitable property to release nucleic acid into the cytoplasm, attributing to the proton sponge effect as well as its high transfection efficiency. The clinical trials that have been performed or that are underway are mainly conducted for the treatment of cancer. Phase II A and phase II B studies were conducted to test the safety and efficacy of BC-819, a DNA plasmid carrying a gene for the diphtheria toxin-A chain along with an H-19 promoter complexed with PEI. The bladder tumour cells express a higher amount of H19 transcription factors. This ultimately results in the expression of the toxin, resulting in the destruction of the tumour cells. The study has been found to be safe with a low level of side effects (Sidi *et al*., 2008). The effect of BC-819 was also seen on unresectable pancreatic cancer, which was started in 2008 with an objective to assess its safety, tolerability, and maximum tolerated dose. A total of nine participants were subjected to different doses (4 and 8 mg). Three out of nine were tested for the 4-mg dose, and the rest were tested with 8 mg for a period of 4 weeks. At the dose 4 mg, one out of three patients was demonstrated with stable

disease. In the case of dosage 8 mg, four out of six represented a stable disease. It was observed that among the two dosages, tested patients showed mild adverse effects (Hanna *et al.*, 2012). Similarly, in another study, PEI-based nanoparticle SNS01-T was developed to induce apoptosis in B-cell cancers via small interfering RNA designed to suppress hypusinated eIF5A with simultaneous overexpression of nonhypusinable eIFA mutant by plasmid-based vectors. Basically, the phase I and phase II study was conducted to test its safety and tolerability by starting with a low dose of the formulation in the first group of patients with relapsed or refractory multiple myeloma or B-cell lymphoma. If tolerated, the subsequent groups would receive a higher dose. The study is still on its way to establish itself as an efficient therapeutic agent (Francis *et al.*, 2014). Polymeric-based gene therapy has also been exploited in other genetic and metabolic disorders such as cardiovascular disease. One such eminent study was done by Taljaard *et al.*, where eNOS-pVAX plasmid complexed with L-PEI was transfected ex vivo in circulating mononuclear cells. These cells were then reinfused into the infarct-related artery in patients in order to overexpress endothelial nitric oxide synthase (Taljaard *et al.*, 2010). Besides, immunocytokine-based therapies have also been investigated in the treatment of various diseases such as ovarian cancers. Interferon-alpha (IFN-α), IFN-γ, IL-2, and IL-12 are the major cytokines that have shown good activity in preclinical models of ovarian carcinoma (Mantia-Smaldone *et al.*, 2012). However, IL-12 was observed to be the most potent immunocytokine that can regulate the cascade of biochemical events leading to immunological response toward the cancer cells (Lenzi *et al.*, 2007). Thus a novel therapeutic agent, EGEN-001, was introduced, comprised of the IL-12 expressing gene in a synthetic gene delivery system comprised of polyethyleneglycol-polyethyleneimine-cholesterol (Fewell *et al.*, 2005). Initially, in the phase I study, EGEN-001 was administered alone in 13 patients with platinum-resistant recurrent ovarian cancer at escalating doses for four weeks. It was observed that CA-125 levels decreased in 6 out of 13 patients, and plasmid DNA levels were predominantly observed in the peritoneal region with a subsequent increase in the IFN-γ levels. Further, EGEN-001, in combination with carboplatin and docetaxel, was brought into the phase IA study to treat patients with platinum-sensitive recurrent ovarian cancer. In this study, an intraperitoneal catheter was implanted subcutaneously rather than using the percutaneous method to decrease the occurrence of peritonitis. Overall, the combinational therapy was well tolerated at all the doses, and multi drug resistance (MTD) was not identified. In general, these two studies demonstrated feasibility and safety of the drug EGEN-001 with 35% of the patients exhibiting stable disease condition. Moreover, 30% of the patients received one cycle or less of the combinational therapy, which could have probably limited its ability to assess the antitumour efficacy of EGEN-001 (Anwer *et al.*, 2010, 2013). Later, another phase I study was conducted, employing EGEN-001 in combination with liposomal doxorubicin. In this case, doxorubicin was mainly selected due to its immunomodulating properties that include modulation of macrophage, natural killer cells, and diminished activity of immune suppressing cells (Casares *et al.*, 2005; Ujhazy *et al.*, 2003).

A PLGA-based therapeutic drug, siG12D LODER, designed to knock down the expression of KRASG12D, was enrolled in a phase 0/I clinical trial by Silenseed to evaluate its safety and tolerability for the treatment of pancreatic ductal adenocarcinoma.

Mutations in KRASG12D are known for causing pancreatic ductal carcinoma. In the study, the formulation was injected into the tumour region using an endoscopic ultrasound biopsy needle. LODER is a biodegradable polymeric matrix, which was used in the study to release drugs in the tumour. The study was conducted for over 8 weeks. Safety and tolerability of the drug were tested in phase 0, while the dose escalation study was performed in the phase I clinical trial (Khvalevsky *et al.*, 2013).

Cyclodextrins (CD), a cyclic oligosaccharide, on the other hand are widely investigated in order to develop a better carrier molecule for the delivery of therapeutic nucleic acids. These CD molecules have been used extensively as a complexing agent due to their beneficial properties such as improvement of solubility, stability, and bioavailability. Hence a nanoparticle-based formulation (CALAA-01) was prepared by Davis and colleagues using cyclodextrin, siRNA (against M2 subunit of ribonucleotide reductase (RRM2)), and transferrin ligand to target the cancer cells. From the results, it was inferred that the formulations could be used for various cancers including melanoma cancer (Davis, 2009; Davis *et al.*, 2010).

12.5.2 Lipid nanoparticles in nucleic acid delivery

Lipid-based nanoparticles are one of the most exploited vectors among most of the available nonviral vectors. These include liposomes/lipoplexes and solid lipid nanoparticles with a diameter <100 nm (Li and Szoka, 2007). These have been extensively used in drug and nucleic acid delivery such as siRNA (Gomes-da-Silva *et al.*, 2012). Cationic liposomes are noticed to play a major role in delivery systems, attributing to their ability to condense negatively charged nucleic acid into the cationic lipids that ultimately protects the nucleic acid from degradation and aids in the transfer of nanoparticles into the cell by interacting with the negatively charged cell membrane. Besides, these have been considered to be more biocompatible and biodegradable. For instance, liposomal drugs such as Doxil (doxorubicin liposome) have acclaimed FDA approval for the treatment of HIV-related cancers such as Kaposi's sarcoma and other cancers, which include breast cancer, ovarian cancer, and solid tumours. Similarly, ambisome (a combination of liposome and amphotericin) was approved as an efficient antibiotic against severe fungal infections. In the case of nucleic acid delivery, there are no formulations available in the market due to their toxicity and stability issues. However, there are number of formulations such as Lipofectamine 2000 (life technologies), oligofectamine (Life technologies), and TransIT-2020 (Mirus Bio) that have been regularly used in laboratories for transfection assays *in vitro* (Adler-Moore and Proffitt, 2002; Hoekstra *et al.*, 2007; Jeong *et al.*, 2011; Torchilin, 2005).

12.5.3 Stable nucleic acid lipid nanoparticle (SNALP)

In 2009, Tekmira Pharmaceuticals launched an siRNA formulation targeted against hypercholesterolemia named TKM-ApoB (PRO-040,201). TKM-ApoB was systemically delivered to knock down the expression of Apo-B lipoprotein that constitutes the main apolipoprotein of low density lipoprotein (LDL). Thus inhibiting the expression of Apo-B can significantly decrease cholesterol levels in hypercholesterolemic

patients. A phase I study was conducted with a single-blinded, placebo, and ascending dose of the designed drug in both male and female subjects with hypercholesterolemia. This was conducted in order to determine the safety, tolerability, and pharmacokinetics, as well as pharmacodynamics, of the formulations. Among 23 patients, a particular group of people (17) were treated with the drug and the rest with placebo. Interestingly, in the course of treatment, it was observed that at a higher dosage, some showed flu-like symptoms while in others cholesterol was reduced up to a certain level. Therefore due to differences in the results, the phase I trial was terminated (Clinical Trials). Similarly, another lipid nanoparticle (LNP) formulation (ALN-PCS) possessing siRNA targeting against PCSK9 (Proprotein convertase subtilisin/Kexin type 9) regulates LDL receptor. On silencing the gene, the expression of LDL receptors increases, consequently decreasing the blood LDL levels (Abifadel *et al*., 2010; Cariou *et al*., 2011; Clinical Trial: ALN-PCS; Farnier, 2011). Furthermore, several other nucleic acids have been investigated including LNA antisense oligonucleotide for silencing PCSK9 in preclinical studies employing HepG2, HuH7, and β-TC3 cell lines, along with intravenous administration in mice. The study showed promising results with 60% reduction in the PCSK9 mRNA level that further aids in the development of a more potent LNA formulation for human therapeutic intervention (Gupta *et al*., 2010). In 2010, another siRNA drug named TKM-PLK1 (polo-like kinase) was developed that was targeted against deregulated PLK1, which at normal conditions contributes its major role in mitosis for the segregation of chromosomes. The deregulated function of PLK1 can lead to the development of tumours and chromosomal instability. The outcome of the study showed positive results, and the study (phase I/II) was completed in 2015 (Clinical Trial: ALN-VSP02; Clinical Trial: TKM-PLK1; Ramanathan *et al*., 2014; Reagan-Shaw and Ahmad, 2005). For the treatment of hepatocellular carcinoma, vascular endothelial growth factor (VEGF) and kinesin spindle protein (KSP) siRNA formulation (ALN-VSP) with LNP was designed by Alnylam that contains the first-generation cationic lipid DLinDMA. Because KSP and VEGF are essential in tumour progression, siRNA targeting against these genes can preferentially ameliorate the disease. In phase I clinical trial, 41 patients tolerated the dose levels ranging from 0.1 to 1.5 mg/mL. Thus the drug at 1mg/mL concentration was taken as an optimum dose for phase II studies. The phase I results displayed convincing therapeutic data where the disease abated even in patients with liver metastasis from endometrial carcinoma. These were clearly evident from the biopsy samples of tumours in the treated patients (Clinical Trial: ALN-VSP02).

In another study, Alnylam conducted a phase I study of ALN-TTR01 (siRNA drug with a first-generation SNALP carrier) to test its safety, tolerability, pharmacokinetics, and pharmacodynamics, typically in patients with transthyretin (TTR)-mediated amyloidosis. TTR, also called prealbumin, exists as a tetramer in a physiological state that mainly functions as a transport protein for thyroxine in blood plasma with the help of a retinol-binding protein. Mutations in TTR gene cause instability in the tetramer that consequently leads to misfolding and the formation of protofibrils and fibrils. These amyloid deposits are cytotoxic and cause damage to several organs such as gut, heart, eyes, and kidneys. To date, liver transplantation has been used as the major intervention to overcome the disease. In contrast, ALN-TTR01 showed

dose-dependent silencing of TTR-mRNA. A randomized, placebo-controlled, single-blind, single-dose escalation study was conducted. Up to 36 patients were tested with TTR in Portugal, Sweden, France, and the United Kingdom. It was observed that at a dose of 1 mg/kg, there was a significant reduction in serum TTR levels. With this positive result, Alnylam has headed towards the development of a more potent formulation, ALN-TTR02, using second generation LNP that has shown >10-fold potency *in vivo* (Clinical Trial: ALN-TTR01; Nagasaka, 2012).

12.5.4 Lipid nanoparticles

Alnylam Pharmaceuticals has also investigated blood-related and genetic disorders. ALN-AT3SC was initiated for the treatment of haemophilia patients, especially for those who develop inhibitory antibodies. In the study, antithrombin was knocked down. Consequently, the propensity to bleed decreases (Clinical Trial: AT3-SC). Similarly, TD101 was formulated for another rare genetic autosomal disorder, Pachyonychia congenital. The disorder mainly affects the skin, nails, oral mucosa, hair, and teeth. Therefore siRNA against keratin 6A N171K administered intralesionally was found to be safe and efficient. However, intense pain was noticed during delivery. Thus a more reliable formulation with lipid-based carriers, as well as dissolvable needles, are being developed (Leachman *et al*., 2010). In another study, EphA2, a transmembrane protein belonging to tyrosine-kinase family, is known to overexpress in many cancers such as melanoma, breast, prostate, oesophageal, and lung carcinoma. It was observed that 76% of samples from 79 patients overexpressed EphA2, whih was associated with high stage and high grade disease. Higher overexpression of this protein led to shorter survival of protein, thus EphA2 proved to be an important marker for ovarian cancer. Henceforth, siRNA-based strategies were developed to knock down the function of EphA2 using a neutral (1,2-Dioleoyl-sn-glycero-3-phosphocholine) DOPC liposome. Currently, the drug is in phase I clinical trial, recruiting patients for the study with an aim to determine its safety and maximum tolerated dose (Clinical Trial: EphA2; Hess *et al*., 2001; Zelinski *et al*., 2001).

In 2009, a liposomal−siRNA drug named Atu027 was introduced by Silence Therapeutics for the treatment of solid tumours. Phase I study was conducted in order to determine the safety, tolerability, and pharmacokinetics of the drug. A total of 34 patients were recruited and were subjected to the drug for a period of 28 days. Basically, siRNA mainly targets protein kinase N3 gene (PKN3) that plays a key role in PI3 kinase signalling. As PKN3 is an effector molecule of PI3 kinase signalling cascade, it can be silenced to control tumoural angiogenesis. In general, this drug imparts its function by stabilizing the endothelial vessels mediated changes in the vascular endothelial cadherin. It was observed that the drug was tolerable up to a level of 0.336 mg/kg with less toxicities. Atu027 showed positive results with disease stabilization in 41% of patients and a decrease in the metastasis among a few patients. Furthermore, Atu027 in combination with gemcitabine (Atu027-I-02) was employed for the treatment of advanced/metastatic pancreatic cancer. Thus a phase I and II study was conducted to determine its safety as well as activity of the combinational formulation. The drug was found to be safe and the study was completed in 2016 (Clinical Trial: Atu027; Clinical Trial: Atu027-I-02; Möpert *et al*., 2012; Santel *et al*., 2010).

A novel therapeutic drug, DCR-MYC-102 (denotes a siRNA against myelocytomatosis oncogene synthesized by Dicerna pharmaceuticals), was introduced in a phase I/II trial by Dicerna Pharmaceuticals in 2014 with an intention to treat patients with hepatocellular carcinoma (HCC). For this, a synthetic double-stranded RNA in a stable lipid particle suspension was formulated to target the oncogene MYC that is responsible for growth of the tumour and malignancies. In general, the phase I/IIb study is a multicentered, dose escalation study, designed to test the safety and tolerability of the patients with HCC who do not tolerate sorafenib or who do not have access to the drug. The drug DCR-MYC was administered as intravenous (IV) infusions once a week for 2 weeks following a break of 1 week. In the ongoing phase, 14 patients are subjected to an escalating dose (0.125−0.68 mg/kg) to determine MTD. In a similar fashion, DCR-MYC-101 was introduced in a phase I clinical trial to study its role in patients with advanced solid tumours and haematological malignancies. The drug was given as an IV infusion in 37 patients. It was observed that the antitumour activity was seen in two out of three patients possessing pancreatic neuroendocrine tumours with 34% reduction in the tumour size (Clinical Trial: DCR-MYC; Clinical Trial: DCR-MYC 2). Nitto Denko Corporation initiated another drug, METAVIR F3-4 (ND-L02-s0201), in a phase Ib/II clinical trial for the treatment of patients with moderate to extensive hepatic fibrosis. A randomized study with dose escalation was conducted using vitamin A-coupled lipid nanoparticle containing siRNA against HSP47. The study results showed tolerance in advanced fibrosis patients and histological improvement (METAVIR F3-4).

12.6 Conclusion

The application of nanotechnology is widely increasing in different disciplines. Currently, there are more than 20 nanotherapeutics in clinical use, in which lipid nanoparticles have been predominant. While most of the nanocarriers are polymers, LNPs and dendrimers are in preclinical and clinical phases. Moreover, more than 80% of the nanotherapeutics have aimed at developing anticancer formulations. Thus with continuous efforts, nanotechnology is believed to flourish in the medicine sector in the coming years.

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WOODHEAD PUBLISHING SERIES IN BIOMEDICINE

Nucleic acids have been widely explored for the treatment of cancer and infectious diseases or to block cell proliferation and thereby caused diseases. Nucleic acids–based therapeutics depends on the progress of safe and effective carriers for systemic administration. Nanomedicine has facilitated availability of vectors with diminished cytotoxicity and enhanced efficacy.

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