Biomaterials Science

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Journal:	*Biomaterials Science*
Manuscript ID	BM-REV-07-2016-000441
Article Type:	Review Article
Date Submitted by the Author:	01-Jul-2016
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Recent Progress in Nanomaterials for Gene Delivery Applications

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Abstract

Nanotechnology-based gene delivery is the division of nanomedicine concerned with the synthesis, characterization, and functionalization of nanomaterials to be used in targeted-gene delivery applications. Nanomaterial-based gene delivery systems hold great promise for curing fatal inherited and acquired diseases, including neurological disorders, cancer, cardiovascular diseases, and acquired immunodeficiency syndrome (AIDS). However, their use in clinical applications is still controversial. To date, the Food and Drug Administration (FDA) has not approved any gene delivery system because of the unknown long-term toxicity and the low gene transfection efficiency of nanomaterials *in vivo*. Compared to viral vectors, nonviral gene delivery vectors are characterized by a low preexisting immunogenicity, which is important for preventing a severe immune response. In addition, nonviral vectors provide higher loading capacity and ease of fabrication. For these reasons, this review article focuses on applications of nonviral gene delivery systems, including those based on lipids, polymers, graphene, and other inorganic nanoparticles, and discusses recent advances in nanomaterials for gene therapy. Methods of synthesizing these nanomaterials are briefly described from a materials science perspective. Also, challenges, critical issues, and concerns about the *in vivo* applications of nanomaterial-based gene delivery systems are discussed. It should be noted that this article is not a comprehensive review of the literature.

1. Introduction

Gene therapy is aimed at altering or modifying defective and/or missing gene sequences in order to cure acquired and/or inherited diseases, including genetic disorders, cancer, cardiovascular diseases, and acquired immunodeficiency syndrome (AIDS). Although no gene delivery therapeutics have yet been approved by the Food and Drug Administration (FDA) (1, 2), many clinical trials on the use of gene therapy to cure various inherited and acquired diseases have been conducted. Until now, medicine has treated fatal diseases that result mainly from missing, defective, and/or mutated genetic material through procedures such as symptomatic treatment, radioactivity, and chemotherapy. In contrast, gene therapy provides a new treatment modality of altering the genetic information within cells.

A gene therapy formulation is made up of two main elements, a gene carrier agent and genetic material. The carrier agent protects the genetic material and introduces targeted gene delivery properties with controlled release kinetics. The carrier must be designed to increase transfection efficiency, which is correlated with the proportion of the encapsulated nucleic acids having the ability to transform a target cell to a desired state. Regarding the desired properties, the fundamental challenge is to develop effective, nontoxic, non-immunogenic, noncarcinogenic vectors to deliver genetic material into cells.

Since naked deoxyribonucleic acid (DNA)/ribonucleic acid (RNA) molecules are not able to transfect a cell before being degraded by lysosomes in the endocytic pathway, it is necessary to encapsulate nucleic acids in a carrier system to protect them against enzymatic degradation. Encapsulating DNA/RNA molecules in a carrier vehicle involves using electrostatic interactions between the negatively charged DNA/RNA molecule and the positively charged carrier agent. Upon arrival in a cytoplasm or nucleus, the carrier vehicle should be degraded so that it releases nucleic acids in order to transfect the cell.

There are two main types of vectors used in gene delivery applications, viral and nonviral. Virus capsid proteins have evolved to transfect cells and are more efficient as gene delivery vehicles than nonviral vectors; however, viral vectors raise safety concerns about severe off-target immunogenicity, inflammatory responses, and toxicity (1). In contrast, synthetic gene delivery vehicles have low immunogenicity since patients do not have preexisting immunogenicity

against nonviral vectors (2,3). In addition, nonviral vectors are easier to scale up and synthesize commercially.

There are a number of nanomaterials used for gene delivery applications which are based on lipids, polymers, graphene, carbon nanotubes (CNTs), nanospheres, mesoporous nanoparticles (NPs), and other types of inorganic NPs. Each has its own advantages and disadvantages as a gene delivery platform, and functionalization of these materials with organic and/or inorganic molecules can improve their gene delivery efficiency and lower their cytotoxicity. It has been shown that functionalized nanomaterials are the most promising gene delivery platforms thanks to their small size, targeted delivery of nucleic acids, sustainment of gene delivery effect in target tissue, and superior stability of genetic material (4,5).

The surfaces of nanomaterials are functionalized with small molecules, polymers, and/or biomolecules to modify their physical and/or chemical properties, including charge density, hydrophobicity, and binding affinity to a certain type of cell surface protein/receptor. The physicochemical properties of nanomaterials are also correlated with their size, so control over the size distribution of NPs is important in synthesizing NPs with similar degradation kinetics, cellular uptake mechanisms, and transfection abilities. To ensure effective and safe gene delivery, a number of parameters related to physicochemical properties must be controlled. These include biodegradability, charge density, solubility, molecular weight, crystallinity, hydrophobicity, rigidity, and pKa value of cationic NPs (6).

Viral gene delivery vehicles are more effective at transfecting a target cell; however, their severe immunogenicity limits their use in medical applications *in vivo*. Nonviral gene delivery vectors provide lower immunogenicity, lower toxicity, easier preparation, and higher loading capacity than viral vectors (7). For these reasons, this review article focuses on nonviral gene delivery systems. It discusses their potential in gene delivery applications and introduces recent progress in nanomaterials for gene therapy.

2. Lipid-Based NPs in Nonviral Gene Delivery Applications

Lipid-based NPs are among the major gene delivery vehicles, and their first use for gene delivery was carried out by Felgner et al. in 1987 (8). Lipid-based NPs are made up of four major domains: a cationic polar head group, a hydrophobic domain, a linker, and a backbone domain

(Figure 1) (5). The cationic head group attracts negatively charged phosphate groups on a DNA molecule to form a complex called a lipoplex, which plays an important role in the self-assembly of DNA and lipid NPs. The hydrophobic portion of a lipid NP is composed of a steroid or an alkyl chain, and its length and type affect its transfection efficiency (9). The linker group connects the polar head group with the hydrophobic portion and determines the chemical stability, biodegradability, and transfection efficiency of the lipid NP (5). The backbone domain acts as a scaffold separating the head group from the hydrophobic domain. It can be manipulated by introducing novel side chains to enhance targeting, cell uptake, and trafficking of lipid NPs (10,11).

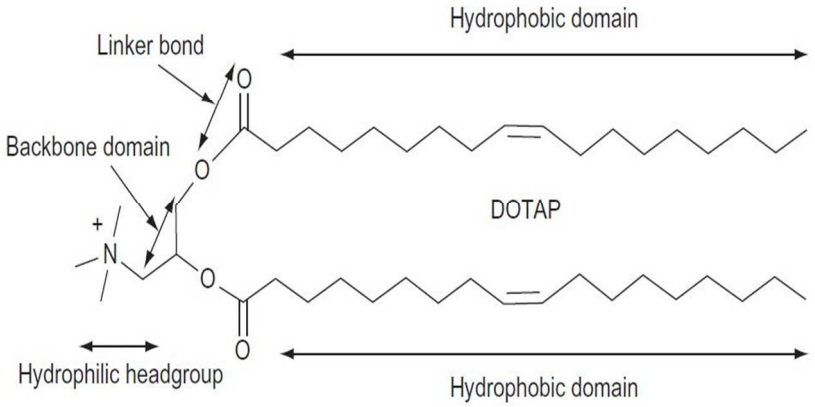

Figure 1. A representative line molecular structure of 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP); reprinted with permission from ref. (5). Copyright 2011 Elsevier B.V.

A vast number of cationic lipids were discovered by 1987, including quaternary ammonium detergents, cationic derivatives of cholesterol and diacylglycerol, lipid derivatives of polyamines, N-[l-(2,3-dioleyl)propyl]-N,N,N-trimethylammonium chloride (DOTMA) and 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP). DOTMA and DOTAP are among the most studied lipid-based NPs. Various formulations of lipid-based NPs have been generated by tuning head group size and hydrocarbon tail length to increase the transfection efficiency (12). However, it has been shown that the use of DOTAP and DOTMA for gene delivery is not efficient enough to transfect a target cell *in vivo*. This is due to the relatively high positive charge density on the liposome surfaces of DOTAP and DOTMA. This high positive charge density gives a poor separation of DNA from lipid NPs and causes poor gene transfection efficiency (13).

Mével et al. introduced a novel cationic lipid, N,N-dioctadecyl-N-4, 8-diaza-10-aminodecanoylglycine amide (DODAG), and a neutral co-lipid, 1,2-dioleyl-sn-glycerol-3-phosphoethanolamine (DOPE) (Figure 2) (14). It has been shown that DODAG has a higher transfection efficiency than DOTMA in OVCAR-3 (ovarian cancer cells), IGROV-1 (a cell line originating from ovarian carcinoma), and HeLa (an immortal cancer cell line) and lower cytotoxicity both in the presence and in the absence of serum (14). The higher transfection efficiency of liposomes containing DOPE is achieved using a tertiary amine group to lower the surface charge of the liposome in order to ease DNA release from the lipoplex (15). Slightly charged liposome surfaces lower the aggregation of lipoplexes and increase transfection efficiency (16).

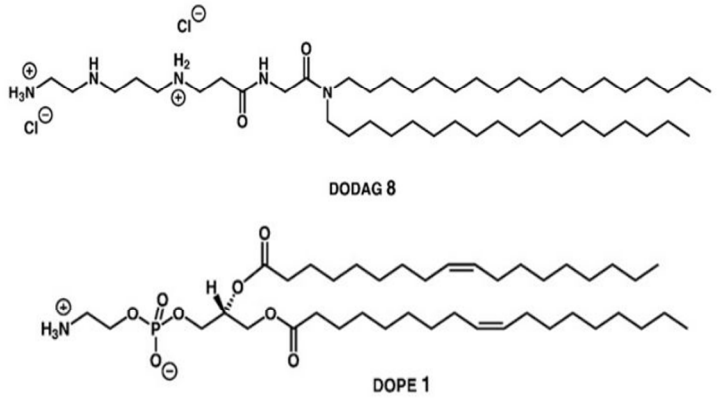

Figure 2. Schematic illustration of cationic lipids of DODAG 8 and DOPE 1; reprinted with permission from ref. (14). Copyright 2009 Elsevier B.V.

Cationic liposomes and DNA/RNA molecules form lipoplexes to protect genetic material from enzymatic degradation, increase stability of the vector system, and interact with the cell through electrostatic interactions between cationic liposomes and the negatively charged cell membrane (5,17). The internalization of lipoplexes is carried out via endocytosis (18). There are a number of endocytosis pathways, including caveolae- and clathrin-independent, caveolae-mediated, and macropinocytosis, and use of these pathways for the internalization process primarily depends on lipoplex diameter (Figure 3) (18). Although the diameter of caveolae vesicles varies between 50 and 100 nm, they can internalize structures up to 300-400 nm (18). In contrast, clathrin vesicles can only internalize structures up to 250 nm (18). However, optimal gene delivery of nanoparticles is reached in the range of 50 nm to 100 nm in diameter (19).

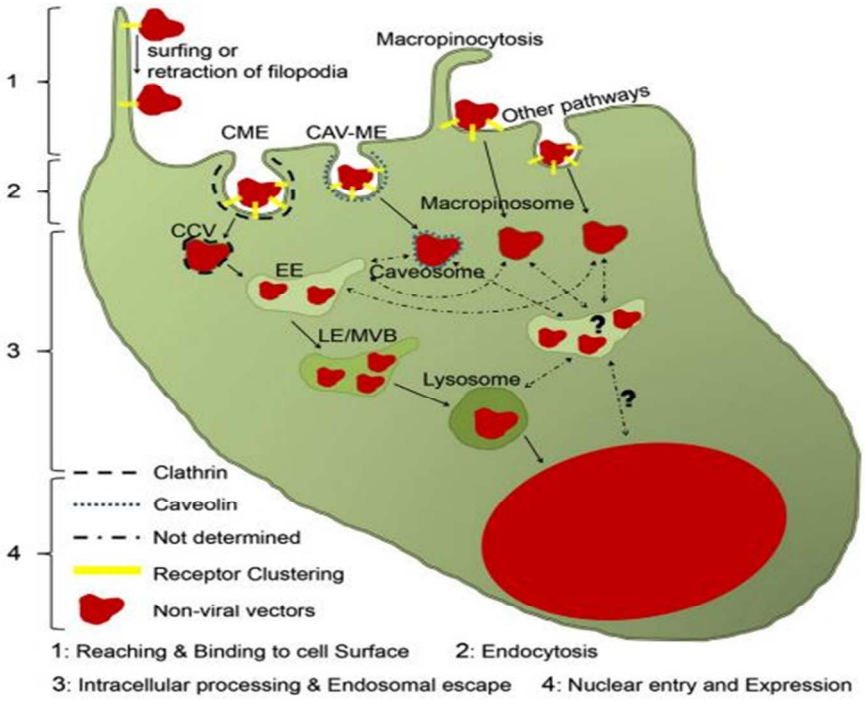

Figure 3. Schematic illustration of entry pathways and cellular barriers to nanocarrier-cell interaction. (1) The cell surface binding of nanocarriers is carried out through the mechanism of filopodia or through direct interaction. (2) After the nanocarrier/cell surface interaction, the cargo may enter the cell via endocytic pathways including clathrin-dependent and clathrin-independent endocytosis. (3) After cellular entry, the nanocarrier is released into the cell via mechanisms including lipid mixing and non-bilayer-induced membrane (lipoplex) perturbation, also called the proton sponge effect. (4) Finally, the nanocarrier is delivered into the nucleus, where it promotes gene expression; reprinted with permission from ref. (18). Copyright 2012 Elsevier B.V.

Although each endocytosis pathway promotes cellular entry of the gene carrier agent, transfection efficiency varies among endocytosis pathways (20,21). In one study, it was shown that the gene transfection efficiencies of lipid-based carrier agents of DOTAP/ dioleoylphosphocholine (DOPC) and 3β-[N-(N,N-dimethylaminoethane)-carbamoyl] (DCChol)/DOPE on chinese hamster ovary cells vary significantly depending on the endocytosis pathways (22). In another study, chloropromazine, an amphiphilic drug preventing clathrin vesicle formation, was used to inhibit the clathrin-mediated internalization pathway so that lipoplexes would be internalized via the cavealae pathway (23). As a result, there was an

approximately 1.3-fold increase in the gene transfection efficiency of Tat-modified lipoplexes (23). Studies have indicated that the gene transfection efficiency of lipopexes varies among the internalization pathways of endocytosis. Therefore, even a successful internalization of lipoplexes via endocytosis may not lead to effective gene transfection.

Addressing the problem of endosomal escape, Gujrati et al. developed a multifunctional cationic lipid-based carrier, (1-aminoethyl)iminobis[N-(oleicylcysteinyl-1-amino-ethyl)-propionamide]) (ECO) (24). ECO is made up of three units including two cysteine-based linker groups, a protonable ethylendiamine headgroup, and two oleic acid lipid tails. The positively charged ethylendiamine headgroup of ECO forms a polyplex with negatively charged siRNA. Disulfide bridges formed between the free thiol groups stabilize ECO/SiRNA NPs. Cysteine-based linker groups provide room for functionalization to improve biocompatibility and targeted delivery of ECO/SiRNA NPs (24). Because of the pH-sensitive nature of CEO/SiRNA, acidification of the endosome induces endosomal escape of ECO/siRNA into cytosol. After the endosomal escape of ECO/siRNA, endogenous glutathione reduces disulfide bonds between ECO molecules to release siRNA into cytosol (Figure 4) (24). The optimal N/P ratio for balancing cytotoxicity and the effective gene silencing effect on a U87 glioblastoma cell line is formulated 10 (24).

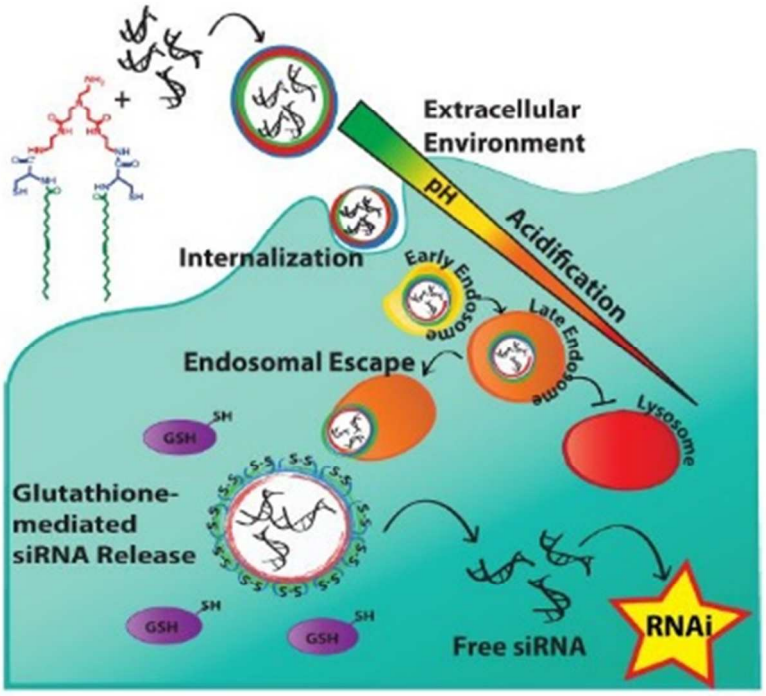

Figure 4. Schematic illustration of ECO/siRNA polyplex formation, internalization, and endosomal escape steps. Electrostatic interaction between the cationic head group of ECO and anionic siRNA forms polyplexes. Because of the pH-sensitive nature of CEO/SiRNA, acidification of endosome induces endosomal escape of ECO/siRNA into cytosol. After the endosomal escape of ECO/siRNA, endogenous glutathione reduces disulfide bonds between ECO molecules to release siRNA into cytosol; reprinted with permission from ref. (24). Copyright 2014 ACS Journals.

Cationic lipid-induced toxicity needs to be avoided by shielding lipid NPs with polyethylene glycol (PEG). PEG shielding can provide an extended circulation time, with a half-life of 1-10 h, by preventing reticuloendothelium system uptake (25,26) and allowing better stability and increased targeted gene delivery because of the availability of surface modifications, compared to pristine lipid-based NPs (27). Naicker et al. reported that PEGylated liposomes increase the stability of cationic lipid NPs by shielding the positive surface charge density of liposomes and promote biocompatibility better than non-PEGylated liposomes (27). In addition, the transfection efficiency of lipoplexes on HEpG2 (human liver carcinoma) cells is increased via asialoglycoprotein receptor (ASGP-R)-mediated targeting of PEGylated liposomes (27).

Repeated administration of PEGylated liposomes induces the accelerated blood clearance (ABC) effect, which shortens their circulation time *in vivo* (28). It has been reported for various animal models that the circulation time of PEG-conjugated liposomes is shortened when they are administered repeatedly (29-34). To overcome the ABC phenomenon, various PEGylated liposome formulations have been proposed, including G–diacylglycerol lipids (PEG–S-DAGs) (35), cholesteryl hemisuccinate (CHEMS)-conjugated PEGs (36), and a pH-sensitive cleavable PEG-lipid derivative of mPEG-Hz-CHEMS (37). Among the proposed PEGylated liposome derivatives, mPEG-Hz-CHEMS is the most promising candidate for lessening the ABC effect and preventing liver accumulation of liposomes, since mPEG-Hz-CHEMS is more easily cleaved and degraded at physiological pH than pristine PEG or the other proposed PEGylated liposome derivatives (37).

Small interfering RNA (siRNA) holds great promise for silencing the expression of specific genes. It is designed as a complementary match with a target mRNA sequence. When siRNA is released into a cell, it interacts with the target mRNA to activate RNA-induced silencing

complex (RISC) (38). In siRNA delivery, increasing the stability and circulation half-life of the delivery system is crucial to increasing gene delivery efficiency and the silencing effect. Similar to DNA delivery systems, the PEGylation of RNA polyplexes holds promise for increasing stability and enhancing the *in vivo* tumor gene silencing effect (39). Sarett et al. have shown that PEGylated siRNA-palmitic acid polyplexes have balanced cationic and hydrophobic contents, which promotes doubled circulation half-life, and increased siRNA biodistribution compared to the unmodified siRNA-palmitic polyplexes (39).

Synthesis Methods of Lipid NPs	Advantages	Disadvantages	Ref.
High-shear homogenization Hot homogenization Cold homogenization	Widespread and practical	Poor dispersion quality	(42-44) (45,46) (47)
Ultrasonication/high-speed homogenization Probe ultrasonication Bath ultrasonication	One of the most practical synthesis methods	Broad particle size distribution and potential metal contamination	(48)
Solvent emulsification/evaporation	Avoidance of excessive heat application	Use of chlorinated organic solvents: biosafety concern	(41,49-52)
Microemulsion-based preparations	Better particle size control	Low-concentration process: solvent needs to be removed	(53-58)
Supercritical fluid technology	Particles yielded as a dry powder: avoidance of solvent removal, use of mild pressure and temperature conditions	Low solubility of polar particles	(59-61)
Spray drying method	Cheaper alternative to lyophilization	Application of high-temperature shear forces: particle melting and aggregation	(55)
Double emulsion method	Common technique: many adjustments and adaptations	Large particle size, low entrapment efficiency	(62,63)

Table 1. Methods of Synthesizing Lipid-Based NPs

There are a variety of methods for synthesizing lipid-based NPs, as shown in Table 1 (40). The most practical method is ultrasonication, but it generates particles with a broad particle size distribution up to the micrometer range. Homogenization is a widespread and practical alternative to the ultrasonication method; however, particle coalescence is a problem because of poor dispersion quality (40). With the microemulsion-based NP synthesis technique, smaller NPs (<100 nm) are produced within solvents: the NPs are distributed into the aqueous phase (acetone), while larger particles are produced using more lipophilic solvents (41).

In a recent study, cationic solid lipid NPs (SLNs) were synthesized using the double emulsion method in order to characterize their transfection efficiency, cytotoxicity, and stability during storage and after lyophilization (64). No significant change was observed in the stability of SLNs in terms of zeta potential, polydispersity index, or hydrodynamic diameter when they were stored at 4^{o}C for 30 days in amber glass flasks (64). HeLa cells in a 1% SLN solution show about 70% viability; however, they show only 10% viability in a 10% SLN solution (64). This indicates the cytotoxicity of SLNs is concentration-dependent. In another study, it was shown that two-tailed cationic lipids such as dimethyldioctadecylammonium bromide, N,N-di-(b-stearoylethyl)-N,N-dimethyl-ammonium chloride, and tetradecyltrimethylammonium bromide have lower cytotoxicity than one-tailed cationic lipids such as cetylpyridinium chloride and tetradecyltrimethylammonium bromide (65). However, the transfection efficiency of one-tailed cationic lipids is better than that of two-tailed cationic lipids (65).

3. Polymeric NPs in Nonviral Gene Delivery Applications

Effective gene delivery vehicles should form complexes with negatively charged DNA/RNA molecules to provide gene packaging that protects genetic material from degradation in the endocytic pathway. Second, a carrier vehicle should be easily modified to provide targeted gene delivery and cellular uptake. In addition, it should be biodegradable so that it releases nucleic acids into cytoplasm or nucleus in a controlled manner. There is a wide range of polymeric NPs that can be used as gene delivery vehicles, and some of them provide these properties, such as poly (2-dimethylaminoethyl methacrylate) (PDMAEMA), poly-L-lysine (PLL), and polyethyl-enimine (PEI).

They are used in a vast number of promising gene delivery applications because they provide controlled release kinetics and adjustable charge distribution via the copolymerization of different polymers. The molecular weights (MW) and chain lengths of polymers have substantial impacts on their physiochemical characteristics. High-MW and long-chain polyplexes have better nucleic acid encapsulation properties, cellular uptake and transfection efficiency than short-chain polyplexes (66,67). However, high-MW and long-chain polymers cause increased immune response and accumulate in living organisms. Therefore, the optimal sizes of polymeric NPs need to be determined for specific applications to balance transfection efficiency and the cytotoxicity of polymeric gene carriers. For example, chitosan, a linear polysaccharide formed by randomly distributed β-(1-4)-linked D-glucosamine monomers to form a natural polymer, holds promise in gene delivery because of its biocompatibility, biodegradability, and nontoxicity (68). In one study, the optimum size range for chitosan was reported to be between 5 and 40 kDa (67). This study indicates that each polymeric NP gene carrier should be specifically formulated to optimize the transfection efficiency of the polymer while inducing little or no cytotoxicity or immunogenicity.

Because of the anionic nature of DNA/RNA molecules, cationic polymers can generate electrostatic interactions with genetic material to form a complex of polymeric NPs and DNA/RNA molecules called a polyplex (66,69). Polyplexes are developed to form nano-sized polymer complexes between nucleic acids and cationic polymers (66,69,70). Hydrophobic and electrostatic interactions between cationic polymers such as PDMAEMA and the negatively charged phosphate groups on the DNA/RNA backbone prevent the enzymatic degradation of genetic material and promote cellular entry (69,71). However, Wong et al. report that cationic polymers may cause a high degree of cytotoxicity (69). Moreover, a strong electrostatic attraction between polymeric NPs and genetic material may lower the release kinetics once the polyplexes are taken up by the cell (69).

Since biodegradable polymers can be degraded to shorter oligomeric and monomeric components because of their ester linkages (i.e., polyesters), they are preferred over non-biodegradable polymers to minimize the accumulation of polymeric NPs in living organisms (72). The degradation kinetics of polymeric NPs are highly influenced by the physicochemical nature of the intracellular microenvironment. According to studies, major challenges in gene

delivery are poor encapsulation efficiency of polymeric NPs, DNA degradation upon gene delivery, and overly slow or fast release kinetics of the encapsulated gene (69,73,74). Moreover, during the formation of lipoplexes, genetic material may be degraded by exposure to organic solvents and/or extreme conditions (75). Therefore, the polyplexes of nucleic acids with polymers should be formed under mild conditions (room temperature, neutral pH, etc.). Smart hydrogels are alternatives that can collapse and swell in response to relatively small changes in temperature and pH, providing better control over DNA/RNA encapsulation and release processes.

The complex formation of DNA/RNA molecules with cationic polymers is a combination of electrostatic interactions and encapsulation. It requires polymers having both cationic moieties and biodegradability. PLL is an example of a biodegradable cationic natural polymer, but its defects, including low transfection efficiency due to a lack of rapid endosomal escape, hamper its use in gene delivery applications (76,77). The inadequate properties of PLL are largely due to nucleic acids dangling on its surfaces making it vulnerable to enzymatic degradation (69,78,79). In a study addressing these problems of PLL in gene delivery applications, PLL was copolymerized with an amphiphilic octadecane (C18)-modified hyperbranched polyglycerol derivative (HPG-C18) through a click reaction to form a star-shaped copolymer that could be used in the codelivery of docetaxel and matrix metalloproteinase-9 (MMP-9) siRNA plasmid (80). Compared to PEI-25k, the formed star-shaped polymer provided improved gene delivery efficiency, better gene packing, and lower cytotoxicity through segment flexibility and local cationic charge density (80).

Vector unpacking and DNA/RNA release are recognized as key concerns in designing an effective gene delivery vehicle (81). The degradation of biodegradable polymers drives the dissociation of the genetic material from the carrier polymer after cellular entry and induces gene delivery into the target site. The degradation site of a polyplex is critical to its delivering genes to the correct site in the cell. To achieve a successful DNA/RNA release, a polymeric gene carrier should remain stable in the endosome. Since the inside of an endosome is acidic, to protect the genetic material against enzymatic degradation, a polyplex should not be degraded in an acidic microenvironment. It should be degraded upon release from the endosome, to release DNA/RNA

into cytoplasm. Thus, polyplexes should be highly stable in the acidic conditions in the endosome but be degraded in a neutral pH to release DNA/RNA into the cytoplasm.

Release kinetics is another concern in transfecting cells because rapid gene transfection increases efficiency and requires lower vector doses, which is critical to minimizing cytotoxicity and immunogenicity (82,83). Figure 5 illustrates the intracellular gene delivery stages (66). The first step is the interaction between a polyplex and a cell membrane in which the cargo is internalized through macropinocytosis, phagocytosis, or receptor-mediated endocytosis (caveolae and clathrin) mechanisms. If the polyplex is digested in the endosome, the encapsulated genetic material is also digested and removed from the cell through the exocytosis mechanism.

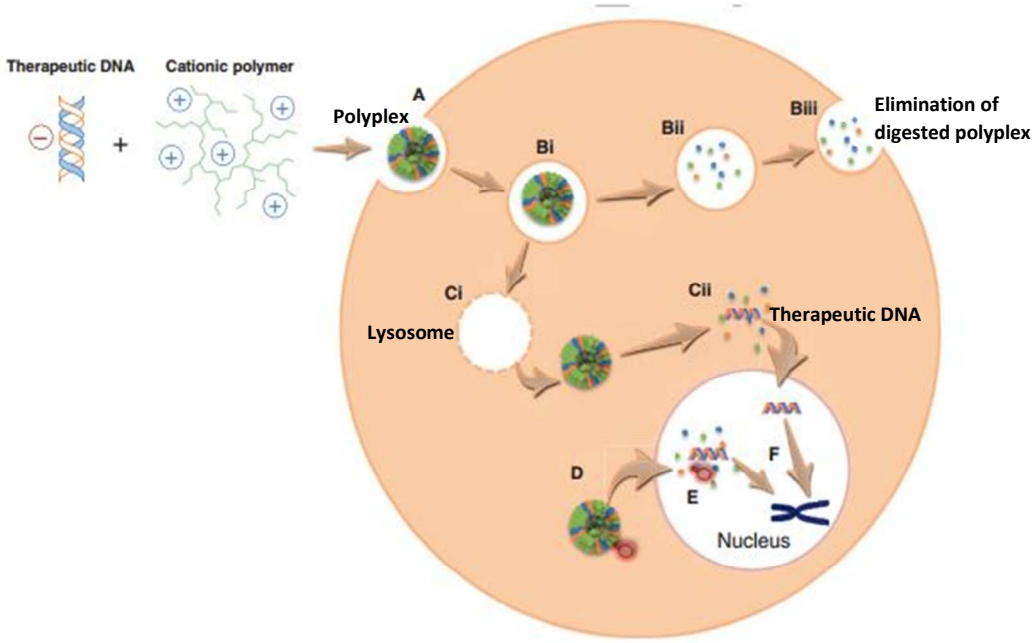

Figure 5. Stages of intracellular delivery of therapeutic DNA. (A) Interaction of polyplex and cell membrane in which the cargo is internalized through macropinocytosis, phagocytosis, or receptor-mediated endocytosis (caveolae and clathrin) mechanisms. (Bi) Internalized cargo is engulfed in a membranous sac called the early endosome. (Bii) If the cargo is trapped in the endosome, it is digested in the late endosome and/or lysosome and (Biii) eliminated from the cell by exocytosis. (Ci) Alternatively, if the cargo escapes from the lysosome via the proton sponge effect, (Cii) gradual degradation of the polymeric matrix by cytoplasmic enzymes promotes DNA release into cytoplasm. (D) Accordingly, nuclear internalization of the polyplex can be promoted via nuclear localization signal (NLS) peptides. (E) DNA is released into cellular

nucleus through polyplex degradation, (F) and the host genome is transfected by therapeutic DNA molecules; reprinted with permission from ref. (66). Copyright 2013 Elsevier B.V.

Therefore, the successful endosomal escape of the polyplex is crucial to inducing the proton sponge effect (Figure 6) (84) to release the gene into the cytoplasm. Alternatively, polyplexes can be decorated with nuclear localization signal (NLS) peptides so that they are internalized into cellular nuclei, which may further increase transfection efficiency.

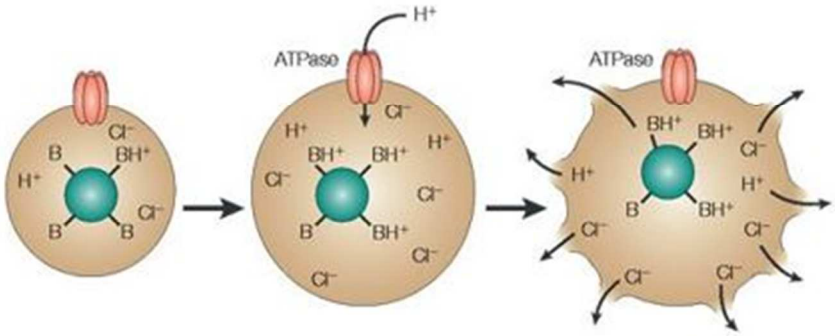

Figure 6. Proton sponge effect: Protonatable groups of polyplexes release protons into the endosome, which induces the passive diffusion of chloride ions into the endosome. This increases the ionic concentration, causing water entry and subsequent swelling of the endosome; reprinted with permission from ref. (84). Copyright 2005 Nature Publishing Group.

Since negatively charged and neutral NPs cannot form electrostatic interactions with negatively charged DNA/RNA backbones, they are not suitable for gene delivery systems. Cationic polymers can form polyplexes with DNA/RNA molecules and interact with negatively charged cell membranes. Furthermore, cationic polyplexes can pass through the cell membrane via electrostatic interactions (85). In addition, the net positive charge of cationic polyplexes is an advantage for endosome escape: it induces the proton sponge effect, which prevents the degradation of nucleic acids and increases gene transfection efficiency (85).

Since PEI-based polyplexes show better transfection efficiency both *in vitro* and *in vivo* than other types of polymers, PEI is a representative example of the use of cationic polymers in effective, efficient gene delivery applications (86,87). DNA/RNA molecules and cationic polymers can self-assemble, condense, and neutralize them to form nanoscale polyplexes (70). Since polymers can be easily functionalized and copolymerized, they have wide-ranging properties and versatility. The versatility of polyplexes increases their potential for use in gene

delivery applications; their other advantages are narrow MW distribution, high stability, and high protection against nucleases (88). Moreover, polyplexes have cationic residues which can pass the vesicular membrane to enhance the transfection efficiency (89).

The MW of polymers can affect their physical properties, including degradation rate and stability. Therefore, the broad MW distribution of polymeric NPs produced by using addition polymerization is a drawback. However, cationic polymers can be synthesized in a variety of ways. The first is condensation polymerization, which is used to synthesize PLL by generating peptide bonds between lysine residues. Another polymerization method is the ring-opening polymerization of 2-ethyl-2-oxazoline, which can be used to produce linear polyethylenimine (PEI) (90). It may be a problem to produce a narrow size distribution of polymeric NPs using ring-opening polymerization, since many monomers come together to form the polymer in an uncontrollable manner. Branched PEI can be synthesized using acid-catalyzed polymerization of oxazoline monomers (90,91). One study showed that emulsion polymerization, a type of addition polymerization, can be used to synthesize poly(2-dimethylaminoethyl methacrylate) (PDMAEMA) (92). Natural cationic polymers like chitosan and cyclodextrin can be synthesized via the modification of natural polymers chitin and starch, respectively (93). In another study, Li et al. used a star-shaped polymer made up of a cationic poly[2-(dimethylamino) ethyl methacrylate] (PDMAEMA) shell and zwitterionic poly[N-(3-(methacryloylamino) propyl)-N,N-dimethyl-N-(3-sulfopropyl) ammonium hydroxide] (PMPD) to encapsulate doxorubicin (DOX) and the p53 gene in micelles during micelle formation of the star-shaped polymer (94). The proposed star-shaped gene delivery system increases caspase-3 activity and reduces the DOX side effect (94). Alternatively, size exclusion chromatography steps may be carried out to achieve a narrower size distribution of particles after the synthesis of polymers; however, these additional steps increase manufacturing cost.

Biodegradable polymers, such as poly-lactic co-glycolic acid (PLGA) copolymer and polycaprolactone (PCL), are widely used in biomedical applications because of their excellent biocompatibility, biodegradability (95,96), and low cytotoxicity (74). Since pristine PLGA is not cationic, it has a low affinity for forming a polyplex with a negatively charged DNA molecule. Further modifications of PLGA need to be carried out to form cationic PLGA NPs (97). Other challenges in using PLGA NPs as gene carriers are the use of harsh manufacturing conditions

and their poor DNA encapsulation behavior (98). Although polyethylene glycol (PEG) has a lower biodegradability than PLGA, its high flexibility and hydrophilicity make it promising for gene delivery applications (99). Moreover, PEG coating (shielding) can provide extended circulation time and better stability *in vivo* (15).

Polymer coating and copolymer NPs are promising tools for extending circulation time, increasing gene transfection efficiency, and minimizing the cytotoxicity of gene delivery vehicles. One of the most promising vaccine candidates against porcine reproductive and respiratory syndrome (PRRS) in one study was created by encapsulating the ORF5 gene of porcine reproductive and respiratory syndrome virus (PRRSV) in poly(D, L-lactide-co-glycolide)/polyethylenimine (PLGA/PEI) NPs (100). Since application of the ORF5 gene alone gives inadequate transfection efficiency because of the enzymatic degradation of genetic material, the ORF5 gene was encapsulated in PLGA/PEI nanoparticles in order to protect the genetic material and provide sustained gene delivery (100). In addition, naked DNA and DNA with a number of gene carrier agents, including PLGA-DNA, branched polyethylenimine (BPEI)-DNA, starburst polyethylenimine(SPEI)-DNA, PLGA/BPEI-DNA, and PLGA/SPEI-DNA, were tested, and the most significant increase in humoral and cellular immune response against PRRS was obtained using the PLGA/BPEI-DNA gene delivery system (100). In another study, biodegradable and biocompatible polymeric nanopharmaceuticals (PNPs) were formulated by conjugating PLGA and siRNA via an intracellular cleavable disulfide linker (PLGA-siRNA) (101). Additionally, PLGA was conjugated with PEG to improve the pharmacokinetics of the PNPs; a cation was complexed with siRNA to avoid the high negative zeta potential of siRNA; and, finally, polyvinyl alcohol (PVA) was conjugated to prevent the aggregation of PNPs (101). It has been shown that knockdown in mice bearing human colorectal xenograft HT-29 tumors occurs after 6 hours and reaches a maximum of 50% after 168 hours of administration (101).

One of the most promising applications of gene therapy is against cancer. A combined chemo-gene therapy approach is promising and aims to deliver chemotherapeutic drugs along with a plasmid DNA/siRNA which transfects cancer cells to make them more vulnerable against chemotherapeutic drugs. Moreover, the polymeric NP conjugated chemo-gene therapy approach provides targeted delivery and controlled release kinetics and allows the use of a lower dosage of chemotherapeutic drugs than the conventional therapy. Double-walled microspheres of PLGA

cores and poly(L-lactic acid) (PLLA) shell layers were used to deliver DOX along with chitosan/p53-encoding plasmid, and it was shown that p53-encoding plasmid is able to transfect cancer cells to activate caspase-3, which further enhanced the anti-proliferation efficacy of DOX in HepG2 cells (102). However, the encapsulation of chemotherapeutic drugs and genetic material in the same cargo and delivery of the therapeutics together may cause interference between the drug and the DNA molecules which reduces gene transfection efficiency (103). The interference effect of chemotherapeutic drugs teniposide, a podophyllotoxin derivative; cis-diamminedichloroplatinum(II) (CDDP), an anticancer drug containing platinum; and temozolomide, an alkylating agent used as a prodrug, were studied, and the mechanism of the chemotherapeutic drug action was found to affect the degree of the interference between the drug and the DNA molecules (103). Since teniposide and CDDP damage the DNA double helix to inhibit the DNA replication process, their interference effect on the gene transfection efficiency is greater than that of the prodrug, temozolomide (103). Therefore, the order of polyplex formation is important to protecting the DNA molecule from damage due to chemotherapeutic drug activity: direct physical interaction between the encapsulated DNA molecule and the chemotherapeutic drug needs to be minimized.

In addition to the co-delivery of DNA and chemotherapeutics, the synergistic effects of siRNA and an antitumor drug hold great promise for the design of better treatment modalities against cancer (104). In one study, PDMAEMA was conjugated with PEG and polycaprolactone (PCL) to fabricate PDMAEMA-based amphiphilic nanomicelles, mPEG−PCL-graft-PDMAEMA (PECD), to be used in DOX and siRNA co-delivery applications (104). It has been shown using fluorescence tracking that DOX-loaded (PECD-D) can conjugate with siRNA and co-deliver siRNA and DOX *in vitro* and *in vivo* (104). Since the cancer microenvironment is acidic, designing pH-sensitive delivery systems has potential for synergistic tumor therapy. A pH-sensitive triblock copolymer micelle, N-succinyl chitosan–poly-L-lysine–palmitic acid (NSC–PLL–PA), is fabricated for use with DOX-siRNA co-delivery in synergistic tumor therapy (105). Rapid release of siRNA and DOX is achieved in hepatocellular carcinoma multidrug-resistant (HepG2/ADM) cells because the triblock copolymer micelle is not stable in the acidic microenvironment of tumors and releases its cargo into the tumor cells, significantly inhibiting tumor growth (105).

In addition to synthetic polymers, a positively charged natural polymer, chitosan, has promising applications in gene delivery because of its biocompatibility, biodegradability and low toxicity, as shown in Table 2 (106-108). Chitosan has a positive charge due to its free C2-amino group and can complex with the negatively charged phosphate backbone of the DNA molecule (106). Furthermore, chitosan can be PEGylated with a number of PEG derivatives (106) and/or grafted with synthetic polymers such as polyethylenimine (109) in order to improve its circulation time, gene packing and gene delivery efficiency. Chen et al. showed that grafting chitosan with PEI increases gene transfection efficiency 44 times compared to pristine chitosan and 38 times compared to pristine PEI in human epithelial type 2 (HEp-2) cells (109). In another study, siRNA was conjugated with polyethylenimine-grafted chitosan oligosaccharide (CSO-PEI) to suppress endometriotic lesion formation (110). To increase gene silencing efficiency, CSO-PEI/siRNA is conjugated with hyaluronic acid (HA) because of its specific binding to CD44 (110). It has been shown that (CSO-PEI/siRNA)HA gives a more significant accumulation in an endometriotic lesion than CSO-PEI/siRNA and significantly diminishes endometriotic lesion size (110).

Table 2. Advantages and disadvantages of use of common cationic polymers in gene delivery applications.

Name	Advantages	Disadvantages
Poly-L-lysine (PLL)	Biodegradable peptide-based structure: similar to protamine and other amphiphilic peptides	Poor transfection efficiency
Polyethylenimine (PEI)	High positive charge density: increased loading capacity and transfection efficiency	High toxicity
Poly-amido-amine (PAMAM) dendrimers	Low toxicity, high transfection efficiency, and ease of manufacturing	Poor biodegradability
Chitosan	Natural polymer: biodegradable and	Low transfection efficiency due

	digestible	to poor endosomal escape Poor solubility in aqueous solutions
Cyclodextrin	Excellent biocompatibility and biodegradability	Difficulty in processing

4. Graphene-Based Nanomaterials in Nonviral Gene Delivery Applications

As a newly emerging class of nanomaterials, nanoparticle-based gene delivery systems have aroused increasing interest because of their unique structures and functionalities aimed at reducing drug toxicity and enhancing gene delivery efficiency. Specifically, great interest has been raised in the synthesis of graphene-based nanomaterials since their invention because of their wide range of applications. It is widely established that the morphology of nanomaterials has a significant impact on their performance and therefore provides enormous opportunities for enhancing the performance of their applications. The large surface area, excellent thermal and electrical conductivities, and ease of functionalization of their surfaces make graphene-based nanomaterials a promising gene/drug delivery platform carrying active agents and targeting specific tissue types. On the other hand, interaction between biomolecules and graphene oxide (GO) *in vivo* forms protein corona on the surface of NPs and decreases cell uptake and lowers biocompatibility by inducing immune response (111,112). In a study, dose-dependent cytotoxicity of GO nanosheets on human breast cancer cell line of MDA-MB-231 has been observed higher than 100 mg/mL concentration of GO nanosheets (113). Since naked GO induces immune response and causes cytotoxicity to macrophages due to interaction between blood proteins and GO surface, surface modification of GO with PEG or bovine serum albumin could lower GO cytotoxicity *in vivo* (114,115).

Increasing the gene packing property of gene delivery systems is an important concern, and GO) provides a large surface area for encapsulating DNA molecules (116). However, GO is

negatively charged, and electrostatic repulsion between the negatively charged phosphate backbone of the DNA molecule and GO needs to be shielded via surface modification of GO using cationic polymers such as polyethylenimine (PEI). Thanks to the availability of a wide range of surface modifications, graphene has emerged as one of the most promising nanomaterials for diverse applications in nanomedicine, and many groups have focused on developing various kinds of GO-based drug/gene delivery systems, including ours. Our group has synthesized a series of graphene-based nanomaterials which are promising for applications in gene delivery and the intracellular tracking of delivery platforms (117-119). For example, a graphene-based gene delivery vehicle was reported to offer tremendous durability in diagnosing life-threatening disease (117). The proposed gene delivery vehicle exhibits good performance for single-stranded DNA delivery (Figure 7) (117). It has also been shown that a graphene-based platform enhances single-stranded DNA adsorption and protects the DNA molecule from enzymatic cleavage in complex cellular and biofluid samples. Lin's laboratory extended the graphene-based platform to the exploration of intracellular delivery routes and *in situ* molecular probing applications (118,119).

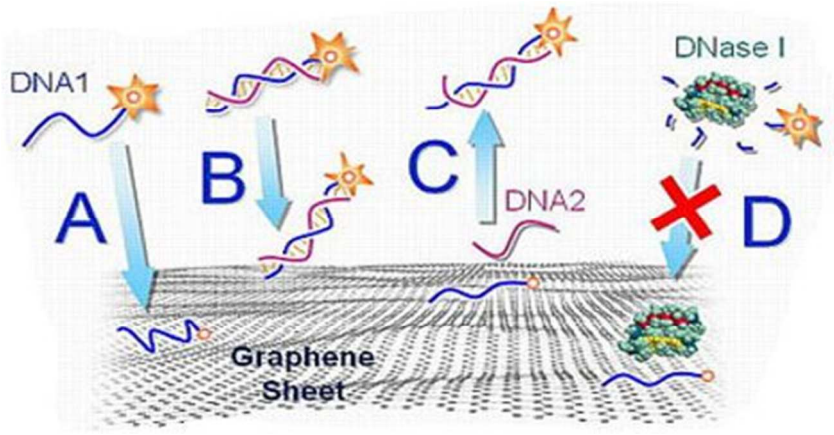

Figure 7. An illustration of how fluorescence-tagged DNA interacts with functionalized graphene. Both (A) single-stranded DNA (ssDNA) and (B) double-stranded DNA are adsorbed onto a graphene surface, but the interaction is stronger with ssDNA, causing the fluorescence intensity of the ssDNA to decrease via the fluorescence quenching of graphene. C) Complementary DNA nears the ssDNA and causes the adsorbed ssDNA to detach from the graphene surface. D) DNA adsorbed onto graphene is protected from being degraded by enzymes; reproduced with permission from ref. (117). Copyright 2010 John Wiley & Sons.

Single-layered GO and reduced GO, characterized by planar covalent-network solids, possess not only an ultrahigh surface area but also a tunable band gap, which provides a host of properties for molecular shuttling. This high molecular loading efficiency is due to the specific binding between GO and DNA/drug molecules, which makes GO an excellent platform for the immobilization of nucleotides on its surface. The introduction of specific ligands onto GO surface provides selectivity towards specific target cells. In addition, the properties of GO can be harnessed in composites through incorporation with other materials because of synergistic contributions among components. For example, PEI, a positively charged polymer, can interact electrostatically with negatively charged phosphate groups of DNA or RNA and form a complex of GO and DNA molecules. Since PEI has low biocompatibility and high cytotoxicity, it needs to be functionalized so as to avoid its cytotoxicity. Zhang et al. fabricated layered PEI-grafted GO for the sequential delivery of small interfering RNA (siRNA) (120). In that study, GO was covalently functionalized with PEI through amide bond formation of N-ethyl-N'-[3-(dimethylamino)propyl]carbodiimide (EDC) chemistry. PEI-GO is more favorable than bare GO for gene packaging and exhibits a better gene transfection efficiency of siRNA delivery. Recently, using the same method, Feng et al. synthesized PEG and PEI co-conjugated ultrasmall nano-GO nanocomposites (NGO-PEG-PEI) and then immobilized siRNA on the surface of the composite through electrostatic interaction to create a photothermally enhanced gene delivery platform (121). This NGO-PEG-PEI system was shown to have superior stability in salts and serum and better transfection efficiency than pristine PEI or GO-PEI (121). Furthermore, the gene release kinetics of the NGO-PEG-PEI system can be modulated through the application of near-infrared (NIR) laser irradiation to increase transfection efficiency and provide light-controlled localized gene delivery therapy. The transfection efficiency of NGO-PEG-PEI on HeLa cells shows concentration dependence on the ratio of moles of cationic polymer to moles of phosphate groups on the DNA backbone, called the N/P ratio (102,121). Bare PEI has superior transfection efficiency at an N/P ratio of 10; however, its transfection efficiency is lower than that of NGO-PEG-PEI when the N/P ratio is increased, because of the cytotoxicity of bare PEI (121). Polymer coating and copolymerization change particle size, charge distribution, zeta potential, and the extent of weak interactions between polymer chains. Changes in these parameters affect the degradation rate, circulation time, cytotoxicity, and cellular internalization pathway of functionalized GOs. Control over these parameters to modulate gene delivery

efficiency and minimize cytotoxicity in order to optimize formulations is still elusive. Zhou et al. integrated PEI with ultrasmall GO to develop a robust bio-interface, which was used to fabricate an efficient DNA delivery method (122). The interlinkage of highly concentrated DNA molecules with PEI-GO provides an increased transfection efficiency of plasmid DNA into mammalian cells.

It has been shown that chitosan-functionalized GO can be effective for plasmid DNA (pDNA) immobilization and the delivery of cargo into cancer cells (123). Liu et al. employ PEI as a stabilizer to synthesize well-dispersed PEI-GO nanocomposites using π–π stacking interactions. This results in a specific GO–PEI nanocomplex which exhibits less cytotoxicity and a higher transfection efficiency than bare PEI (124). Zhang et al. take advantage of the unique properties of PEI to synthesize PEI-GO nanocomposites with good solubility and biocompatibility, and have used this platform effectively for DNA immobilization and delivery applications (125). Yang et al. showed that the synergistic effect of B cell lymphoma 2 (Bcl-2)-targeted siRNA/GO-PEI nanocomposites can reduce Bcl-2 protein expression to suppress oncogene activity and improve gene delivery efficiency to HeLa cells (126). Yin et al. developed another promising GO-based gene delivery system, functionalized GO-PEG-1-pyrenemethylamine nanocomposites, as a robust bio-interface; this is an efficient siRNA delivery system (127). The introduction of PEG improves the stability of GO, while 1-pyrenemethylamine adsorbed on GO via π–π stacking interactions enhances siRNA loading capacity.

As discussed above, GO-based nanocomposites, including PEI-(reduced)GO, PEI/GO, and PEG/GO, offer great opportunities for creating more complex functional nanostructures to develop novel gene delivery vehicles. Although the GO-based nanocomposites have great potential for the design of better gene delivery vehicles, the *in vivo* fates of these systems are still elusive.

5. Other Nanomaterials in Nonviral Gene Delivery Applications: Nanotubes, Nanoshells, and Mesoporous NPs

In addition to the materials described above, there are other types of nanomaterials, including carbon nanotubes, nanoshells, and mesoporous nanoparticles, receiving significant consideration due to their unique physical, chemical, and electronic properties (128-130). The explosion of studies on graphene-based nanomaterials has also raised interest in using other nanomaterials for

gene delivery applications. Unlike graphene, which is composed entirely of carbon atoms, other nanomaterials can be used to create novel bio-interfaces that are versatile and have diversity of structure, composition, and functionality. Because of their distinct properties and large specific surface areas, these nanomaterials hold great promise for loading larger amounts of gene and drug molecules and providing the functionality to control the fate of drug/gene delivery systems *in vivo*.

Synergy among a large surface area and other properties including electrical conductivity, ease of surface functionalization and the fast heterogeneous electron transfer of carbon nanotubes (CNTs) has been discussed for a broad range of applications for gene therapy (131). In addition, the functionalization of CNTs may further improve their properties to minimize cytotoxicity and increase targeted gene delivery efficiency and loading capacity (132). Large surface areas also endow them with enhanced mass transport and high loading capacity. By virtue of the synergy between the stability and the biocompatibility of functionalized CNTs (fCNTs), excellent immunogenic properties and effective condensation of plasmid DNA can be achieved to create better gene delivery platforms (133,134). With the intercalation of specific molecules between single-walled carbon nanotubes (SWCNTs), functionalized SWCNTs could be used to stimulate drug or gene delivery *in situ* using NIR irradiation (131,135-137). For example, Lu et al. fabricated novel folate conjugated-magnetic multi-walled carbon nanotubes (FA-MN-MWCNTs), and it has been shown that DOX-loaded FA-MN-MWCNTs have better specificity toward U87 human glioblastoma cells than pristine DOX because of the specific ligand-receptor interaction with magnetic targeting and enhanced cytotoxicity to the cancer cells (138). In another study, Cy3-labelled DNA was linked to SWCNTs (Cy3-DNA/SWCNT), and an increase in Cy3 fluorescence due to the release of Cy3-labelled DNA from the fluorescence quencher, SWCNT, was observed (139). Furthermore, the proposed gene delivery system, Cy3-DNA/SWCNT, is functionalized with a polyethylene glycol (PEG) moiety and a folic acid (FA) terminal group to provide selective internalization of the gene delivery system into HeLa cells.

In addition to CNTs, other promising nanomaterials, such as porous nanospheres and silica nanospheres, have been exploited as new gene delivery materials to develop novel gene delivery systems. Radu et al. employed mesoporous silica nanospheres (MSNs) to develop a gene delivery system (140). Plasmid DNA with enhanced green fluorescence protein (eGFP) was

adsorbed onto functionalized MSN via electrostatic assembly (Figure 8) (140). The resulting DNA-MSN nanocomposites were characterized by good biocompatibility and high transfection efficiency when they were used to target and take up neural glia, human cervical cancer cells, and ovarian cells.

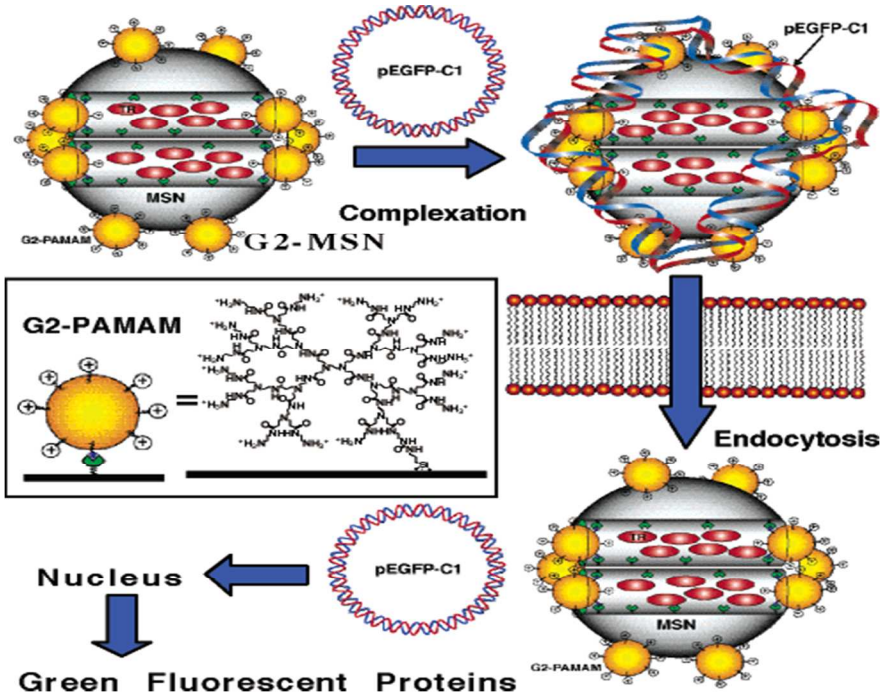

Figure 8. Schematic illustration of a nonviral gene transfection system. G2-PAMAM dendrimer-capped MSN material is loaded with Texas Red (TR) and then complexed with an enhanced green fluorescence protein (Aequorea Victoria) plasmid DNA (pEGFP-C1); reprinted with permission from ref. (140). Copyright 2004 American Chemical Society.

Li et al. took advantage of the unique properties of MSN-based nanocomposite nanomaterials with good solubility and biocompatibility. The resulting nanocomposite was more favorable for the immobilization of siRNA and effective uptake into A549 (a lung cancer cell line) and HeLa cells (141). The low cytotoxicity of MSNs for six days was realized simultaneously. In another work, Kim et al. fabricated monodispersed MSN (MMSN) through hydrothermal synthesis (142). Compared to normal MSN, the obtained MMSN was more favorable for pDNA loading and exhibited high gene delivery efficiency. Using the same method, Pan et al. first synthesized TAT-MMSN and then loaded DOX onto the surface of nanocomposites through electrostatic interaction to deliver it to targeted nuclei and kill cancer cells (143). The resulting nanocomposites displayed high efficiency for cell-nucleus-targeted drug delivery, holding out

great promise for gene delivery in various fields. In another study, Hartono et al. integrated MSN with PDMAEA to construct a robust nanocomposite which was used to fabricate a novel gene delivery vehicle (144). Probe siRNA is linked to the surface of nanocomposites, and enhanced transfection efficiency is observed using poly (acrylic acid) as nanopores. Nanocomposites based on magnetic MSN (Figure 9) (145) and complex PEI/MSN (141) are also promising for fabricating gene delivery vehicles with increased gene delivery efficiency.

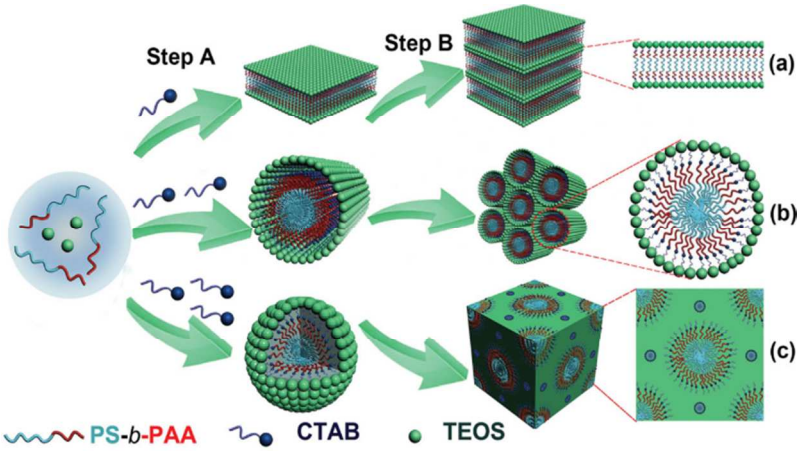

Figure 9. Schematic illustration of the formation of ordered large-pore silica nanospheres with tunable pore structure: (a) lamellar, (b) hexagonal and (c) cubic. Step A: increasing concentration of cetyltrimethyl ammonium bromide (CTAB) induces the morphological transformation of the silicate/polystyrene (PS) b-poly acrylic acid (PAA) micelle aggregates. Step B: orderly packing together, or aggregating assembly, forms long-period lamellar, hexagonal and cubic stacking structures; reprinted with permission from ref. (145). Copyright 2014 John Wiley & Sons.

Functional nanomaterials hold promise as nanoquenchers in gene delivery because of their high quenching efficiencies, large surface area, and good biocompatibility. Two-dimensional nanomaterials have been widely employed as quenchers and delivery systems to achieve multifunctional therapy. Fan et al. investigated the fluorescence quenching efficiency of manganese dioxide (MnO_2) nanosheets for the first time and revealed their different affinities for single-stranded DNA and double-stranded DNA (146). They could efficiently deliver them into cancer cells and achieve gene silencing in the presence of intracellular glutathione. Several delivery platforms based on MnO_2 nanomaterials have been reported. As shown in Figure 10, Zhao et al. systematically studied the interaction of MnO_2 nanosheets with DNA and proposed a universal gene delivery and intracellular imaging strategy (147). More importantly, other therapy

methods, such as magnetic resonance imaging (MRI), can also be facilely combined with this universal platform for further developments in gene therapy.

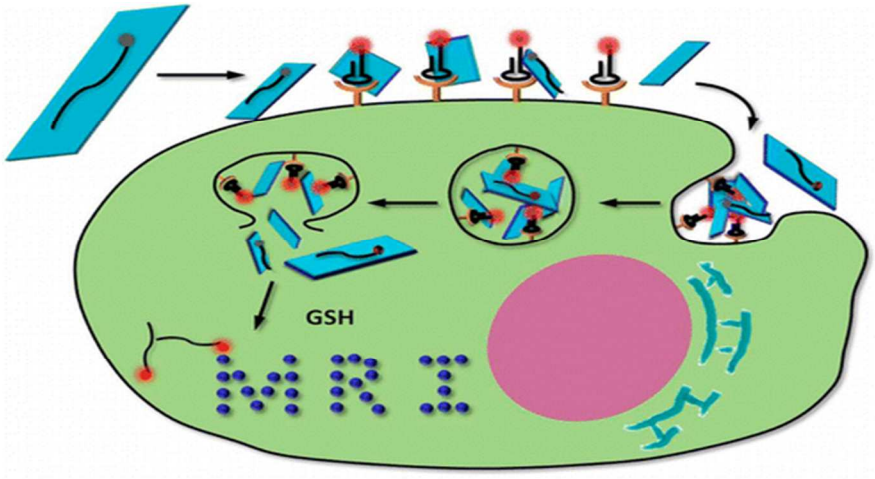

Figure 10. Schematic illustration of the activation mechanism of the manganese dioxide (MnO_2) nanosheet/DNA nanoprobe for fluorescence/MRI bimodal tumor cell imaging: a redoxable MnO_2 nanosheet is modified to be used as a DNA nanocarrier, fluorescence quencher, and intracellular glutathione (GSH)-activated MRI contrast agent. Binding of aptamers to the target cell causes partial fluorescence recovery and induces endocytosis of nanoprobes. Once the cargo reaches the cytoplasm, GSH reduces the MnO_2 nanosheets to increase the fluorescence signal intensity further and generate Mn^{2+} ions suitable for MRI; reprinted with permission from (147). Copyright 2014 American Chemical Society.

Although few available examples illustrate the challenges for using graphene or 2D materials beyond graphene for gene delivery, the cytotoxicity caused by these nanomaterials needs to be addressed and the drawbacks associated with exposure to these nanomaterials must be determined before they are considered for clinical uses. A recent review discussed the variation of the cytotoxic response of 2D materials with dose, surface group, particle size and shape (148). It was found that in exfoliated transition metal dichalcogenide nanosheets WS_2 induced the lowest cytotoxic effect to A549 cancer cells, while other graphene-like 2D materials exhibited a dose-dependent cytotoxic effect on cell viability (149). A comprehensive morphology study on cytotoxic responses to three different types of MoS_2 samples indicated that aggregated MoS_2 had stronger cytotoxicity to the THP-1 cell line than exfoliated MoS_2 or surface-modified MoS_2. Moreover, an inflammation in lung was observed upon exposure to aggregated MoS_2, indicating

the aggregated nanomaterials may be associated with higher cytotoxicity than thin or single-layer nanomaterials (150). Exfoliated or surface-modified nanomaterials will increase dispersibility and biocompatibility as well as reduce surface reactivity at the cellular interface. Other types of metal- and carbon-based graphene-like 2D nanomaterials, including metal nanosheets and C_3N_4 nanosheets, also displayed low cytotoxicity. Ultrathin palladium nanosheets show negligible toxicity to organs because they hardly accumulate in organs: they can be eliminated through metabolism (151). Similarly, C_3N_4 results in no cytotoxicity at high doses, indicating that it could be a suitable candidate for transition 2D nanomaterials (152,153).

Before these nanomaterials are used in real applications, it is mandatory to study their health and environmental impacts to assess their risk potential. Our understanding of the cytotoxic properties of nanomaterials is still in the initial stage; their long-term impacts on human beings need to be addressed in more detailed research. As we known, CNTs and graphene could result in lung damage caused by sub-chronic granulomatous inflammation due to long-term accumulation and slow degradation (154-156). Graphene and CNTs exhibit great resistance to oxidation, resulting in low biodegradability. C_3N_4 and other 2D graphene-like nanomaterials which possess semiconducting degradability have an inherently semiconducting property, because of which they are widely used in tissue engineering. Two-dimensional materials possess distinct properties; as long as their cytotoxicity is low, they can be advantageous nanomaterials for biological applications. The critical issues that need to be further addressed are their long-term cytotoxic effects and their environmental resistance.

6. Perspectives and Conclusions

Gene therapy provides a new platform for curing illnesses which generally involve the malfunctioning of cellular machinery because of missing, defective, and/or mutated genetic material. Since a naked DNA/RNA molecule is not able to reach a cell before being degraded by lysosomes in the endocytic pathway, gene delivery vehicles are needed to provide protection and transfection ability to nucleic acids. There are two main types of gene delivery vectors, viral and nonviral. Thanks to their low immunogenicity and low toxicity compared to viral gene delivery vectors, nonviral gene delivery systems are safer than viral systems *in vivo*.

Compared to the conventional formulations, which are mainly composed of a single active agent, nonviral gene delivery systems are highly complex. For example, lipid NPs are made up of four

major active components, and each must be modulated to reach an effective gene delivery level. The majority of the studies conducted in the field have used small animal models to test the effectiveness of formulations and dosages. The scaling up of these formulations for clinical use needs to be studied on larger animal models to determine the effective gene delivery dosages. Although there are a number of hypotheses about the intracellular mechanisms of gene delivery systems, there are still knowledge gaps that need to be filled in so that gene transfection efficiency can be increased. In order to study the cellular intake mechanism, nanomaterials can be decorated with a variety of imaging agents, including fluorescent molecules.

Lipid-based NPs are some of the major and most studied nonviral gene delivery systems. However, their low efficiency in gene delivery requires modifications such as PEG shielding. In contrast, cationic polymers interact electrostatically with the negatively charged DNA/RNA backbones and cell membranes to provide efficient DNA/RNA packing and cellular uptake. Their drawbacks are poor MW control, overly slow or fast degradation kinetics, and toxicity of degradation by-products. As for graphene-based nanomaterials, they are promising vectors for nonviral gene delivery applications because of their superior physicochemical properties and potential for targeted gene delivery applications. However, dose-dependent cytotoxicity of GO is need to be avoided by coating its surface with PEG or bovine serum albumin. It has been shown that GO-PEI NPs are promising for providing transfection efficiency and decreased cytotoxicity in gene delivery compared to bare PEI NPs. As a supplementary approach, inorganic NPs may have applications in biolabeling and bioimaging for designing *in vivo* NP tracking systems.

On the other hand, lipid-based NPs do not condense DNA/RNA well and have poor gene delivery performance. Cationic polymer-based NPs have a broad MW distribution, so their degradation kinetics and cytotoxicity problems vary. Thus, a variety of strategies should be used to develop alternative gene delivery platforms such as higher-ordered, multicomponent nanomaterial systems that exploit the strengths of individual components of various types of materials. Moreover, hybrid NPs can be modified with various ligands, including biomolecules, aptamers, and NLS peptides, to increase their cellular and nuclear uptake abilities.

Most of the nanomaterials discussed above have been extensively studied *in vitro*, but only some of them have been successfully tested *in vivo*. The knowledge gaps discussed above contribute to the limited *in vivo* studies. Nanomaterials exhibit lower *in vivo* transfection efficiencies than

expected, which may be due to their poor endosomal escape. Even if they are successfully released into cytoplasm, their intracellular fate in the presence of cytoplasmic nucleases is still elusive. In addition, the long-term cytotoxicity and fate of internalized NPs are not well understood. There is no single NP system that addresses all of these concerns. Therefore, taking advantage of the synergistic effects among different NPs is key to designing superior nanomaterials for gene delivery applications.

Acknowledgements

This work was supported by the Centers for Disease Control and Prevention/National Institute for Occupational Safety and Health (CDC/NIOSH) Grant R21OH010768. Its contents are solely the responsibility of the authors and do not necessarily represent the official views of the federal government.

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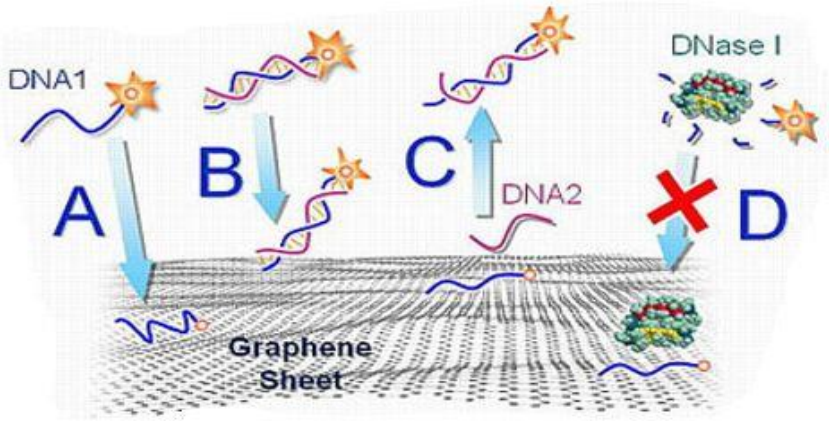

Thanks to the availability of a wide range of surface modifications, graphene has emerged as one of the most promising nanomaterials for gene delivery applications in nanomedicine.