Nonviral Vectors for Gene Delivery

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1. Introduction to Gene Therapy

Gene therapy has gained significant attention over the past two decades as a potential method for treating genetic disorders such as severe combined immunodeficiency,[1] cystic fibrosis,[2] and Parkinson's disease,[3] as well as an alternative method to traditional chemotherapy used in treating cancer.[4] Research efforts are currently focused on designing effective carrier vectors that compact and protect oligonucleotides for gene therapy: free oligonucleotides and DNA are rapidly degraded by serum nucleases in the blood when injected intravenously.[5] Initial research concentrated on using viral carriers, including both retroviruses and adenoviruses, as these vectors exhibited high efficiency at delivering both DNA and RNA to numerous cell lines.[6] However, fundamental problems associated with viral vector systems, including toxicity, immunogenicity, and limitations with respect to scale-up procedures, encouraged the investigation of other potential scaffolds exogenous DNA into targeted tissue.[7]

Nonviral vector systems, including cationic lipids, polymers, dendrimers, and peptides, all offer potential routes for compacting DNA for systemic delivery. However, unlike viral analogues that have evolved means to overcome cellular barriers and immune defense mechanisms, nonviral gene carriers consistently exhibit significantly reduced transfection efficiency as they are hindered by numerous extra- and intracellular obstacles. However, biocompatibility and potential for large-scale production make these compounds increasingly attractive for gene therapy.[8] As a result, a significant amount of research in the past decade has focused on designing cationic compounds that can form complexes with DNA and can avoid both in vitro and in vivo barriers for gene delivery. In the following sections, the main barriers for nonviral gene delivery will be discussed, and the current strategies for overcoming these obstacles will be illustrated by compound class.

1.1. In Vitro Barriers for Cellular Uptake

1.1.1. DNA Complexation

Facile cellular uptake of free DNA via plasma membrane permeation is hindered by the size and negative charge of

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the DNA. While several studies have shown that free DNA can be introduced into cells through electroporation,[9,10] a "gene gun",[11] or direct injection into target tissue,[12] the clinical relevance of these methods is limited. Systemic circulation of free DNA is hindered by nuclease degradation. Complexation of DNA mediated by electrostatic interactions between the negatively charged phosphate backbone of DNA and cationic molecules leads to charge neutralization and a compaction of the nucleotide fragment. It has been shown that the size of the complex formed varies significantly depending on the type of cationic structure used (although preparation conditions including concentration of DNA, pH, type of buffer, and N/P ratio also affect size). For example, cationic oligomers such as cysteine-spermine-cysteine exhibit reversible binding that result in the collapse of single molecules of DNA. The particle size for such complexes varies proportionally to the cubic root of the DNA size.[13] Cationic polymers, on the other hand, typically interact with DNA in an stronger manner which leads to formation of complexes containing multiple DNA molecules. The size of the resulting complex is attributed to the physical properties of the cationic polymer rather than the size of the DNA molecule. The morphology of DNA complexes formed with cationic polymers is independent of the polymer used. For example, complexes derived from DNA and polylysine, polyethylenimine, or various dendrimers form toroidal structures of similar diameters. However, the aggregation behavior of the complexes appears to be influenced by the polymer structure: clustering is observed for less flexible intact dendrimers and polylysine complexes.[14] Additionally, the size of polymer−DNA complexes has been correlated with the molecular weight of the polymer. High molecular weight polylysine (224 kDa) form DNA complexes with diameters ranging from 100 to 300 nm, while low molecular weight polylysine (∼4 kDa) form complexes with diameters between 20−30 nm.[15] Predicting in vitro and in vivo gene transfection efficiency based only on the physicochemical characteristics of the complex is still not possible. Additional properties of cationic vectors that impact cellular uptake, endosomal escape, and nuclear targeting appear equally critical.

1.1.2. Cellular Uptake and Endosomal Escape

Transfection of nonviral DNA complexes generally proceeds by one of two routes based on whether or not the complex is conjugated to targeting ligands. For nontargeting cationic complexes, evidence suggests that the complexes first associate with the cell membrane through electrostatic interactions with anionic cell surface proteoglycans. In 1996, Baldeschwieler et al. showed that for PLL−DNA complexes, inhibition of proteoglycan sulfation using sodium chlorate, removal of cell surface glycosaminoglycans (GAGs) using glycosaminoglycan lyases, or the addition of extracellular GAGs to the transfection media all dramatically inhibited gene transfer.[16] Shortly after, Debs et al. showed similar trends for cationic liposome−DNA complexes.[17] It was shown that the complexes were unable to transfect Raji cells, which lack proteoglycans in vitro. Additionally, intravenous cationic liposome-mediated DNA transfection was inhibited when mice were pretreated intravenously with heparinases, protein lyases that cleave heparan sulfate molecules from cell surface proteoglycans. However, Ruponen et al. showed that DNA cellular uptake varies significantly based on cationic carrier vector, cell type, and amount of cell surface GAGs present.[18] Transfection efficiency was shown to be inhibited by GAGs, suggesting that the internalized complexes may be delivered into intracellular compartments that do not promote transcription.

The cellular uptake of nontargeting cationic complexes has been proposed to proceed through various endocytic routes. In 2004, Behr et al. showed that the internalization of PEI−DNA complexes in HeLa cells was inhibited by both staurosporine, a protein kinase C (PKC) inhibitor, as well as by β-cyclodextrin, which reduced the amount of cholesterol at the cell surface.[19] Additionally, using anti-β-actin conjugated to FITC and rhodamine-conjugated PEI, it was shown that following internalization the complexes were distributed along the actin filament. Based on these results, the group proposed a method of internalization for PEI−DNA complexes that proceeds by the following sequence of events: the complex binds to transmembrane heparan sulfate proteoglycans, known as syndecans, which cluster into cholesterol-rich rafts on the cell surface. This clustering triggers protein kinase C (PKC) phosphorylation followed by binding of the syndecan to the actin skeleton through linker proteins. This binding allows the complex to then be pulled into the cell through phagocytosis, however, it is unclear whether this route promotes translation processes. In HepG2 cells, Pichon et al. showed that uptake of cationic PLL−DNA complexes

can proceed by either clathrin-dependent endocytosis, which was inhibited by chlorpromazine or by macropinocytosis, which was stimulated by phorbol myristate acetate (PMA), a PKC activator, and inhibited by dimethylamiloride (DMA), an Na^+/H^+ antiport inhibitor.[20] However, luciferase activity was only seen for the clathrin-dependent pathway as stimulation of macropinocytosis using PMA afforded minimal protein expression.

Regardless of the route of endocytosis for nontargeting vector−DNA complexes, it has been shown that the size of the complex affects cellular uptake in various cell lines. Amidon et al. showed that uptake of PLGA copolymer-DNA complexes in Caco-2 cells was size dependent, with highest uptake seen for particles with a mean diameter of 100 nm.[21] Labhasetwar et al. showed that cellular uptake of these same complexes in COS-7 and HEK-293 cell lines was higher for particles with mean diameters of 70 nm as compared to particles with mean diameters of 200 nm.[22] Finally, Yao et al. prepared PEI nanogels by a photo-Fenton reaction to create samples with mean diameters of 38, 75, 87, 121, 132, and 167 nm but with similar surface charge.[23] In four different cancer cell lines, highest transfection efficiency was seen for the complexes with 75 and 87 nm mean diameters.[24] These results suggest that optimal size for gene transfer of nontargeting cationic vector−DNA complexes is between 70 and 90 nm.

In addition to nontargeting vector−DNA complexes, significant research has focused on developing vector systems with attached receptor ligands to promote delivery to specific cells and tissues. These ligands include but are not limited to asialoglycoprotein, epidermal growth factor (EGF), folate, integrin, lactose, mannose, and transferrin.[25] Once bound to the receptors on the cell surface, the vector−DNA complexes are internalized by clathrin-dependent endocytosis.

The effect size has on the cellular uptake of receptor-targeting vector−DNA complex may be more pronounced as compared to nontargeting analogues. Because the electrostatic interactions between nontargeting complexes and the cell surface may promote "enforced" endocytosis by sedimentation in in vitro studies, larger complexes with more surface area for these interactions may be incorporated into the cell despite the more favorable uptake for smaller complexes. This may explain why large particles have shown more successful gene delivery than smaller analogues in some cases. This effect is not seen for receptor-mediated gene delivery. In 2003, Aoyama et al. showed that internalization of glycocluster nanoparticles varies significantly with size when charge effects are excluded.[26,27] The optimal mean diamter for gene transfer was reported to be ∼50 nm. This number was supported later by theoretical calculations performed by Gao et al., who determined the optimal size for particles to be 54−60 nm.[28] Other studies showed similar size-dependent variations in cellular uptake when both asialoglycoprotein[29] and transferrin[30] were used as receptor ligands.

ATP-mediated proton accumulation makes the endosomal and lysosomal compartments of cells significantly more acidic (pH 5.0−6.2) than the cytosol or intracellular space (pH than 7.4).[31] Some viruses have evolved to make use of this variation. For instance, the Semliki Forest virus (SFV) undergoes a conformational change in the coat proteins of the particle at low pH to promote endosomal membrane fusion.[32] Nonviral DNA vectors that can utilize the acidic environment of endosomes and lysosomes to escape degradation often exhibit efficient gene transfection. One method of exploiting the low pH environment of lysosomes involves the incorporation of chloroquine into the DNA/vector complex. Chloroquine is a well-known lysosomotropic agent that raises the pH of the lysosomal environment thus inhibiting the enzymes involved in lysosomal degradation.[33] Similarly, the incorporation of membrane-destabilizing peptides, such as synthetic N-terminal peptides of Rhinovirus VP-1 or influenza virus HA-2, into the cationic complex can mediate endosomal release. Under acidic conditions, these peptides arrange to form an amphipathic α-helical structure that can interact with the endosomal membrane to promote complex escape.[34] Alternatively, various macromolecules that have amine groups with low pK_a values have been shown to exhibit "proton sponge" potential. When complexed with DNA and incorporated into the cell, these compounds are capable of buffering the endosomal vesicle, which leads to endosomal swelling and lysis, thus releasing the DNA into the cytoplasm.[35] Once released into the cytoplasm, DNA/carrier vector complexes must overcome additional barriers in the cytosol that hamper delivery of the complex into the nucleus of the host cell.

1.1.3. Cytoplasmic Mobility and Nuclear Entry

The cytosol presents multiple barriers to DNA/vector complexes en route to the nucleus. Mobility of free plasmid DNA based on diffusion in the cytosol is negligible,[36] possibly due to cytoskeletal elements within the cytoplasm that function as molecular sieves and prevent the diffusion of large molecules.[37] Viruses such as adenovirus serotype 5[38] and herpes simplex virus[39] travel through the cytoplasm via microtubule-mediated transport. Cationic carrier-mediated gene delivery generally lacks such assisted transport. Vectors that compact DNA into small particles should aid in the movement of the DNA to the nucleus. DNA fragmentation in the cytoplasm represents another barrier. This fragmentation can be detected using the terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) assay.[40] Cationic carriers may offer protection for DNA from such degradation in the cytoplasm.

To gain access to the transcriptional machinery of the nucleus, plasmid DNA must cross the nuclear membrane. Trafficking between the cytoplasm and the nucleus takes place through pore complexes within the nuclear envelope. Passive diffusion through the nuclear pore complex (NPC) generally occurs only for compounds less than 9−11 nm in diameter.[41] However, protein structures (>20 kDa) are trafficked into the nucleus in an ATP-dependent process triggered by reorganization of short peptide sequences that can be hindered by certain antinucleoporin antibodies and wheat germ agglutinin (WGA).[42] The expression of exogenous plasmid DNA can also be inhibited by WGA, suggesting that gene transfer across the nuclear membrane proceeds via a similar pathway to proteins.[36] Dividing cells often exhibit higher transfectability than nonmitotic cells, indicating that plasmid DNA can reach the nucleus during nuclear envelope disassembly as cell division occurs.[43] Transfection studies of DNA complexed with a cationic vector showed significantly higher levels of gene expression than that of free plasmid DNA, suggesting that a positively charged vector may exert a nuclear-localizing effect.[44] To promote nuclear uptake, nuclear localization sequences (NLS) have been utilized. Regardless of the exact method of nuclear entry, gene sequences complexed with cationic

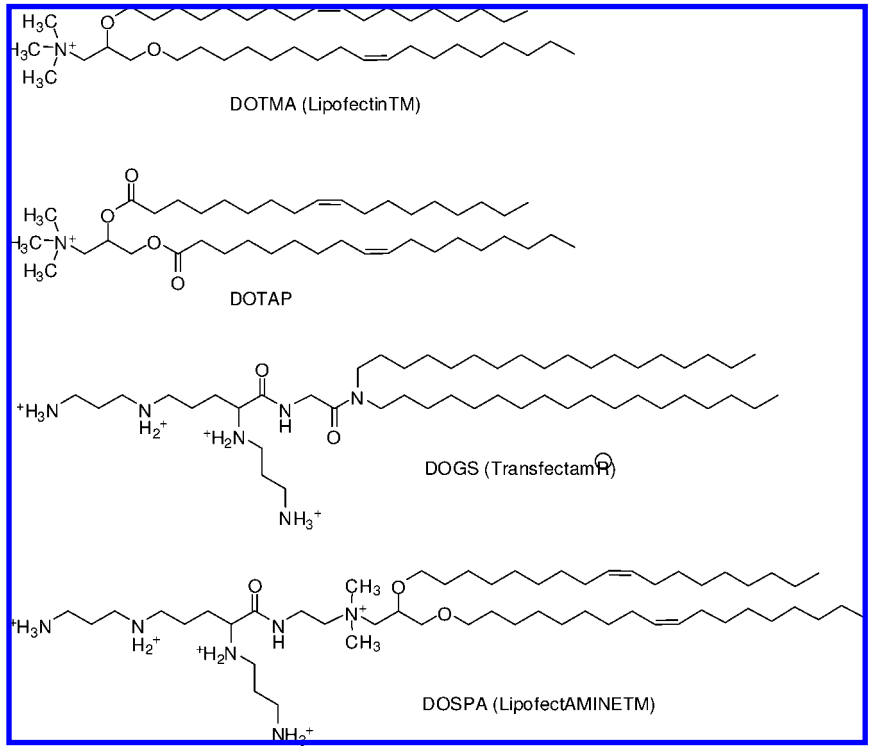


Figure 1. Chemical structures of several commercially available liposome reagents for gene transfection.

vector systems seem to have an advantage over free plasmid DNA for in vitro cell transfection. Unfortunately, successful delivery of DNA in vitro does not always correspond to successful in vivo delivery.

1.2. In Vivo Barriers for Gene Transfer

While cationic DNA carrier systems often exhibit successful gene delivery in vitro, systemic delivery is hindered by complex instability under physiological conditions. The physiological salt concentration (150 mM) often promotes aggregation of cationic complexes, which leads to vascular blockage.[45] Additionally, cationic complexes readily bind with serum proteins such as serum albumin, and the protein binding hinders cellular uptake, promotes aggregation, and possibly encourages phagocytosis.[46] Studies of liposome clearance from the blood have also shown that plasma protein association plays a major role in plasma clearance. Liposomes derived from egg phosphatidylcholine, cholesterol, and dioleoylphosphatidic acid (PC/CHOL/DOPA) bind high levels of proteins and are cleared more readily from circulation than those comprising distearylphosphatidylcholine and cholesterol (DSPC/CHOL), a liposome system that binds much more poorly.[47] These results suggest that in vivo gene delivery can be promoted by reducing salt/serum affects. The most common method of reducing these affects is adorning the periphery of the complex with hydrophilic moieties, particularly poly(ethylene glycol), abbreviated PEG.

2. Lipid-Based Vectors

Liposome-mediated gene transfer was one of the earliest strategies used to introduce exogenous genetic material into host cells. In the mid-1970's, various studies showed the fusogenic potential of liposomes with cell membranes,[48,49] and by 1980, several publications had demonstrated the capability of delivering exogenous globin mRNA,[50−52] chromosomes,[53] and DNA[54−56] into host cells using such carrier systems. The formation of stable, liposome-mediated transformed cell lines was demonstrated by incorporating the thymidine kinase gene into LTK$^-$ cells.[57] By 1987, the term "lipofection" had been coined to describe lipid-based gene transfection.[58] Several commercially available lipid reagents include *N*-[1-(2,3-dioleyloxy)propyl]-*N*,*N*,*N*-trimethyl-ammonium chloride (DOTMA),[58] 2,3-dioleyloxy-*N*-[2(spermine-carboxamido)ethyl]-*N*,*N*-dimethyl-1-propanaminium trifluoroacetate (DOSPA),[59] 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP),[60] and dioctadecylamido-glycylspermine (DOGS;[61] Figure 1).

The mechanism of gene transfer of cationic lipoplexes has been thoroughly reviewed.[62,63] Early work suggested that lipoplexes were delivered into the cytoplasm by direct plasma membrane fusion,[64,65] but it is now agreed upon that liposome-mediated gene transfer proceeds primarily through endocytosis.[66−68] Following cellular uptake, lipoplexes destabilize the endosomal membrane, resulting in a flip-flop reorganization of phospholipids. These phospholipids then diffuse into the lipoplex and interact with the cationic lipids causing the DNA to dissociate into the cytoplasm (Figure 2).[69,70]

Cationic lipids comprise three structural domains: a cationic headgroup, a hydrophobic portion, and a linker between the two domains. Variations in each of these domains produced first DOTMA,[58] then DOGS,[61] followed by DC-Chol.[71] Structure−activity relationships of cationic lipopolyamines elucidated two key trends: (1) the density and nature of the cationic headgroup affects the transfection properties of lipids, and (2) for a given headgroup, the hydrocarbon moiety can be manipulated without predictably impacting gene transfer.[72]

2.1. Cationic Head-Group Manipulations

In manipulating the cationic headgroup of monovalent lipids, some research groups have investigated replacing the

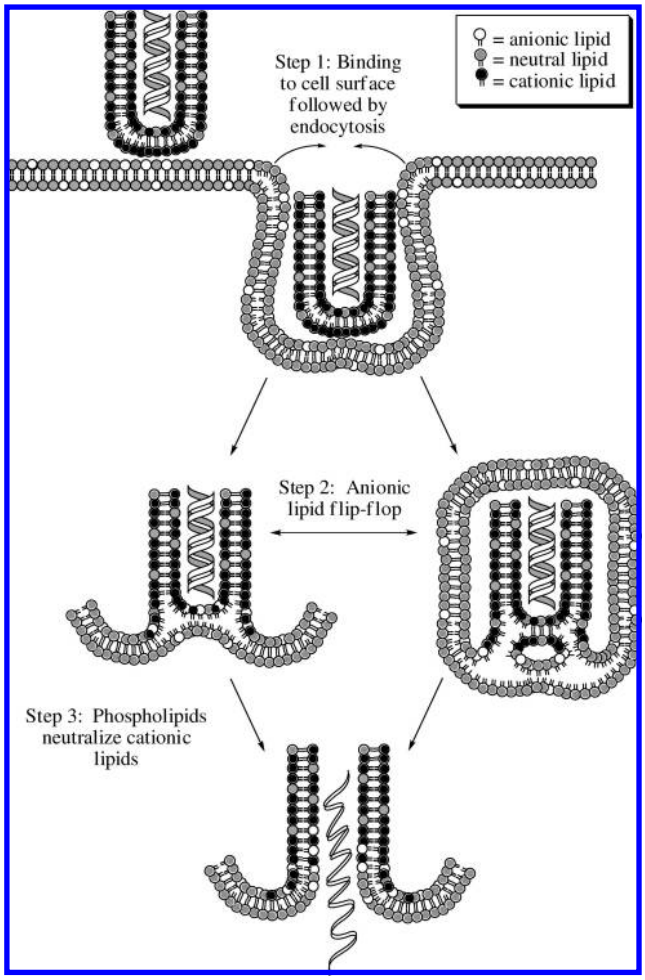


Figure 2. Mechanism of gene transfer of lipoplexes proceeds by a three-step process. The complex is incorporated into the cell through endocytosis. The cationic lipoplex destabilized the endosomal membrane, leading to reorganization of the phospholipids. The reorganized phospholipids neutralize the lipoplex, causing the DNA to dissociate into the cytoplasm. Figure adopted from previous publication by Szoka et al.[69]

ammonium group with different monovalent cationic moieties (Figure 3). Clément and Floch et al. replaced the ammonium cation of phosphonolipids with either phosphonium or arsenium groups. Precedent for the use of such derivatives was set by Stekar et al., who replaced the ammonium group of edelfosine and miltefosine with phosphonium and arsonium functionalities and found significantly reduced toxicity while maintaining antitumor activity.[73] The reduced toxicity of the edelfosine and miltefosine analogues was attributed to the increased atomic radii of As and P as compared N, which resulted in the formation of larger cationic complexes with reduced charge densities. Gene transfer studies conducted by Clément[74] and Floch et al.[75] have shown that phosphonolipids with arsonium and phosphonium cations exhibit significantly lower cytotoxicity than the ammonium analogues. Furthermore, it was determined that, for these arsonium and phosphonium phosphonolipid derivatives, in vitro transfection efficiency in Hela cells increased proportional to the number of of methylene units (n) between the phosphonate group and the cationic moiety ($n = 3 > n = 2 > n = 1$). In addition, in vivo gene transfer studies using lipophosphoramidates showed up to 3600-fold increase in gene transfer efficiency for the phosphonium and arsonium derivatives as compared to commercially available

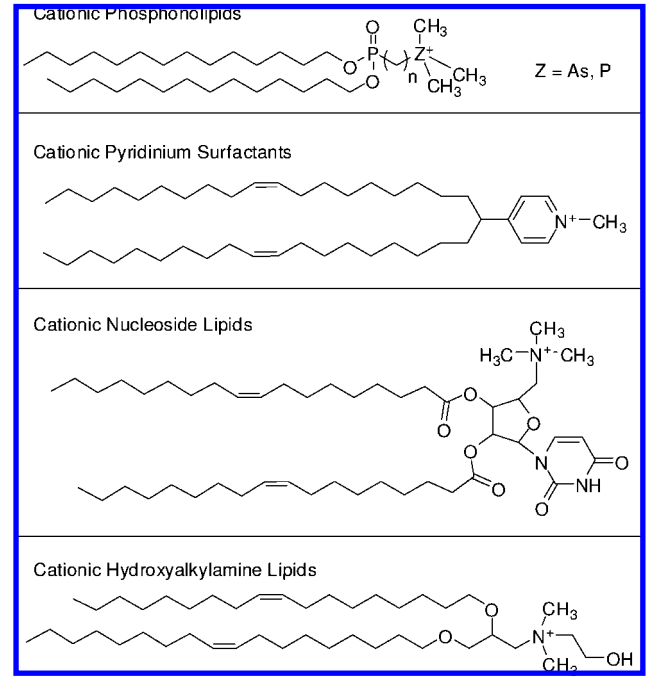


Figure 3. Monovalent headgroup manipulations for cationic lipids.

DOTAP.[76] In addition to replacing the cationic nitrogen atom with phosphonium and arsonium, research has focused on replacing the ammonium groups with more biocompatible amine derivatives. Hoekstra and colleagues have investigated surfactant compounds having a cationic pyridinium headgroup that is capable of delocalizing the cationic charge to reduce toxic effects of the lipid. These compounds were shown to be capable of vesicle formation almost three decades ago,[77] and in vitro gene transfer for a pyridinium-based derivative showed tranfection efficiency that was 3- to 6-fold higher than that of Lipofectin[78] (Table 1).

Grinstaff and co-workers have also attempted to improve transfection efficiency of cationic lipids by replacing the typical ammonium functionality with uridine to form a cationic nucleoside lipid. These complexes are capable of interacting with DNA base pairs via hydrogen bonding and $\pi-\pi$ stacking interactions, in addition to the typical electrostatic interactions between cationic amine and the anionic phosphate groups of DNA. While the uridine derivative exhibited a lesser ability to condense DNA as compared to DOTAP, toxicity was reduced as compared to Lipofectamine 2000. Disappointingly, transfection efficiency of the uridine-derivative was significantly lower than for both DOTAP and Lipofectamine 2000, possibly due to a lower number of DNA molecules per complex or a release of the DNA in the cytoplasm rather than the cell's nucleus.[79] However, the use of high cationic lipid/DNA ratio can improve gene transfer, particularly for the O-ethyl dioleyl uridine amphiphile.[80]

In addition to replacing the ammonium group of monovalent cationic lipids, simply modifying the existing amine moiety can also improve transfection efficiency. The effectiveness of gene transfer for lipids possessing monovalent cations has been shown to be related to hydration potential of the headgroup.[81] The instability of lipoplexes is linked to a decrease in the extent of hydration of the cationic lipid headgroup and this instability can enhance the membrane fusion capacity of the complex.[82,83] Incorporation of a hydroxyalkyl chain onto the ammonium group successfully

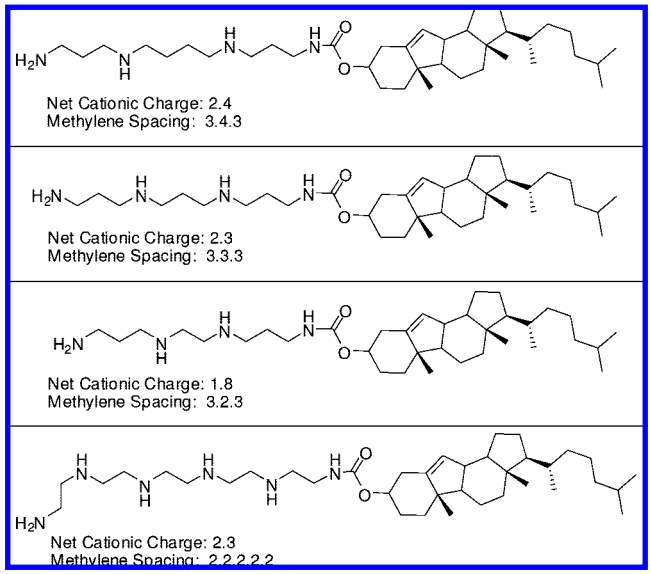


Figure 4. Multivalent cationic headgroup with different methylene spacer lengths.

dehydrates the ammonium cation by promoting hydrogen bonding with neighboring headgroups. The hydroxyethyl derivatives of DOTMA,[81] DOTAP,[84] DC-Chol,[85] and a tetradecylamine-based lipid[86] all showed enhanced transfection efficiency as compared to their methylammonium analogues. In addition, substituted hydroxyethyl portions synthesized from lactic acid,[87] arabinose, and xylose[88] have shown more effective gene transfer than the unmodified ammonium derivatives. Finally, replacing the hydroxyl group of hydroxylethyl-modified lipids to form a β-aminoethyl group has shown improved gene transfer.[89]

As higher numbers of cationic groups increases DNA binding,[90] a shift from monovalent to multivalent headgroups quickly became significant for lipid-mediated gene transfer (Figure 10). Multivalent derivatives of cholesterol lipids[91] as well as DOGS[61] are two early examples of such lipid compounds. Blagbraugh et al. have shown that the spacing of the ammonium groups of a linear multivalent headgroup (Figure 4) strongly influences its transfection efficiency.[92] For instance, cationic lipids with tetraammonium cationic headgroups show reduced transfection efficiency for decreased ammonium spacer lengths (3.4.3 > 3.3.3 > 3.2.3). This trend may result from reduced net cationic charge at pH 7.4 of the cationic lipids with shorter spacer lengths. Additionally, for cationic headgroups with the same net charge at pH 7.4, higher transfection efficiency is seen for compounds that are better able to distribute the charge density (3.3.3 > 2.2.2.2.2). Safinya et al. and Byk et al. have shown this property to be important for branched headgroups as well.[93,94] However, it should be noted that additional ammonium groups can reduce transfection efficiency if the added groups result in a more folded lipid conformation.

In addition to branched and hyperbranched headgroups, Kirby and Feiters et al. have synthesized gemini surfactants with two cationic headgroups and two alkyl chains connected by a tether (Figure 5). The polylysine- and tartaric acid-based headgroups have shown significant transfection efficiency.[95,96] Addtionally, a sugar-based cationic gemini surfactant synthesized by Engberts et al. showed high transfection efficiency that surpassed that of Lipofectamine 2000.[97] It was shown that these surfactant−DNA complexes underwent a

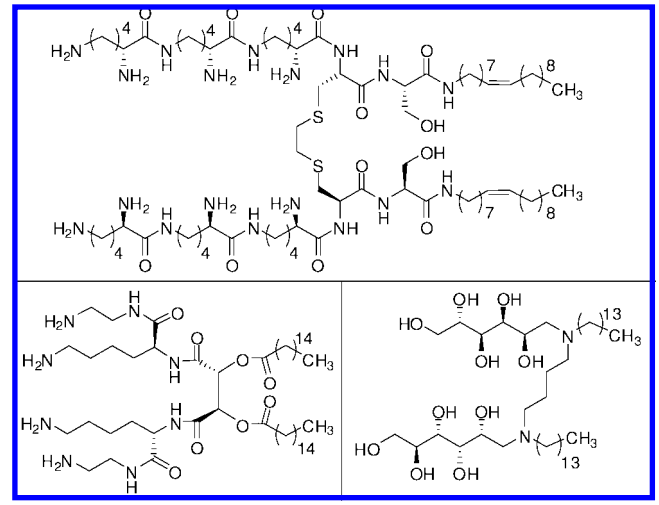


Figure 5. Cationic gemini surfactants.

morphological change from lamellar to inverted hexagonal structures in reduced pH environments, leading to endosomal fusion.[98]

In addition to linear and branched multivalent headgroups, lipids with dendritic cationic headgroups have exhibited significant gene transfer as compared to various commercially available agents (Figure 6). Kono et al. synthesized PAMAM dendron-based lipids for transfections. These compounds showed enhanced transfection efficiency for higher generation headgroups as a result of higher buffering capacity.[99] When the third generation PAMAM dendron-based lipid was mixed with DOPE at a ratio of 1:10, the lipoplex exhibited higher transfection efficiency in the presence of serum than both Lipofectamine and Superfect.[100] This serum stability was increased further by grafting PEG-chains onto the surface of the PAMAM headgroup.[101] Similar to the investigations conducted by Kono et al. using PAMAM, Safinya et al. synthesized polyornithine dendron-based lipids for transfection. When the second generation polyornithine dendron-based lipid was mixed with 1,2-dioleoyl-sn-glycerophosphatidylcholine (DOPC) and complexed with DNA, the lipoplexes exhibited higher transfection efficiency at a low mole fraction of cationic lipid as compared to DOTAP/DOPC lipoplexes.[102,103] Diederich et al. have also investigated the transfection efficiency of polyamine dendritic amphiphiles. These amphiphiles with cationic dendron-based headgroups exhibited significantly higher transfection efficiency that PEI, DOTAP, and Superfect. It was proposed that this increase resulted from a high surface charge density of the headgroup that promoted buffering capacity.[104]

Despite the improved DNA binding of multivalent headgroups, the cytotoxicity of these compounds stimulated investigation of alternative functionalities, including aminoglycosides, which bind to the phosphodiester backbone of RNA via hydrogen bonding interactions with 1,3-hydroxyamine groups.[105] KanaChol, an aminoglycoside lipid synthesized from konamycin (Scheme 1), showed significant gene transfer both in vitro and in vivo.[106] Shortly after the success of KanaChol, a variety of other aminoglycoside derivatives were synthesized[107] and showed gene transfer capability.[108] Most recently, aminoglycoside lipoplexes have been successfully used for siRNA delivery and interference.[109] In addition to aminoglycoside lipid derivatives, lipids containing cationic peptide headgroups have shown transfection efficiency rivaling that of known commercial agents.[110]

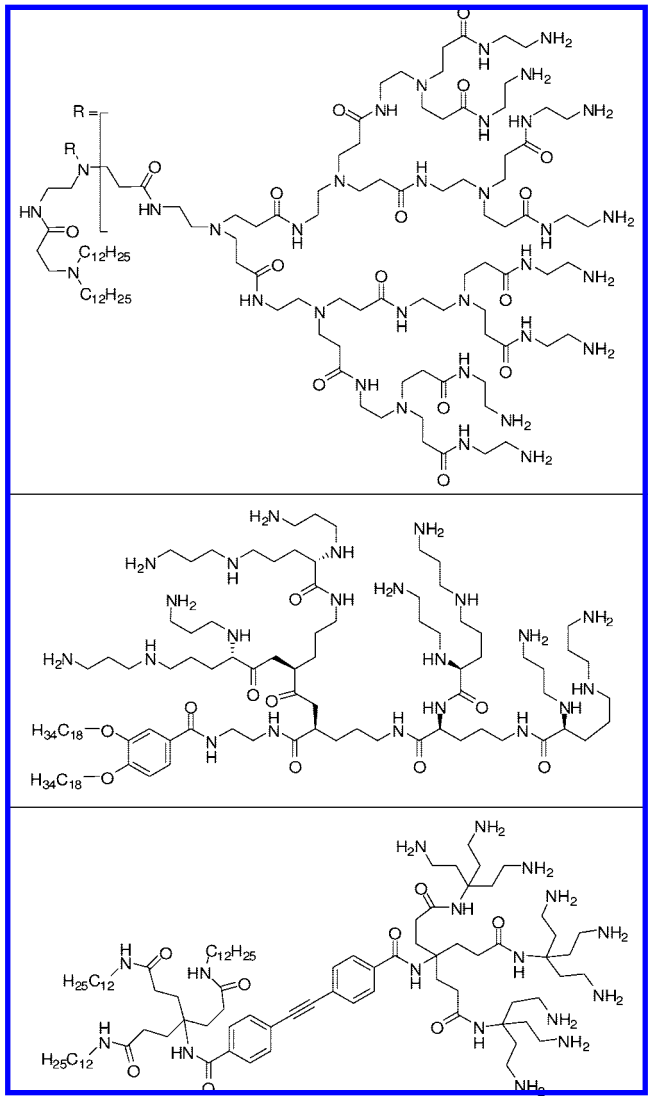
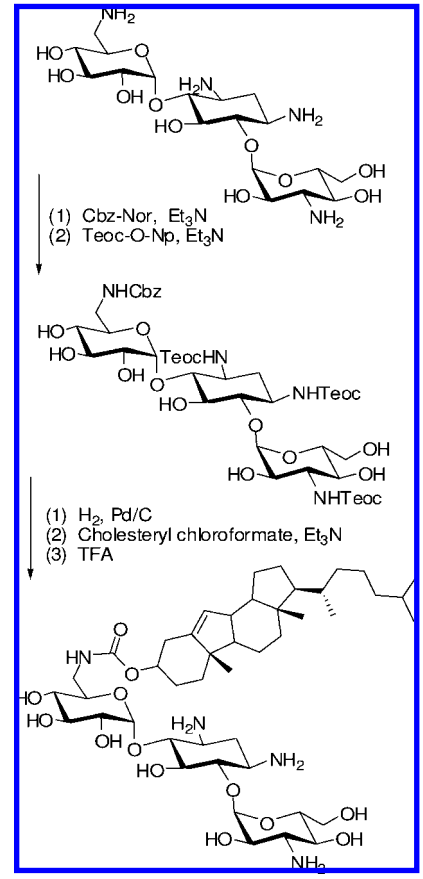


Figure 6. Amphiphilic structures with dendron-based cationic headgroups.

2.2. Hydrophobic Tail-Group Manipulations

Several studies suggest that the length and type of aliphatic chain affect the transfection efficiency of a given lipid. Derivatives based on DOTMA,[84] glycine betaine,[111] alkyl acyl carnitine esters,[112] lactic acid,[87] and DOTAP[113] indicate that gene transfer increases with reduced chain length (C_{14} > C_{16} > C_{18}). Behr et al. found that, for lipospermine derivatives, no correlation exists between lipid chain length and transfection efficiency.[72] Additionally, Byk and Sherman et al. found that a decrease in alkyl chain length from 18 to 12 for a polyamine lipid leads to decreased transfection efficiency as well as increased cytotoxicity in both Hela and NIH3T3 cell lines.[94] Kono et al. found that, for polyamidoamine (PAMAM) dendron-bearing lipids, transfection efficiency was generally higher for longer alkyl chain length derivatives (C_{18}) as compared to those of shorter chains (C_{12}), particularly in the presence of serum.[114] Finally, Camilleri and Kirby et al. have showed that for ϵ,ϵ-linked trilysine-based gemini surfactants, transfection efficiency is higher for longer alkyl chain derivatives (C_{18} > C_{16} > C_{14} > C_{12}). Interestingly, for α,α-linked trilysine surfactants, the longest alkyl chain derivatives exhibited lower transfection efficiency than the shortest alkyl chain analogue of the ϵ,ϵ-linked compounds, indicating that high transfection efficiency

Scheme 1. Synthesis of the Aminoglycoside Lipid, KanaChol, by a Selective Protection/Deprotection Strategy



requires both the correct cationic headgroup and the hydrophobic lipid chains.[95] Several studies have also shown that incorporating two chains with different lengths can improve transfection efficiency potentially by promoting endosomal escape.[113,115]

In addition to varying the length and type of aliphatic chain, various alternative hydrophobic moieties have been used to promote gene transfer (Figure 7). Following the work pioneered by Huang et al.,[71] cholesterol[116−118] and other steroids[119] have been used in place of aliphatic chains to probe the roles of rigidity, biodegradability, and fusogenic capability. Ascribed to rigidity, cholesterol derivatives are particularly advantageous by demonstrating increased liposomal stability upon nebulization, thus making these analogues beneficial for aerosol gene delivery.[120] Furthermore, the use of bile acid-based cationic facial amphiphiles has shown significant transfection efficiency in vitro.[121] These facial amphiphiles can promote gene uptake by the same route as membrane-penetrating peptides but are less toxic, cheaper to synthesize, and more stable.[122] These bile acid-based amphiphiles have also exhibited antibacterial properties, which are relatively unique among gene delivery agents.[123] Diacetylene-based lipids have also been successfully used in place of linear aliphatic chains for lipid-mediated gene transfer.[124] In addition, tetraalkyl lipid chain surfactants have been used for gene transfer as such lipids form cone-shaped vectors that enhance endosomal membrane mixing.[125] These complexes show enhanced gene transfer over *N,N*-dioleyl-*N,N*-dimethylammonium chloride (DODAC), provided the carbon chain length separating the lipid chains was long enough. Finally, highly fluorinated alkyl chains have shown successful gene transfer due to their

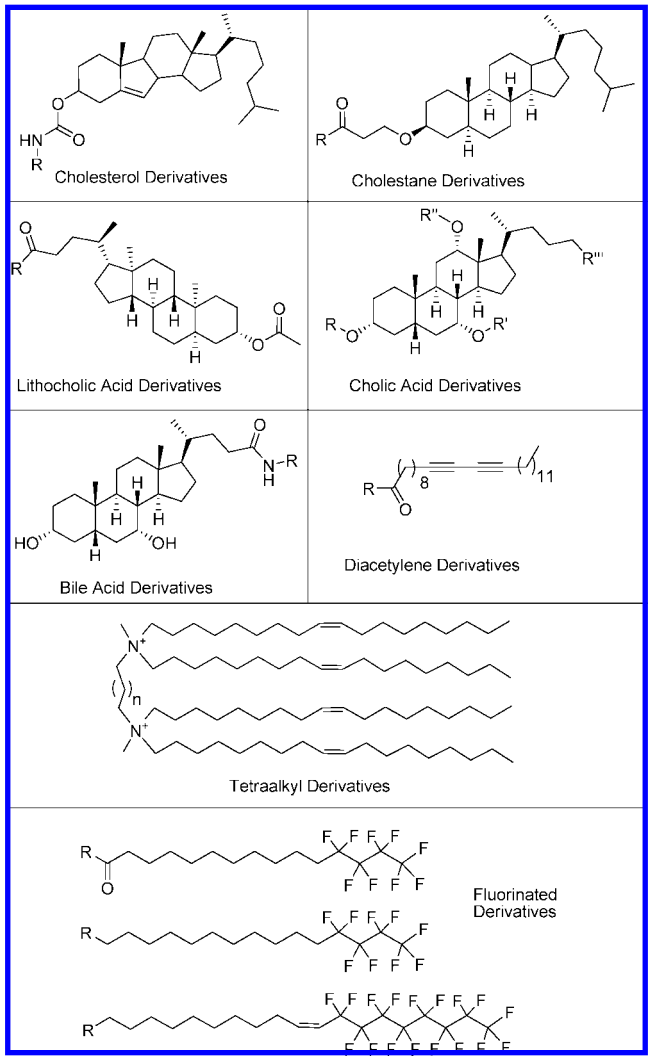


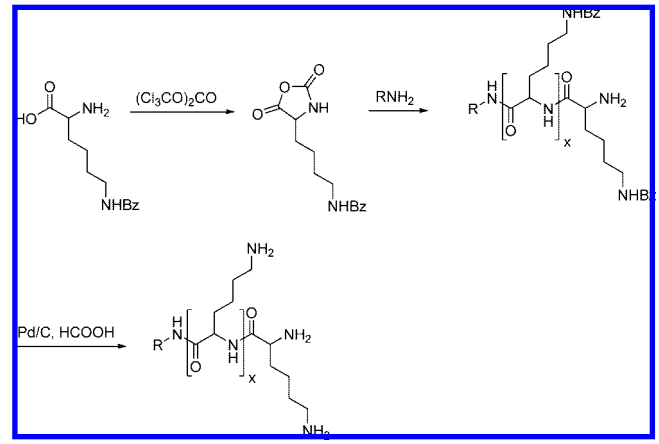
Figure 7. Amphiphilic structures with hydrophobic group modifications. R represents various cationic functionalities.

ability to avoid interactions with hydrophilic and lipophilic biocompounds.[126−129]

2.3. Linking Group Manipulations

For all lipids used in gene transfection, the hydrophobic and cationic portions are joined by several common linkers including amides, carbamates, esters or ethers. Ethers exhibit stability when used as the linking group,[130,131] while esters allow for cleavage once inside the cell.[132] Carbamates have been used as linkers to illicit both of these benefits.[71,133,134] Cleavable lipids are of interest for designing complexes, which can easily release DNA after endocytosis.[135] Such cleavable linkers include photo- and pH-sensitive, redox reactive, and enzymatically degradable groups. While photocleavable linkers have shown significant gene transfer,[136] biologically stimulated release is advantageous, as it does not require outside-stimulus to induce the cleavage. Successful transfection efficiency has been promoted by pH-sensitive linkers including vinyl ethers,[137] ketals,[138] ortho esters,[139−141] and acylhydrazone,[142] as well as redox reactive groups.[143−148] Finally, esters have been incorporated as the labile linker group for a variety of lipids, resulting in successful transfection.[149−152] Recent investigations have shown that the orientation of the ester linkage can have significant effect on the transfection efficiency.[153]

Scheme 2. Synthesis of Poly(L-lysine) Proceeds via Ring-Opening Polymerization of Protected *N*-Carboxy-(*N*-benzyloxycarbonyl)-L-lysine Anhydride



3. Polymeric Vectors

3.1. Poly(L-lysine) (PLL)

In 1975, Laemmli demonstrated the exceptional capability of polylysine to condense DNA.[154] Subsequently, this vector was used for in vitro[155] and in vivo[156] gene transfer. Synthesis of PLL proceeds by conversion of an ϵ-amine protected L-lysine monomer to *N*-carboxy-(*N*-benzyloxycarbonyl)-L-lysine anhydride (Scheme 2).[157] The anhydride undergoes ring-opening polymerization using a primary amine initiator.[158] Control of molecular weight can be achieved through the use of specific feed ratios of monomer to initiator.

At physiological pH, all primary ϵ-amino groups of PLL are protonated, yielding a structure with no buffering capacity to aid in endosomal escape. Akinc and Langer were able to determine the pH environment of PLL−DNA complexes following cellular uptake by covalently double-labeling DNA with fluorescein, a pH sensitive fluorophore, and Cy5, a pH-insensitive fluorophore, and determining the ratio of fluorescein to Cy5 fluorescence using flow cytometry. The average environmental pH surrounding PLL after cellular uptake was found to be between 4.0 and 4.5, indicating that most of the polyplex is contained in the lysosomal trafficking pathway as opposed to being released into the cytoplasm.[159] Endosomal release can be improved with the addition of chloroquine or membrane-active peptide.[160] An alternative method of promoting endosomal lysis involves substituting PLL with histidine groups, yielding conjugate acids with a $pK_a = 6.0$. Such derivatization provides PLL with buffering capacity.[161,162]

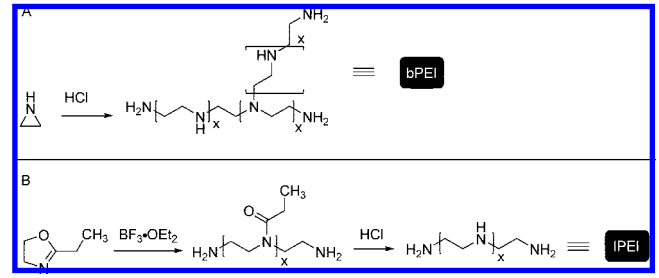
In general, only polylysine structures with molecular weights >3000 Da can effectively condense DNA to form stable complexes, indicating the significance of primary amine number for complex formation.[163] However, a short lysine oligomer, $(Lys)_{16}$, has shown DNA binding capability when coupled to fusogenic peptides and, furthermore, can promote nuclear translocation in corneal endothelial cells.[164−166] This nuclear translocation is not seen for the high molecular weight PLL, which exhibits gene transfer only in cells that undergo mitosis.[167] Despite the effective condensing ability of high molecular weight PLL structures, these compounds exhibit relatively high cytotoxicity.[168] This toxicity has been reduced with the incorporation of imidazole functionality into the poly(lysine) chain[169] as well as through the use of dendritic poly(L-lysine) derivatives.[170]

In addition to increased cytotoxicity, high molecular weight PLL/DNA complexes have shown a tendency to aggregate and precipitate depending on the ionic strength of the solution.[171] One method used to overcome the formation of insoluble precipitates is to form block copolymers of PLL with poly(ethylene glycol) (PEG).[172] PLL−PEG copolymers form complexes with DNA and oligodeoxynucleotides that have reduced sizes regardless of the concentration of NaCl in the buffer solution and are resistant to deoxyribonuclease I (DNase I) digestion.[173,174] In vitro and in vivo studies show successful gene transfer of an antisense glutamic acid decarboxylase mRNA expression plasmid using a PLL−PEG copolymer.[175] Recently, a triblock copolymer of PEG, poly-[(3-morpholinopropyl)aspartamide], and PEI was synthesized by Fukushima et al. When complexed with plasmid DNA, this polymer system exhibited improved buffering capacity compared to PLL−PEG copolymers as well as significant transfection efficiency.[176] In addition to using PEG to prevent aggregation of PLL, the attachment of dextran,[177] poly[*N*-(2-hydroxypropyl)methacrylamide],[178] or the cross-linking agent dimethyl-3,3′-dithiobispropionimidate (DTBP)[179] increase the stability of PLL polyplexes.

In an effort to reduce cytotoxicity and improve release of DNA from the PLL polyplex following endocytosis, various biodegradable polylysine conjugates have been synthesized. Rice et al. condensed DNA with low molecular weight lysine oligomers containing terminal cysteine residues that were cross-linked to form complexes with reducible disulfide linkages. These structures showed significant gene transfer compared to commercially available lipid agents, particularly in HepG2 cell lines.[180] Kataoka et al. also synthesized thiolated PLL-*b*-PEG compounds that could form cross-linked complexes with DNA that exhibited improved colloidal stability[181] and enhanced transfection efficiency compared to PLL and PLL−PEG noncross-linked conjugates.[182] Various groups have incorporated ester functionality into PLL structures to create hydrolyzable derivatives. Park et al. synthesized poly(lactic-*co*-glycolic acid) (PLGA)-grafted poly(lysine) conjugates that showed reduced cytotoxicity and significant transfection efficiency that rivaled that of PLL. Healy et al. synthesized a similar polymeric structure containing a PEG block. This poly(lysine-*g*-(lactide-*b*-ethylene glycol) terpolymer was able to protect DNA from nuclease degradation[183] and exhibited zero-order plasmid release kinetics over a six-week time period.[184] Finally, Kim et al. synthesized ester-linked PLL−PEG multiblock copolymers with various ratios of histidine residues to promote buffering capacity. The compounds exhibited reduced cytotoxicity and improved gene transfer efficiency as compared to underivatized PLL.[185] In vivo biodistribution data of intact complexes revealed a blood circulation time of up to 3 days, suggesting that the PEG chains promote binding of serum proteins that mask the complex from degradation (dysopsonization).[186]

To overcome nonspecific cell targeting, various research groups have derivatized PLL with targeting moieties. Artery wall binding peptide (AWBP) was covalently attached to a PLL-*g*-PEG copolymer. The results showed improved transfection efficiency in both bovine aorta endothelial cells and smooth muscle cells for the AWBP-conjugated copolymer as compared to the nonconjugated PLL-*g*-PEG copolymer. Alternatively, hepatic cell/tissue targeting was induced by conjugating either PLL or PLL−PEG derivatives to galactose,[187−189] lactose,[190] or asialoorosomucoid.[191] Tumor cell targeting was enhanced by conjugating PLL to both folate[192,193] and transferrin.[194−196] Additionally, a terplex system comprised of PLL hydrophobized with C_{18}-steryl groups and complexed with low-density lipoprotein to form a supramolecular gene vector showed improved transfection efficiency in smooth muscle cells (A7R5) as compared to the nontargeting derivatives of PLL and an alternative commercially available transfection agent.[197,198] In addition, complexation of PLL with an apoprotein E derived peptide that targets LDL receptors to transport molecules across the blood−brain barrier (apoEdp) showed capability to deliver DNA to brain cells in vivo.[199] Antigen−antibody interactions can also improve cell targeting. The monoclonal antibody 34A, which targets cell−surface thrombomodulin, was conjugated to the N-terminus of PLL.[200] When injected intravenously, this complex accumulated preferentially in the lungs when compared to standard PLL, which accumulated predominantly in the liver. However, gene expression was only modest for the lung-targeting PLL derivative. Leukemia-specific JL1 antigen was conjugated to PLL to target leukemia T-cells.[201] For the PLL−antibody conjugate, β-galactosidase activity was significantly higher in Molt 4 (human, peripheral blood, leukemia, T cell) cells as compared to Lipofectin and PLL. Finally, polylysine was covalently linked to receptor-associated protein (RAP), a ligand that binds to LDL receptors, and results showed significant gene transfer.[202] It should be noted that poly-D-lysine (PDL) conjugates showed significantly improved gene transfer over PLL conjugates, suggesting that the PDL−RAP conjugates were more stable to lysosomal degradation.

Scheme 3. Synthesis of PEI by (A) Acid-Polymerization of Aziridine To Yield Branched PEI and (B) Ring-Opening Polymerization of 2-Ethyl-2-oxazoline Followed by Hydrolysis To Yield Linear PEI



3.2. Polyethylenimine (PEI)

3.2.1. Homopolymeric PEI

Polyethylenimine, often considered the gold standard of gene transfection, is one of the most prominent examples of cationic polymers capable of gene transfection. Since the first successful polyethylenimine-mediated oligonucleotide transfer conducted by Behr et al. in 1995,[203] PEI has been derivatized to improve the physicochemical and biological properties of polyplexes.[204] Polyethylenimine exists as both a branched and linear structure. Synthesis of branched polyethylenimine (bPEI) proceeds via acid-catalyzed polymerization of aziridine,[205] whereas the linear structure (lPEI) is synthesized via ring opening polymerization of 2-ethyl-2-oxazoline followed by hydrolysis (Scheme 3).[206] Several linear polyethylenimine transfection agents have been made commercially available, including ExGen500 and jetPEI, both linear derivatives of PEI.[207]

The transfection efficiency of PEI has been shown to be due, at least in part, to the "proton sponge" nature of the

polymer.[35] The branched structure of PEI contains nitrogen at every third atom, resulting in a high charge density capacity. Theoretical calculations originally suggested that branched polyethylenimine contains a 1:2:1 ratio of primary/secondary/tertiary amines.[208] Later measurements showed the commercially available compounds have closer to a 1:1:1 ratio, indicative of an even more highly branched structure.[209] As a result of the high density of amines, PEI lends itself to protonation, with the charge density proportional to the pH of the biological environment. At a physiological pH, approximately 80% of the amines remain unprotonated compared to less than 50% unprotonated nitrogens at a pH of 5.[210] This buffering capacity allows PEI polyplexes to avoid lysosomal trafficking and degredation once inside the cell, and the theory has gained widespread acceptance despite challenges to the hypothesis.[211] Sonawane et al. showed reduced acidification and increased swelling and chloride concentration for PEI polyplexes as compared to those of polylysine, supporting the buffering capacity of polyethylenimine.[212] Akinc et al. later showed that, while the removal of protonable amines by N-quaternized PEI actually increases cellular uptake, transfection activity is reduced by 50-fold, thus, quantitatively verifying the "proton sponge" hypothesis.[213]

Transfection efficiency of polyethylenimine has been studied over a wide range of molecular weights. While Godbey et al. showed that transfection efficiency of PEI polyplexes increases with increased molecular weight ranging from 600 to 70000 Da,[214] high molecular weight polymers also result in significantly higher cytotoxicity.[215] Kissel et al. showed that this increased toxicity is caused by aggregation and adherence on the cell surface, which results in significant necrosis.[216] The optimal molecular weight for PEI polyplex formation is typically between 5 and 25 kDa.[204] Low molecular weight PEI can transfect cell lines more effectively than the high molecular weight counterparts only if higher PEI amine/DNA phosphate (N/P) ratios are used.[217]

In addition to the molecular weight, the degree of branching of polyethylenimine has been shown to affect DNA complex formation and stability. Dunlap et al. showed that linear PEI is less effective at condensing DNA compared to the branched form for similar molecular weights.[218] In addition, the stability of polyplex formation is higher for complexes with more primary amines, making branched PEI a seemingly more suitable transfection vector.[219] However, despite the lower complexation capability of linear PEI, multiple in vivo studies have shown this topology of polyethylenimine to be a particularly effective gene transfer agent.[220−222]

3.2.2. Variations to PEI Structure

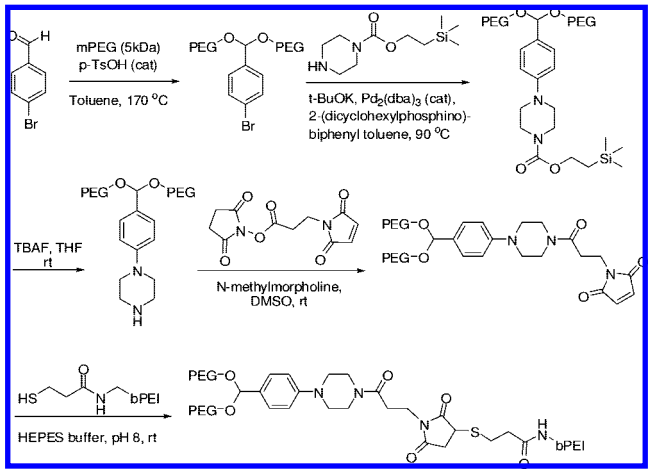
An extensive variety of modifications to PEI structure have been employed in an effort to improve transfection efficiency of the polymer. Perhaps the most utilized of these modifications is PEGylation, which creates a hydrophilic exterior that reduces interactions of the polyplex with plasma proteins and erythrocytes. Various synthetic strategies exist to conjugate PEG to PEI. In a two-step procedure, PEG can be activated with either epoxide[223] or isocyanate groups[224] followed by reaction with the amino groups of PEI. Alternatively, commercially available NHS (*N*-hydroxysuccinimide) activated PEG can be appended onto polyethylenimine.[45] Bifunctional NHS-activated PEG with a vinyl sulfone group on the opposite end allows for further functionalization of the PEI−PEG block copolymer with targeting moieties such as arg-gly asp (RGD) peptides to target integrin receptors on endothelial cell[225] or galactose to target hepatocytes.[226]

The length and density of PEG chains conjugated to PEI have an affect on transfection efficiency in vitro. DNA, oligonucleotides, and ribozymes are most effectively transfected when a high density of relatively short (550 Da) PEG chains are grafted onto PEI.[224,227] Interestingly, siRNA gene knockdown was most effective when a low density of longer (5000 Da) PEG chains were conjugated to PEI.[228] Furthermore, while in vivo studies of PEI-*g*-PEG copolymers have shown increased circulation time and reduced toxicity, no gene expression was detected with doses of 25 μg pDNA in mice.[229] This reduced gene expression has been suggested to be caused by two factors. First, PEI-*g*-PEG copolymers have a decreased surface charge that results in reduced interaction with the cell membrane, thus hindering the first step of the intracellular trafficking of PEI polyplexes.[230] Kwon et al. devised a unique strategy to overcome this charge neutralization barrier by eliminating the covalent binding of PEG to PEI.[231] The group conjugated NHS-activated biotin to PEI in one reaction and conjugated 5 kDa PEG−succinimidyl propionate (PEG5k-SPA) to chicken avidin in a separate reaction. The two copolymers were then linked by biotin−avidin interactions. When exposed to high salt conditions in vitro, the noncovalent interaction between biotin and avidin remained stable, suggesting the potential for prolonged systemic circulation as seen with covalently conjugated PEI−PEG copolymers. The addition of excess biotin reduced the degree of PEGylation and improved binding to the cell surface in vitro.

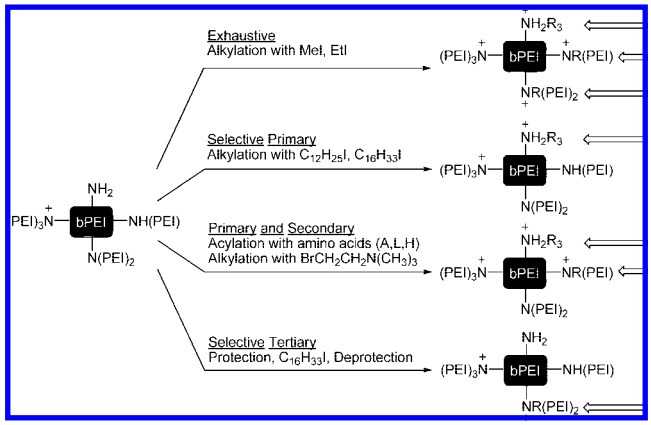
The reduced transfection efficiency observed with PEI−PEG conjugates has also been attributed to hindered gene transfer steps that occurr after cellular uptake as opposed to reduced interactions with the cell membrane.[232] This belief is supported by studies that demostrated that PEI−PEG conjugates with cell-targeting ligands are still internalized less effectively than unmodified polyethylenimine.[233] While the neutral PEGylated compounds exhibit reduced aggregation, endosomal escape is attributed to cationic charge of the vector. To create a virus-mimicking neutral vector that could undergo changes within the endosome environment to become cationic, Knorr et al. synthesized an acetal-based PEGylation reagent with a maleimide terminus that could be coupled to mercaptan-modified PEI (Scheme 4).[234] The hydrolysis of acetal-conjugated PEG chains had a half-life of 2 h at physiological conditions but was reduced to approximately 3 min at pH 5.5. In vitro results from this study showed the transfection efficiency of the acetal-based PEGylated PEI to be comparable to unmodified PEI, suggesting a potential for improved efficiency in vivo.

Besides PEGylation, several other modifications to PEI have been made to improve transfection efficiency. Thomas and Klibanov have investigated the affect of a number of amine-modifications on the in vitro transfection efficiency of 25 kDa PEI (Scheme 5).[235] Quaternary amine structures were introduced by reacting PEI with methyl and ethyl iodide. In both the presence and absence of serum, these structures showed reduced transfection efficiency, supporting the "proton sponge" hypothesis. This prerequisite of protonable amines for significant transfection efficiency was confirmed by reacting PEI with 2-(bromoethyl)trimethylammonium bromide to create a structure with N-quaternized

Scheme 4. Synthesis of Acetal-Based PEGylation Reagent with a Maleimide Terminus that is Coupled to Mercaptan-Modified bPEI



Scheme 5. PEI-Modified Structures Investigated by Thomas and Klibanov



terminal amines, while maintaining protonable secondary and tertiary amines that was as effective at transfection in the absence of serum. The effect of the hydrophilic−hydrophobic balance of polyplexes was investigated by reacting PEI with the hydrophobic amino acids alanine and leucine as well as by alkylating the primary amino groups with dodecyl and hexadecyl halides. Moderate enhancement of hydrophobicity with moieties such as alanine improves transfection efficiency, but significant increase in hydrophobicity, as with leucine and long aliphatic chains, is deleterious. Alkylation of the tertiary amino groups was less harmful and actually showed improved gene transfer in the presence of serum, indicating that the position of alkylation is significant.

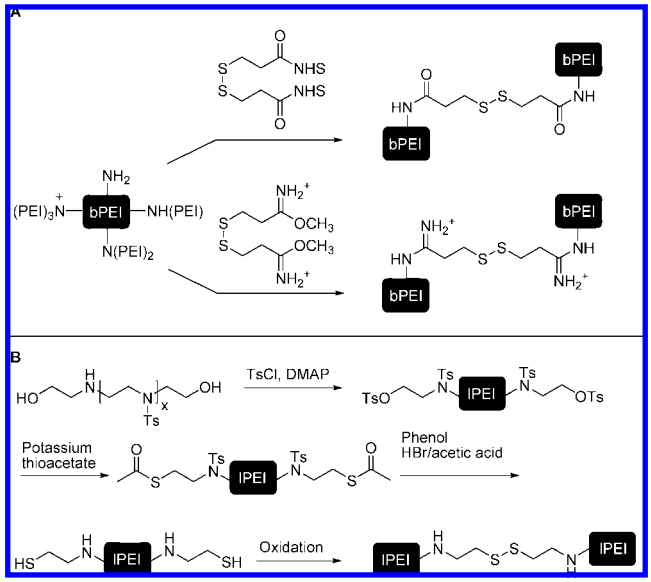
Various other groups have modified the amine functionality of PEI to improve transfection efficiency. Wang et al. modified branched polyethylenimine (1.8 and 10 kDa) by linking cholesterol to the amines. Selective secondary amine functionalization was achieved by first protecting the primary amines of bPEI with carbobenzyloxy (Cbz) groups followed by treating the protected bPEI derivative with cholesteryl chloroformate and then deprotecting with 10% palladium on activated carbon. Alternatively, both the secondary and the primary amines were linked to cholesterol by reacting unprotected bPEI with cholesteryl chloroformate.[236,237] For low molecular weight bPEI (1.8 kDa), transfection efficiency increases and toxicity is reduced with the addition of cholesterol, regardless of whether or not the primary amine is modified. Pun et al. synthesized cyclodextrin (CD)-modified PEI derivatives (branched and linear) to reduce toxicity.[238] The CD-modified PEI was condensed with fluorescently labeled plasmid (pEGFPLuc plasmid labeled with YOYO-1) and delivered into prostatic carcinoma (PC3) cells. While the cytotoxicity of both CD−bPEI and CD−lPEI was reduced with the increasing density of cyclodextrin, transfection efficiency of CD−bPEI was lower than that of the unmodified analogue despite higher cellular uptake established by flow cytometry. However, the transfection efficiency of both CD−bPEI and CD−lPEI were higher than the unmodified analogues in the presence of chloroquine, suggesting that cyclodextrin conjugation hinders endosomal escape. Based on the ability of cyclodextrin to form inclusion complexes, Pun et al. synthesized adamantane-terminated PEG, which could be noncovalently conjugated to the CD−PEI. In vivo studies revealed no toxicity up to 120 μg DNA, a dose that is lethal for linear PEI−DNA complexes. Biodistribution data showed the highest tumor accumulation in the liver, followed by the lungs then the kidneys, with gene expression observed in only the liver.[238] In an alternative method, Bae et al. improved the transfection efficiency of bPEI by synthesizing a dexamethasone-conjugated low molecular weight (2 kDa) bPEI derivative.[239] Dexamethasone causes nuclear pore complexes to dilate upon exposure, creating "giant pores" through which typically impermeable macromolecular structures can pass.[240] In the gene transfer studies, dexamethasone−PEI (2 kDa) exhibited comparable transfection efficiency to 25 kDa PEI and PAMAM when used at high polymer/DNA weight ratios but exhibited almost no toxicity typical for larger macromolecules.

Mikos et al. reported that the cytotoxicity of PEI derives from two mechanisms. Free PEI can cause cell death prior to cellular internalization by membrane destabilization. Alternatively, 7−9 h after cellular internalization (when DNA has been released from the complex), free PEI can induce cellular stress responses such as endothelial cell activation.[241] In an effort to minimize the latter process, analogues that break down into less-toxic low molecular weight structures after cellular uptake have become appealing. Synthesis of biodegradable PEI compounds has involved either the incorporation of reducible disulfide linkages or ester conjugation. Lee et al. synthesized reducible PEI derivatives by treatment of low molecular weight PEI (800 Da) with either dithiobis(succinimidylpropionate) or dimethyl-3,3′-dithiobispropionimidate (Scheme 6A). These compounds showed transfection efficiency less than that of 25 kDa PEI, but exhibited significantly reduced cytotoxicity.[242] Park et al. synthesized linear reducible PEI derivatives which exhibited high cell viability like the branched analogues and transfection efficiency similar to that of PEI (Scheme 6B).[243]

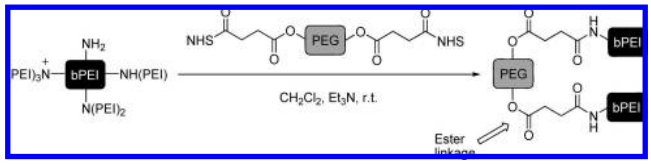
In addition to disulfide linkages, PEI derivatives with acid-labile ester linkages have been explored by multiple research groups to create biodegradable gene carriers. Kim et al. synthesized biodegradable PEI−PEG conjugates by reacting low molecular weight PEI (600, 1200, 1800 Da) with PEG succinimidyl succinate (2000 Da) to form polymeric structures (Scheme 7). When condensed with pDNA, these PEI−PEG conjugates showed reduced cytotoxicity as compared to 25 kDa PEI and improved gene transfer as compared to 1.8 kDa PEI.[244]

Kissel et al. incorporated PEG−polycaprolactone (PEG−PCL) grafts onto the periphery of hyperbranched PEI structures. Following ring-opening polymerization of ϵ-caprolactone using monomethoxyl poly(ethylene glycol) es-

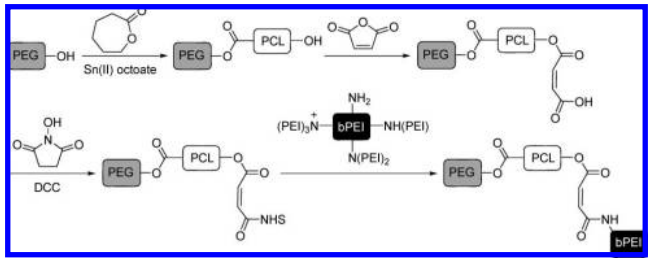
Scheme 6. Synthesis of (A) Branched and (B) Linear PEI Derivatives with Disulfide Linkers



Scheme 7. Synthesis of Biodegradable PEI−PEG Copolymers Using Branched PEI and PEG Succinimidyl Succinate



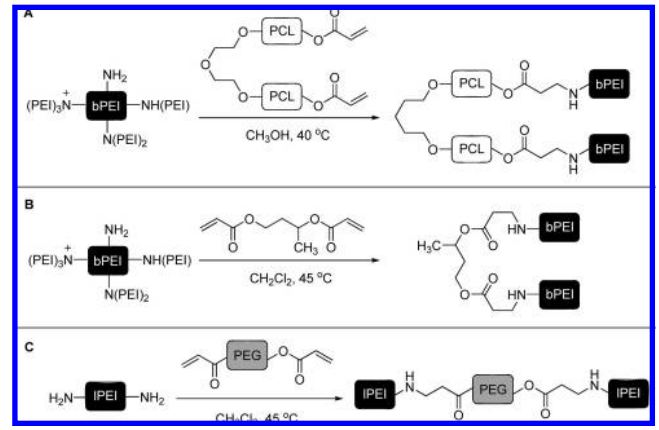
Scheme 8. Synthesis of Hyperbranched PEI-*g*-PCL-*b*-PEG



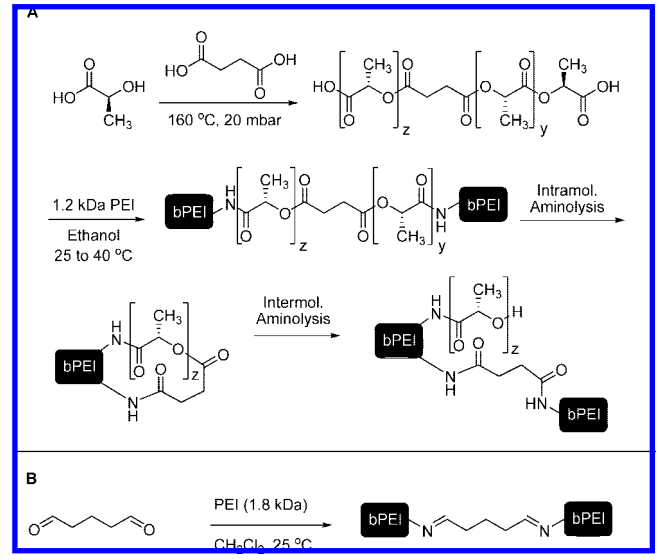
terification with succinic anhydride yielded an acid-terminated polymer that was then activated with *N*-hydroxysuccinimide and conjugated with hyperbranched PEI (Scheme 8). These structures showed reduced cytotoxicity and some of the polyplex structures exhibited improved transfection efficiency compared to 25 kDa PEI.[245] The enhanced gene transfection efficiency of these PEG−PCL−PEI copolymers was later improved by creating inclusion complexes between the PEG−PCL grafts and α-cyclodextrin.[246]

Cho et al. investigated in vivo delivery of PCL−PEI conjugates synthesized via Michael addition of PEI (600, 1200, 1800 Da) and polycaprolactone diacrylate (Scheme 9A). Results showed that these PEI derivatives exhibit significantly higher transfection efficiency compare to 25 kDa PEI when delivered using aerosol administration.[247] Alternatively, Pack et al. synthesized PEI derivatives with ester linkages by reacting low molecular weight branched PEI (800 Da) with 1,3-butanediol diacrylate or 1,6-hexanediol diacrylate (Scheme 9B). These compounds showed significantly lower cytotoxicity and transfection efficiency that was 2−16-fold higher than 25 kDa PEI.[248] Work conducted by Wagner et al., who synthesized PEI derivatives similar to those of

Scheme 9. Synthesis of Biodegradable (A) PEI−PCL, (B) PEI−Diacrylate, and (C) PEI−PEG Derivatives by Michael Additions



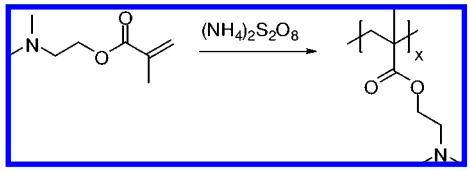
Scheme 10. Synthesis of Biodegradable PEI Derivatives with (A) Amide and (B) Imine Linkers



Pack by conjugating oligoethylenimine with hexanediol diacrylate, showed that the reaction temperature affects the ester/amide ratio of the resulting polymer. Lower reaction temperatures (20 °C) lead to products with a higher ester/amide ratio, resulting in more rapid degradation. This rapid degradation improved transfection efficiency as compared to the analogues synthesized at higher temperatures (60 °C).[249] Cho et al. synthesized linear PEI-alt-PEG using a Michael-type addition with low molecular weight PEI (423 Da) and PEG diacrylates (Scheme 9C). These compounds exhibited reduced cytotoxicity and improved gene transfer in HepG2 and MG63 cells compared to 25 kDa PEI.[250]

In addition to the reducible disulfide linkages and the hydrolyzable ester linkages, hydrolyzable amide and imine linkages have been investigated. Kissel et al. synthesized oligo(L-lactic acid-*co*-succinic acid) and reacted this compound with low molecular weight branched PEI (1200 Da; Scheme 10A). While these amide-linked compounds exhibited reduced cytotoxicity compared to 25 kDa PEI and improved transfection efficiency compared to 1.2 kDa PEI, degradation time was significantly higher than that of ester analogues.[251] Kim et al. synthesized imine-linked PEI by treating low molecular weight branched PEI (1.8 kDa) with glutadialdehyde (Scheme 10B). While these structures showed

Scheme 11. Synthesis of PDMAEMA Proceeds via Radical Polymerization of 2-(Dimethylamino)ethyl Methacrylate



reduced cytotoxicity, transfection efficiency was lower than that of 25 kDa PEI.[252]

3.3. Polymethacrylate

Due to its inherent cationic charge, poly[2-(dimethylamino)ethyl methacrylate] (PDMAEMA) offers significance as a gene transfer agent. Synthesis of PDMAEMA proceeds by radical polymerization of 2-(dimethylamino)ethyl methacrylate initiated by ammonium peroxydisulphate (Scheme 11).[253] Initial evaluations of this compound showed highest transfection efficiency with acceptable cytotoxicity at PDMAEMA/pDNA ratios of 6/1 (w/w) for polymer structures with molecular weights greater than 300 kDa.[254] The successful in vitro transfection efficiency of PDMAEMA polyplexes is attributed to the ability of the polymer to destabilize endosomes as well as to dissociate easily from the plasmid once delivered into the cytosol.[255] However, while in vitro and ex vivo studies showed successful gene transfer, preliminary in vivo transfection activity against OVCAR-3 cells using intraperitoneally injected PDMAEMA-polyplexes was negligible.[256] Because ovarian cancer results in the formation of excess fluid in the peritoneal cavity known as ascites fluid, Storm et al. supposed that this fluid hindered in vivo gene transfer. When OVCAR-3 cells were transfected in vitro in the prescence of varying concentrations of ascites fluid, transfection efficiency was significantly decreased. Furthermore, hyaluronic acid, a polyanionic carbohydrate present in high concentrations in ascites fluid, significantly decreased in vitro transfection efficiency. Together, these results suggest that in vivo transfection efficiency using PDMAEMA−DNA complexes injected intraperitoneally is negatively affected by hyaluronic acid present in ascites.[256] When PDMAEMA-polyplexes were injected intravenously into mice, significant accumulation in the lungs ensued. Because the lungs are the first organ encountered following tail vein injections, Oussoren et al. proposed that polyplex or erythrocyte aggregation in the small capillaries (7 μm in diameter) of the lungs caused the predominant lung uptake. In vitro investigations showed limited PDMAEMA-polyplex aggregation in the presence of albumin, but significant erythrocyte aggregation occurred. Consequently, the significant accumulation of PDMAEMA−DNA complexes in the lungs following intravenous injections appears to result from the formation of aggregates caused by erythrocytes.[257,258]

The mechanism of gene transfer for methacrylate polyplexes has been shown to proceed by both clathrin- and caveolae-dependent pathways.[259] Results suggest that if either of the pathways was blocked with specific inhibitors, polyplex uptake is still feasible: both pathways can be used to incorporate genetic material into cells. However, gene expression was inhibited by the blocking of the caveolae-dependent pathway, while transfection activity was unaltered by inhibition of the clathrin-dependent pathway. This suggests that although the clathrin-dependent pathway can be utilized to incorporate PDMAEMA polyplexes into the cell, delivery to the cell nucleus is not necessarily promoted. Caveolae-dependent uptake appears to be vital for effective gene transfer of PDMAEMA polyplex.

Various modifications to PDMAEMA structure have been investigated in an attempt to further improve transfection efficiency. Hennink et al. attempted to improve the endosome lysis capability of PDMAEMA by incorporating an additional tertiary amino group in each monomeric unit to promote the "proton sponge" effect.[260] While the modified polymeric structure had reduced cytotoxicity, transfection efficiency was significantly lower than the unmodified polymer. This result suggests that the "proton sponge" hypothesis may not be universally applicable to all polymers that can buffer the acidic endosomal compartments. Schacht et al. changed various percentages of the ammonium groups of PDMEMA to pyridine, imidazole, and carboxylic acid functionalities to improve endosomal escape. Modifying PDMEMA with pyridine significantly reduced transfection efficiency, while imidazole and carboxylic acid derivatization eliminated transfection, further illustrating the imperfection of the "proton sponge" hypothesis.[261] The synthesis of methacrylate polymers capable of penetrating the cell membrane was attempted by incorporating guanidinium side groups that emulates an arginine-rich peptide.[262] However, while the guanidinylated polymer showed improved transfection efficiency, cellular uptake was shown to proceed by endocytosis as opposed to direct membrane passage.

Attempts to reduce the cytotoxicity of PDMAEMA have involved copolymerization of 2-(dimethylamino)ethyl methacrylate with other hydrophobic and hydrophilic structures. Methyl methacrylate (MMA), a hydrophobic monomer unit, *N*-vinyl-pyrrolidone (NVP), a hydrophilic monomer unit, and ethoxytriethylene glycol methacrylate (triEGMA), a hydrophobic and hydrophilic monomer unit, were all copolymerized with PDMAEMA. The PDMAEMA−MMA copolymer showed reduced transfection efficiency due to increased cytotoxicity. However, the PDMAEMA−triEGMA and the PDMAEMA−NVP copolymers both showed reduced cytotoxicity with the latter exhibiting improved transfection efficiency.[263] Variations to the PDMAEMA−triEGMA and PDMAEMA−NVP copolymer structures showed that increased ratios of comonomer decreased cytotoxicity, but also reduced the capability of the polymer to condense DNA. However, PDMAEMA−NVP copolymers polymerized to high conversion showed significant improvement in transfection efficinency and cytotoxicity, possibly due to the synergistic effect of the DNA condensing PDMAEMA structure and the prevention of complex aggregation by NVP polymer segments.[264] The formation of aggregates was inhibited by both the grafting of PEG chains onto 2-(dimethylamino)ethyl methacrylate-based polymers[265,266] as well as by coating PDMAEMA polyplexes with anionic lipids, although it should be noted that the lipid coating also reduced transfection efficiency.[267]

To promote cellular uptake of PDMAEMA-based complexes in specific cell lines, various studies have looked at incorporating a targeting agent onto the periphery of PDMAEMA. To target cancer cell lines, Hennink et al. investigated two targeting agents. First, a tumor-targeting Fab′ fragment of mAB 323/A3 was incorporated onto the periphery of a lipid-coated PDMAEMA polyplex. After a 48 h exposure, cellular uptake and transfection efficiency was notably improved as compared to the unconjugated analogues in human ovarian carcinoma (OVCAR-3) cell

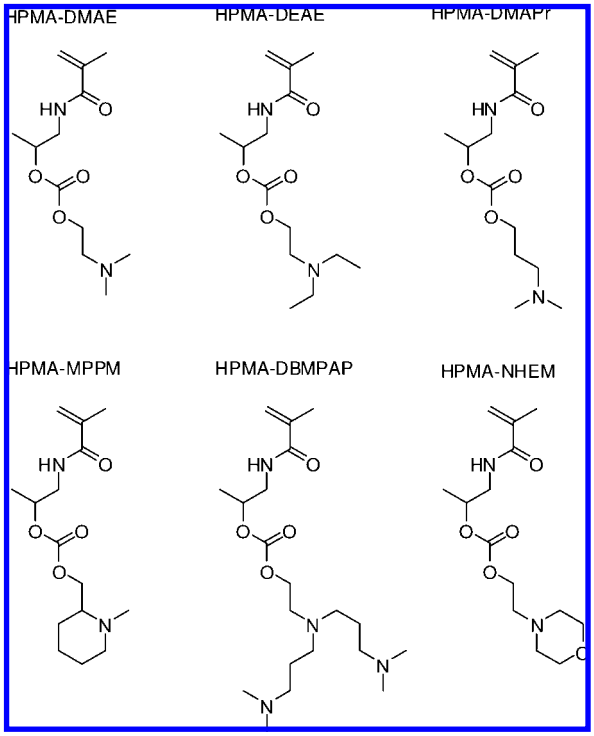


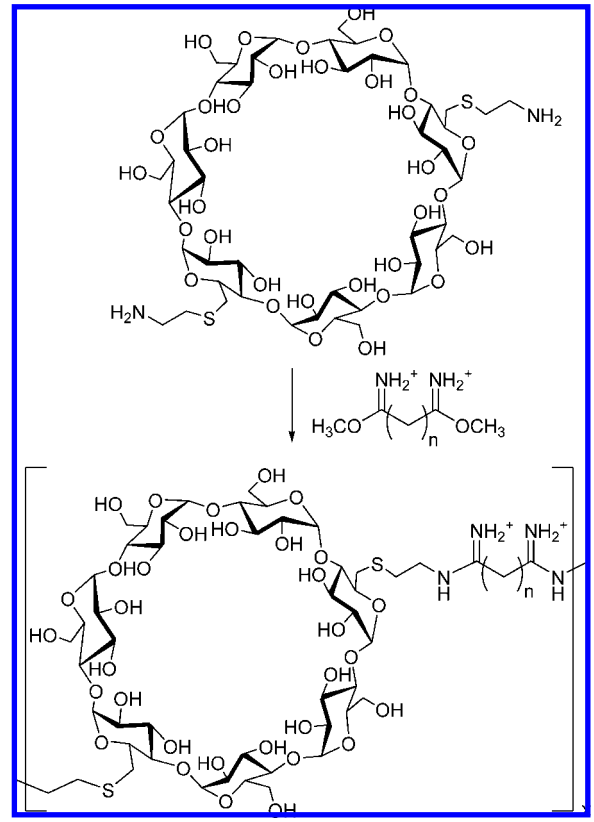
Figure 8. Monomer units for biodegradable methacrylate-based polymers.

lines.[267] Additionally, folate-targeted PDMAEMA-based polyplex showed markedly improved transfection efficiency as compared to unconjugated derivatives in the same cell line.[268] In addition to tumor-targeting, hepatocyte-targeting agents, including galactose[269] and lactose,[270] were incorporated onto the terminus of a PDMAEMA−PEG copolymer. Both modified structures exhibited improved transfection efficiency in HepG2 cells. In a later study, a lactosylated ABC triblock copolymer incorporated a poly(silamine) block to improve endosomolytic properties of the PDMAEMA−PEG copolymer and to eliminate the need for the incorporation of either hydroxychloroquine or the fusogenic peptide KALA.[271]

In recent years, designing methacrylamide polymers with hydrolyzable cationic side chains to create biodegradable gene carriers has become appealing. Hennink et al. synthesized a methacrylate-based polymer with carbonate functionality (pHPMA−DMAE) from which DNA was released after 48 h at pH 7.0 but to which DNA remained bound at pH 5.0. The degradable structure showed improved transfection efficiency in the presence of INF-7, an endosomal membrane disrupting peptide (Gly-Leu-Phe-Glu-Ala-Ile-Glu-Gly-Phe-Ile-Glu-N-Gly-Trp-Glu-Gly-Met-Ile-Trp-Asp-Tyr-Gly).[272] In later studies, methacrylate-based polymers with variations of the hydrolysis-sensitive cationic side groups were synthesized (monomer units shown in Figure 8), and several derivatives of the new series (pHPMA−DEAE, pHPMA−MPPM, and pHPMA−BDMPAP) showed lower cytotoxicity and transfection efficiency that was serum independent and superior to 25 kDa PEI.[273]

Most recently, a copolymer of poly(hydroxylethyl methacrylate-*co*-hydroxylethyl methacrylate propargyl alcohol) was grafted with PDMAEMA−N_3 via click chemistry to form a brush copolymer structure with a biodegradable carbonate linker. This material showed reduced toxicity and improved gene transfer in the presence of INF-7 when compared to both PDMAEMA and linear PEI.[274] While the incorporation of hydrolyzable carbonate groups improved gene transfer of methacrylate polymers, the incorporation of ester functionality eliminated gene transfer capabilility as a result of reduced cellular uptake and endosomal release.[275]

Scheme 12. Synthesis of β-Cyclodextrin-Based Polymers Proceeds via Polymerization of 6A,6D-Dideoxy-6A,6D-di(2-aminoethanethio)-β-cyclodextrin Hexahydrate with Dimethylsuberimidate



3.4. Carbohydrate-Based Polymers

3.4.1. β-Cyclodextrin

To overcome the cytotoxicity of many nonviral gene delivery agents, Davis et al. have looked at incorporating β-cyclodextrin into cationic polymers, as studies have shown cyclodextrins to have significant biocompatability.[276−279] Synthesis of linear β-cyclodextrin-based polymers proceeds via the polymerization of a bifunctional β-cyclodextrin monomer, such as a (2-aminoethanethio)-β-cyclodextrin derivative, with an additional bifunctional comonomer, such as the HCl salt of dimethylsuberimidate, as shown in Scheme 12.[280] Transfection results obtained with cyclodextrin-based polymers showed efficiency that rivals that of PEI and Lipofectamine at N/P ratios above 10 as well as limited toxicity in both fibroblast (BHK-21) and epithelial (CHO-K1) cell lines at N/P ratios as high as 70 in the presence of serum.[281] It was later shown that the length of the alkyl chain (*n*) between cyclodextrin monomer units affects polyplex cytotoxicity: cytotoxicity generally decreases with longer chain lengths consistent with a decreased charge density.[282] For a series of compounds with alkyl chain lengths (*n*) ranging from 4 to 10, cytotoxicity was lowest, and transfection efficiency was highest for polymers with 6, 7, or 8 methylene units. The high toxicity/low transfection efficiency of the polymer with 10 methylene units was attributed to

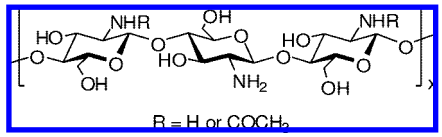


Figure 9. Chemical structure of chitosan.

reduced solubility, indicating the importance of balancing low charge density and high solubility.

Structure−activity relationships of β-cyclodextrin-based polyplexes later showed that increasing the hydrophiliciy of the cyclodextrin spacer unit (alkyl vs alkoxy) can reduce cytotoxicity, possibly as a result of increased hydration around the cyclodextrin moieties and higher flexibility.[283] Additionally, it was determined that, as the cation is moved further from the cyclodextrin group, toxicity increases.[284] Quaternary ammonium cations were shown to transfect cells less efficiently than the amidine analogues due to reduced endosomal escape.[285] However, later intracellular trafficking studies showed that β-cyclodextrin-based polyplexes do not exhibit buffering capacity in the endosomal environment unless derivatized to form imidazole-terminated structures.[286] This suggests that for β-cyclodextrin polyplexes, endosomal escape, and high transfection efficiency cannot always be correlated to buffering capacity.

Like most other cationic vector systems, in vivo use of β-cyclodextrin was shown to be hindered by the formation of aggregates at high ionic strengths. While PEGylation can generally reduce the formation of aggregates, it also reduces the cationic charge density of the polymer. Due to the ability of β-cyclodextrin to form inclusion compounds,[287] Davis et al. complexed adamatane-terminated PEG chains with β-cyclodextrin polymers to reduce aggregation behavior without affecting cationic charge density.[288] Treating β-cyclodextrin polymers with either adamantane-PEG-galactose[288] or adamantane-PEG-transferrin[289] copolymer has shown successful cell-targeted gene transfer potential. In vivo studies using the transferrin-conjugated β-cyclodextrin polymers showed that these structures could form complexes with siRNA that inhibited metastatic Ewing's tumor growth in mice.[290]

3.4.2. Chitosan

The biodegradability, biocompatibility, and cationic potential[291] of chitosan has helped it become one of the most prominent, naturally derived nonviral vectors for gene transfer.[292,293] Chitosan is produced by deacetylation of chitin to form a polymer composed of D-glucosamine and *N*-acetyl-D-glucosamine subunits linked by β(1,4) glycosidic bonds (Figure 9).[294,295] Mumper et al. pioneered the early efforts for gene delivery using chitosan in the mid-1990's.[296] Investigations indicated that molecular weight of chitosan polymers can strongly influence gene transfer efficiency. Rolland et al. showed that the size of chitosan polyplexes increases with increased molecular weight of chitosan.[297] However, Leong et al. later showed that, regardless of the increased polyplex size, high molecular weight chitosan forms more stable complexes with DNA due to a chain entanglement effect. High molecular weight polymers can more effectively entrap DNA than low molecular weight analogues.[298] Transfection efficiency in A549 cells increases with the molecular weight of chitosan polyplexes (213 kD*a* > 98 kD*a* > 48 kD*a* > 17 kDa).[299] Additional studies of chitosan derivatives containing deoxycholic acid groups (DAMC) showed similar trends.[300] An ethidium bromide

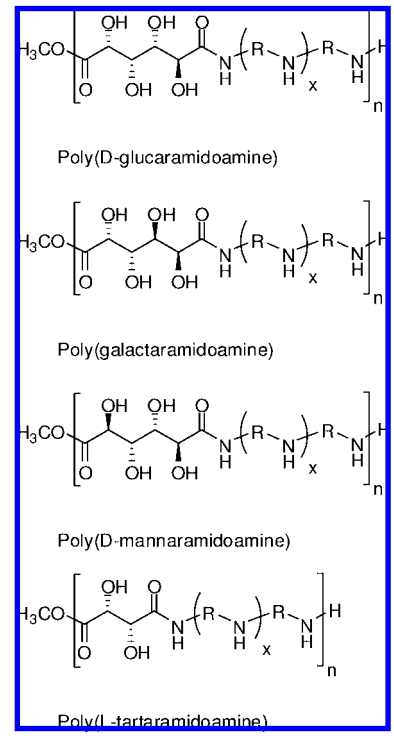
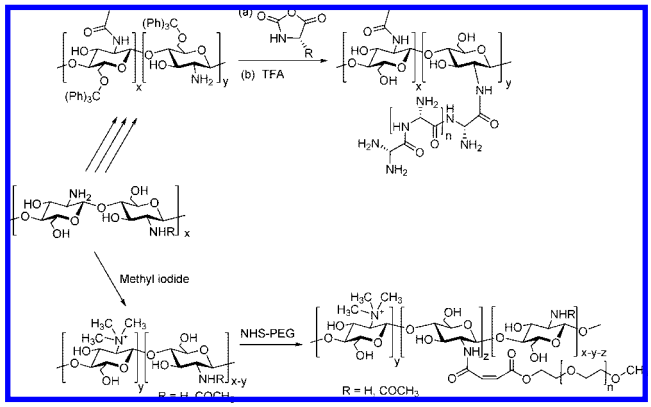


Figure 10. Poly(glycoamidoamines) containing various hydroxyl group number and stereochemistry.

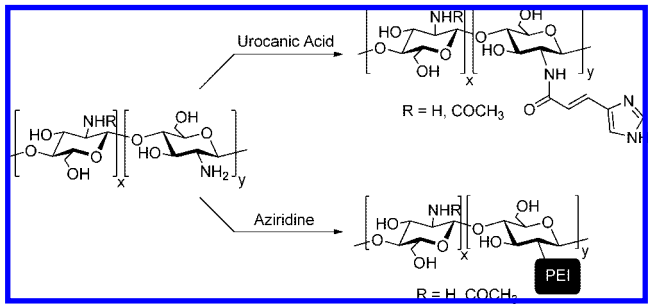
exclusion assay showed increasing complexation for higher molecular weight derivatives. However, the highest molecular weight deoxycholic acid derivative did not show the greatest gene transfer activity, possibly due to hindered DNA release.

In addition to molecular weight, several other factors have been shown to affect the transfection efficiency of chitosan polyplexes, including the N/P charge ratio, pH, the degree of deacetylation, and cell type. The optimum N/P ratio was shown to be 5 for chitosan polyplexes.[301] This value varies with the molecular weight and degree of deacetylation of the polymer used.[302] Increased deacetylation generally improves transfection efficiency in vitro for various cell lines.[298] Mechanistic studies indicate that the success of highly deacetylated compounds results from the higher complex stability when compared to less deacetylated derivatives. Stability is attributed to the increased charge density of the deacetylated polymer.[299] However, in vivo studies have shown more successful gene transfer using a moderate rather than high degree of deacetylation.[298] This result stresses the importance of balancing the protection of the DNA from nuclease attack by stable complex formation versus facile release of DNA from the complex. Optimal transfection efficiency of chitosan polyplexes can be achieved between pH 6.8 and 7.0.[303] Above pH 7.5, DNA was shown to dissociate from the complex, thus preventing cellular uptake and transfection efficiency. Below pH 6.5, cellular uptake was significant but transfection efficiency was low, possibly due to hindered endosomal release.[301] Chitosan-based polyplexes have also shown transfection efficiency that varies with the cell type used: higher gene transfer is seen in HEK293 cells as compared to HT-1080, Caco-2, MG63, or mesenchymal stem cell lines.[304,305] The variation in gene transfer activity of different cell lines is attributed to differences in cellular uptake due to cell-specific plasma membrane compositions as well as differences in chitosan-degrading enzymes present within the endosomal compartments of the cells.

Scheme 13. Modifications to Chitosan To Improve Cationic Charge Density



Scheme 14. Synthesis of Chitosan Derivatives that Improve Buffering Capacity

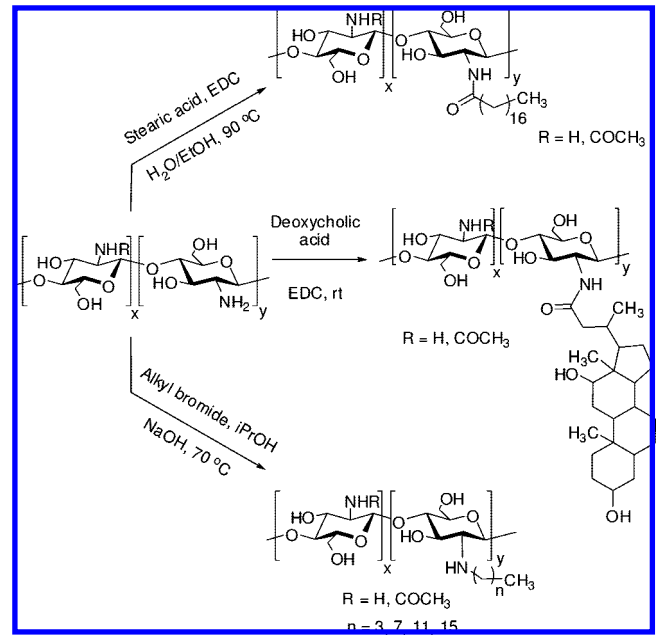


To improve transfection efficiency of chitosan polyplexes, numerous modifications to the polymer structure have been made (Scheme 13). Several strategies were utilized to improve the cationic property of chitosan. The first of these strategies involved N-quaternization of chitosan terminal amines to increase charge density, which resulted in improved transfection efficiency despite higher cytotoxicity.[306,307] This higher cytotoxicity of the trimethyl chitosan derivatives was reduced by grafting of the trimethyl chitosan polymer with PEG.[308] This same increased charge density was achieved by grafting chitosan with polylysine.[309] The chitosan-*g*-PLL polymer exhibited better DNA-binding ability, reduced cytotoxicity, and increased transfection efficiency as compared to both polylysine and 25 kDa PEI.

To improve the buffering capacity of chitosan-based polyplexes, two important modifications were investigated (Scheme 14). The chitosan polymer was conjugated with varying ratios of urocanic acid. Results showed that these imidazole-containing derivatives reduced cytotoxicity and significantly enhanced transfection efficiency as compared to chitosan, illustrating the role of the "proton sponge" effect.[310] This same buffering capacity was achieved by conjugating chitosan with polyethyleneimine.[311] Transfection efficiency of the chitosan−PEI derivative rivaled that of 25 kDa PEI, but cytotoxicity was significantly reduced for the chitosan−PEI derivative.

To reduce the aggregation of chitosan polyplexes and improve interactions with cell surfaces, hydrophobic moieties such as deoxycholic acid,[312] stearic acid,[313] and alkyl chains[314] have been conjugated to chitosan (Scheme 15). The transfection efficiency of the deoxycholic acid derivatives varied with molecular weight of the chitosan. Both low (5 kDa) and high (200 kDa) molecular weight deoxycholic acid−chitosan polyplexes showed low gene transfer activity that was attributed to complex instability and reduced DNA

Scheme 15. Synthesis of Chitosan Derivatives To Reduce Aggregation and Improve Transfection Efficiency

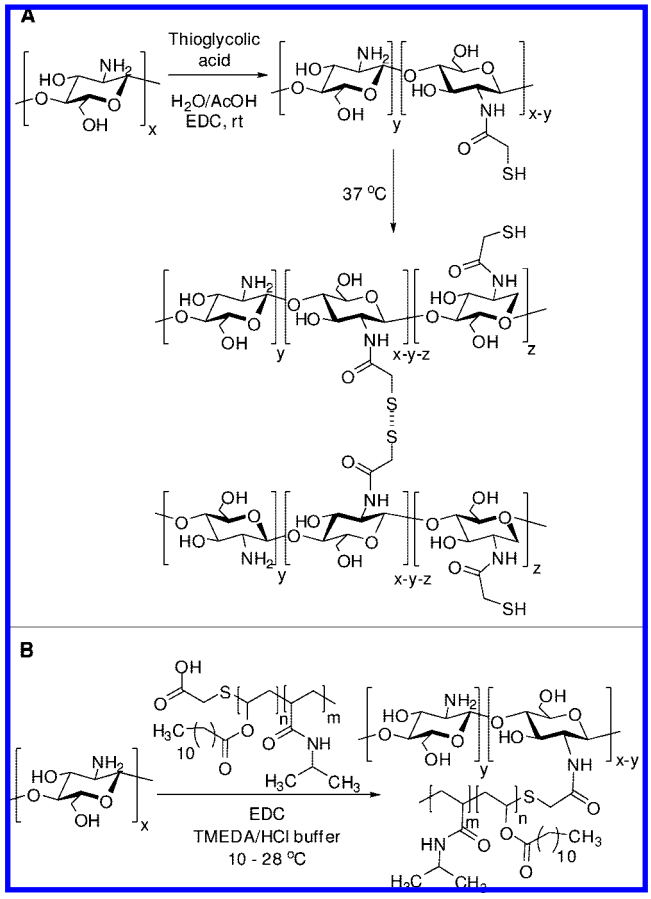


release, respectively. Highest transfection efficiency was achieved for 40 kDa deoxycholic acid−chitosan complexes. The transfection activity of the stearic acid−chitosan complexes increased about 8-fold compared to underivatized chitosan polyplexes. Transfection efficiency of the alkylated chitosan derivatives were 5- to 7-fold higher than unmodified chitosan.

To further improve transfection efficiency of chitosan, a thiolated derivative that could form reducible disulfide linkages was synthesized (Scheme 16A).[315,316] For in vitro studies, the thiolated chitosan derivatives showed improved cellular uptake compared to chitosan. This enhancement was attributed to the formation of disulfide bonds between the thiolated chitosan derivative and plasma membrane proteins. The inactiviation of the thiol groups using 5,5′-dithiobis-(2-nitrobenzoic acid) (DNTB) led to reduced transfection efficiency, thus supporting this hypothesis.[316] Cross-linking the thiolated chitosan derivatives resulted in sustained gene expression in vitro when compared to both unthiolated chitosan and Lipofectin.[315] These cross-linked chitosan derivatives also showed sustained gene expression for cells in the bronchoalveolar lavage (BAL) fluid 14 days after intranasal administration in mice. Cotransfecting a chitosan-plasmid complex with a chitosanase (csn) gene capable of degrading the polymer also improved the transfection efficiency of chitosan.[317] This improved gene transfer activity was attributed to intracellular unpacking of the DNA following slight csn gene expression. To improve endosomal release of chitosan complexes, chitosan was coupled to an *N*-isopropylacrylamide/vinyl laurate copolymer to create a thermoresponsive copolymer (PNVLCS; Scheme 16B). By reducing cell culture temperature to 20 °C 18 h after transfection, transfection efficiency of PNVLCS−DNA was increased when compared to PNVLCS complexes that were incubated at 37 °C only.[318] However, such thermoresponsive derivative offers little advantage for in vivo studies.

The delivery of chitosan complexes to specific cell types was achieved by conjugating chitosan to various cell-targeting ligands. Hepatic cell-targeting using galactose,[319,320] lactose,[321,322] or a trisaccharide[323] showed improved gene

Scheme 16. Synthesis of (A) Thiolated Chitosan Derivative and (B) *N*-Isopropylacrylamide/Vinyl Laurate-Chitosan Derivative



transfer to HepG2 cells. Complex stability was further enhanced for galactosylated complexes by conjugating chitosan−galactose polymers with either PEG[324] or dextran.[325] Tumor cell-targeting was promoted by conjugating chitosan with folate.[326]

3.4.3. Poly(glycoamidoamine)

In an effort to combine the successful transfection properties of both PEI and chitosan, polymers with carbohydrate moieties along a linear amino-backbone have been explored by Reineke et al. Synthesis of these polymers proceeds by polycondensation of a carbohydrate diester with a diamine monomer unit.[327] Initial gene transfer studies using poly(glycoamidoamines) with D-glucaric acid as the carbohydrate moiety showed almost no cytotoxicity and significant transfection efficiency that improves with increased chain length of the amine-containing monomer unit.[328] In later studies, it was shown that the stereochemistry of the hydroxyl groups of the poly(glycoamidoamine) also affects the transfection efficiency by altering the stability of the polyplex structures (shown in Figure 10).[329,330] Based on competitive displacement assays with heparin, it was shown that the order of DNA binding affinity is L-tartarate > meso-galactarate > D-glucarate > D-mannarate. It should be noted, however, that the galactarate-based polymers showed higher transfection efficiency than the tartarate analogue despite the lower binding affinity, which was proposed to result from better cell−surface interactions.[330] In a separate study, polyplex aggregation was shown to be inhibited in the presence of serum, resulting in significant gene transfer, when trehalose

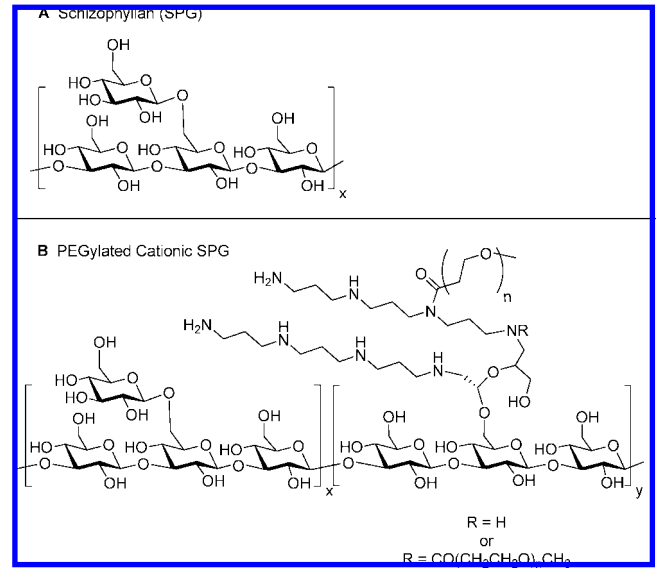


Figure 11. Chemical structures of schizophyllan and PEGylated cationic derivatives.

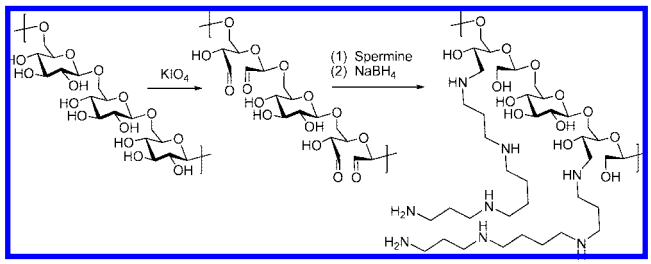
was used as the carbohydrate moiety in poly(glycoamidoamine) stuctures.[331]

More recent studies have investigated the structure−activity relationships of poly(glycoamidoamines). Results indicated that toxicity increases as the cation is further removed from the carbohydrate moiety[284] and quaternary ammonium cations exhibit lower gene transfer activity when compared to the amidine analogues.[285] Cytotoxicity decreases with increased carbohydrate size.[284] In a later investigation, the following conclusions were reached: (1) for polyplexes with the same carbohydrate group but varied amine stoichiometry, cellular uptake is the most influential factor for gene delivery, with higher number of amino groups promoting greater uptake; (2) for polyplexes with the same stoichiometry of amine monomer but varied carbohydrate units, buffering capacity and higher amine density facilitate greater cellular uptake; and (3) for polymers with the same carbohydrate and amine stoichiometry but varied amine spacers, gene delivery correlates with higher complex stability.[332] These results highlight the limitations of using the "proton sponge" theory in making predictions of gene delivery efficiency, as complex stability and cellular uptake are crucial factors. A more recent study compared the transfection efficiency of branched and linear poly(glycoamidoamines) and found that, generally, branched structures were less toxic due to a decrease in secondary amine density but also less efficient at gene delivery.[333]

3.4.4. Schizophyllan

Schizophyllan, a β-(1→3)-glucan with one β-(1→6)-glycosyl side chain per three glucose residues, is a polysaccharide structure shown to have potential antitumor effects (Figure 11).[334] In addition to its use as a cancer-targeting agent, studies performed by Shinkai et al. have shown that schizophyllan can bind to polynucleotides through nonionic hydrogen bond interactions between bases and a single schizophyllan chain (s-SPG).[335] Based on these results, schizophyllan and various derivatives have been successfully used in vitro to promote the delivery of CpG DNA, an oligonucleotide that stimulates Th1 immune responses in mammalian cell lines.[336] Later, schizophyllan was conjugated to ovalbumin, and this schizophyllan−ovalbumin derivative

Scheme 17. Synthesis of Dextran−Spermine Conjugates



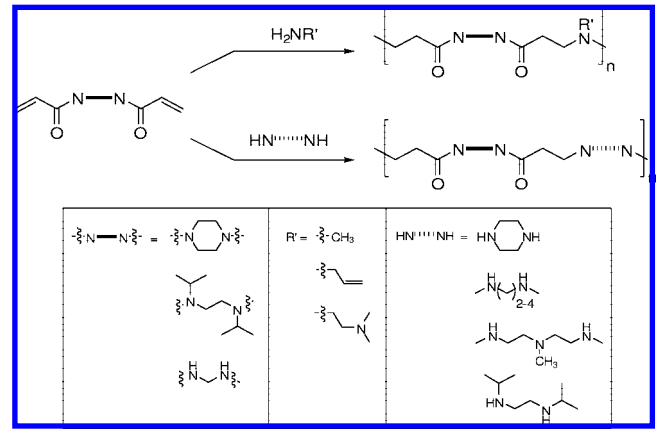
was complexed with CpG DNA and delivered into a macrophage-like cell line (J774.A1) to successfully elicit an antigen-specific immune response.[337] Plasmid DNA complexed to PEGylated cationic schizophyllan derivatives was successfully delivered into cells with greater efficiency than PEI complexes. These constructs exhibited higher long-term gene expression when compared to readily hydrolyzable dextran analogues due to a slower rate of degradation.[338] In vivo studies showed that intraperitoneal injections of SPG/CpG−DNA complexes increased the immune response in mice 2- to 9-fold that of uncomplexed CpG DNA.[339]

One drawback of using schizophyllan-mediated gene delivery is that the DNA must include a long poly(2′-deoxyadenylic acid) [poly(dA)] or polycytidylic acid [poly(C)] tail, as only homonucleotide sequences bind to schizophyllan. To overcome this barrier, ternary complexes of PEI, SPG, and CpG DNA with no poly(dA) tail were formed.[340] CpG−DNA was neutralized with cationic PEI, and the PEI−CpG DNA complex was encapsulated by SPG to form a ternary structure exhibiting less aggregation behavior than unencapsulated PEI−CpG DNA complexes. The neutral ternary complex showed high transfection efficiency mediated by dectin-1,[341] a transmembrane receptor that recognizes β(1,3)-linked glucans. Due to the high expression of dectin-1 on the surface of myeloid cells,[342] this method of DNA transfection has promising capability for the in vivo targeting of antigen presenting cells.

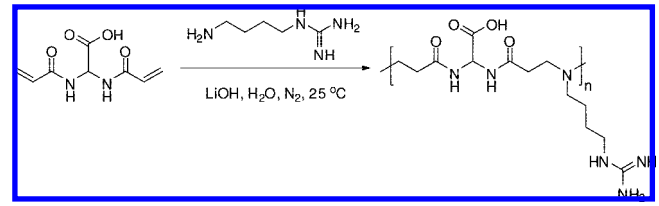
3.4.5. Dextran

Derivatives of the polysaccharide dextran have been applied across the fields of chemistry and biology. For decades, diethylaminoethyl−dextran (DEAE−dextran) has been a commonly used dextran-based polymer for gene transfer.[343] More recently, however, dextran−spermine polycations have been investigated. These structures are synthesized by oxidizing dextran with potassium periodate followed by reductive amination with spermine (Scheme 17).[344] These conjugates showed transfection efficiency that rivals that of both Transfect and DOTAP.[344] In vitro gene transfer was optimized using dextran-spermine derivatives (6000−8000 Da) with 25−30% of the spermine groups conjugated at both ends to form branched polymeric structures.[345] Quaternary ammonium derivatives of dextran−spermine conjugates show reduced transfection efficiency, possibly due to the hindered release of DNA from a too strongly bound complex.[346] Recently, gene delivery to mesenchymal stem cells was enhanced when the dextran−spermine complex was impregnated into a poly(glycolic acid)-reinforced collagen sponge (3D) rather than administered to cells grown on a tissue culture plate (2D). This improved transfection efficiency was attributed to greater cell surface area available in the 3D constructs.[347] In vivo results showed that PEGylated dextran−spermine conjugates successfully mediate gene transfer when administered either intramuscularly or intravenously at a polycation to DNA weight-mixing ratio of 5.[348]

Scheme 18. General Synthesis of Linear poly(amido-amine) via Polyaddition of Aliphatic Primary Monoamines or Bis(secondary amines) and Bisacrylamide



Scheme 19. Synthesis of Cationic Amphoteric PAA

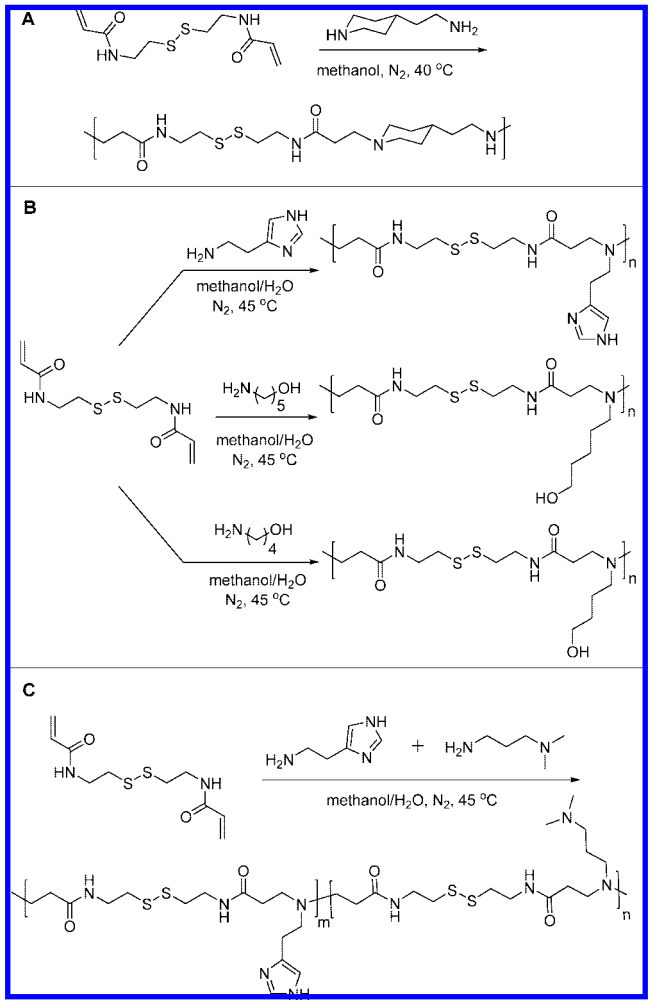


3.5. Linear Poly(amido-amine) (PAA)

Both cationic and amphoteric linear poly(amido-amines) have been investigated for gene transfer. Synthesis of these structures proceeds by hydrogen-transfer polyaddition of aliphatic primary monoamines or bis(secondary amines) and bisacrylamides to form polymer structures with amido- and tertiary amino functional groups (Scheme 18).[349] Based on viscosimetric titrations, Barbucci et al. showed that the protonation of PAA reduces the conformational freedom of the polymer and leads to a more rigid structure.[350] Ferutti et al. showed the hemolytic activity of PAA increased at low pH due to this change in conformation upon protonation, suggesting that these polymers could function as endosomolytic agents.[351] The in vitro cytotoxicities of amphoteric PAA structures, determined using MTT and hemolysis assays, were low when compared to 56.5 kDa PLL and 70 kDa PEI due to the overall negative charge of the amphoteric PAA.[352] When PAA was injected into tumor-bearing mice, the negative charge increased circulation time of the polymer and tumor accumulation by the enhanced permeation and retention (EPR) effect.[351]

Gene transfection studies of amphoteric PAA compounds showed transfection efficiency that rivaled Lipofectin, 70 kDa PEI, and LipofectACE.[353] More recently, a cationic amphoteric PAA structure was synthesized by polyaddition of (4-aminobutyl)guanidine and 2,2-bis(acrylamido)acetic acid (Scheme 19). Despite the significant cationic charge at pH 7.4, the cationic amphoteric PAA exhibited low hemolytic activity and cytotoxicity.[354] Additionally, this polymeric structure showed negligible clearance by the reticuloendothilial system (RES), indicating that it possesses stealth-like properties comparable to anionic PAA compounds.[355] The expression of the green fluorescent protein gene transfected

Scheme 20. Synthesis of (A) Bioreducible PAA Structure with Disulfide Linkages, (B) Bioreducible PAA Structure with both Hydroxyl and Histidine Side-Chain Functionalities, and (C) Random and Block Copolymers of PAA with Histidine- and Tertiary Amine-Terminated Side Chains



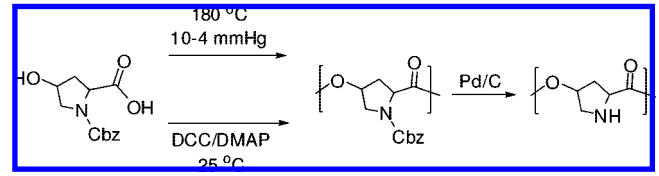
into Hela cells using cationic amphoteric PAA rivaled that of JetPEI based on cytofluorimetric analysis.[355]

PAA structures with disulfide linkages in the bisacryamide monomer unit to create a reducible polymeric structure have been synthesized by Engbersen et al. (Scheme 20A). These structures showed reduced cytotoxicity and improved gene transfer as compared to branched 25 kDa PEI.[356] Manipulations to the side chain of these biolabile PAA structures (Scheme 20B) showed that both hydroxyl and histidine functionalities further reduce the cytotoxicity and improve transfection efficiency of PAA polyplexes.[357] Random and block copolymers containing histidine- and tertiary amine-terminated side chains (Scheme 20C) improved transfection efficiency compared to homopolymers containing these side chains as well as mixtures of these homopolymers. Covalent attachment of functional groups that promote gene transfer activity (i.e., histidine for buffer capacity and tertiary amines for DNA binding) is suggested to be more beneficial than simply mixing the functionalities.[358]

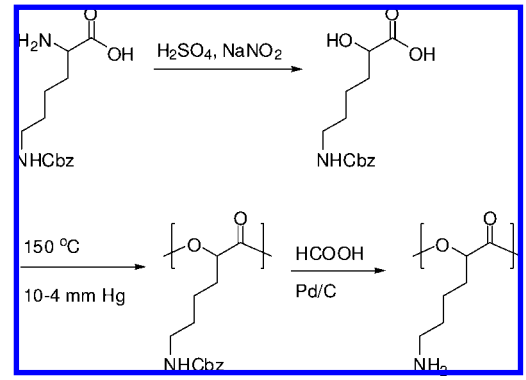
3.6. Biodegradable Polymers

In addition to the biodegradable PEI, PLL, PAA, and methacrylate-based macromolecules, various other degradable compounds have been synthesized and investigated for gene transfer. These compounds include poly(amino acids), poly(amino esters), and phosphorus-containing polymers. Each of these structures contains functionalities in the polymer chain that hydrolyze under physiological conditions.

Scheme 21. Synthesis of Poly(4-hydroxy-L-proline ester)



Scheme 22. Synthesis of poly[α-(4-aminobutyl)-L-glycolic acid]



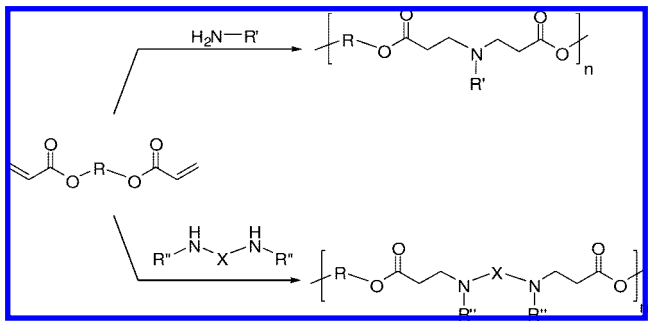
3.6.1. Poly(4-hydroxy-L-proline ester)

The first hydrolytically degradable cationic polymer, poly(4-hydroxy-L-proline) (PHP), was synthesized in 1999 by both Langer et al.[359] and Park et al.[360] Synthesis of this compound proceeds by polymerization of N-cbz-4-hydroxy-L-proline followed by deprotection (Scheme 21).[361] Results from the transfection studies showed that PHP could condense DNA effectively at a polymer/DNA ratio of 3:1 (w:w) and that this polymer had significantly reduced cytotoxicity as compared to PEI or PLL.[359] The polymer degrades to less than half the original molecular weight within two hours in an aqueous solution of pH 7.0 at 37 °C due to aminolysis by the secondary amine groups, and shows complete degradation to monomeric units after 3 months. However, when condensed with DNA, the PHP/DNA complex shows no degradation for at least 4 h under the same conditions due to the fact that the amine groups of the polymer are involved in electrostatic interactions with the phosphate backbone of DNA.[360] Gene transfer efficiency of PHP rivals (or surpasses) that of PLL even in the presence of fetal bovine serum.[360] Huang et al. showed that when PHP is copolymerized with D,L-lactide, the resulting polymer can form DNA-loaded microspheres capable of sustained gene delivery and expression for a period of at least 7 days.[362]

3.6.2. Poly[α-(4-aminobutyl)-L-glycolic acid] (PAGA)

Poly[α-(4-aminobutyl)-L-glycolic acid] is a biodegradable polyester analogue of PLL. Synthesis of PAGA proceeds by conversion of the α-amino group of N_ϵ-cbz-L-lysine to a hydroxyl group followed by polymerization then deprotection (Scheme 22).[363] This biodegradable polymer degrades to less than half the original molecular weight within 30 min in an aqueous solution of pH 7.3 at 37 °C and completely degrades to monomeric units within 6 months.[364] Initial in vitro gene

Scheme 23. General Synthesis of poly(β-amino ester)[a]



[a] Proceeds via condensation of a diacrylate ester with either a primary amine or bis(secondary amine). R groups include both linear and cyclic aliphatic groups, ethyleneoxy-groups, and aromatic functionalities. R′ groups include linear and cyclic aliphatic groups and hydroxyl-terminated aliphatic groups. Diamine monomers (R′′NHXNHR′′) include both piperidine and 4,4′-trimethylenedipiperidine.

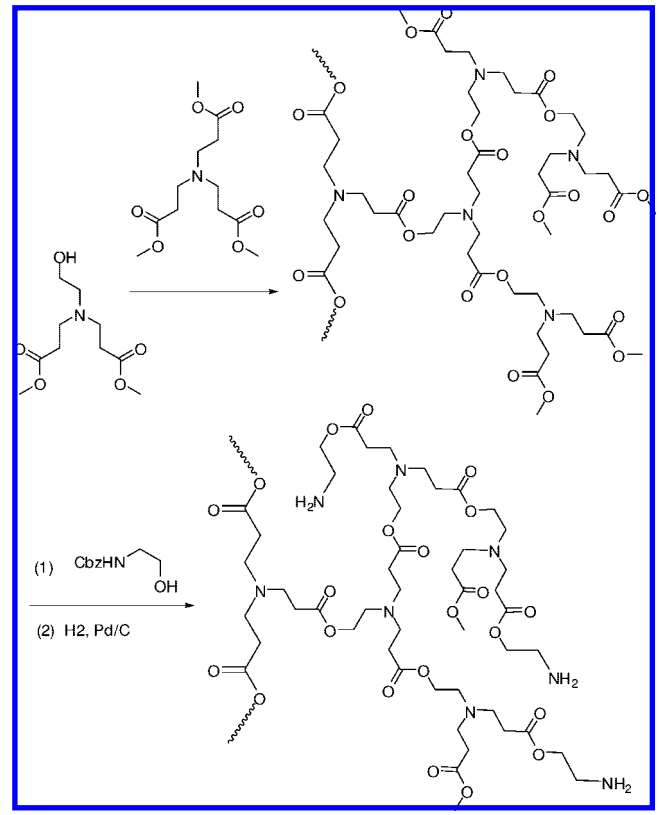
transfer studies showed that PAGA/DNA complexes (N/P charge ratio of 60) to have no cytotoxicity and approximately 3-fold higher transfection efficiency in the presence of chloroquine as compared to PLL/DNA complexes, due to a more rapid release of the DNA as a result of polymer degradation.[363] In vivo studies showed that complexes of PAGA and mouse IL-10 (mIL-10) plasmid could effectively reduce the occurrence of severe insulitis in nonobese diabetic (NOD) mice (15.7%) compared to naked DNA (34.5%).[365] Additionally, complexes of PAGA and a plasmid encoding both IL-4 and IL-10 were shown to effectively prevent the onset of both diabetes[366] and insulitis[367] in NOD mice. Additional studies have investigated the antitumor effect of IL-12 plasmid/PAGA complexes. The PAGA/DNA complexes elicited 7-fold higher IL-12 protein levels and reduced tumor growth as compared to naked plasmid DNA injections.[368,369]

3.6.3. Poly(amino-ester)

Langer et al. conducted the first investigations into using poly(β-amino esters) for gene delivery. Synthesis of these compounds involves the conjugation of either a primary amine or a bis(secondary amine) monomer with a diacrylate ester (Scheme 23). In general, poly(β-amino esters) completely degrade to monomeric units within 5 h and exhibit no negative effects on cell viability.[370] A library of these poly(β-amino esters) showed members with gene transfer efficiencies that rival PEI, PLL, and Lipofectamine 2000 in both COS-7[371] and HUVEC cell lines.[372] Recently, hepatic cell-targeting was promoted by synthesizing poly(β-amino esters) with a thiol-reactive side chain capable of being derivatized with the thiol-containing peptide arginine-glycine-aspartate-cysteine (RGDC).[373]

The biophysical characterization of a library of poly(β-amino esters), based on fluorescence-based flow cytometry and plasmid DNA covalently labeled with both fluorescein and Cy5, showed that the dominant limiting factor for gene transfer was cellular uptake followed by the ability to escape the lysosomal trafficking pathway.[374] Polymers with imidazole moieties or two amines in close proximity were most efficient at avoiding lysosomal degradation due to higher buffering capacity. In a unique high-throughput, semiautomated synthesis and screening of over 2300 poly(β-amino esters), the use of hydrophobic diacrylate monomer units improved transfection efficiency despite the potentially higher cytotoxicity.[375] Additional structure−activity relationships of these polymers showed that molecular weight, end groups,

Scheme 24. Synthesis of Hyperbranched Poly(amino ester)

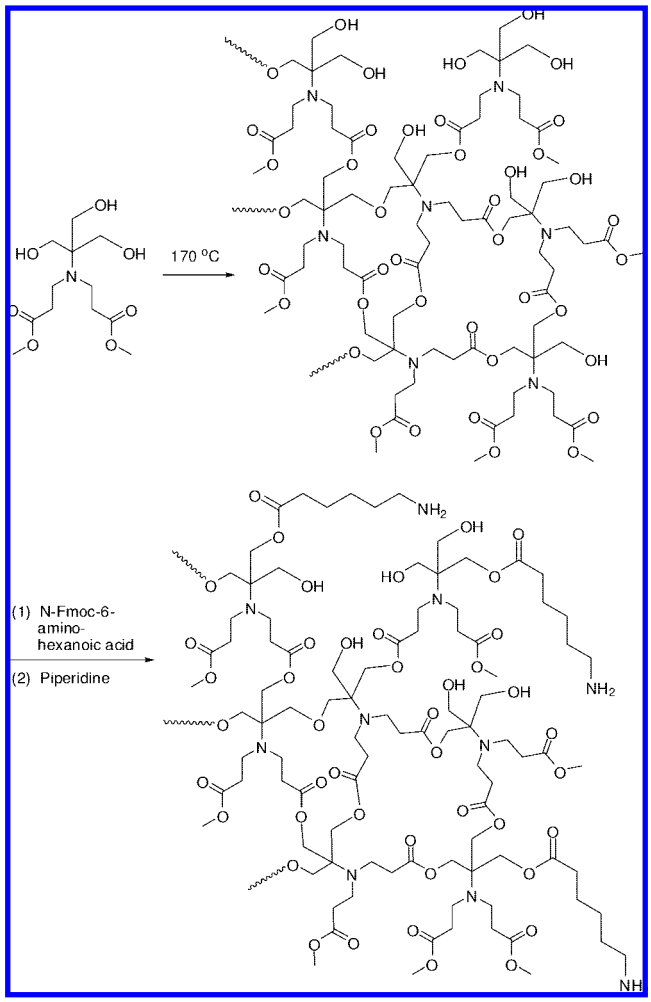


and polymer/DNA ratio effect transfection efficiency.[376] Only poly(β-amino esters) capped with amine moieties exhibit significant gene transfer activity. All polyplexes showed the highest gene transfer for the highest molecular weight polymers, but the optimal polymer/DNA ratio varied according to cytotoxicity of the complex. Poly(β-amino esters) capped with diamine chains exhibit high transfection efficiency despite higher cytotoxicity due to more effective DNA binding.[377]

Complementing the work of Langer and co-workers, Park et al. investigated hyperbranched poly(amino esters) for gene transfer. These compounds were synthesized by reacting a monomer bearing one hydroxyl group, two methyl ester groups, and one tertiary amine group with a generation 0.5 polyamidoamine (PAMAM) core (monomer/core ratio 200/1) using $Al(OiPr)_3$ as a catalyst. The reaction was terminated using N-Cbz-ethanol amine followed by deprotection (Scheme 24).[378] The cytotoxicity of these polymers was significantly lower than that of PEI or PAMAM and the transfection efficiency was higher than that of PAGA.[378] Other groups have synthesized additional hyperbranched structures that also showed significant capacity for gene transfer.[379−382]

In addition to hyperbranched poly(amino esters), Park et al. have synthesized network-type poly(amino esters) (nt-PAE) to increase the degradation time of the polyplex. Initial synthesis proceeded by preparing the monomer by reacting methyl acrylate with tris(hydroxymethyl) aminomethane. Bulk polycondensation reactions afforded cross-linked structures (Scheme 25) that exhibited transfection efficiency superior to that of PEI, particularly in the presence of serum.[383] Later, nt-PAEs were conjugated with either aminohexanoic acid or lysine.[384−386] The rate of degradation of nt-PAE was determined by fluorescence microscopy using the PicoGreen method. Briefly, nt-PAE

Scheme 25. Synthesis of Network-Type Poly(amino acid)[a]



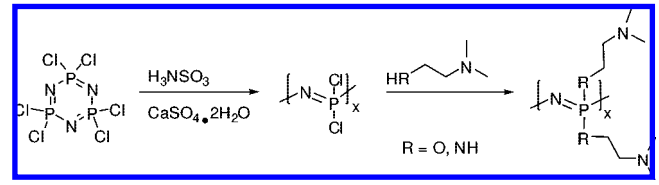
[a] Proceeds by melt polymerization.

was complexed with DNA and at various time intervals was exposed to PicoGreen, which exhibits enhanced fluorescence upon binding to double-stranded DNA. As the polymer degrades, more DNA is free to bind with PicoGreen, leading to enhanced fluorescence. Results indicated that nt-PAE degraded slowly, with DNA complexing ability that lasted 5−10 days.[386] These compounds all showed minimal toxicity and transfection efficiency rivaling that of PEI and PAMAM in various cell lines.[385,386]

3.6.4. Phosphorus-Containing Polymers

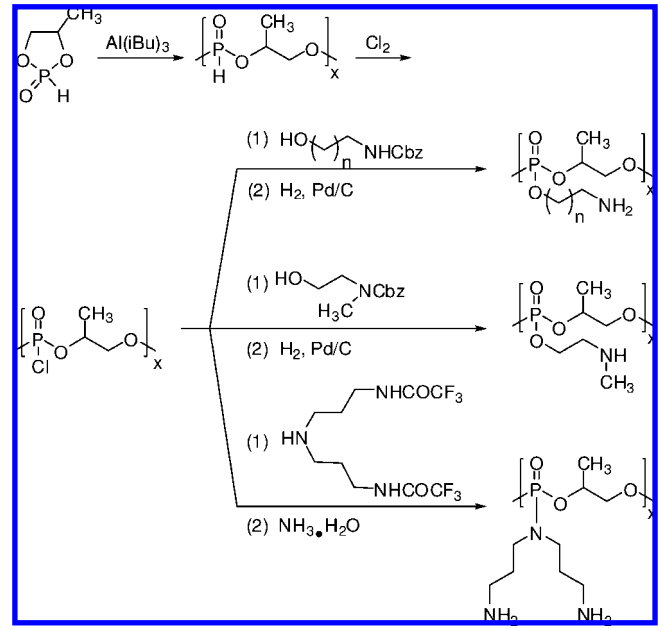
Degradable phosphorus-containing polymers used for gene delivery include poly(phosphazenes) (PPZ), poly(phosphoesters) (PPE), and poly(phosphoramidates) (PPA). The first of these classes, the poly(phosphazenes), are synthesized via ring-opening polymerization of hexachlorocyclotriphosphazene, as described by Magill,[387] followed by the introduction of either 2-dimethylaminoethanol (DMAE) or 2-dimethylaminoethylamine (DMAEA) following procedures described by Allcock (Scheme 26).[388] These compounds exhibited slow acid-catalyzed degradation with half-lives of between 7 and 24 days based on the side chains and showed low cytotoxicity and improved gene transfer in vitro compared to PDMAEMA.[389] Poly(DMAEA-phosphazene)/DNA complexes share preferential gene expression in tumor tissue in vivo, suggesting the benefit of this polyplex for cancer therapy.

Scheme 26. Synthesis of Poly(phosphazene)[a]



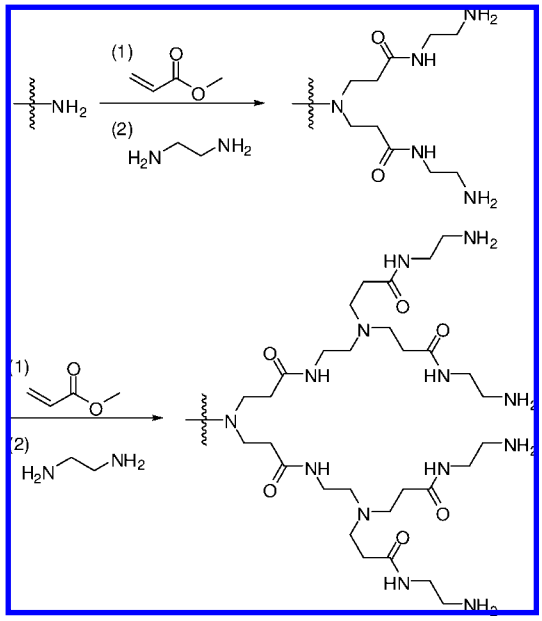
[a] Proceeds by ring-opening polymerization of hexachlorocyclotriphosphazene followed by a substitution reaction with either DMAE or DMAEA.

Scheme 27. Synthesis of PPE and PPA by Ring-Opening Polymerization of 4-Methyl-2-oxo-2-hydro-1,3,2-dioxaphospholane Followed by Cholorination and then Substitution with Either an Alcohol or Amine



Polyphosphoesters and polyphosphoramidates have been synthesized by ring-opening polymerization of 4-methyl-2-oxo-2-hydro-1,3,2-dioxaphospholane followed by chlorination, then substitution with either an alcohol or amine following methods described by Penczek et al. (Scheme 27).[390] Polyphosphoesters degrade to less than half the original molecular weight within 7 days at 37 °C and completely degrade to monomer units within 10 days.[391] Transfection efficiency of polyphosphoesters with amine-terminated side chains, while lower than that of PEI, surpassed that of PLL, particularly in the presence of serum.[391] Additional studies showed that the side chain affects the rate at which DNA is released from the complex. For complexes containing alkyl side chains with terminal amines, the rate of DNA release increases for longer chain length, suggesting that hydrophobicity reduces hydrolysis of the polymer structure.[391,392] In addition, it was shown that polymers with secondary amine-terminated alkyl side chains release DNA more quickly than the primary amine analogues. This more rapid release was attributed to faster side-chain cleavage via aminolysis by the more nucleophilic secondary amine.[392] This rapid DNA release hinders in vitro gene transfer, but shows higher transfection efficiency in vivo for reasons that are still unclear.[392] Polyphosphoramidates with spermidine side chains showed transfection efficiency that rivaled that of commercially available PEI.[393]

Scheme 28. Synthesis of PAMAM Dendrimers



4. Dendrimer-Based Vectors

4.1. Polyamidoamine Dendrimers (PAMAM)

Due to ease of synthesis and commercial availability, polyamidoamine (PAMAM) dendrimers have become the most utilized dendrimer-based vectors for gene transfer. Synthesis of these compounds proceeds by a repetitive sequence involving Michael addition of a nucleophilic core (e.g., ethylene diamine or ammonia) to methyl acrylate followed by an amidation of the resulting ester with an amine functionality (e.g., ethylene diamine; Scheme 28).[394] The first gene transfer studies using PAMAM to form a dendrimer−DNA complex (termed dendriplex) was conducted by Haensler and Szoka in 1993, and since then, numerous groups have utilized PAMAM and PAMAM derivatives for transfection.[395]

Studies conducted by Baker et al. showed that high transfection efficiency is achieved using G5-G10 PAMAM, but within this range, the highest gene transfer efficiency varies with cell line.[396] Additionally, it was shown that nearly all PAMAM-based DNA transfection (>90%) is carried out by low-density complexes whose formation varies based on dendrimer−DNA charge ratio and dendrimer generation.[397] Ottaviani et al. showed that at low dendrimer/DNA charge ratios, r ($0 < r < 1$), small changes to DNA conformation occur, but the complex remains soluble and not compact. At intermediate charge ratios ($1 < r < 100$), insoluble complexes/aggregates form due to DNA charge neutralization. Finally, at high ratios ($r > 100$), resolubilization occurs due to a salting-in effect.[398] By investigating the interactions of PAMAM−DNA complexes using ethidium bromide probes, Turro et al. determined that complexes consist of both "tightly bound DNA" regions with no nucleotide preference and "linker DNA" regions. Higher generation structures contain larger fractions of "tightly bound DNA" regions and are able to more effectively condense DNA.[399] The same trend has been observed for PAMAM−siRNA[400] and PAMAM−ribozyme[400] complexes. Baker et al. have shown that the complexation of DNA with PAMAM prevents nuclease degradation.[401]

Mechanistic studies show that PAMAM promotes two events that aid in cellular delivery and endosomal release of DNA. Smith et al. used fluorescene probe dilution assays to demonstrate that high generation PAMAM dendrimers (G5−G7) induce lipid mixing and leakage from anionic vesicles formed with a 3:7 ratio of 1-palmitoyl-2-oleyl-sn-glycero-3-phosphate (POPA) and phosphatidylethanol-amine (PE). When POPA/PE vesicles that contained the resonance energy transfer pair of NBD-PE and Rh-PE were mixed with PAMAM and PLL, the vesicles exposed to PAMAM showed approximately 2-fold higher lipid mixing compared to those exposed to PLL. This increased lipid mixing was attributed to the ability of the spherical PAMAM structure to bend the anionic membrane through electrostatic forces and induce packing stresses, leading to lipid mixing.[402] Smith et al. suggest that this ability makes PAMAM useful for promoting cellular penetration. Additionally, Verkman et al. treated PAMAM with tetramethylrhodamine succinimidyl ester iminothiolane to create a structure that could be conjugated to Cl^--sensitive BAC-labeled dextran through a disulfide linkage. Following cellular uptake, endosomal chloride accumulation increased significantly and the pH of the surrounding environment increased, indicating the occurrence of endosomal swelling/lysis. This result was attributed to the significant buffering capacity of PAMAM, underscoring the relevance of the "proton sponge" theory for PAMAM-mediated gene delivery.[212]

Various alterations to the basic PAMAM dendrimer structure have been investigated in an effort to improve transfection efficiency. These alterations can be categorized with respect to cytotoxicity, complex formation, cell binding, endosomal release, and cell-targeting. Reduction of cytotoxicity of PAMAM has been achieved by neutralizing the surface of the dendrimer (Figure 12). Park et al. synthesized an internally quaternized PAMAM dendrimer having a hydroxyl periphery. While these compounds exhibited significantly reduced cytotoxicity compared to unmodified PAMAM and PEI, transfection efficiency was reduced as well.[403] Park et al. later synthesized a triblock copolymer consisting a PEG core with two PAMAM substructures on either side. Results from this study indicated that this modified dendrimer exhibited little cytotoxicity and high transfection efficiency compared to PEI.[404]

In addition to reducing cytotoxicity, different attempts have been made to improve PAMAM/DNA complex formation. Szoka et al. heated a series of PAMAM dendrimers in *n*-butanol/H_2O to afford degraded structures that exhibited increased transfection efficiency (Figure 13A). Improved gene transfer was attributed to a more flexible structure that could form compact complexes with DNA but would swell and release DNA more readily than unmodified PAMAM upon a decrease in pH.[405] The degraded PAMAM structure, now commercially available as Superfect, has become an important standard against which gene transfection efficiency is compared. In addition to Superfect, other more flexible PAMAM dendrimers have been synthesized by PEGylation of the PAMAM periphery (Figure 13D)[406] or by the introduction of a trimesyl core (Figure 13C).[407] These other derivatives exhibited significant transfection efficiency. Alternatively, Harada et al. synthesized a PAMAM dendron with a PLL tail (Figure 13B), which showed selective DNA condensation with the PLL segment due to the higher affinity of DNA with cationic polymers having high pK_a values, while the PAMAM segment offered buffering capacity not seen for homopolymeric PLL complexes.[408]

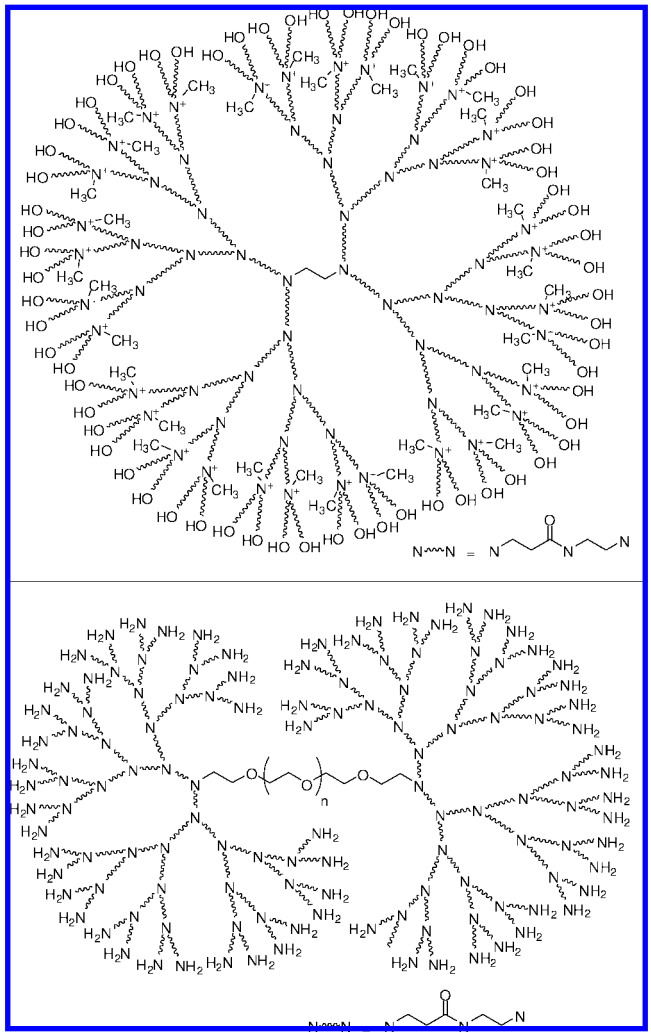


Figure 12. Modifications to reduce cytotoxicity have incorporated a hydroxyl periphery or a PEG core.

In addition to improved complex formation, various attempts have been made to help increase interactions between PAMAM dendriplexes and the plasma membrane. In initial studies, Szoka et al. determined that conjugating GALA, a membrane-destabilizing peptide, to the surface of PAMAM improved gene transfer 2- to 3-fold.[395] In addition, Park et al. have shown that surface modification of PAMAM or PAMAM-PEG-PAMAM triblock copolymer with L-arginine significantly improves gene transfection efficiency.[409,410] Kono et al. have shown that the incorporation of a hydrophobic amino acid such as phenylalanine[411] or alkyl lipid chains[411] onto the periphery of PAMAM dendrons improves gene transfer compared to commercially available PAMAM. This result was attributed to increased hydrophobic interactions between the plasma membrane and PAMAM dendriplex. Juliano et al. observed that Oregon green 488-conjugate PAMAM dendriplex showed improved gene transfection efficiency compared to the unmodified analogues, indicating a similar trend with regard to hydrophobicity.[412]

The release of DNA from PAMAM dendriplexes after endocytosis was improved by Uekama et al. by incorporating cyclodextrin onto the surface of PAMAM to promote hemolysis and lysosomal collapse. Initial studies showed that the α-cyclodextrin conjugates provided up to 100 times the transfection activity of unconjugated PAMAM.[413] In later studies it was shown that, for PAMAM−CD conjugates, generation affects transfection efficiency, with G3 dendriplexes exhibiting the highest gene transfer activity.[414] Furthermore, by increasing the number of α-cyclodextrin per dendrimer (1.1, 2.4, 5.4), it was shown that gene transfection efficiency was highest for the conjugate with a degree of substitution of 2.4.[415]

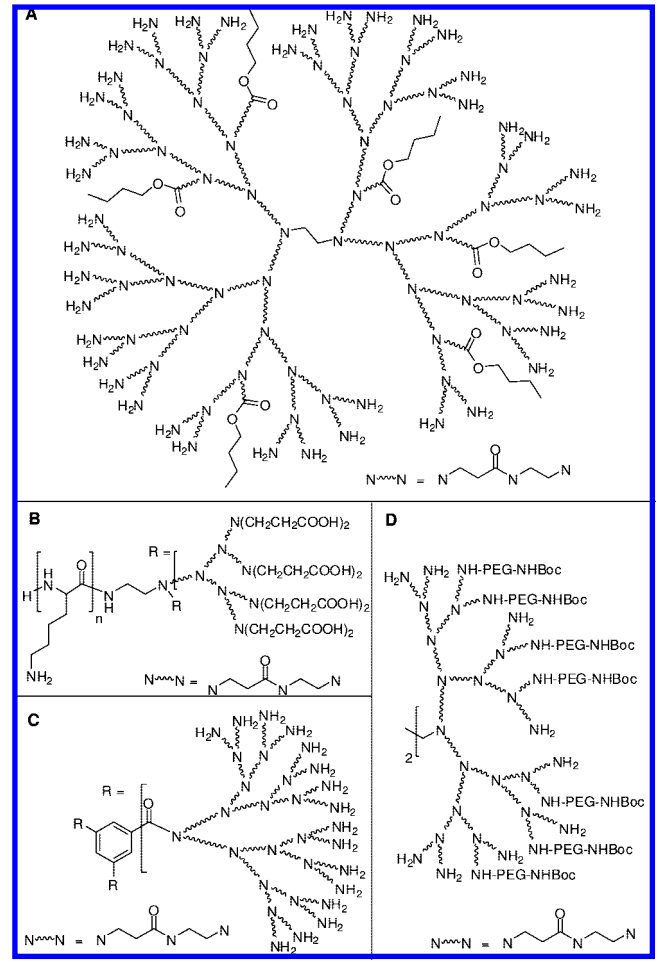


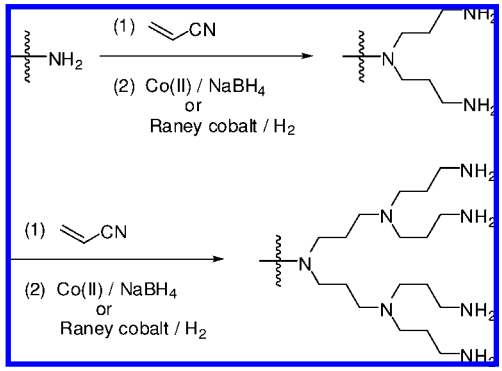
Figure 13. Schematic representations of (A) PAMAM dendrimer randomly fragmented using n-buntanol as solvent, (B) PAMAM−PLL conjugate, (C) PAMAM with trimesyl core, and (D) PEGylated−PAMAM.

Finally, various efforts have focused on incorporating cell-targeting capability into PAMAM-based dendriplexes. Uekama et al. incorporated mannose[416] or galactose[417] residues onto the periphery of cyclodextrin−PAMAM conjugates in an attempt to promote cell-specific uptake. While these showed improved transfection efficiency for the mannose and galactose derivatives, cellular uptake was not dependent on the expression of sugar-specific receptors on the cell surface, indicating inefficiencies in cell-targeting. Recently, however, Wood et al. have shown tumor-targeted gene delivery exceeding that of PEI by conjugating a short peptide ligand (Trp-Ile-Phe-Pro-Trp-Ile-Gln-Leu) that targets glucose-regulated protein-78 (GRP-78), a particular tumor antigen, with a PAMAM dendrimer.[418]

4.2. Poly(propylenimine) Dendrimers (PPI)

In the late 1970s, Vögtle, et al., reported the synthesis of the first cascade molecule, poly(propylenimine). The synthetic route involved the repetitive sequence of a Michael-type addition of a primary amine nucleophile to acrylonitrile followed by subsequent reduction using cobalt and sodium borohydride (Scheme 29).[419] Despite the successful synthesis

Scheme 29. Synthesis of Poly(propylenimine) Dendrimers



of this first dendrimer-based structure, industrial application was limited due to difficulties associated with nitrile reduction. However, later efforts of Mülhaupt and Wörner as well as de Brabander-van der Berg and Meijer showed yields could be increased by using Raney-cobalt catalyst for reduction under more controlled reaction conditions; thus, large scale synthesis and commercialization were achieved.[420,421] Based on the structure of PPI, which has basic amine peripheral groups (pK_a 9−11) and more acidic internal ammonium functionalities (pK_a 5−8),[422] potential for gene transfer was soon realized. Initial studies conducted by Kabanov et al. showed that PPI binds to DNA via electrostatic interactions with only the peripheral amine moieties.[423] Tertiary amine groups at the interior of the complex are unable to interact with DNA, leaving them available to function as a "proton sponge" in an endosomal environment. Later studies showed that, while all generations of PPI are capable of condensing DNA into compact particles, higher generation structures condense to form cationic, water-soluble complexes.[424]

Uchegbu et al. have investigated the gene transfer activity of PPI and showed that low generation (G2, G3) PPI/DNA complexes have the highest transfection efficiency and minimal cytotoxicity.[425] Later studies showed that these low generation complexes could also mediate efficient delivery of an antisense oligonucleotide targeting the epidermal growth factor receptor (EGFR).[426] Improved DNA binding was achieved by N-quaternization of a series of PPI dendrimers. In vivo studies of the low generation (G2) N-quaternized PPI derivative showed significantly reduced toxicity and resulted in liver targeted gene expression as opposed to accumulation in the capillaries of the lungs seen with Exgen 500.[427] This difference in tissue targeting may result from decreased circulation time of PPI as compared to Exgen 500. Schätzlein et al. showed the potential anticancer properties of PPI dendriplexes by complexation of PPI with a TNFα expression plasmid. Results showed transgene expression in tumor tissue that ultimately lead to tumor regression and 100% survival of studied mice.[428] Park et al. have also synthesized pseudorotaxane-terminated PPI dendrimers via conjugation of PPI with mono-Cbz-protected diaminobutane followed by deprotection then treatment with cucurbituril (Scheme 30).[429] These derivatized PPI compounds showed reduced cytotoxicity and gene transfer activity of high generation (G5) PPI structures that rivaled that of PEI.[430] Additionally, the ability to functionalize cucurbituril with amine, alcohol, or sulfhydryl groups offers the potential for peptide ligand conjugation. Recently, Park et al. have conjugated arginine functionality onto the periphery of PPI (G2). These arginine-conjugated structures had reduced toxicity and significantly higher transfection efficiency compared to both 25 kDa PEI and unmodified G2 PPI.[431] Toxicogenomic investigations conducted by Akhtar et al. showed that PPI-dendrimers (G2, G3) can alter the expression of endogenous genes in both A431 and A549 cell lines, suggesting that these structures may impact cellular function in a manner unrelated to their gene transfer capability.[432]

4.3. Poly(L-lysine) Dendrimers

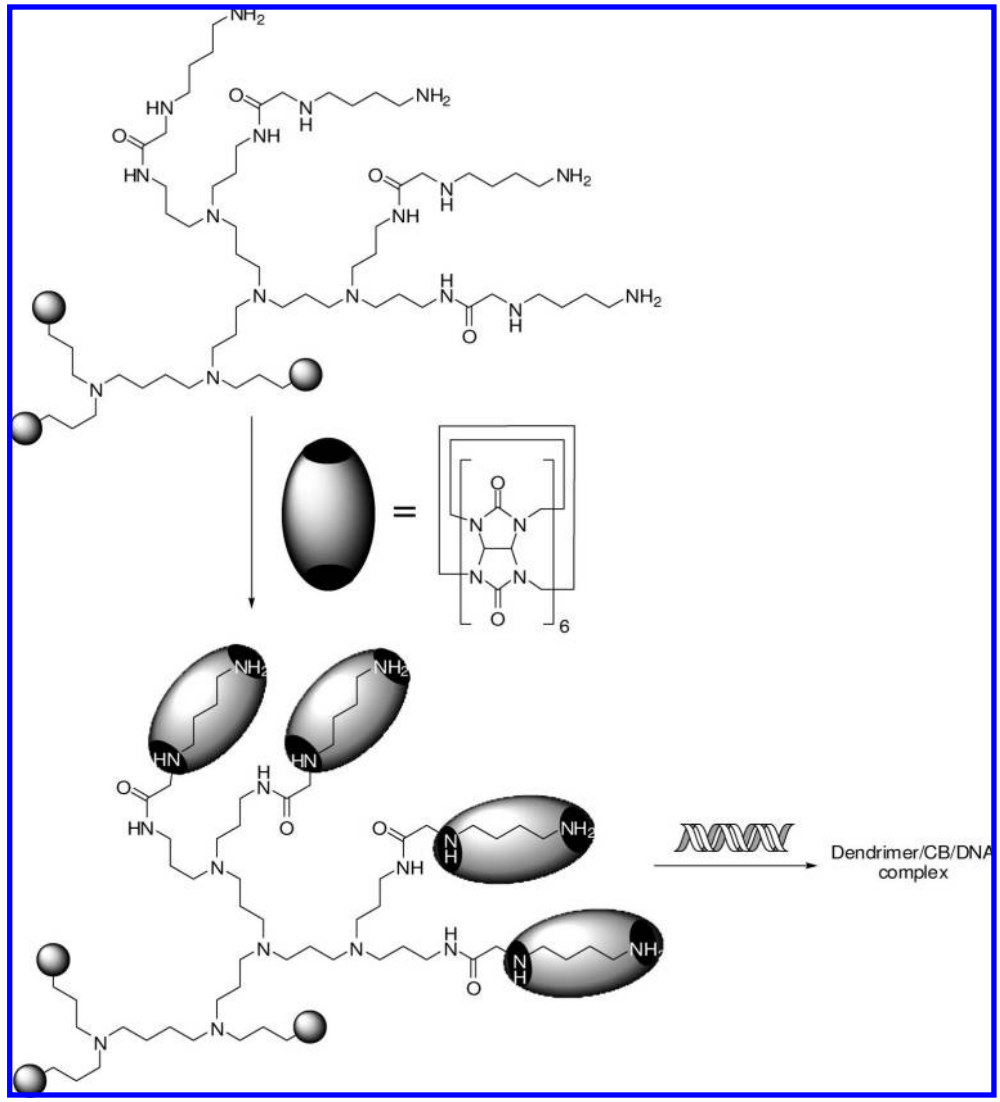
One of the main drawbacks of linear PLL-based gene transfer is the relatively high cytotoxicity of the polymer. To overcome this obstacle, poly(L-lysine)-based dendrons (DPL) have been synthesized. The first synthesis of DPL introduced by Denkewalter et al. followed a divergent route from a two-directional asymmetric core derived from L-lysine and benzhydrylamine. Coupling between the core and a Boc-protected lysine derivative activated by a *p*-nitrophenylester followed by an acid deprotection afforded the first generation DPL structure, which could undergo additional coupling steps to afford higher generation structures (Scheme 31).[433]

Initial studies of gene transfer using DPL vectors (G2, G3) showed high gene transfer efficiency in both COS7[434] and BHK cell lines[435] at a dendrimer/DNA charge ratio of 5:1. Later studies using higher generation DPL structures (G5, G6) showed transfection efficiency that rivaled that of Superfect and Lipofectin. The sixth generation structure also exhibited complex stability in the presence of serum.[170] In vivo studies of this same DPL structure showed long circulation time and significant tumor accumulation, however, no gene expression was observed possibly due to polymer/DNA binding that was too strong to allow for DNA release postendocytosis.[436] Recent studies have shown that DPL compounds can also mediate efficient antisense oligonucleotide[437] and siRNA gene knockdown.[438,439]

Investigations into the complex formation of DPL have revealed a number of interesting properties. First, Niidome et al. investigated the relationship between the structure of a DPL dendriplex and transfection efficiency. For the sixth generation DPL dendriplex, the size increased with time: large complexes (>1 μm) exhibited the highest transfection efficiency.[440] Although cellular uptake of DPL dendriplexes is 4-fold less efficient than for linear PLL, DPL-mediated gene expression is 100-fold higher. This improved gene transfer activity despite reduced cellular uptake was attributed to improved endosomal release and a higher availability to RNA polymerase inside of the nucleus do to less compact structures of DPL complexes.[441]

Various modifications to DPL structure have been investigated to improve transfection efficiency. Niidome et al. replaced terminal lysine residues of DPL with either arginine or histidine. The arginine derivatives exhibited significant DNA-binding and transfection activity that was 3- to 12-fold higher than unmodified DPL. The histidine derivatives, on the other hand, exhibited poor complexation, and no gene transfer activity was realized unless complexes were formed under acidic conditions (pH 5.0).[442] In an alternative investigation, Lu et al. synthesized poly(L-lysine) dendrimers with a cubic octa(3-aminopropyl)silsesquoxane core. These compounds exhibited significantly high transfection efficiency that was attributed to a more globular nature that promoted better DNA compaction.[443] Finally, biodegradable DPL nanoparticles have been synthesized to improve the release of DNA inside the cell. Florence et al. condensed

Scheme 30. Conjugation of PPI with Cucurbituril Followed by the Formation of a Dendrimer, Cucurbituril, DNA Ternary Complex



DNA with DPL compounds containing lipid chains. These complexes were then encapsulated in biodegradable PLGA nanoparticles (~200 nm), which exhibited sustained DNA release.[444] These nanoparticles were later used to introduce a plasmid containing the gene for protective antigen (PA) of *Bacillus anthracis*, the causative agent of anthrax, by intramuscular injection. Although results showed enhanced immunogenicity, indicated by increased anti-PA IgG antibody production, the absence of a toxin neutralizing antibody suggested that the mice lacked protection against the two binary toxins associated with anthrax.[445]

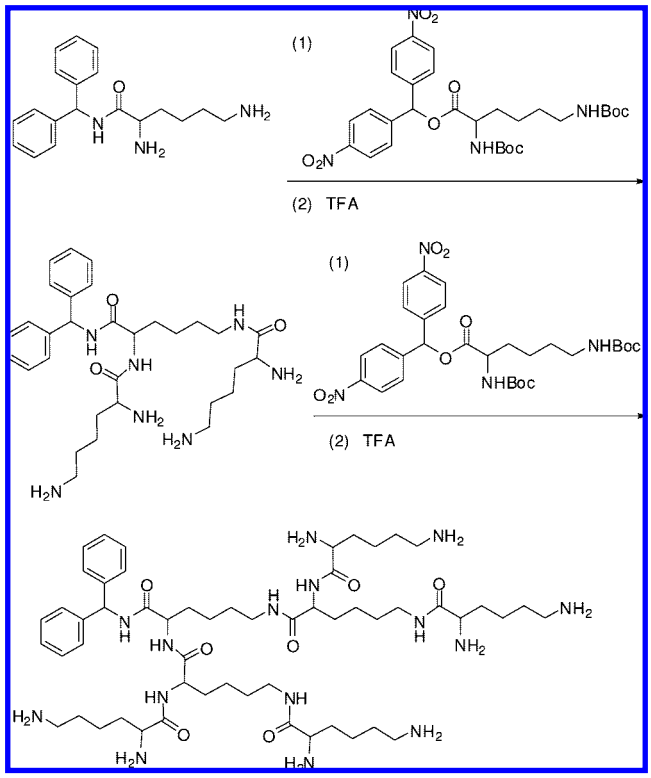
4.4. Phosphorus-Containing Dendrimers

In the past decade, synthetic strategies for phosphorus-containing dendrimers have generated macromolecular structures with significantly higher generation number than that of either PAMAM or PPI. Synthesis of these compounds proceeds by the reaction of hexachlorocyclotriphosphazene with 4-hydroxybenzaldehyde. Reaction with methylhydrazine followed by the addition of diphenylchlorophosphine affords a dendron structure, which can undergo a Staudinger-type reaction with azide functionality to afford a first generation dendrimer structure (Scheme 32). This reaction sequence can be repeated to afford higher generation dendrimers.[446,447] In an effort to employ phosphorus-containing dendrimers for gene transfer, a unique class of compounds with amines were synthesized. The amine-terminated polyaminophosphine dendrimers showed significant gene transfer efficiency, particularly in the presence of serum[448] due to reduced aggregation behavior.[449] More recent studies have shown that incorporating anionic oligomers into the plasmid DNA solution prior to complexation with dendrimer results in the formation of less-condensed phosphorus-based dendriplexes, which exhibited significantly increased gene transfer activity.[450]

4.5. Carbosilane Dendrimers

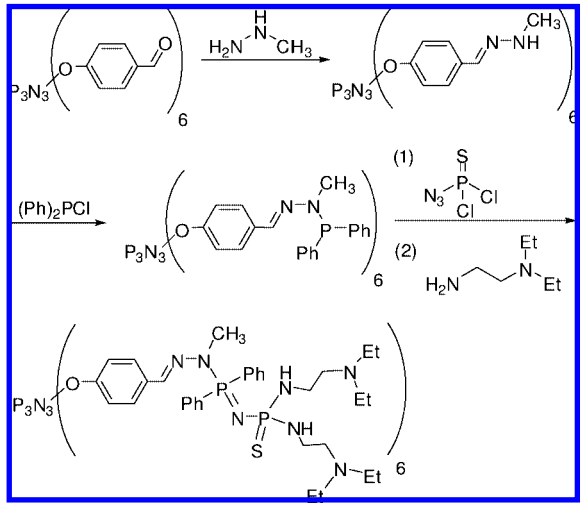
Within the past few years, several investigations have probed the use of carbosilane-based dendrimers for gene transfection. The general synthetic procedure for the formation of carbosilane-based dendrimers involves the treatment of tetraallylsilane core with methyldichlorosilane or chlorodimethylsilane in a hydrosilylation step and vinyl magnesium bromide in an alkenylation step.[451] The formation of amine-terminated carbosilane dendrimers involves the alcoholysis of the Cl−Si terminated structures.[452] In vitro biocompatibility studies showed that second generation carbosilane dendrimers exhibit relatively low cytotoxicity and have the potential to form complexes with oligonucle-

Scheme 31. Synthesis of Poly(L-lysine) Dendrimers[a]



[a] As originally proposed by Denkewalter et al.

Scheme 32. Synthesis of First Generation Polyaminophosphine Dendrimer

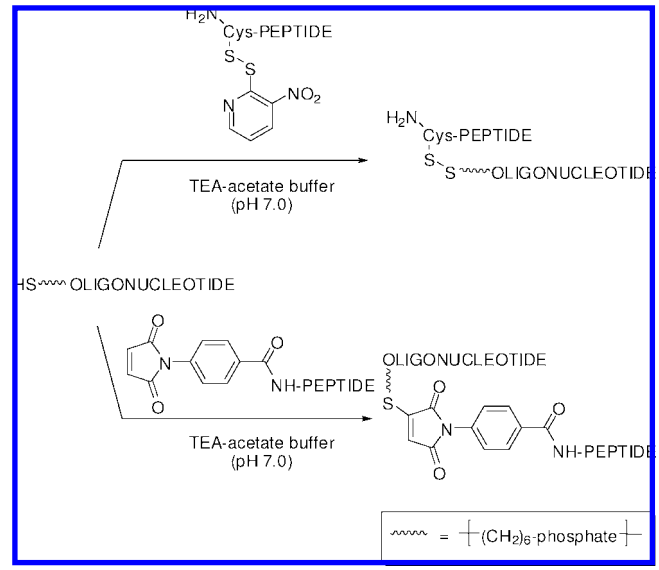


otides.[452] Preliminary data from confocal microscopy showed that carbosilane dendrimers can undergo cellular uptake and intracellular distribution of fluoresceine-labeled oligodeoxynucleotides.[453] Additionally, complexation studies in the presence of serum proteins have shown that these carbosilane structures are capable of protecting short nucleic acids from degradation.[454] Despite these initial results, further investigations of gene transfer activity using carbosilane-based vectors are still needed.

5. Polypeptide Vectors

Peptide−oligonucleotide conjugates offer a unique strategy of delivering genetic material into cells with high efficiencies and cell-specificity. These peptide vectors are able to deliver

Scheme 33. Conjugation of Peptides and Oligonucleotides Using either Disulfide or Thioether Linkages



oligonucleotides into cells by utilizing short sequences of basic amino acid residues which readily cross the plasma membrane. These amino acid sequences, called protein transduction domains or cell-penetrating peptides, are generally divided into two classes: (1) lysine-rich peptides, such as the amphipathic MPG peptide and transportan, and (2) arginine-rich peptides, such as the homeodomain of antennapedia (Antp) and trans-activating transcriptional activators (TAT).[455] Generally, in peptide−oligonucleotide delivery strategies, the peptide is covalently linked to the oligonucleotide construct rather than complexed via electrostatic interactions. Synthesis of these peptide−oligonucleotide complexes follows several strategies. Haralmbidis et al. first synthesized peptide−oligonucleotide constructs by solid-phase synthetic approaches.[456] In this method, derivatized controlled pore glass (CPG) with amine terminal groups was used to create the polypeptide $(AlaLys)_5Ala$ using FMOC peptide synthesis methodology, and then an oligonucleotide was attached to the peptide at the 3′-hydroxyl position using phosphoramidite chemistry (Scheme 33). These structures showed a decrease in oligonucleotide degradation due to the positively charged peptide at the 3′-position. Similar synthetic strategies that used base-labile protecting groups such as 1-(4,4-dimethyl-2,6-dioxocyclohex-1-ylidene)ethyl (Dde) groups[457] or tosyl groups[458] were later developed to avoid the acid deprotection conditions required to remove Boc-protecting groups in the presence of the oligonucleotide. An alternative method to synthesized peptide−oligonucleotide conjugates involves separate syntheses, deprotections, and purifications of the oligonucleotide and peptide sequences followed by conjugation of the two fragements using various linking groups, including disulfides and thioethers (Scheme 10).[459] In general, this conjugation method requires milder conditions than solid phase strategies, but also requires multiple purification steps that result in lower yields. Unlike the covalent attachement of peptide−oligonucleotide complexes, peptide−DNA complexes are generally formed by electrostatic interactions similar to those used to form lipoplexes or polyplexes. Complexation of peptides and siRNA can proceed via either electrostatic or covalent interactions. The following sections will focus on individual cell-penetrating peptides and their uses in gene delivery.

5.1. Tat-Based Peptides

Tat protein is an 86−102 amino acid sequence organized into three domains: (1) cationic regions involved in controlling the rate of gene expression, (2) cysteine-rich regions involved in DNA binding, and (3) basic amino acid regions involved in promoting the crossing of the cell membrane.[460] However, it has been shown that much shorter amino acid sequences incorporating the basic amino acid regions of the Tat protein allow for cellular uptake,[461] with transduction possible using only residues 47−57 (Tyr-Gly-Arg-Lys-Lys-Arg-Arg-Gln-Arg-Arg-Arg). While the cellular uptake of free Tat-peptides has been shown to proceed by an energy-independent pathway,[461] the transfection of Tat−DNA complexes proceeds by endocytosis.[462] However, while Tat−DNA complexes were shown to undergo caveolae-dependent cellular uptake in both HepG2 and CHO1 cell types, transfection of the BGM cell line exhibited an alternative route of endocytosis.[462] One of the earliest strategies for gene therapy using Tat-peptides involved inhibiting the expression of P-glycoprotein.[463] This transmembrane protein, encoded by the MDR1 gene, functions as a pump to remove drugs that have been internalized by cells, and overexpression of this protein in certain cancer cells often leads to ineffective chemotherapy treatments.[464] By covalently attaching the Tat−peptide with anti-MDR antisense oligonucleotide, it was shown that in vitro P-glycoprotein expression could be significantly inhibited, particularly in the presence of serum, which could potentially lead to a 2- to 3-fold increase in drug uptake.[465] However, it was later determined that the effectiveness of the Tat−oligonucleotide conjugates is less than that of oligonucleotide−lipid conjugates with respect to Luciferase induction assays in the absence of serum.

After the initial success of Tat-based oligonucleotide transfection, various modifications were used to improve gene transfer. Torchilin and Volodina et al. prepared Tat−liposomes by covalently attaching the Tat−peptide to *p*-nitrophenyl-carbonyl-PEG-phosphatidyl ethanolamine liposomes. These Tat−liposome conjugates delivered a plasmid designed for expression of green fluorescent protein, and in vitro results showed fluorescence similar to that obtained with Lipofectin, despite higher cytotoxicity. In vivo results showed that Tat−liposome plasmid complexes result in significantly higher gene expression as compared to Tat-free liposome plasmid complexes when injected directly into tumor tissue.[466] Müller and Rosenecker et al. have shown that ternary complexes of Tat−peptide, plasmid DNA, and PEI or Superfect exhibited significantly higher gene transfer efficiency when compared to either PEI or Superfect complexes if Tat−peptide was conjugated with DNA followed by complexation with the cationic polymer. This increase in gene transfer is suggested to result from significant cellular uptake and endosome lysis mediated by the PEI/Superfect moieties followed by translocation into the cell nucleus mediated by the Tat−peptide.[467]

Miyamoto et al. reported similar improvements in gene transfer mediated by Tat−polylysine conjugates. These conjugates, however, required the addition of chloroquine to achieve the highest gene transfer efficiency, suggesting that Tat−peptides are inefficient at promoting endosomal escape despite having significant plasma and nuclear membrane fusion capabilities.[468] Wang et al. recently overcame this barrier by covalently attaching 10 histidine residues to the C-terminus of the Tat−peptide. This complex exhibited up to 7000-fold higher gene transfection efficiency as compared to the unmodified Tat−peptide and rivaled that of 25 kDa PEI but with lower cytotoxicity.[469] Also, Hänze et al. observed significant transfection efficiency of Tat−RGD conjugated peptides in the presence of Lipofectamine. The cellular uptake of this complex was determined to proceed via caveoli-dependent endocytosis. Improved gene transfer in the presence of Lipofectamine may be due to enhanced endosomal escape mediated by the lipid moiety.[470] Gopal et al. conjugated the Tat−peptide with the cationic peptide μ (mu), a 19-amino acid sequence peptide (Met-Arg-Arg-Ala-His-His-Arg-Arg-Arg-Arg-Ala-Ser-His-Arg-Arg-Met-Arg-Gly-Gly). The results showed highest transfection efficiency in the presence of the commercially available cationic lipids Lipofectamine and DC/Chol.[471]

5.2. Antennapedia Homeodomain Peptide

Synthesized in the early 1990's by Prochiantz et al., the antennapedia homeodomain is a 60-amino acid polypeptide corresponding to the *Drosophilia melanogaster* antennapedia homeobox sequence.[472] It has been shown that while the third α-helix of the antennapedia homeodomain is involved in promoting translocation, the 60-amino acid structure could be reduced to a 16-mer peptide (pAntp, residues 43−58, Arg-Gln-Ile-Lys-Ile-Trp-Phe-Gln-Asn-Arg-Arg-Met-Lys-Trp-Lys-Lys) and still exhibit significant cellular uptake.[473] Cellular uptake of pAntp proceeds by a nonendocytic pathway: internalization occurs even at 4 °C.[473] Additionally, the translocation of the reverse helix of pAntp and a helix composed of D-enantiomers indicates that internalization of pAntp is not receptor-mediated. The replacement of several amino acids with proline disrupted the α-helical structure of pAntp but did not hinder cellular uptake, suggesting that the α-helical conformation is not required.[474] Based on this data, Derossi, Chassaing, and Prochiantz have suggested that internalization of pAntp proceeds via electrostatic interactions between the basic amino acid residues of the peptide and the cell membrane, which destabilize the membrane and lead to the formation of inverted micelles that deliver the peptide across the membrane and into the cytoplasm.[475] This mechanism of cellular uptake was supported by Brasseur et al. who showed that, while pAntp does not possess significant membrane-destabilizing properties,[476] charge neutralization of pAntp by the phospholipid bilayer can induce hydrophobicity and promote entrance into the lipid bilayer, which leads to membrane destabilization provoked by the Trp residue.[477]

Early transfection studies involving pAntp-mediated oligonucleotide delivery showed effective cellular uptake and transfection efficiency with minimal cytotoxic effects and serum-independence. These original pAntp−oligonucleotide complexes afforded less oligonucleotide delivery than Lipofectin.[478] Pritchard et al. overcame this inefficiency by conjugating pAntp with L4F, a water-soluble amphipathic peptide with a high binding affinity for lipids (amino acid sequence Asp-Trp-Phe-Lys-Ala-Phe-Tyr-Asp-Lys-Val-Ala-Glu-Lys-Phe-Lys-Glu-Ala-Phe). When the group complexed GFP−DNA with Lipofectamine 2000 and then pretreated the lipoplex with pAntp−L4F, the ternary complex exhibited significantly higher transfection efficiency than either of the peptide−DNA conjugates or the Lipofectamine 2000−DNA complex. This increased transfection efficiency was attributed to a more rapid cellular uptake of pAntp−L4F−Lipofectamine 2000−DNA complexes when compared to the nonpeptide conjugated analogue.[479] Based on data collected by Laczkó et al. using circular dichroism measurements, pAntp binds

to oligonucleotides via electrostatic interactions that result in significant conformational changes. However, in the presence of anionic micelles, pAntp was shown to maintain its α-helical conformation despite oligonucleotide binding.[480] The significantly improved gene transfer efficiency of pAntp−L4F−Lipofectamine 2000−DNA complex was attributed to this preserved α-helical structure. Similar DNA−polymer−pAntp nanoparticles have been used to promote gene transfer in brain capillary endothelial cells (BCEC). Results from this study showed that pAntp enhanced gene transfer efficiency in BCECs only for polyplexes capable of delivering DNA on their own (PEI−DNA and PAMAM−DNA).[481] The fact that nontransfecting polyplexes (Lipofectamine 2000−DNA and chitosan−DNA) showed no enhanced gene transfer in BCECs when complexed with pAntp suggests that pAntp promotes nuclear uptake more effectively than cell membrane-penetration.

5.3. MPG Peptide

The MPG peptide is a synthetic compound containing a hydrophobic N-terminal region derived from the fusion sequence of HIV gp41 (Gly-Ala-Leu-Phe-Leu-Gly-Phe-Leu-Gly-Ala-Ala-Gly-Ser-Thr-Met-Gly-Ala) and a hydrophilic region derived from the NLS of the SV40 large T antigen (Pro-Lys-Ser-Lys-Arg-Lys-Val). Complexation of MPG with oligonucleotides was shown to proceed via electrostatic interaction between the basic residues of the NLS region and the phosphonate backbone of the oligonucleotide. Multiple peptides interact with a single oligonucleotide strand to form particles aggregates of oligonucleotide sequences and peptides.

Originally, the cellular uptake of MPG−oligonucleotide complexes was shown to proceed by an energy-independent pathway, suggesting a mechanism that does not require endocytosis.[482] More recently, analyses based on circular dichroism (CD) and Fourier transform infrared (FTIR) spectroscopy have shown that when MPG peptide is complexed with oligonucleotides, the hydrophobic region partially folds into a β-sheet structure. This β-sheet structure promotes cellular uptake by inserting into the plasma membrane and forming a transmembrane pore-like structure.[483]

When used as a vector for the transfection of plasmid DNA, the cellular uptake of MPG complexes was shown to be more efficient than Lipofectamine when performed at high charge ratios, unaffected by the presence of serum, and nontoxic to cells.[484] A single mutation of the NLS region of MPG (Lys to Ser) significantly inhibits gene transfer prior to cellular mitosis. This reduced transfection efficiency was caused by an inhibited binding of the mutated MPG derivative with importin α, the nuclear protein involved in recognition of and binding to the NLS of a peptide.[485] Interestingly, the mutated MPG/siRNA complex showed enhanced gene knockdown compared to MPG/siRNA due to increased siRNA release into the cytoplasm,[485] which has been reported to be the active site of siRNA.[486]

5.4. Transportan Peptide

In 1996, Langel et al. synthesized the 27-amino acid peptide galparan.[487] This structure consists of the first 13 amino acids from the N-terminus of galanin (Gly-Trp-Thr-Leu-Asn-Ser-Ala-Gly-Tyr-Leu-Leu-Gly-Pro) that effectively target cell surface galanin receptors. The C-terminus of galparan consists of the 14-amino acid sequence mastoparan (Ile-Asn-Leu-Lys-Ala-Leu-Ala-Ala-Leu-Ala-Lys-Lys-Ile-Leu), a wasp venom peptide toxin that forms pores within the plasma membrane to promote cellular uptake. Two years later, Langel and Pooga et al. synthesized transportan, a galparan analogue with the Pro^{13} residue replaced with Lys.[488] When conjugated with biotin at Lys-13, this compound showed cellular uptake that occurred below 15 °C, indicating that the uptake mechanism was not based on endocytosis. Additionally, when cells were pretreated with phenylarsine oxide, an inhibitor of galanin receptor-based cellular uptake, the transportan-biotin complex still entered Bowes' melanoma cells, indicating that the uptake was not receptor-mediated. Based on this data, cellular uptake was proposed to proceed by the formation of inverted micelles in a similar mechanism as suggested for pAntp.

A more recent investigation into the mechanism of transportan−protein cellular uptake conducted by Pooga et al. has suggested alternative routes of translocation.[489] Only a small portion of internalized transportan−protein complexes were detected in the cytoplasm in close proximity to the plasma membrane, suggesting that direct passage across the plasma membrane by inverted micelles is not the dominant form of translocation. The group also determined that clatharin-mediated endocytosis does not contribute significantly to transportan−protein cellular uptake since treatment of both Hela and Bowes' cell lines with transportan−protein and transferrin, a marker for clatharin-mediated endocytosis, did not results in significant cointernalization. Caveolae-mediated endocytosis was ruled out as a significant contributing factor to transportan−protein cellular uptake due to the lack of a significant accumulation of vesicles at the Golgi apparatus, a typical destination for caveolae-mediated cellular uptake. Based on size of the most common vesicles, the Pooga et al. suggested that macropinocytosis is the most probable mode of cellular uptake for transportan−protein complexes. Such mechanism of translocation seems likely for transportan−oligonucleotide sequences, although the transportan cargo can effect the membrane destabilizing effect of a transportan derivative.[490]

While only a limited number of studies have been conducted using transportan as a carrier for delivering both siRNA and plasmid DNA into cells, results indicate that this peptide exhibits potential for gene therapy. Eccles et al. synthesized transportan with an N-terminal cytsteine residue and covalently attached these structures to siRNA targeting the firefly luciferase transgenes via disulfide linkages. When Chinese hamster ovary cells that stably expressed luciferase were transfected with transportan−siRNA complexes, gene knockdown was significantly higher than that obtained using Lipofectamine 2000, and this decreased luciferase expression remained stable for a significantly longer period of time (24 h of stability for Lipofectamine 2000 versus 3 days for transportan).[491] Additionally, transportan 10 (TP10), an analogue of transportan in which the Gly-Trp-Thr-Leu-Asn-Ser sequence has been removed to reduce cytotoxicity, was linked to linear PEI (60 kDa) via a succinimidyl *trans*-4-(maleimidylmethyl) cyclohexane-1-carboxylate (SMCC) cross-linker. This TP10−PEI-mediated DNA delivery showed 2-fold improved in gene transfer efficiency as compared to PEI (60 kDa) complexes in three different cell lines.[492] Because transportan showed no ability to complex with DNA and transfect cell lines in the absence of PEI, the improved gene delivery of the TP10−PEI complexes was attributed to enhanced endosomal release.

6. Nanoparticles

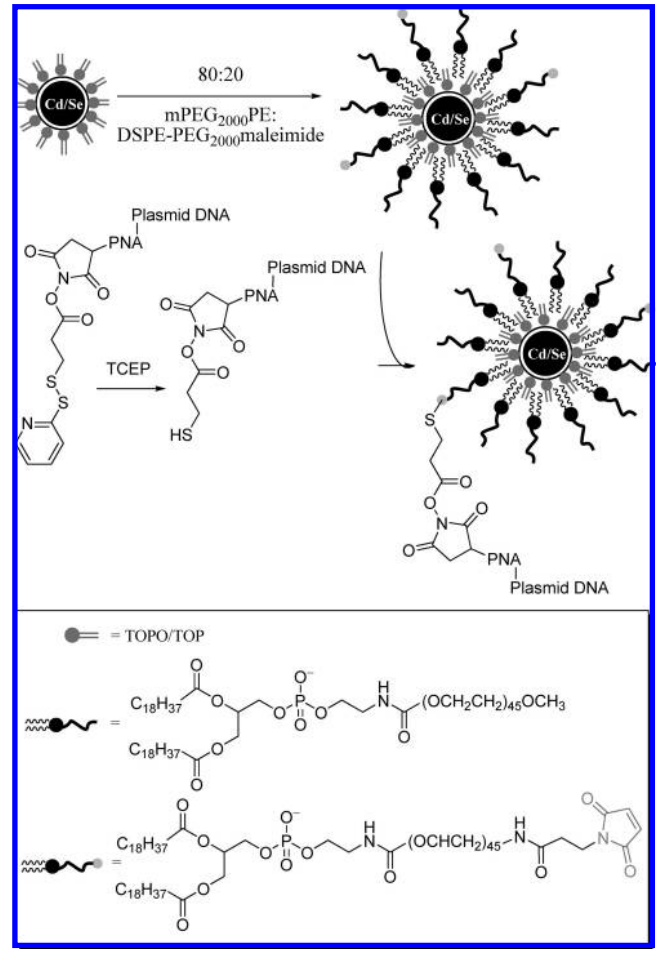
6.1. Quantum Dots

Recently, Förster resonance energy transfer (FRET) using quantum dot-based donor molecules has become an attractive method for the labeling and tracking of proteins inside of the cell. One reason for the interest in using quantum dots (QD) over other organic fluorophores, such as fluoroscein, is that quantum dots can be excited over a wider range of wavelength and the emission spectra is more narrow and symmetrical. Therefore, quantum dots can be excited at a shorter wavelength than the emission wavelength, resulting in less signal overlap.[493] Additionally, quantum dots are less prone to photobleaching and have significantly longer fluorescence lifetimes when compared to organic fluorophores.[493] Finally, while fluorescently labeled DNA complexes can show the association of the carrier and DNA, determining the point of dissociation is hindered by the fact that the two components must diffuse to a certain distance before being detected as distinct entities. Quantum dot-based FRET analysis of protein shows donor−acceptor separation distances of less than 100 Å, indicating potential for monitoring the unpacking of DNA nanoplexes after endocytosis.[494]

Burgess et al. first showed that quantum dots could be covalently conjugated to plasmid DNA for transfection studies.[495] The group encapsulated CdSe/ZnS quantum dots with triocytylphosphine oxide/trioctylphosphine (TOPO/TOP) followed by a mixture of 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-*N*-[methoxy(polyethylene glycol)-2000] and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-*N*-[maleimide(polyethylene glycol)-2000] (80:20 ratio). Peptide nucleic acid-*N*-succinimidyl-3-(2-pyridylthio)propionate (PNA-SPDP) was incorporated into pGeneGrip (gWIZ-LUC), plasmid DNA that has PNA binding sites ($[AG]_{20}$CCATGG-$[AG]_{20}$) and codes for luciferase. The disulfide bonds of the modified plasmid were reduced using tris-(2-carboxyethyl)phosphine (TCEP) followed by reaction with the maleimide functionality of the encapsulated QD (Scheme 34). Transfection studies using these QD−DNA conjugates and Lipofectamine 2000 as the carrier vector showed maximum cellular uptake at 6 h, with approximately 75% of the DNA located in the cytoplasm and 25% located in the nucleus. After 10 h, approximately $^2/_3$ of the DNA was located in the nucleus of the cell, and this nuclear localization was proportional to gene expression. The QD−DNA complexes showed minimal cytotoxicity. Leong and Wang et al. have conducted additional intracellular trafficking studies of polyplexes using quantum dot-labeled DNA.[496,497] In these studies, plasmid DNA encoding for green fluorescence protein was biotinylated using polyethylene oxide-psoralen-biotin, and these analogues were conjugated to streptavidin-functionalized quantum dots. The QD-labeled DNA was complexed with Cy5-labeled chitosan and introduced into HEK293 cells. The trafficking of the complexes within the cell was monitored using confocal microscopy. At 24 h after transfection, the intact complexes were localized around the cell nucleus. At 48 h after transfection, most complexes had released DNA. At 72 h after transfection, QD-labeled DNA was seen within the nucleus of a cell expression green fluorescence protein.

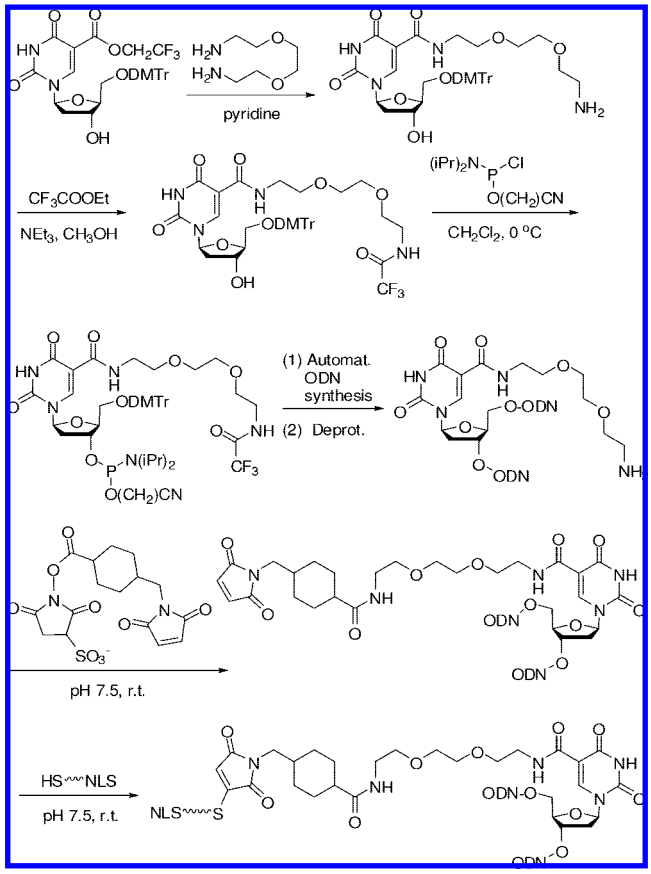
Quantum dot-labeling has also been utilized by Bhatia et al. for intracellular trafficking of siRNA.[498] These studies, however, required cationic transfection agents (Lipofectamine 2000, Superfect, or a translocation peptide) to promote gene transfer, indicating that the quantum dot functioned only as a labeling moiety. However, Bhatia et al. recently functionalized quantum dots with both siRNA and the tumor-targeting F3 peptide (Cys-aminohexanoic acid-Ala-Lys-Val-Lys-Asp-Glu-Pro-Gln-Arg-Arg-Ser-Ala-Arg-Leu-Ser-Ala-Lys-Pro-Ala-Pro-Pro-Lys-Pro-Glu-Pro-Lys-Pro-Lys-Lys-Ala-Pro-Ala-Lys-Lys). In the HeLa cell line, these tumor-targeting QD-siRNA conjugates showed up to ~29% EGFP gene knockdown when cellular uptake was followed by the addition of an endosome escape agent (Lipofectamine 2000).[499]

Scheme 34. Synthesis of Covalent Linking of Plasmid DNA to Lipid-Encapsulated Quantum Dot



Despite the success of QD-labeled DNA transfection, it has been shown that chemical modifications to DNA can reduce transfection efficiency. Harashima et al. synthesized oligonucleotide sequences with a modified uracil base. This modified uracil was reacted with 4-(*N*-maleimidomethyl)-cyclohexane-1-carboxylic acid *N*-hydroxysuccinimide and conjugated with the SV40 large T antigen NLS peptide (Pro-Lys-Lys-Lys-Arg-Lys-Val-Glu-Asp-Pro-Tyr-Cys; Scheme 35). Plasmid DNA was digested using BglII and HindIII and then ligated with the oligonucleotide−NLS conjugates. When microinjected into the nucleus of the cell, these modified DNA compounds showed reduced transgene expression when compared to the unmodified analogues, indicating the potentially deleterious effect of chemically modifying DNA with quantum dots.[500]

Scheme 35. Synthesis of Linearized DNA Conjugated to Nuclear Localization Signal (NLS) Peptide and Various Oligonucleotide (ODN) Sequences



6.2. Gold Nanoparticles

The incorporation of DNA-coated metallic nanoparticles into cells was first accomplished using particle acceleration devices. In early studies, DNA was precipitated onto tungsten nanoparticles, and these nanoparticles were deposited onto polypropylene macroparticles. A gun powder explosion was used to push the macroparticles down a 0.22 caliber barrel. A polycarbonate disk positioned at the end of the barrel stopped the polypropylene macroparticle, but the tungsten nanoparticles continued past the disk and into cells.[501] Shortly after, a similar device was developed in which pressurized helium gas provided a shock wave to propel gold nanoparticles into tissue.[502] This new device showed 4-fold improvement in luciferase expression in mouse skin when compared to the gun powder-driven analogue and was made commercially available by Bio-Rad as PDS-1000/He. However, the shock wave generated by the helium gas in this device can damage the target cells and tissue. Therefore, a modified helium-driven particle acceleration devise was designed in which a hammering bullet is pushed by helium gas into a vibration plate coated with DNA particles on the opposite side. The force of the hammering bullet on the vibration plate could force the DNA particles from the vibration plate toward the target cells with minimal shock wave damage.[503] Additionally, arc-discharge guns have been used in particle acceleration devices. In these systems, gold nanoparticles (1.5−3 μm in diameter) are coated with DNA, and the particles are deposited onto a Mylar film. The film is placed between two electrodes that generate an electric discharge that causes the mylar film to accelerate toward a mesh screen. The mylar film is stopped by the screen, and the gold particles are projected from the film toward the target cells.[504] The velocity of the particles can be changed by adjusting the discharge voltage, the DNA−gold particle density, or the DNA−gold particle size.[505,506]

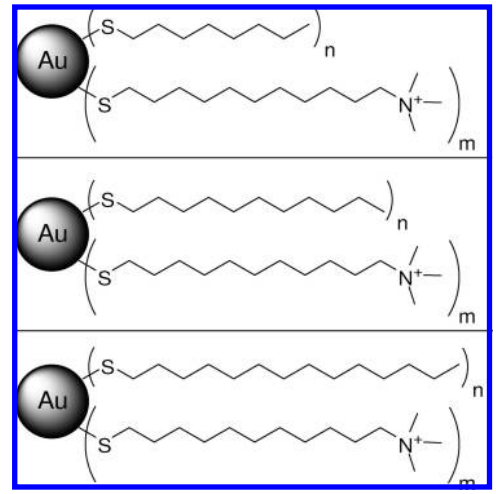


Figure 14. Gold nanoparticles functionalized with various ratios of alkanethiols and trimethylammonium thiol.

The particle bombardment method was one of the earliest strategies used to introduce exogenous DNA into cells. In 1989, McCabe et al. introduced foreign DNA into soybean plants by electric discharge particle acceleration using DNA-coated gold particles.[507] The genetically modified plants showed that the exogenous DNA could be inherited in a Mendelian manner over two generations. The following year, Yang et al. showed that the same method could be used to introduce the chloramphenicol acetyltransferase (CAT) and β-galactosidase genes into both CHO and MCF-7 cell lines in vitro. Also, in situ bombardment of various rat organs showed high gene expression in both skin and liver tissue.[505] The success of gene transfer using particle bombardment is most utilized for genetic immunization applications using skin as the target organ.[508,509]

Despite successful gene transfer using particle acceleration, this method is useful in targeting only certain organs, primarily the skin. Recently, research has focused on modifying the surface of gold nanoparticles to allow for cellular entry via the endocytic pathway rather than the particle bombardment method. Rotello et al. synthesized mixed monolayer protected gold clusters (MMPCs) by reacting 2 nm gold particles with various ratios of alkanethiols and trimethylammonium thiols (Figure 14).[510] Highest transfection efficiency was seen for particles having 68% cationic coverage (amine/alkyl chain). Transfection efficiency improved proportional to alkyl chain length. The most efficient of these modified nanoparticles showed transfection efficiency that was ~8-fold higher than that of 60 kDa PEI.

Klibanov et al. synthesized gold nanoparticles conjugated with 2 kDa PEI.[511] These PEI−Au particles showed 15- and 6-fold higher transfection efficiencies than 2 kDa PEI and 25 kDa PEI, respectively. This transfection efficiency was further increased by creating a ternary complex of DNA, PEI−Au nanoparticles, and dodecyl-terminated 2 kDa PEI. However, cytotoxicity of the PEI−Au nanoparticles was higher when compared to 2 kDa PEI for reasons that are unclear. Later, plasmid DNA that encodes for murine IL-2 was conjugated to positively charged colloidal gold nanoparticles (PGN) that contained approximately six primary amines per particle.[512] At a PGN−DNA ratio of 2400:1,

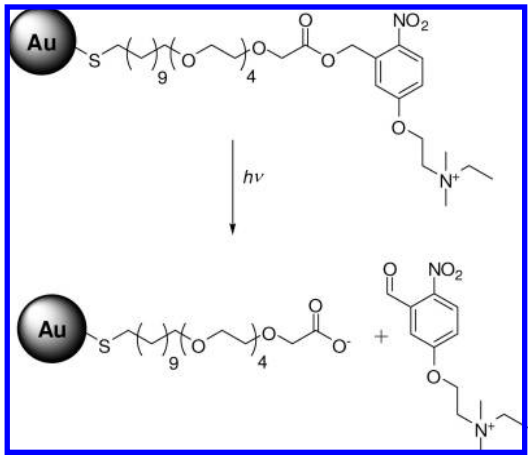


Figure 15. Structure of photolabile cationic gold nanoparticle.

complexes showed 3.2- and 2.1-fold higher gene expression levels than 25 kDa PEI−DNA (10:1) and 2 kDa PEI−DNA (80:1) complexes, respectively. Feldheim et al. covalently conjugated various peptides to bovine serum albumin (BSA) attached this BSA−peptide conjugate to the surface of gold nanoparticles.[513] When transfected into different cell lines, these structures showed varying degrees of nuclear targeting based on the peptide and cell line studied. Gold particles targeted with the NLS of the large T antigen of the SV40 virus showed no nuclear transport, regardless of the cell line used. However, gold particles targeted with the integrin binding domain protein showed nuclear transport in both HeLa and HepG2 cell lines despite the fact that this protein does not interact with the nuclear pore complex. These results stress the importance of of designing transfection vectors that balance the ability for nuclear targeting with endosomal escape properties. Rosi, et al., conjugated either tetrathiol- or monothiol-modified antisense oligonucleotides (antisense sequence A-A-AAAAAAAACTGCCGTCGCACGTCG-A-G, where hyphens indicate phosphorothioate linkages) to 13 nm gold particles.[514] These nanoparticles showed higher knockdown of EGFP gene expression when compared to antisense DNA delivered by both Lipofectamine and Cytofectin.

To improve the efficiency of gold nanoparticle-mediated oligonucleotide transfection, some efforts have focused on designing DNA−gold nanoplexes with labile bonds that can be cleaved once the complex is in the cytosol. Rotello et al. synthesized gold nanoparticles with terminal amine groups linked to the gold particle by photolabile *o*-nitrobenzyl ester-functionalized thiol ligands (Figure 15). The inhibition of DNA transcription due to complexation with the gold particle was monitored prior to and after exposure to UV light (λ = 350 nm). Before UV irradiation, only 5% translation efficiency was seen for the photolabile gold nanoplexes when compared to uncomplexed DNA. However, after 8 min of UV exposure, up to 75% translation efficiency was observed for the gold nanoplexes. Additionally, both fluorescence and confocal microscopy showed that significant nuclear localization of the DNA occurred after the photolabile gold nanoplexes were incorporated into a mouse embryonic fibroblast cell line and exposed to UV irradiation.

Similarly, efforts have focused on utilizing intracellular concentrations of glutathione to mediate the release of thiol-conjugated oligonucleotides from gold nanoparticles. Nagasaki et al. complexed poly(ethylene glycol)-*b*-poly(2(*N*,*N*-dimethylamino)ethyl methacrylate) copolymers with gold nanoparticles, and these particles were then treated with either HS−siRNA, which bonded to the particle through a thiol−Au interaction, or siRNA, which bonded to the particle through electrostatic interactions.[515] Both complexes showed inhibition of luciferase expression in human hepatoma cells, but the gold particles containing HS−siRNA showed more effective inhibition than the gold particles containing siRNA (65 and 25%, respectively). This increased inhibition was attributed to glutathione-mediated release of siRNA into the cytoplasm. Rotello et al. also showed that the inhibition of DNA transcription of T7 RNA polymerase by gold particles with terminal amine groups linked to the particle through Au−thiol interactions is reversed in the presence of glutathione.[516]

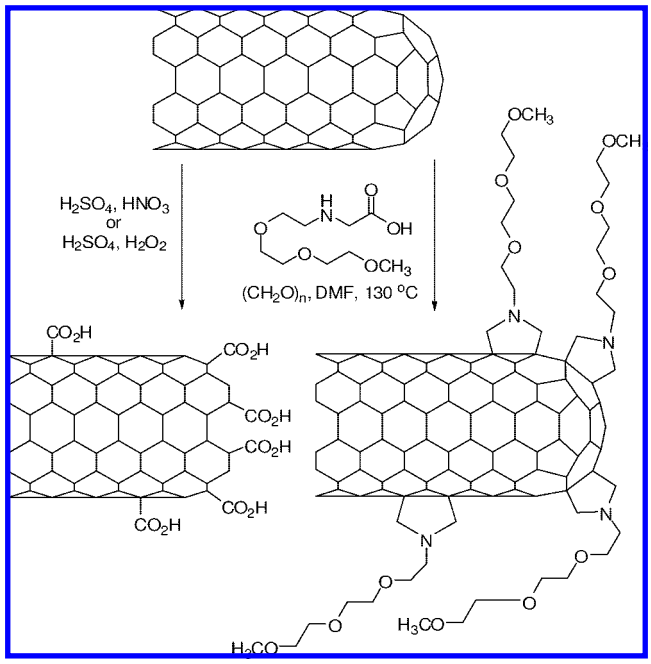
6.3. Silica Nanoparticles

Surface-modified silica nanoparticles are attractive candidates for gene delivery for several reasons. Compared to cationic carriers, colloidal silica nanoparticles are inert and exhibit less cytotoxicity, and compared to liposomes, silica particles are more stable with respect to physical stresses such as aerosolisation.[517] Additionally, the physicochemical properties of modified silica nanoparticles have been shown to be unaffected by freeze-drying in the presence of various lyoprotective agents, offering an appealing method for particle storage.[518]

Saltzman et al. showed that unmodified silica nanoparticles could be used in conjugation with commercial transfection agents to enhance β-galactosidase gene expression by up to 750%.[519] This enhancement was attributed to increased accumulation of DNA at the cell surface. Large silica particles enhanced gene expression more significantly than small particles; this effect was attributed to a faster settling of the larger particles onto the cell surface.[520] However, Gemeinhart et al. used flow cytometry to show that the percent of cells containing DNA after transfection was not significantly different for Superfect−DNA complexes and Superfect−DNA−silica ternary nanoplexes, suggesting that the higher β-galactosidase expression of Superfect−DNA−silica nanoplexes is caused by more than just increased sedimentation.[521] Cellular trafficking studies have shown that both ternary silica nanoplexes[522] and organically modified silica nanoplexes[523] are incorporated into the cell by the endocytic pathway and result in nuclear localization of the DNA only, suggesting that the high gene expression of the silica nanoparticles cannot be attributed to enhanced crossing of the nuclear pore complex.

Various modifications to the surface of silica nanoparticles have been investigated to allow for DNA complexation without the use of commercial transfection agents. Chen et al. modified silica nanoparticles with sodium chloride.[524] These particles showed transfection efficiency that exceeded that previously reported for liposome-mediated transfection. The DNA-binding capacity of silica nanoparticles with cationic surface groups was first investigated by Lehr et al.[517] The group modified silica nanoparticles with both *N*-(2-aminoethyl)-3-aminopropyltrimethoxysilane (AHAPS) and *N*-(6-aminohexyl)-3-aminopropyltrimethoxysilane. Agarose gel electrophoresis showed that these nanoparticles could effectively immobilize DNA at nanoparticle/DNA weight ratios between 10 and 100. These particles showed significant transfection efficiency in Cos-1 cell lines, particularly in the presence of serum and chloroquine.[525] The increased transfection efficiency in the presence of serum was attributed to

Scheme 36. Covalent Functionalization of Carbon Nanotubes Proceeds by Acid-Catalyzed Oxidation or by 1,3-Dipolar Cycloaddition Reactions



physical stabilization of the nanoplexes or enhanced cell binding. The positive effect of chloroquine was suggested to result from increased buffering capacity. Similar cationic silica nanoparticles have been effective at complexing with antisense oligonucleotides and inhibiting survivin protein expression.[526] In 2004, Lin et al. reported the first use of mesoporous silica nanoparticles for gene transfer.[527] These particles permit the encapsulation fluorescent dyes inside the mesoporous structure. Second generation PAMAM was covalently bound to the surface of these silica nanoparticles to promote electrostatic interactions with plasmid DNA. These cationic mesoporous silica nanoplexes showed higher gene transfer efficiency than PolyFect, SuperFect, and Metafectene. This high efficiency was attributed to increased sedimentation. In vivo studies conducted by Prasad and Stachowiak et al. showed that sterotaxic injections of organically modified silica nanoplexes resulted in transfection efficiency that equaled or surpassed that obtained with herpes simplex virus 1 vectors and showed less tissue damage.[528] This success is a significant landmark in nonviral gene transfer, as such carriers typically exhibit low in vivo gene expression when compared to viral analogues.

6.4. Carbon Nanotubes

Single-walled carbon nanotubes (SWNT) and multiwalled carbon nanotubes (MWNT) were first discovered in the late 1950s, and in recent years, these structures have been used for gene therapy applications. While these carbon structures are highly insoluble, both noncovalent and covalent functionalization techniques have been utilized to solvate these structures.[529] Covalent functionalization proceeds by two methods: the oxidation of the carbon nanotubes in acidic conditions to afford acid-terminated structures or 1,3-dipolar cycloaddition reactions using α-amino acid derivatives and paraformaldehyde (Scheme 36). Noncovalent functionalization typically involves either hydrophobic or $\pi-\pi$ stacking interactions between the carbon nanotube and either surfactants, nucleic acids, peptides, polymers, or oligomers.

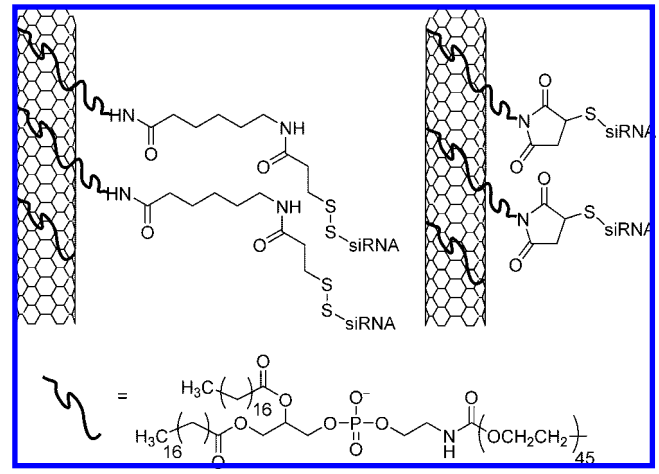


Figure 16. Schematic representations of carbon nanotubes noncovalently modified with phospholipid−PEG chains and then linked to siRNA though thioether or disulfide bonds.

Bianco et al. synthesized ammonium-functionalized single-walled and multiwalled carbon nanotubes by 1,3-dipolar cycloaddition reactions to form cationic nanotubes that could bind to DNA through electrostatic interactions to form nanoplexes (f-SWNT and f-MWNT, respectively).[530] Cellular uptake of these nanoplexes in HeLa cell lines was shown to proceed by a nonendocytic route, as cellular uptake was not inhibited by sodium azide or 2,4-dinitrophenol, which typically hinder energy-dependent cellular processes. In CHO cell lines, the f-SWNT nanoplexes yielded significant β-galactosidase expression at f-SWNT/DNA ratios between 2:1 and 6:1. Results from scanning electron microscopy and surface plasmon resonance showed that the multiwalled carbon nanotubes bind to DNA more tightly than the single-walled analogues due to increased surface area. Additionally, DNA binding capacity could be increased by replacing the ammonium cation with a lysine group that would have an increased charge density.[531] In complementary studies, Cai et al. synthesized vertically aligned carbon nanotubes by plasma-enhanced chemical vapor deposition.[532] Because these structures contain nickel particles at their tips, they can be magnetically manipulated to penetrate cells through a nanotube spearing procedure. When these nanotubes were complexed with plasmid DNA encoding for EGFP, the nanotube spearing technique promoted high transfection efficiency, particularly in nondividing neuron cells.

In addition to DNA transfection, various studies have used carbon nanotubes to deliver siRNA into cells and cause gene knockdown. Dai et al. noncovalently functionalized carbon nanotubes with phospholipid−poly(ethylene glycol) (PL−PEG) moieties containing either maleimide or amine terminal groups.[533] The maleimide-terminated PL−PEG conjugates were reacted with thiol-terminated siRNA. The amine-terminated PL−PEG analogues were reacted with sulfosuccinimidyl 6-(3′-[2-pyridyldithio]propionamido)hexanoate (sulfo-LC-SPDP), followed by reaction with thiol-terminated siRNA to form the structures illustrated in Figure 16. Both nanoplexes inhibited the gene encoding lamin A/C protein in HeLa cells more effectively than Lipofectamine−siRNA lipoplexes. The disulfide-linked constructs showed higher gene knockdown levels when compared to the thioether linked analogues. These same structures also showed capacity to deliver siRNA in vitro into both human T-cells and primary cells, two cell lines that are generally unable to be

transfected using liposome-mediated delivery.[534] Recently, Zolk et al. reacted oxidized carbon nanotubes with either hexamethylenediamine then coated these functionalized particles with poly(diallyldimethylammonium) chloride (PDDA). When complexed with siRNA through electrostatic interactions, these nanoparticles exhibited 80% inhibition of the targeted genes, ERK1 and ERK2, in vitro.[535]

Based on the in vitro success of carbon nanotube-mediated gene silencing, Yang et al. investigated the in vivo potential of these structures. Oxidized carbon nanotubes with acid chloride terminal groups were covalently attached to mono-Boc-protected hexamethylenediamine followed by deprotection to yield amine-terminated single-walled nanotubes (SWNT).[536] These structures were conjugated with siRNA targeting either CD80 or SOCS1 expression. CD80 is a costimulatory protein expressed by antigen-presenting cells to activate T-cells, while SOCS1 is an intracellular signal regulator. When delivered by tail-vein injections into mice bearing Lewis lung carcinoma (LLC), CD80siRNA/SWNT showed significant inhibition of CD80 expression in myeloid immunosuppressive cells, a cell type known to negatively affect antitumor immune responses. In addition, tail-vein injections of SOCS1siRNA/SWNT resulted in enhanced antigen-presenting function of dendritic cells, leading to suppressed tumor growth. Later SWNT were conjugated to telomerase reverse transcriptase siRNA (TERTsiRNA). Telomerase is associated with the ability of cells to reproduce indefinitely and is detected in cancer but not most normal cell lines. When TERTsiRNA/SWNT nanoplexes were injected intralesionally into mice bearing HeLa xenograft growth, tumor growth was suppressed nearly 6-fold when compared to the control.[537] These in vivo results suggest carbon nanotube-medaited siRNA delivery offers significant promise for anticancer gene therapy.

6.5. Lipid-Based Nanoparticles

6.5.1. Solid Lipid Nanoparticles (SLNs)

Following the success of drug delivery mediated by solid lipid nanoparticles, the potential for using SLNs for gene delivery was realized. These particles are advantageous as they can be produced on a large scale in a cost-effective manner and exhibit lower cytotoxicity when compared to polymeric agents. Additionally, these compounds can be sterilized or prepared aseptically following current protocol of the pharmaceutical industry.[538]

In 2001, Kneuer et al. reported the first successful DNA transfection using a cationic SLN.[539] These structures were synthesized by melting Compritol ATO 888 (glycerol behenate) and then mixing the melted lipid with a hot aqueous solution containing the surfactants Tween 80 (2.8%) and Span 85 (1.2%), as well as the cationic structures *N*,*N*-di-(β-steaorylethyl)-*N*,*N*-dimethylammonium chloride (eq1) and cetylpyridinium chloride. The mixture was homogenized at 85 °C using a Laboratory 60 homogenizer, followed by processing, filtering, and sterilization. When complexed with DNA through electrostatic interactions, the SLN−DNA nanoplex could effectively transfect COS-1 cell lines, although gene expression was lower when compared to both PLL and PEI. However, when compared to the well-known lipid transfection agent DOTAP, SLN−DNA nanoplexes that used cetylpalmitate as the solid lipid matrix showed superior transfection efficiency when used in conjugation with chloroquine.[540]

To improve the gene transfer efficiency of SLN, Rudolph et al. synthesized ternary complexes of SLN, DNA, and TAT_2 peptide (Cys-[Tyr-Gly-Arg-Lys-Lys-Arg-Arg-Gln-Arg-Arg-Arg-Gly]$_2$) by precompacting DNA with TAT_2 and then complexing this negatively charged compound with cationic SLN.[541] The SLN used either eq1 or DOTAP as the surfactant. The ternary complexes showed 101- and 145-fold higher gene expression in human bronchial epithelial cells (16HBE14o) than the binary complexes of SLN_{DOTAP}−DNA and SLN_{eq1}−DNA, respectively. Furthermore, the TAT_2−SLN_{DOTAP}−DNA complexes showed 3-fold higher gene expression when compared with 25 kDa PEI. However, when the TAT_2−SLN_{DOTAP}−DNA nanoplex was delivered by jet nebulization into mouse lungs, gene expression was 80- to 100-fold lower when compared to PEI polyplexes. This low transfection efficiency was attributed to the inability of the SLN−TAT_2 complex to protect DNA from degradation during the nebulization procedure. Ritthidej et al. also modified SLN to improve gene transfer by varying the cationic lipid content.[542] When the lipid content was modified to include significant amounts of cholesterol, transfection efficiency was improved. However, these modified SLN−DNA complexes exhibited only moderate transfection capability when compared to the commercially available lipid Fugene 6. Kim et al. synthesized solid lipid nanoparticles using tricaprin as the solid lipid matrix, Tween-80 and DOPE as the surfactants, and DC-Chol as the cationic agent.[543] When complexed with plasmid DNA encoding for p53-EGFP, a protein that aids in tumor suppression, in vitro transfection efficiency in human nonsmall cell carcinoma cells (H1299) was higher when compared to Lipofectamine. Additionally, in vivo administration by tail-vein injections showed prolonged gene expression in the liver, lung, spleen, and kidney for SLN−DNA nanoplexes.

Recently, Carlo et al. investigated the potential of using cationic SLN to deliver siRNA into cells.[544] SLNs composed of Compritol as the solid lipid matrix, Pluronic F68 as the surfactant, and dimethyldioctadecylammonium bromide (DDAB) as the cationic agent were complexed with RNA through electrostatic interactions. These complexes showed resistance to RNase T1 degradation and exhibited no cytotoxicity. Future studies evaluating the transfection efficiency of these SLN−RNA complexes are pending.

6.5.2. Cerasomes

Cerasome, a lipid bilayer structures with a ceramic surface, was first prepared in 1999 by Kikuchi et al. by the vortex mixing of [3-(((*N*,*N*-dihexadecylamino)-succinyl)amino)propyl] triethoxysilane in 1 mM aqueous HCl.[545] These complexes were later modified to contain cationic moieties that could self-assemble through electrostatic interactions with anionic analogues.[546] This electrostatic aggregation indicated potential for complexation with anionic oligonucleotides. In 2006, Kikuchi and Aoyama et al. complexed DNA with cerasomes composed of a trialkoxysilyl quaternary ammonium lipids (Figure 17). These structures were shown to be 10^3 times more efficient at delivering DNA than the nonceramic liposome analogue and were not affected by the prescence of serum.[547]

Recently, Aoyama and Kikuchi et al. complexed cationic cerasomes with DsRed2-specific siRNA.[548] When introduced into HeLa cells that stably expressed the fluorescent protein DsRed2, the cerasome−$siRNA_{DsRed2}$ complexes showed up to 70% gene knockdown that was unaffected by the presence

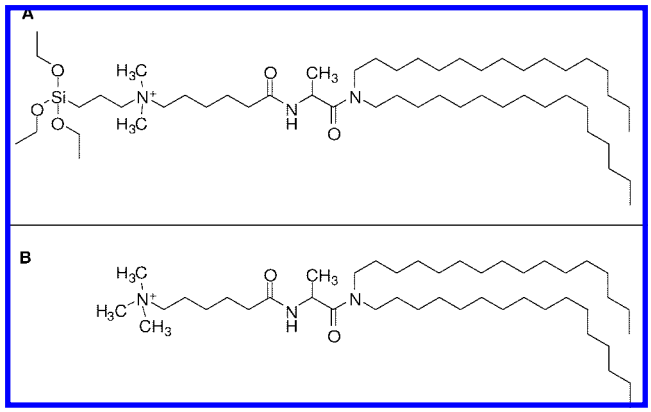


Figure 17. Structures of (A) trialkoxysilyl quaternary ammonium lipids used to form cationic cerasome and (B) cationic lipid used to form nonceramic liposome analogue.

of serum. Based on the success of the cerasome-based gene transfection, further studies, both in vitro and in vivo, are warranted.

6.6. Polymeric Hydrogels

Due to the relatively poor stability of both lipoplexes and polyplexes, the potential for oral delivery of oligonucleotides is hindered. To improve the stability of polyplexes, various groups have investigated cross-linking polycation chains using emulsification/solvent evaporation techniques to form a structure in which DNA is trapped.[549] NanoGel was one of the first of such structures to be reported. NanoGel was synthesized by the cross-linking of PEI and bis-carbonyldiimidazole-activated poly(ethylene oxide) (PEO) using the emulsification evaporation method.[550] In this method, the bis-activated PEO was dissolved in dichloromethane, added to a 1% aqueous solution of 25 kDa PEI, and sonicated for 30 min. Following purification procedures, these NanoGel particles were conjugated with phosphorothioate antisense oligonucleotides that target mdr1-gene inhibition. Inhibition of the mdr1-gene, which is associated with the expression of P-glycoprotein efflux pump in multidrug resistant cancer cell lines, should result in higher cellular accumulation of the NanoGel−antisense oligonucleotide complex. Results from the study showed that these nanoplexes exhibited significantly higher gene transfer activity when compared to free oligonucleotide. Additionally, the transfer of these NanoGel−oligonucleotide complexes across Caco-2 cell monolayers occurred up to 26-fold more efficiently than for free oligonucleotide, suggesting the potential for oral delivery.

Since the initial success of NanoGel, various other groups have investigated the potential for hydrogel-mediated oligonucleotide delivery. DeSimone et al. synthesized polymeric nanogels by the inverse microemulsion of 2-acryloxyethyltrimethyl-ammonium chloride (AETMAC), 2-hydroxyethylacrylate, and poly(ethylene glycol) diacrylate.[551] When conjugated with DNA, the structures composed of 25% AETMAC showed significant ability to deliver the DNA into HeLa cell lines. Fréchet et al. synthesized acid-labile polyacrylamide nanoparticles by inverse microemulstion polymerization of *N*,*N*-bisacryloylbis(2-aminoethoxy)-[4-(1,4,7,10-tetraoxaundecyl)phenyl]methane and acrylamide in the presence of a β-galactosidase reporter plasmid containing 6.7% of the CpG dinucleotide, which induces immune response in macrophages.[552] Transfection efficiency of these nanoplexes was evaluated in RAW 264.7 macrophages. When the cells were exposed to 0.1 μg DNA-loaded polyacrylamide nanoparticles, transfection efficiency was 3.75-fold higher when compared to the transfection efficiency of 1 μg free plasmid DNA. Finally, Park et al. synthesized reducible PEG/DNA nanogels by complexing EGFP−plasmid DNA with thiol-functionalized six-arm branched PEG and then cross-linking the complex using dithio-bis-maleimidoethane (DTME).[553] The PEG/DNA nanoparticles were coated with the cationic KALA peptide and transfected to HeLa cells. While GFP protein expression was observed, transfection efficiency was lower than that of both Lipofectamine and PEI.

7. Nonviral Therapeutics in Clinical Trials

Although adenoviral- and retroviral-mediate transfections are currently the most widely used strategies for gene therapy in clinical trials, the use of nonviral vectors has increased by approximately 3% within the last four years.[573,574] Lipid- and polymer-mediated gene delivery has been used to successfully target both genetic diseases as well as cancer. For instance, a number of clinical trials using the plasmid vector DMRIE/DOPE, a hydroxyalkyl derivative of DOTMA with side chain variations, have have been investigated for treating cancer. In 1997 Stopeck et al. completed a phase 1 clinical trial that assessed the safety and efficacy of Allovectin-7, a plasmid DNA encoding the genes HLA-B7 and β2-microglobulin complexed with DMRIE/DOPE, in treating metastatic melanoma.[554] Results showed no dose-limiting toxicity, and plasmid expression leading to clinical responses was documented. In 2001, a phase 2 trial consisting of six intratumoral injections of 10 μg of Allovectin-7 over a 9 week period in patients suffering from metastatic melanoma was shown to cause regression in 18% of patients.[555] In a later phase 2 trial, it was shown that Allovectin-7 could be safely administered at high doses of up to 2 mg injections.[556] Currently, Allovectin-7 is in a phase 3 clinical trial for patients with stage III or stage IV melonoma.

Allovectin-7 has also been utilized by Fox et al. in a phase 1/2 clinical trial using gene-modified autologous tumor vaccines.[557] In the study, autologous tumor cells of patients suffering from stage IV melanoma or renal cell cancer (RCC) were transfected ex vivo with plasmid DNA encoding HLA-B7 and β2-microglobulin complexed with DMRIE/DOPE. The modified autologous tumor cells were administered intradermally into patients adjacent to superficial draining lymph nodes to increase the number of tumor-specific T-cells. After 7−10 days, the treated lymph nodes were surgically removed, and lymphocytes were activated and expanded with anti-CD3 and IL-2. The cells were then reinjected intravenously into the patients followed by intravenous IL-2 therapy. Tumor-specific cytokine secretion was observed for vaccinated patients, but no tumor regression was documented.

DOSPA/DOPE has also been used to treat cancer in clinical trials. In 1997, Lindemann et al. conducted a phase I clinical trial to vaccinate patients suffering from advanced malignant tumors using autologous tumor cells and a fibroblast cell line that had been transfected with interleukin-2 gene complexed with DOSPA/DOPE (3:1).[558,559] Results from the study showed that four out of fifteen patients experienced little to no disease progress for several months after vaccination.

Various clinical trials using DC-Chol/1,2-dioleoyl-sn-glycero-3-phosphoethanolamine) (DCC) as a plasmid vector have been investigated. In 1993, Nabel et al. utilized DCC

Table 1. Various Nonviral Transfection Agents Used in Clinical Trials

construct	delivery vector	disease	status	section	ref.
Allovectin-7	DMRIE/DOPE (1:1)	melanoma	phase 3	2.1	554−556
Allovectin-7	DMRIE/DOPE (1:1)	melanoma and renal cell cancer	phase 2	2.1	557
	DOSPA/DOPE (3:1)	solid tumors	phase 1	2	558,559
tgDCC-E1A	DC-Chol/DOPE (1.5:1)	head and neck cancers	phase 2	2.2	560,561
tgDCC-E1A	DC-Chol/DOPE (1.5:1)	ovarian cancer	phase 1	2.2	562
LIPO-HSV-1-tk	DC-Chol/DOPE (3.7)	glioblastoma multiform	phase 2	2.2	563
	DC-Chol/DOPE (1.5:1)	cystic fibrosis	phase 1	2.2	564,565
	DOTAP	cystic fibrosis	phase 1	2	566
GL-67:DOPE-pCF1-CFTR	GL-67/DOPE (1:2)	cystic fibrosis	phase 1	2.1	567
BC-819	in vivo jetPEI	bladder cancer	phase 2b	3.2.1	568
EGEN-001	PEG−PEI−Chol	ovarian cancer	phase 1	3.2.2	569
DermaVir	PEI mannose and dextrose	HIV	phase 2	3.2.2	570
	PEGylated 30mer PLL	cystic Fibrosis	phase 2	3.1	571
CALAA-01	cyclodextrin-based polymer	solid tumors	phase 1	3.4.1	572

to introduce a plasmid encoding for the protein HLA-B7 into five patients suffering from stage IV melanoma.[575] Results showed increased immune response to HLA-B7, and tumor regression was documented for one patient. In 2001, a phase 1 clinical trial was conducted by Murray et al. to treat patients suffering from either breast or head and neck cancer using DCC complexed with plasmid DNA containing the E1A gene, a sequence that encodes for proteins that inhibit HER-2/neu, a gene shown to enhance tumorigenicity, metastasis, and resistance to chemotherapeutic agents.[560] Results showed down-regulation of HER-2/neu in two out of five patients who overexpressed HER-2/neu at the beginning of the trial, and up to 10 patients showed either disease stability or minor response to treatment. In 2002, a phase 2 trial using the same tgDCC−E1A complex to treat patients with head and neck cancer showed complete response in 1 patient (4.2%), minor response in two patients (8.3%), and disease stability for seven patients (29.2%).[561] A phase I trial using DCC−E1A to treat patients suffering from advanced cancer of the breast or ovary was conducted by Hortobagyi et al. in 2001.[562] Results from the study showed E1A gene expression, which resulted in down-regulation of HER-2/neu. Currently, phase 1/phase 2 trials using tgDCC−E1A and paclitaxol to treat patients with ovarian cancer are under investigation.

In a phase 1/2 clinical trial, Voges et al. treated patients with recurrent glioblastoma multiform by delivering a liposome-gene complex comprised of the HSV-1-*tk* gene and DC-Chol/DOPE (3:7) intratumorally using convection-enhanced delivery followed by systemic administration of ganciclovir (GCV).[563] The administration of the HSV-1-*tk*/GCV enzyme/prodrug combination was previously shown to be a successful route for suicide gene therapy.[576] In the clinical trial conducted by Voges et al. tumor size was monitored preoperative and postoperative using gadolinium-diethylenetriaminepentacetic acid (Gd-DTPA) as a contrast agent for magnetic resonance imaging (MRI) and 2′-fluoro-2′-deoxy-1-β-D-arabinofuranosyl-5-[^{124}I]iodo-uricil as an imaging agent for positron emission tomography (PET). It was shown that DC-Chol/DOPE-mediated gene delivery resulted in a greater than 50% reduction in tumor volume in six out of the eight patients treated.

In addition to cancer treatment, lipid-mediate transfection has also been used to target cystic fibrosis (CF). In 1995, Caplen et al. treated nine patients with cystic fibrosis with a DC-Chol/DOPE-condensed cDNA encoding the CF transmembrane conductance regulator (CFTR).[564] Temporary improvement of the chloride defect was seen in approximately one-third of the patients. A similar study was conducted in 1997 by Gill et al. using the same lipoplex.[565] Results showed improved CFTR function in 75% of the patients. In 1997, Porteous et al. introduced cDNA endocing for CFTR and condensed using DOTAP to the nasal epithelium of eight CF patients.[566] Results showed transient correction of the chloride defect in 25% of the treated patients. Finally, in 1997, Zabner et al. conducted a phase 1 clinical trial to treat patients suffering from cystic fibrosis using GL-67/DOPE-mediated CFTR gene delivery, and partial correction in the Cl^- transport defect of the CF patients was shown.[567]

In addition to lipid-mediated gene transfer, polymers have also been used for transfection in clinical trials. In 2004, Ohana et al. utilized jetPEI (22 kDa) to complex a plasmid that was used to treat two patients with recurrent superficial transitional cell carcinoma of the bladder.[568] The plasmid encoded for the expression of diphtheria toxin A under the H19 promoter gene, an oncofetal gene commonly expressed in various tumor tissue. When 2 mg plasmid−jetPEI complex was injected intravesically into the two patients once a week for nine weeks, video imaging showed nearly complete tumor regression.

Fewell et al. synthesized a PEG−PEI−cholesterol (PCC) derivative in 2005 that is currently being investigated in clinical trials. In 2008, PCC was used to complex an IL-12 expressing plasmid and was used in two early phase 1 clinical trials to treat patients with recurrent ovarian cancer.[569] Intraperiotoneal injections of the PCC−DNA complex resulted in 31% stable disease and 69% progressive disease after four weekly treatments. When patients were treated with both PCC−DNA complex as well as docetaxel and carboplatin, all patients demonostrated partial response to treatment.

DermaVir, a topical immunization strategy for HIV, is composed of plasmid DNA encoding for all genes of the HIV virus (minus the integrase gene) that is condensed with PEI mannose and dextrose.[570] In a phase 1 clinical trial conducted by Lisziewicz et al. involving 9 HIV+ individuals on fully suppressive highly active antiretroviral therapy (HAART), it was shown that the DermaVir patch could significantly increase the number of HIV-specific T-cells at doses of 0.1, 0.4, or 0.8 mg DNA. The magnitude of increase was dose dependent. Currently, the DermaVir patch is in a phase 2 clinical trial.

In addition to PEI, polylysine is another polymeric vector that has been used clinically to compact and deliver a therapeutic gene. In 2004, Konstan et al. used PEG−PLL to deliver a plasmid encoding for CFTR intranasally into twelve patients suffering from cystic fibrosis.[571] Results showed partial or complete restoration of CFTR chloride channel function for up to six days after treatment.

Recently, a polymer based on cyclodextrin (CD). has made it to clinical trials for siRNA delivery. Davis et al. designed a CD-based polymer that can bind to siRNA through electrostatic interactions. The complex can be stabilized with PEG−adamantane groups and can be targeted to tumor tissue with transferrin protein targeting ligand. In preclinical evaluation of the CD−siRNA complex (CALAA-01). in nonhuman primates, no signs of toxicity were seen after multiple injections of the complex.[572] Currently, the CD−siRNA complex is being evaluated in phase 1 clinical trials for treating solid tumor cancers.

While viral-mediated gene transfer still dominates the field of gene delivery with respect to clinical trials, nonviral transfection agents have still shown success in targeting diseases such as cystic fibrosis, HIV, and various cancers. Viral vectors are more efficient that nonviral analogues based on transfection efficiency per gene delivered, but safety of viral vectors is a significant concern. Unlike viral vectors, nonviral mediated gene delivery has not elicited any substantial toxicity or immune responses in various clinical trials as described above. With extensive effort being put into designing nonviral vectors with higher gene transfer efficiency, synthetic gene carriers may become superior to viral analogues in clinical trials in the near future.

8. Conclusion

The past decade has seen tremendous progress in the design and synthesis of nonviral vectors for gene delivery as well as application of a battery of techniques from which structure−property trends have emerged. Common design principles have also crystallized. For example, with few exceptions, nearly all synthetic vectors have achieved the positive charge necessary for electrostatic complexation with DNA by means of ammonium cations. The structure and density of the amine groups clearly affects transfection efficiency, particularly for polymer and dendrimer-based compounds. Increasing the number and charge density of the amines typically improves transfection efficiency. However, the increased charge density generally promotes high cytotoxicity. The cytotoxicity can be reduced by using histidine or guanidine functional groups that better distribute the positive charge that results in higher transfection efficiency. The quaternization of amines is a commonly used method for increasing the cationic charge density for a given vector. However, the effect of quaternizing amine groups still remains illdefined. For both chitosan and polypropylenimine, quaternization results in higher transfection efficiency, while quaternization of polyethylenimine, cyclodextrin, poly(glycodiamines), schizophyllan, and polyamidoamine was shown to be deleterious.

Nonspecific cellular uptake has been achieved by the inclusion of hydrophobic groups, including steroidal moieties, alkyl chains, and hydrophobic amino acids, and these modifications generally result in higher transfection efficiency. Additionally, cellular uptake of silica-based nanoplexes has been attributed to settling of the dense particles on the cell surface. However, the possibility of promoting this effect with other synthetic vector systems may not be achievable. Promoting the uptake of vector−DNA complexes by specific cell types has been achieved by conjugating the vector with a number of cell targeting agents, particularly folate for cancer cell targeting and galactose for hepatic cell targeting.

As nearly all synthetic vectors are incorporated into the cell by means of the endocytic route, the first intracellular barrier is the escape of the vector−DNA complex from the acidic environment of the endosome prior to lysosomal degradation. The most common method of promoting endosome lysis has been the inclusion of chloroquine with the vector−DNA complex. Alternatively, improving the buffering capacity of the vector by including both primary and tertiary amines has been investigated to increase endosomalysis, but the effect of such modifications is still unclear. While PEI, chitosan, poly(amino esters), and PAMAM all exhibit high transfection efficiency attributed to the ability to buffer the acidic endosomal compartment, PDMAEMA shows lower transfection efficiency with the addition of tertiary amines.

While the cytosol is the prime location for the delivery and activity of siRNA, gene transfer requires that DNA be delivered to the nucleus. Compared to achievements in overcoming other barriers to gene transfer, the methods for promoting nuclear localization remain vague. While utilizing synthetic vectors to minimize the complex size appears to improve delivery to the nuclear region, most studies show that DNA uptake by the nucleus proceeds without the vector (i.e., only DNA is incorporated into the nucleus). Also, complexation of DNA with MPG, a peptide with a NLS, showed only moderate improvement to transfection efficiency when compared to Lipofectamine, a vector that is unable to target the nucleus. The significantly higher transfection efficiency seen for viral-mediated DNA delivery when compared to that mediated by synthetic vectors may result primarily from the ability of viruses to utilize the cytoskeleton for intracellular trafficking to the nucleus. Due to only moderate success achieved in overcoming this barrier, future efforts may begin focusing more and more on surmounting this obstacle.

If physicochemical understanding of the field is punctuated ultimately by the number of successful clinical trials afforded by these efforts, the community has great reason to be optimistic.

9. Acknowledgments

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