PART VII

BIODEGRADABLE POLYMERS FOR EMERGING CLINICAL USES

CHAPTER 15

BIOCOMPATIBLE POLYMERS FOR NUCLEIC ACID DELIVERY

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CONTENTS

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15.1 INTRODUCTION

The completion of the human genome project and discovery of inhibitory ribonucleic acid (RNA) has produced new excitement and hope in treatment modalities that involve gene manipulation, anticipating benefits to humanity in ways that were unimaginable before. Despite these promising new developments, barriers in nucleic acid delivery remain the major hurdle in the pursuit of nucleic acid therapeutics. The promise of viral vectors for deoxyribonucleic acid (DNA) delivery was hampered by serious safety concerns in the clinic despite good preclinical data. Nonviral delivery systems such as cationic liposomes were considered a safe alternative to viral vectors, but lack of convincing clinical data due to poor transfection efficiency, acute toxicity, and structural rigidity to chemical functionalization slowed their progress in the clinic. Delivery systems that are structurally amenable to chemical modifications are preferred since multiple delivery attributes must be incorporated to overcome the delivery challenges. Synthetic polymers fit this profile due to the high degree of structural variation inherent in this class of delivery systems. Several types of polymeric carriers for nucleic acid delivery have been described in the literature, including (a) cationic polymers that condense nucleic acid into nanoparticles via electrostatic interaction, (b) nonionic polymers that protect nucleic acid from nuclease action and facilitate nucleic acid entry to the target cell, and (c) biodegradable polymers that serve as nucleic acid depots and release their payload as they erode over time and under specific physiological stress. Most of the early-generation polymeric carriers failed to reach the clinic due to suboptimal activity, which has lead to their chemical functionalization with molecular entities designed to overcome the delivery limitations. For example, poly-*L*-lysine (PLL), despite its extensive use for gene delivery, failed to advance to clinical development due to high polydispersity, poor in vivo stability, high cytotoxicity, and dependence on endosomal disruptive agents for activity. The discovery of polyethyleneimine (PEI) offered a good alternative to PLL due to its high endosomal disruptive activity, which is attributed to its high secondary amine content. Despite better transfection activity than PLL, PEI has not advanced to significant clinical development due to toxicity concerns, which has led to the design of new PEI approaches focusing on backbone modifications to improve biocompatibility and transfection activity. The lessons learned from the early-generation carriers have proven useful in designing new and improved systems.

This chapter focuses on delivery polymers that have been described in the last five years. Special emphasis is given to systems with promising in vivo activity. Greater emphasis is given to the DNA delivery systems. However, a brief overview of RNAi literature is also provided. The structural design, AQ1 physicochemical properties, and preclinical evaluation of different polymeric carriers is described in Sections 15.2–15.4 and progress in clinical development is described in Section 15.5.

15.2 CATIONIC POLYMERS

A common structural characteristic of all cationic polymers is the presence of protonable amines, which at physiological pH provide electrostatic interaction with negatively charged DNA molecule and form polymer–DNA complexes (polyplexes). This electrostatic interaction forces the DNA structure to collapse into nanoparticles and assume a positive surface charge. The magnitude of size reduction and increase in charge density varies with polymer structure, number and location of protonable amines, pK_a of the amines, DNA–polymer molar ratio, and the presence of functional groups but is largely independent of DNA size, nucleotide sequence, and physical form [1–5]. It is generally believed that the size of polyplexes should be <150 nm for optimal cell uptake through endocytosis [6–8]. However, this may not be the only mechanism for particle uptake since much larger complexes also transfect cells in culture [9, 10]. The density of positive charge on polyplexes depends on the concentration of the polycation. Generally, high polymer:DNA molar ratio is required to achieve good DNA compaction, physical stability, nuclease protection, cell surface interaction, and transfection [10–13]. Nevertheless, DNA cationization is also one of the major reasons for poor in vivo performance. The positively charged polyplexes are subjected to interaction with anionic macromolecules such as sulfated glycosaminoglycans, hyaluronan, or other anions in the biological milieu promoting DNA release from polyplexes and ultimately the degradation by nucleases [14–17]. Considerable attention has been given to circumvent this problem by modifying existing cationic polymers or designing new systems to withstand the in vivo barriers. A detailed description of the conventional predecessor systems and their new and improved derivative polymeric carriers is provided in the following sections. The discussion is circumscribed to delivery polymers that have advanced into clinical development or those that have exhibited promising results in preclinical studies.

15.2.1 Polyethyleneimine

Since the demonstration of its DNA delivery properties by Boussif in 1995 [18], PEI has become the most widely used polymeric delivery system for nucleic acids. A distinct advantage of PEI over early-generation cationic polymers is its ability to escape from the endosomes due to its relatively dense amine-containing structure (Fig. 15.1). The overall protonation level of PEI doubles from pH 7 to pH 5 [19–21], resulting in PEI becoming heavily protonated within the endosome. This so-called proton sponge effect results in the influx of chloride ions to neutralize the compartmental charge, which is then followed by a corresponding destructive influx of water to dilute the high salt concentration. The resulting endosomal lysis occurs without damage to the nucleic acid cargo.

AQ1 PEIs vary in molecular weight and backbone configuration. LPEI contains
AQ1 only secondary amines, while branched BPEI contains primary, secondary, and

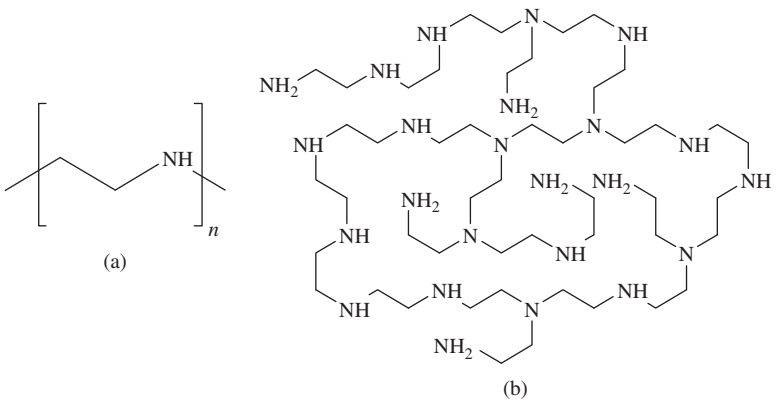

FIGURE 15.1 Structure of (a) LPEI and (b) BPEI.

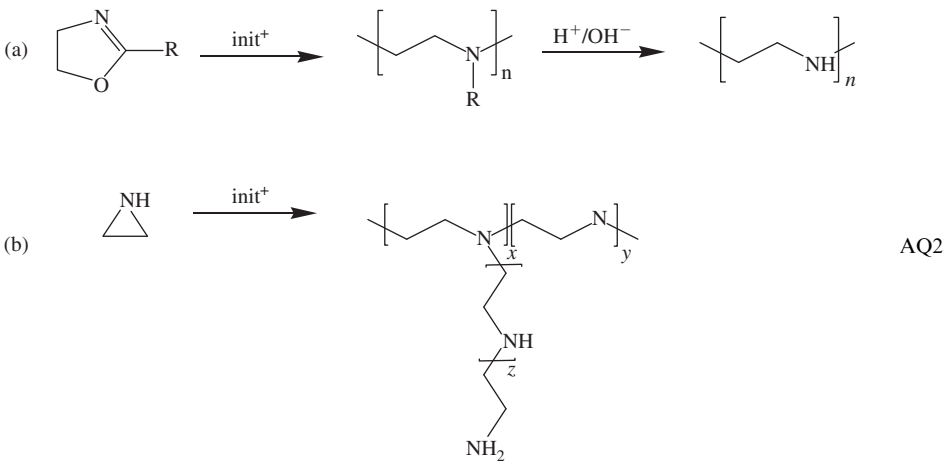

FIGURE 15.2 Synthetic routes for (a) LPEI and (b) BPEI.

tertiary amines in the ratio of 1 : 2 : 1, respectively. These structural differences influence the synthesis schemes and transfection properties of PEIs. LPEI may be synthesized by the cationic ring-opening polymerization of oxazolines in the presence of an initiator. The resulting polymer contains side chains that are hydrolyzed by sulfuric acid and then precipitated as the polysulfate salt. BPEI may be synthesized by the cationic polymerization of ethyleneimine (Fig. 15.2), and the pure product obtained by precipitation. Although under certain conditions PEI may be analyzed using high-performance liquid chromatography (HPLC), the most widely used method of molecular weight and polydispersity

characterization is intrinsic viscosity. An overview of the various different types of PEIs is described in the subsequent sections.

The effect of PEI molecular weight and structural configuration on transfection activity and cytotoxicity has been studied in vitro and in vivo. Both enhancement and reduction in PEI activity has been observed with increasing PEI molecular weight. Goodbey et al. [20] found higher transfection efficiencies with increasing molecular weight of PEI (70 kDa >10 kDa > > > 1.8 kDa) in vitro. Kunath et al. [22] reported high transfection efficiency and reduced toxicity of low-molecular-weight PEI (5 kDa) in several different cell lines. Abdallah et al. [23] found reduced transfection efficiencies with increasing molecular weight of PEI (25 kDa >50 kDa >800 kDa) in vivo. The influence of backbone configuration (linear or branched) on PEI transfection activity and toxicity has also been examined. Initially, BPEI was reported to yield higher transfection activity than LPEI following intravenous delivery to the kidney [24]. However, in later reports, LPEI was found to be more active and less toxic than BPEI [25–27]. The higher transfection efficiency of LPEI is thought to be due to its inherent kinetic instability under salt conditions.

Both LPEI and BPEI have been examined extensively in preclinical studies. Their clinic development has been hampered by poor in vivo stability and acute systemic toxicity. Dissociation of PEI–DNA complexes by negatively charged proteins in blood and extracellular matrix is believed to be the principal mechanism of their low in vivo activity [28]. Acute inflammatory response, characterized by activation of lung endothelium, platelet aggregation, and liver damage, has also been observed following systemic delivery of PEI–DNA complexes [26, 29]. While systemic delivery of nucleic acid with PEIs faces significant delivery and safety challenges, local delivery has generally been found to be more amenable. Gene delivery of diphtheria toxin with LPEI (22 kDa) in human and murine bladder carcinoma was well-tolerated, efficacious, and localized at the injected site [30]. Despite some success in the clinic, the future of PEI as a versatile nucleic acid delivery system remains uncertain unless a substantial improvement is made in its in vivo stability and biocompatibility. Several PEI functionalization approaches have been examined in this regard. These include attachment of tissue-specific or biocompatible components to the PEI backbone, intermolecular crosslinking of low-molecular-weight backbones via degradable linkages, and encapsulation of PEI complexes into biodegradable microspheres. Each of these different approaches to functionalize PEIs is described in the subsequent sections.

15.2.2 Functionalized PEIs

For PEI to be clinically viable it must be made more efficient and tolerable in vivo. Several modifications of PEI have been reported to accomplish this. In one of the approaches, PEI was conjugated with biocompatible polymers such as poly(ethylene glycol) (PEG), polycaprolactone (PCL), chitosan, or cyclodextrins. In another approach, low-molecular-weight PEI was crosslinked to

give a larger total molecular weight polymer to improve in vivo stability. Since the crosslinking was achieved with degradable bonds, the size increase is not permanent and the resulting polymer is potentially more biocompatible than the conventional high-molecular-weight PEIs. PEI was also conjugated with targeting ligands to improve tissue specificity. Each of these approaches is described in the following sections.

Copolymers of PEI. Biocompatible polymers such as PEG, dextran, chitosan, cyclodextrin, PCL or poly(*N*-(2-hydroxypropyl) methylacrylamide have been conjugated to PEI to improve its hydrophilicity, stability, and safety for in vivo application. PEG is a widely used polymer for drug delivery [31]. Strong hydration and high conformation flexibility of PEG provide for steric stabilization of the PEG conjugates in the biological milieu. The principal advantages of PEG conjugation are good serum stability, decreased renal clearance, and overall better pharmacokinetics and bioavailability [32–35]. PEG conjugation to cationic lipids, PLL, poly(DMAEMA-NVP), peptides, and other DNA delivery systems have been shown to reduce positive charge density, improve salt stability, and reduce cytotoxicity of the delivery systems [32, 36, 37]. Merdan et al. [38] have characterized the effect of PEG conjugation on the physical stability, in vivo distribution, and transfection activity of BPEI (25 kDa)–DNA complexes. BPEI conjugation with PEG (2000 Da) did not affect particle size but reduced the positive zeta potential (+26 mV) to neutral values. Intravenous administration of PEGylated polyplexes resulted in reduced toxicity, improved organ deposition, and lowered transfectability as compared to the non-PEGylated BPEI. Hong et al. [39] examined the effect of PEG conjugation density relative to BPEI. PEGylation with 10% NHS-activated PEG (2000 Da) produced significantly higher improvement in PEI tolerability and transfection activity than with 6% PEG. Lipiodolized emulsion of BPEI–PEG (10%) successfully delivered plasmid DNA into skeletal muscle following intravenous administration into surgically occluded rat femoral artery. The magnitude of gene expression was three-fold higher than that of the BPEI control. These results demonstrate that gene transfer into skeletal muscle by intravenous route is feasible with emulsified PEG–BPEI administered into locally occluded blood vessels. Neu et al. [40, 41] examined the effect of larger PEGs than those described in the above two studies. PEG (20 kDa) or PEG (30 kDa) was grafted on to BPEI (25 kDa) in di-block (PEG–BPEI) or tri-block (PEG–BPEI–PEG) configuration. Due to the higher chain terminus dilution inherent in higher molecular weight polymers, the more reactive linking chemistry involving diisocyanates used here may be more advantageous, rather than the NHS-activated esters used by Hong et al. [39] for lower molecular weight PEGs. All four PEG–BPEIs [PEG (20 kDa)–BPEI, PEG (20 kDa)–BPEI–PEG, PEG (30 kDa)–BPEI, and PEG (30 kDa)–BPEI–PEG] exhibited significantly lower zeta potential, better salt stability, and higher blood concentrations when formulated with DNA. The best results were obtained with PEG (30 kDa) in both configurations, which

suggests that PEG size and incorporation density are important factors. Despite good surface modification, PEGylation did not improve transfection activity as its high occluded volume hinders the electrostatic attraction between positively charge polyplexes and the anionic cell surface. However, Fewell et al. [42] reported a PEGylated BPEI that had significantly higher transfection activity than a control BPEI. A low-molecular-weight BPEI (1800 Da) was functionalized with cholesterol chloroformate and a low-molecular-weight NHS-activated PEG (550 Da) at different PEG : BPEI ratios, and the authors found a PEG–dependent improvement in gene transfer to solid tissues. A 10-fold enhancement over BPEI–cholesterol was achieved at a PEG : BPEI molar ratio of 2 : 1. Increasing the PEG concentration higher than 2 : 1 : 1 (PEG : BPEI : cholesterol) ratio led to a significant decrease in transfection efficiency. PEGylation also lowered the particle zeta potential, which was consistent with the charge neutralization effect exerted by the PEG molecule. The enhancement of BPEI in vivo activity in this study could be due to the use of low-molecular-weight BPEI and low-molecular-weight PEG. Previous studies used much higher molecular weight PEG and did not find significant improvement in transfection activity. Fewell et al. [42] demonstrated that covalent modification of a low-molecular-weight BPEI with PEG and cholesterol significantly increases the in vivo activity of BPEI. Together, the cholesterol and PEG modification of BPEI produced approximately 20-fold enhancement in the activity of BPEI (1800 Da), a poorly active transfection agent alone. The PEG–BPEI–cholesterol (PPC) polymer has been successfully used for interleukin-12 (IL-12) gene delivery and anticancer efficacy in several subcutaneously implanted and peritoneally disseminated mouse cancer models. The safety and tolerability of escalating doses of IL-12 plasmid (10, 50, and 250 μg DNA) and PPC complexes at N : P ratio of 11 has been demonstrated by intraperitoneal and subcutaneous administration in normal mice [43]. Biodistribution studies following intraperitoneal or subcutaneous administration showed DNA delivery by PPC was primarily localized at the injection site and only a small amount escaped into the systemic circulation, demonstrating PPC suitability for local gene delivery [44]. Intracranial delivery of IL-12 plasmid–PPC in mice with B16 glioma produced significant IL-12 expression in tumor tissue and prolonged animal survival without causing significant toxicity [45]. The clinical development of PPC-mediated IL-12 gene delivery for the treatment of recurrent ovarian cancer is described in Section V of this chapter.

Another approach to functionalization of low-molecular-weight PEI is to crosslink it with a biocompatible polysaccharide. Tang et al. [46] described the use of β-cyclodextrin for this purpose. Cyclodextrins are attractive targets for PEI functionalization due to their low immunogenicity and low toxicity. Activated β-cyclodextrin was reacted with BPEI (600 Da) in the presence of base and then purified into dry powder. The final molar ratio of BPEI : β-cyclodextrin was calculated to be 1 : 1 based on relative integration of characteristic peaks in the proton nuclear magnetic resonance (^{1}H-NMR) spectrum. The molecular weight of the copolymer was determined to be 61

kDa by gas-phase chromatography (GPC), corresponding to an average degree of polymerization of 35 units for each BPEI–β-cyclodextrin molecule. Although the degradation of the sugar residues was not investigated, albumin was shown to degrade the newly formed carbamate bonds between each PEI–sugar unit significantly within 2 days. The BPEI–β-cyclodextrin was able to condense the DNA into nanoparticles of < 200 nm with a corresponding zeta potential of +40 mV at N : P ratio of 20. In neuronal cells, the efficiency of luciferase gene transfer was 100-to 1000-fold higher than that of BPEI (600 Da) and comparable to that of BPEI (25 kDa). Intrathecal administration of DNA–BPEI–β-cyclodextrin complexes produced two- to four fold higher gene transfer than that of DNA complexes with BPEI (600 Da). The exact mechanism by which cyclodextrins enhance transfection efficiency is not yet understood. Disruption of biological membranes by complexation with phospholipids and cholesterol is believed to be one of the mechanisms by which cyclodextrins mediate their action.

Chitosan is another polysaccharide that has been used for functionalization of PEI. Rather than graft chitosan onto PEI, Wong et al. [47] used the primary amines of chitosan to initiate aziridine polymerization, resulting in a copolymer containing a chitosan backbone and BPEI side chains. Polymerization was efficient, as PEI grafts were observed at 100% of chitosan amines. BPEI-*g*-chitosan fully condensed plasmid DNA into 200- to 400-nm particles of positive zeta potential at high N : P ratios. In comparison, unmodified chitosan (3400 Da) failed to condense plasmid DNA. BPEI-*g*-chitosan had lower toxicity and two- to fivefold higher transfection activity than BPEI (25 kDa) in vitro. Direct infusion of BPEI-*g*-chitosan–luciferase plasmid complexes into the bile duct of rat liver at 0.8 mg/kg plasmid dose produced significant gene transfer in liver, the efficiency of which was 3, 58, and 141 times higher than that of chitosan, BPEI (25 kDa), and naked DNA, respectively. The expression was highly localized in the bile duct and negligible in the lungs, spleen, kidney, and heart. Higher transfection activity was achieved in vivo when particles were prepared at low N : P ratio and displayed large particle diameters. Conversely, higher transfection activity was achieved in vitro when particles were prepared at high N : P ratios and displayed small particle diameters. This discrepancy was believed to be due to difference in DNA concentration used in the in vitro and in vivo formulations. Efficient DNA release from BPEI-*g*-chitosan at low N : P ratios is thought to be one of the reasons for higher transfection efficiency in vivo.

Crosslinked PEIs. One of the approaches to improve PEI transfection activity without augmenting toxicity is to crosslink low-molecular-weight PEI through degradable linkages to create larger and degradable structures. For example, Gosselin et al. [48] synthesized several degradable polymers by crosslinking BPEI (800 Da) via dithiobissuccinimidylpropionate or dimethyl-dithiobispropionimidate linkers. These crosslinked polymers gave significantly higher transfection activity than that of the BPEI monomer but also exerted

significant cytotoxicity. The in vivo properties of these crosslinked polymers were not determined. Thomas et al. [49] synthesized a degradable crosslinked polymer from a mixture of 400 and 2000 Da BPEI, which gave higher in vivo transfection activity than BPEI (25 kDa). Since no in vivo safety data is available, it is difficult to ascertain true potential of this polymer in the clinic. Matar et al. [50] reported a disulfide-crosslinked polymer of LPEI (3600 Da), which had a total average molecular weight of approximately 8 kDa and in vitro and in vivo transfection activity of significantly higher magnitude than that of the LPEI monomer. The crosslinked polymer expressed 20-fold higher transfection activity than that of the monomer LPEI and produced significantly lower toxicity than BPEI (25 kDa) in vitro. Following intravenous administration, plasmid/crosslinked polymer complexes elicited significant gene transfer in lungs, while complexes prepared with LPEI monomer did not yield measurable activity. The transfection activity of the crosslinked LPEI was 2.5 times and 70 times higher than that of LPEI and BPEI (25 kDa), respectively [50]. This crosslinked polymer also exhibited better tolerability than both LPEI and BPEI following in vivo administration, demonstrating that the crosslinking of low-molecular-weight LPEI via degradable linkage is a viable approach to improving PEI transfection activity without augmenting toxicity. This degradable crosslinked polymer of LPEI is distinct from a crosslinked polymer of BPEI [51] with respect to water solubility and transfection activity. The crosslinked polymer of LPEI [50] was highly soluble in aqueous solution and showed transfection activity that was superior to its monomer, whereas the crosslinked polymer of BPEI [51] was poorly soluble in aqueous solution (presumably due to excessive intramolecular crosslinking) and only marginally better than the corresponding monomer in transfection activity. Matar et al. [50] used the *t*-butoxycarbonyl (BOC) anhydride protection step to minimize excessive crosslinking between the monomer amines. The difference between the water solubility and transfection activity of LPEI- and BPEI-based crosslinked polymers shows that the PEI geometry and synthesis scheme are important factors to consider in designing the crosslinking strategy. Neu et al. [52] have described a different approach to PEI crosslinking in which BPEI (25 kDa)/plasmid DNA complexes were stabilized with a crosslinking agent, dithiobis (succinimidylpropionate) (DSP). The size of DNA particles prepared from this crosslinked BPEI increased at higher crosslinking density. This is presumably due to the relatively high local concentration of ester-reactive primary and secondary amines resulting in high intramolecular looping of the linkers. Consequently, this reactivity promoted particle aggregation due to muting of the surface amines and lowering of charge repulsion. Upon intravenous administration the crosslinked polyplexes gave significantly higher tissue plasmid concentrations than control polyplexes of unmodified BPEI. Park et al. [53] synthesized a degradable crosslinked PEI with small molecular weight linear ethyleneimine oligomers (423 Da) using comparatively long-chain bis-acrylate-terminated PEG linkers. Such amine-acrylate reactions are a convenient way to generate labile ester linkages between PEIs in that the

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reactive amines are still available for protonation after linking. The resultant poly(ester amine) (PEA) displayed lower cytotoxicity and higher transfection activity in certain cell lines when compared to BPEI (25 kDa). Unlike the previous example in which reactive amines are consumed leading to aggregation [52], the reactive amines in this case retained their cationic nature, which generated repulsion between the particles and hence prevented aggregation in aqueous solution. In addition, due to the internal ester linkages, these polymers degraded rapidly at 37°C in phosphate-buffered saline (PBS) with a half-life of 72 h. DNA complexes prepared with the crosslinked polymer exhibited 100- to 250-nm particle size and 20−30 mV zeta potential, depending upon the N : P ratio. The in vitro gene transfer activity of the crosslinked polymer was significantly higher, and cytotoxicity was lower compared to BPEI (25 kDa). Aerosolization or intravenous administration of luciferase plasmid with the crosslinked polymer yielded high levels of gene expression in the lungs, liver, spleen, heart, and kidney, and expression levels were higher than those from DNA complexes with BPEI (25 kDa).

Ligand-Modified PEI for Tissue Targeting. As described earlier, most PEI polyplexes are rapidly intercepted by serum proteins when administered into systemic circulation. Only a fraction of the injected formulation escapes the surveillance system and enters nonspecifically into the first-pass organs where barriers of the extracellular matrix further attenuate the passage to the target cells. To achieve gene targeting to a specific tissue by systemic route, the carrier must exhibit stability in serum and extracellular matrix, evade immune recognition, and express tissue-specific homing devices to minimize nontarget uptake. A number of strategies have been described to target gene delivery to a specific tissue using tissue-specific ligands. These strategies are described below.

Many of the targeting ligands used for PEI functionalization are based on peptides and polypeptides. RGD peptides are one of the most prominent ligands for tumor targeting due to the fact that their receptor (fibronectin-binding peptide) is overexpressed on tumor cell surfaces [54]. Sakae et al. [55] conjugated an RGD peptide to PEG with an anionic terminus. The anionic RGD−PEG conjugate was adsorbed onto the positively charged BPEI (25 kDa)−DNA polyplexes via electrostatic interaction. The concept behind this approach was to reduce the cationic surface of the polyplexes in order to minimize particle opsonization by serum proteins and then to incorporate RGD peptide to promote specific binding to RGD receptor overexpressed on tumor surface. The addition of RGD−PEG lowered the zeta potential of the DNA complexes from +37.8 to −25.4 mV. The particle size (150 nm) of the ternary complexes was stable over several hours without aggregation. The ternary complexes were also resistant to albumin-induced aggregation while binary complexes of BPEI−DNA were not. High levels of GFP gene transfer were observed in subcutaneously implanted tumors, liver, and lungs after tail vein injection of the RGD-coated complexes. Much lower levels were obtained with uncoated complexes. Although this study attempted to address

a fundamental problem in the systemic use of cationic polyplexes, it lacks sufficient information on the in vivo stability and target specificity of the delivery system. In addition to its use for surface modification, the RGD ligand has also been directly attached to the PEI polymer [56, 57]. In this approach, RGD was attached to the BPEI (25 kDa) backbone through a heterobifunc-
AQ1 tional PEG (NHS–PEG–VS). The PEG was first conjugated to the peptide amine terminus and the resultant RGD–PEG–VS was then conjugated to BPEI at various molar ratios (1, 5, 10, 20). The RGD–PEG–BPEI/DNA polyplexes displayed a uniform particle distribution with an average particle size of 180 nm. In tumor-bearing mice, tail vein injection of RGD–PEG–B-PEI/DNA complexes yielded tumor-specific uptake of the plasmid DNA with
AQ4 tumor specificity linearly related to the degree of RGD incorporation [58]. The RGD-conjugated transfection complexes were efficacious as their intravenous delivery carrying IL-12 and Flk-1 plasmid in tumor-bearing mice reduced tumor metastases and improved animal survival.

Human immunodeficiency virus (HIV) derived TAT, a potent membrane penetrating peptide, has been used for improving gene delivery in vivo. Kleeman et al. [59] utilized TAT peptide to enhance the transfection efficiency of BPEI (25 kDa) for local gene delivery to the lungs. The conjugation of BPEI to TAT peptide containing a terminal cysteine residue was achieved via a heterobifunctional NHS–PEG–VS (3400 Da). BPEI was attached through the NHS group and TAT through the VS group. BPEI–PEG–TAT was purified by removal of the unreacted material by ultrafiltration. The degree of PEG substitution was 2.0% for PEG and 0.8% for TAT as a fraction of all amines present in the BPEI backbone. In 5% glucose media, TAT–PEG–BPEI/DNA polyplexes displayed a relatively constant particle size of 100 nm and resisted aggregation in high-salt media (150 mM NaCl) over 24 h. The complexes also protected DNA from degradation upon incubation in bronchial alveolar fluid and DNase solution. Intratracheal instillation of TAT–PEG–BPEI/DNA complexes in mice was well-tolerated and produced significantly higher gene expression than the BPEI control.

Nerve growth factor peptide has been used for gene targeting into neuronal
AQ1 tissue [60]. A low-molecular-weight BPEI (600 Da) was functionalized with nerve growth factor loop 4 hairpin. A ternary complex of plasmid DNA, BPEI, and a 39-amino-acid peptide containing NGF loop 4 motif and DNA-interacting lysine 10mer stretch was prepared. The resulting polyplexes were 180 nm in diameter and displayed a +22-mV zeta potential. Transfection of cortical neurons with targeted complexes yielded 1000-fold higher gene transfer than with nontargeted complexes. In vivo gene transfer following intrathecal administration in rats produced gene transfer in NGF receptor-expressing dorsal root ganglia. The level of gene transfer with the TAT–ligand complexes was 59-fold higher than that with the nontargeted complexes. In a cell viability assay, targeted complexes did not display significant toxicity compared to BPEI (25 kDa).

Rudolph et al. [61] have described the use of triiodo-L-thyronine (T3) for targeting gene delivery to the liver. The T3–PEG–BPEI was synthesized by

reacting T3 with PEG-bis acid in the presence of initiators and then with BPEI (25 kDa). T3−PEG−BPEI/DNA complexes of particle size 11−27 nm were prepared for gene transfer studies in vitro and in vivo. T3-specific gene transfer was achieved in HepG2 cells as verified by reduction in transfection efficiency by excess T3. Intravenous injection of T3−PEG−BPEI complexes in mice resulted in sevenfold higher gene expression than PEG−BPEI control in the liver. High levels of gene expression were also obtained in lungs, spleen, thyroid gland, heart, and kidneys from both ligand and nonligand complexes without significant difference.

Elfinger et al. [62] used lactoferrin (Lf) to target lactoferrin receptors expressed on the surface of bronchoepithelial cells. Periodate-oxidized Lf was mixed with BPEI (25 kDa) at different ratios. Lf-modified BPEI successfully condensed plasmid DNA into <100 nm particles of 20−30 mV zeta potential. The transfection efficiency of Lf−BPEI polyplexes in bronchial epithelial cells was fivefold higher than that of Lf-free BPEI polyplexes. The cytotoxicity of Lf−BPEI polyplexes was lower than that of BPEI−DNA complexes. In addition, Lf conjugation was verified by the reduction of transfection activity of Lf−BPEI polyplexes by an excess of free Lf. In A549 cells, which do not express high levels of Lf receptors, the transfection efficiency of Lf−BPEI was comparable to that of BPEI alone. Increasing the Lf incorporation reduced the transfection efficiency in both alveolar and bronchial epithelial cells.

In another approach, a monoclonal antibody was used to target solid tumors with PEI−DNA complexes. Moffatt et al. [63] developed a unique targeting strategy where the targeting ligand was attached to preformed PEI−DNA complexes to ensure ligand exposure to particle surface. A monoclonal antibody (mAb) against prostate-specific membrane antigen J591 was used to target plasmid DNA to prostate tumors. A linking chemistry utilizing the high affinity interaction between phenyl(di)boronic acid (PDBA) and salicylhydroxamic acid, conjugated the antibody through a PEG linker to BPEI (25 kDa). Initially, a heterobifunctional PEG (H_2N−PEG−COOH) was capped with PDBA to form the PDBA−PEG−COOH conjugate, which was then
AQ1 NHS-activated to form PDBA−PEG−NHS. The DTT-reduced J591 antibody was reacted with PDBA−PEG−NHS and the unreacted PDBA−PEG−NHS was removed by dialysis. Intravenous administration of antibody-targeted complexes into mice with subcutaneously implanted prostate tumors resulted in tumor-specific gene transfer, which was 10- to 30-fold higher than that of nontargeted complexes. High tumor specificity of this targeted system is surprising since the targeted complexes had highly positive zeta potential (28−42 mV) and large particle size (600−800 nm). The same group later used
AQ1 this postcomplexation approach to attach CNGRC peptide to BPEI (25 kDa). The CNGRC peptide binds to tumor cells that express aminopeptidase N or CD13 [64]. Physical characterization of the complexes was not performed, and it was assumed that the CNGRC targeted particles have a similar profile as that of the antibody-targeted complexes [63]. The tumor specificity of the CNGRC-modified complexes was demonstrated in CD31 positive cells in vitro.

Intravenous administration of the CNGRC-targeted complexes in tumor-bearing mice produced up to 12-fold higher gene expression in the tumor than in the lungs. The transfection efficiency of targeted complexes was significantly higher than that of nontargeted complexes.

Natural saccharides including lactose, galactose, and mannose have been examined for gene targeting. Lactose and galactose have been used for liver targeting due to their specific affinity for asialoglycoprotein receptors on liver hepatocytes while mannose has been used for targeting antigen presenting cells via surface mannose receptors. Kim et al. [65] conjugated galactose to PEG–BPEI (25 kDa) for liver targeting. Unlike most reports of ligand attachment to distal ends of PEG linkers, Kim et al. [65] attached lactobionic acid (galactose-gluconic acid) (LBA) and mPEG-COOH (2000 Da) independently to BPEI (25 kDa) backbone. This one-pot synthesis does not require the isolation and purification of the activated acid form of LBA. The NHS-activated form of LBA is generated *in situ*, and is followed by the subsequent reaction with a primary amine group of BPEI. The attachment of mPEG-COOH was performed in an identical procedure. The LBA–BPEI–PEG conjugates were purified by extensive dialysis and lyophilized to give the final product. Two forms of the conjugate were synthesized, one with 10% PEG and the other with 50% PEG. The LBA content in both polymers was 10 mol%. PEGylation increased the particle size of LBA–BPEI–PEG to >200 nm and decreased the zeta potential from 19.1 to 5.75 mV. The liver specificity of the
AQ1 targeted systems was confirmed in vitro using ASOR-high and -low expressing cells. Intravenous administration of LBA–BPEI–PEG/DNA complexes in mice with liver tumors was well tolerated and produced significant gene uptake and expression in rapidly dividing liver tumor cells but not in nondividing normal liver cells.

In another approach, poly(vinyl pyrrolidone) (PVP) was coupled to BPEI-galactose to improve delivery safety [66]. BPEI (25 kDa) was first conjugated with galactose residues via reductive amination, then with monocarboxylic
AQ1 acid-terminated PVP in varying ratios via NHS/EDC coupling. Galactose content was determined to be 4.4 mol% and the PVP content estimated to be 4.0, 6.0, and 7.5 mol% compared to BPEI. The galactosylated-BPEI-*g*-PVP (GPP)/DNA complexes showed good DNA binding ability and high DNA protection from nuclease attack. The particle size of DNA complexes decreased with increased charge ratio with a minimum value of around 59 nm at the charge ratio of 40 for the GPP–DNA complex (PVP content: 4.1 mol%). The GPP showed low cytotoxicity and the transfection efficiency of GPP–DNA complexes at charge ratio of 40 in HepG2 cells was higher than that of BPEI–DNA alone.

Sugar ligands have also been examined for gene targeting to lung epithelium [67, 68]. LPEI was covalently conjugated to lactose, glucose, or mannose and evaluated for gene delivery to lung epithelium by nebulization. All three sugar conjugates of LPEI were as efficient as the unsubstituted LPEI but were considerably less cytotoxic. The polyplexes were taken up primarily by alveolar

epithelial cells. Interestingly, despite their high positive zeta potential, the particles were not taken up by alveolar macrophages. Fluorescence microscopy studies showed prenuclear decomplexation of glycosylated complexes, raising concern about the efficiency of nuclear delivery of this sugar-targeted system. Lisziewicz et al. [69] synthesized mannose-LPEI (22 kDa) to target an HIV antigen DNA to mannose receptors on antigen presenting cells in the skin. Topical application of mannosylated LPEI particles (100 nm) containing HIV DNA in naïve rhesus macaques induced virus-specific CD4 helper and CD8 memory T cells. The treatment also produced virological, immunological, and clinical benefits in SIV-infected macaques during chronic infection and acquired immunodeficiency syndrome (AIDS) [70]. When administered in combination with antiviral drugs, mannosylated particles augmented SIV-specific T-cell responses and enhanced control of viral load rebound during treatment interruptions. In the intermittent antiviral therapy, viral load significantly rebounded during the treatment interruption cycles. In contrast, intermittent treatment with combination therapy induced a progressive containment of viral load rebound during treatment interruption cycles in the majority of animals. The HIV vaccine based on mannosylated particles is now in human clinical trials.

Encapsulation of PEI Polyplexes. The PEI approaches described above deal with exposing the tissue to large concentrations of DNA formulations, which could be an important factor contributing to activation of the immune system and rapid clearance of the material. In this section, we have described approaches where PEI-based transfection complexes were delivered from a reservoir under a controlled delivery rate to allow a slow release of the transfection material over a long period of time. A slow release of transfection complexes may be more effective and tolerable for certain gene therapy applications. In some instances, PEI is simply added to encapsulating microspheres to achieve a favorable DNA release kinetic. For example, Nguyen et al. [71] has described a formulation approach to improve encapsulation properties and transfection activity of microsphere systems. Blending poly(orthoesters) (POE) with BPEI (25 kDa) at 0.04 wt% doubled the total release time of plasmid DNA and enhanced gene transfection efficiency of the microspheres up to 50-fold without any significant cytotoxicity. Upon degradation, the microspheres released DNA as complexes with BPEI. Addition of BPEI into the POE microsphere induced 50–60% greater phenotypic maturation and activation of bone-marrow-derived dendritic cells in vitro. Addition of a cationic polymer into POE is a simple approach for modulating the DNA release kinetics and gene transfection efficiency of POE microspheres. Combined with the ability to induce maturation of antigen-presenting cells, POE–BPEI blended microspheres may be excellent carriers for DNA vaccines.

Turk et al. [72] reported a diblock copolymer for temperature-regulated gene transfer properties. This copolymer is composed of poly(*N*-isopropylacrylamide) (NIPA) and PEI with temperature-sensitive and DNA binding

properties, respectively. Carboxyl-terminated NIPA was coupled to LPEI (25 kDa) using standard EDC chemistry, and both the plasmid DNA encapsulation efficiency and release kinetics could be controlled by changing the temperature of the polyplex solutions. Particle sizes ranged from 400 to 1000 nm and remained relatively unchanged upon heating the samples from 25 to 37°C. In addition, zeta potentials of 8−13 mV at polymer : DNA weight ratios were nearly identical to those observed for LPEI (25 kDa) alone. GFP expression was increased from 30 to 50% in vitro as the temperature of the culture environment was lowered to 28 from 37°C. The increase in expression was 10−15% after both subcutaneous and intratibial injection of polyplexes.

Lei et al. [73] used matrix metalloproteinase (MMP)-degradable PEG hydrogels for encapsulation of DNA−BPEI complexes and transfection of mesenchymal stem cells. Mesenchymal cells were seeded inside hydrogels composed of four-arm PEGs terminated with cysteine-reactive vinyl sulfone groups. Crosslinking was accomplished via cysteine-terminated MMP-specific peptide segments in the presence of plasmid DNA−BPEI (25 kDa) polyplexes, which resulted in in situ loading of the hydrogels. Polyplex loading did not significantly affect the viability of mesenchymal cells in transfection. Significant DNA release was observed only after the complexes were treated with trypsin, with over 90% release after 48 h. Transgene expression was reported throughout the 21-day incubation period with maximal values observed on the seventh day.

15.2.3 Cationic Polyesters

The most widely studied polymers in nucleic acid delivery applications that degrade in pharmaceutically relevant time frames are cationic polyesters. The main chain ester bonds may result in in vivo half-lives ranging from minutes to several days. The amine component of the polymers may be primary, secondary, and/or tertiary. The sensitivity to hydrolytic and aminolytic degradation is strongly dependent upon the local environment and may be influenced by the concentrations of amine groups in the polymer, relative hydrophobicity of the polymer and its tendency toward aggregation, and the ratio of ester groups to total polymer molecular weight.

The most basic approach to the synthesis of polymers that contain both amines and esters, so called PEAs, is to form graft-copolymers composed of a polyamine and a polyester. Arote et al. [74] synthesized a low-molecular-weight PEI-based system that contains long stretches of ester-containing segments in the form of polycaprolactone (PCL) for the administration of plasmid DNA to the mouse lung. A range of BPEI−PCL copolymers were synthesized, each containing alternating stretches of BPEI and PCL in varying ratios. PCL was first converted from the diol to the bis-acrylate with acryloyl chloride to give PCL with a molecular weight of 530 Da. Several different molecular weight BPEIs (600, 1200, 1800 Da) were combined with bis-acrylate-PCL to give BPEI-*b*-PCL copolymers. The authors reported gradual variation in the molecular

weights of the copolymers through tuning of the stoichiometric ratio of BPEI to PCL from 1 to 4. Maximum molecular weights were obtained from polymerizations in which the BPEI : PCL ratio was 1 : 1, with molecular weights decreasing at 2 : 1 and 4 : 1. The rate of degradation for the copolymers was also measured at 37°C in PBS and the molecular weights found to decrease by 30% after one day, with extended degradation down to 50% over 10 days. Although the authors attribute the degradation solely to hydrolysis of the PCL ester linkages, it is also likely that the relatively high concentrations of primary and secondary amines from the BPEI segments caused additional chain cleavage via aminolysis of the PCL ester linkages. This additional degradation route was observed in other PEAs containing primary amines [36]. When formulated with DNA at N : P ratios of >10 : 1, those of the higher BPEI molecular weight (1200 and 1800 Da) gave particles sizes <200 nm at N : P ratios up to 30 : 1, while PCL−BPEI (600 Da)/DNA complexes had particle sizes >300 nm. However, the PCL−BPEI (600 Da)/DNA complexes exhibited lower toxicity and higher transfection efficiency than BPEI (25 kDa) in several different cell lines. Aerosolization of PCL−BPEI (600 Da)/GFP plasmid in mice yielded GFP expression. The magnitude of lung transfection with PCL−BPEI(600 Da)/DNA was significantly higher than with BPEI (25 kDa)/DNA complexes.

Similar PEAs may be synthesized with more precise control over the composition by the alternating addition of amine- and ester-containing monomers. Langer et al. [75] reported the development of a technique to synthesize large libraries of PEAs based on the conjugate addition of small-molecule amines to bis-acrylates [76, 77]. Each individual polymer within the library of over 2000 polymers had within its backbone alternating units containing an ester and a tertiary amine. Some members of the group also had secondary or tertiary amines within the side chains, as well as many other structurally unique features including branched structures and aromatic rings. In the high-throughput screening method employed, roughly 2% of the polymers synthesized had equal or higher transfection efficiencies than BPEI (25 kDa) in COS-7 cells. PEAs of this type were shown to be >80% degraded after 24 h at pH 5−7 [77]. Although several polymers in this library possessed high transfection activity, the lack of primary amines in the basic structure, although preventing more rapid degradation, could result in less stable polyplexes with plasmid DNA. Green et al. [78] made further combinatorial changes to the most active members of the previously established library and compared the activity in cell culture, primary cells, and systemic treatment of ovarian cancer. The properties of all the polymers in the library were tuned by adjusting the diacrylate : amine ratio in the reaction mixture. A group of the most active polymers in the library (C32, D60) were synthesized to contain acrylate-terminal groups. To further vary the structure in an attempt to enhance the properties of the polymers, a group of small bis-amine-terminated compounds, some containing short ethylene glycol repeat units, were added to the parent polymers to create an additional library of polymers. These advanced polymers were synthesized by dissolving the parent diacrylate-terminated polymer in dimethyl sulfoxide

(DMSO), and adding to them DMSO solutions of the amine end-capping reagents. In general, C32 polymers terminated with primary amine derivatives containing no ethylene glycol spacers were more active than those containing the PEG spacer. These active derivatives were found to bind DNA more tightly than the parent C32 polymer, and also formed smaller complexes with DNA at the same N : P ratios (~150 nm). Intraperitoneal administration of C32-117/pCAGluc DNA complexes in MISIIR/TAg female transgenic mice bearing bilateral ovarian tumors produced luciferase expression; however, C32/pCA-Gluc complexes were much less active. The efficiency of gene transfer with C32-117 complexes was >100-fold higher than with C32 complexes.

15.2.4 Dendrimers

The majority of polymers and polymeric systems utilized as delivery systems for nucleic acids are linear in nature. Others, such as BPEI, may be synthesized in a relatively uncontrolled manner that results in a branched, or dendritic, architecture. A true dendrimer, however, is built in steps referred to as "generations," which are unique, isolatable, and well-defined polymeric structures. The most widely studied dendrimer is poly(amido amine) (PAMAM), which consists of an alkyl-diamine core and tertiary amine branches containing amide linkages [79]. PAMAM dendrimers are commercially available in generations from 0 to 10 (G0 to G10) with different core types and functional surface groups, which has led to their use as slightly more degradable alternatives to BPEI. Little in vivo data has been reported on the degradation
AQ4 products of PAMAM dendrimers, however, Tang et al. [80] have demonstrated extensive and specific solvolysis of the amide linkages by refluxing in 1-butanol over 43 h. Mamede et al. [81] developed complexes employing PAMAM dendrimers (G4) and avidin-biotin systems (Av-bt) for liver targeting of antisense oligonucleotides. This fourth-generation dendrimer contained 64 active primary amine surface groups and had a molecular weight of 14 kDa. The dendrimers were reacted with NHS-activated biotin and mixed with avidin-bound, In-111-labeled oligonucleotides. PAMAM-bound particles showed high accumulation in the liver (50.95% at 15 min, 47.88% at 60 min) compared to naked particles, which showed low uptake in organs other than
AQ1 the kidneys (21.48% ID/g at 15 min, 18.48% ID/g at 60 min).

Although PAMAM dendrimers have been in use for over a decade, their toxicity in in vivo applications is a primary drawback [82]. Molecular functionalization has been employed to reduce dendrimer toxicity and improve specificity of delivery. Huang et al. [83, 84] functionalized fifth-generation (G5) PAMAM dendrimers with Lf and Tf to enhance plasmid DNA delivery to the brain. Dendrimers were synthesized with a PEG spacer [NHS−PEG−maleimide (NHS−PEG−MAL) (3400 Da)] between the surface amine groups and the peptide ligands in order to enhance the effectiveness of the ligands and to shield the surface-positive charges of the dendrimer. Lf and Tf, modified with surface thiols, were attached separately to the distal end of

the PAMAM–PEG–MAL, giving PAMAM–PEG–Lf and PAMAM–PEG–Tf, respectively. The extent of the conjugation reaction was examined by visualizing the mobility of Lf- and Tf-containing structures in a polyacrylamide gel electrophoresis (PAGE) experiment. Intravenous administration of PAMAM–PEG–Lf/GFP DNA complexed at a 10 : 1 N : P ratio produced significantly higher gene uptake and expression than PAMAM–PEG–Tf/DNA complexes in the brain. Interestingly, gene transfer in the liver, spleen, kidneys, and lungs was higher with PAMAM–PEG–Tf/DNA complexes than with PAMAM–PEG–Lf/DNA complexes, demonstrating high brain specificity of Lf compared to Tf.

A related dendrimer based on lysine was synthesized by Okuda et al. [85] and its ability to target tumors via intravenous injection examined. These bio-inspired dendritic polymers are theorized to derive their reduced toxicity to their strictly peptidic linking arrangement. Hexamethylenediamine was used as the dendrimer core, and an alternating series of amine protection, HBTU–HOBT coupling, and amine deprotection steps were used to build succeeding generations with lysine. Lysine dendrimers up to the sixth generation (G6) were synthesized in this manner. The G6 dendrimers were then PEGylated by reaction with mPEG–NHS (5000 Da). The degree of PEGylation was determined by the barium–iodine method and found to be directly proportional to the amount of mPEG–NHS added in the reaction mixture. The unmodified dendrimers were found to have particle diameters of 6 nm. At a degree of PEGylation of 10, the size increased slightly to 8 nm, and even more dramatically at a degree of PEGylation of 76 to 17 nm. The zeta potentials reported for the dendrimers were 20, 0, and −7 mV, respectively. Although no data was reported when these lysine-based dendrimers were complexed with nucleic acids, the ability of a delivery system to enhance uptake at tumor sites after systemic injection without producing significant immune response has potential to be an important improvement upon dendrimer technology. Biodistribution studies in normal and tumor-bearing mice showed rapid clearance of unmodified dendrimers from the bloodstream and nonspecific accumulation in the liver and kidney. In contrast, the PEGylated derivatives showed better retention in blood and low accumulation in organs dependent upon the degree of PEGylation. In addition, the 76-PEG dendrimer was accumulated effectively in tumor tissue, presumably via the enhanced permeability and retention (EPR) effect. Moreover, multiple administrations did not affect the biodistribution characteristics of a second dose of the same dendrimer.

Ribeiro et al. [86] encapsulated a bacterial DNA antigen with slightly different lysine-based dendrimer and embedded them within PLGA particles using the standard double emulsion method. Two different dendrimers were synthesized, each containing low generation numbers totaling 7 lysine units and 18 total amine groups and differing by the presence or absence of three C_{18} units grafted to the core of the dendrimer. The intact delivery system was formed by mixing the dendrimer with DNA, then with poly(vinyl alcohol) (PVA), and finally PLGA to form the dendriplexes. Particle sizes and zeta potentials were not influenced by the N : P ratio, and ranged from 400 to 450

nm and −15 to −19 mV for the C_{18}-containing variant and 500−600 nm and −13 to −18 mV for the C_{18}-lacking variant. Immunization of mice with the dendriplexes produced superior antibody response in comparison to animals immunized with the PLGA particles alone.

Repeated reaction/purification steps involved in a variety of dendrimer syntheses can influence the stability of such molecular arrangements containing more degradable ester linkages. In an effort to reduce dendrimer degradation during synthesis, pseudodendrimers have been reported by Russ et al. [87] utilizing amine/acrylate chemistry. The pseudodendrimers were synthesized in two steps: (1) Michael addition of low oligoethyleneimine (OEI; 800 Da) to dioldiacrylates and (2) modification of the surface acrylates with various oligoamines. In the first step, OEI was coupled with a 20-fold molar excess of either 1,2-ethyleneglycoldiacrylate (ED), 1,4-butanedioldiacrylate (BD), or 1,6-hexanedioldiacrylate (HD). In the second step, the acrylate-coated cores were modified at the surface with different oligoamines including ethanolamine (E), spermidine (Sp), spermine (S), and OEI (O). The group of polymers displayed a range of molecular weights from 1200 to 60,000 Da, and when formulated with plasmid DNA, displayed a range of particle sizes from 160 to 900 nm and a range of zeta potentials from −8.8 to 29.0 mV. A primary concern was the stability of the ester linkages to aminolysis during the extended reaction and purification times. The remaining percentage of ester content of the polymers was as low as 71% for the ED−O combination and as high as 98% for the HD−Sp combination (although several of the polymers retained >90% of esters). The stability of the HD−O variant was measured in aqueous media at pH 7.4 and 37°C and found to be comparable to other PEAs. Within the first 24 h, only 75% of the original ester bonds remained, after which the level decreased linearly over the next 7 days to <20%. The DNA-binding ability, cytotoxicity, and transfection efficiency were influenced by the pseudodendritic core characteristics and different surface modifications. In vitro reporter gene expression levels were similar to high-molecular-weight LPEI and BPEIs. Intravenous administration of HD−O/DNA polyplexes in mice yielded gene transfer in tumor tissue at levels comparable to that obtained with LPEI. However, HD−O was better tolerated than LPEI and transgene expression was more tumor specific and much lower in all other investigated organs, particularly in the lungs.

Schatzlein et al. [88] have developed lower generation poly(propylenimine) dendrimers (DAB 8, generation 2 and DAB 16, generation 3), and have demonstrated effective DNA delivery in vivo. DAB dendrimers are structurally analogous to low-molecular-weight BPEIs. Methyl quaternary ammonium derivatives of DAB 4 (generation 1, 4 surface primary amines), DAB 8, DAB 16, and DAB 32 were synthesized to give Q4 (generation 1, 4 surface methyl quaternary amines), Q8, Q16, and Q32, respectively. Quaternization of DAB 8 proved to be critical in improving DNA binding, based on data from ethidium bromide exclusion assays and dendrimer−DNA colloidal stability tests. Particle sizes for the various complexes increased with increasing dendrimer

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molecular weight up to 300 nm, and all formulations were decidedly positively charged at N : P ratios of 3–5 : 1. The improved colloidal stability had a major effect on vector tolerability, as Q8/DNA formulations were well tolerated upon intravenous injection while a similar DAB 8/DNA dose was lethally toxic by the same route. Quaternization also improved the in vitro cell biocompatibility of DAB 16/DNA and DAB 32/DNA dendrimer complexes by about fourfold but not that of the lower generation DAB 4/DNA and DAB 8/DNA formulations. In contrast to previous reports with nonviral gene delivery systems, the intravenous administration of DAB 16/DNA and Q8/DNA formulations resulted in liver-targeted gene expression as opposed to the lung-targeted gene expression obtained with the control polymer [Exgen 500 (LPEI)].

15.2.5 Synthetic Polypeptides

Polypeptides were one of the first polymer types to be investigated for complexation and delivery of nucleic acids. These biopolymers are biodegradable, primarily through enzymatic processes and possess nearly identical backbones but with a wide range of functional side chains. The most widely used polypeptide for gene delivery is PLL, due to the side-chain terminal primary amine that has a relatively high pK_a in the range of 9–10, which results in a net positive charge at physiological pH. PLL with different molecular weights has been evaluated for gene transfer activity [89–91]. Although PLL binds very well to nucleic acids via electrostatic attraction, transfection levels achieved with PLL alone are relatively modest due to aggregation, lack of endosomal disruptive properties, and toxicity [92]. To improve both in vitro and in vivo gene transfer levels through increased complex stability and reduced toxicity, PLL has been modified by the grafting of PEG to the termini or to the side chains [93, 94]. In other cases, the intermittent addition of other cationic amino acids such as arginine and histidine within the polypeptide chain has led to improved performance. PLL segments have also been incorporated into polypeptides or shorter oligopeptides containing other amino acids to confer nucleic acid binding properties [95–97].

One of the most clinically advanced synthetic polypeptide-based systems was reported by Konstan et al. [98] for the treatment of cystic fibrosis [99]. The PEG–PLL block copolymer was formulated with plasmid DNA encoding the cystic fibrosis transmembrane regulator (CFTR) gene and delivered to the mucosal lining of the lungs. The PEG–PLL block copolymer consisted of a 30-unit PLL block terminated by a single cysteine residue (CK30) and a linear mPEG block containing a terminal maleimide group (PEG10K–MAL) (10 kDa). CK30–PEG was prepared by linking the lysine block to the mPEG block through the thiol-maleimide linkage. 4,4′-Dithiopyridine release assays were performed to confirm 100% substitution of CK30 with mPEG–MAL. CK30–PEG/DNA nanoparticles self-assembled in the form of rods having dimensions of 200 nm × 20 nm, and showed extended stability in saline. The nanoparticles possessed only slight positive charge and gave a zeta potential of

4 mV at an N : P ratio of 2 : 1 [100]. The authors also performed calculations based on the theoretical volumes occupied by both plasmid and the polymer in water and described the polyplexes as containing a single molecule of plasmid DNA. After intranasal or intratracheal administration of CK30–PEG/DNA nanoparticles to mouse airways, luciferase expression was observed to be 200-fold higher than plasmid-only doses after 48 h. In addition, only at elevated doses was the toxicity profile of CK30–PEG/DNA nanoparticles discernable from saline [101]. The safety, tolerability, and gene transfer activity of identical formulations carrying plasmid encoding the cystic fibrosis gene in cystic fibrosis patients is described in Section 15.5.4.

Itaka et al. [102] have developed a system composed of a PEG-poly(aspartic acid) block copolymer that has been modified to contain lysinelike cationic amines. These modified copolymers were used to deliver plasmid DNA encoding osteogenic differentiation-inducing factors to bone scaffolds. PEG-poly(β-benzyl-L-aspartate) (PEG–PBLA) was synthesized by the ring-opening polymerization of β-benzyl-L-aspartate *N*-carboxyanhydride from the terminal primary amino group of α-methoxy-ω-amino PEG (12 kDa). The *N*-terminal amino group of PEG–PLBA was acetylated to obtain PEG–PLBA-Ac. The polymer was modified by the addition of diethylenetriamine (DET) in order to add a triamine structure to the side chains of the polymer. The aspartic acid side chains were quantitatively converted to the DET derivative and contained a secondary amine and a terminal primary amine designed to both bind nucleic acids and to buffer the endosome. PEG-*b*-poly[Asp-(DET)] formed spontaneous micelles with DNA. The particles had average diameters of 80–90 nm and zeta potentials of 0–3 mV [103]. In vitro gene transfer of mouse calvarial cells with PEG-*b*-poly[Asp-(DET)]/DNA complexes expressing a constitutively active form of activin receptorlike kinase 6 (*caALK6*) and runt-related transcription factor 2 (*Runx2*) produced significant gene transfer with low cytotoxicity. The osteogenic differentiation induced by gene transfer with PEG-*b*-poly[Asp-(DET)]/DNA of calvarial cells was higher compared to BPEI (25 kDa) or FuGENE6. The polyplex nanomicelles were efficiently incorporated into and released from calcium phosphate cement scaffolds and transfected surrounding cells. In a skull bone defect mouse model, local delivery of *caALK6* and *Runx2* genes from nanomicelles incorporated into the calcium phosphate cement scaffold resulted in substantial bone formation covering the entire lower surface of the implant without any sign of inflammation.

15.2.6 Polysaccharides

Chitosan. Chitosan, a linear polysaccharide composed of β (1–4)-linked 2-amino-2-DEOXY-*b*-D-glucose and the *N*-acetylated analog isolated from chitin within the exoskeleton of crustaceans, was first described as a delivery system for nucleic acids in 1995 by Mumper et al. [104]. Both the native and deacetylated versions were later examined for in vivo plasmid delivery [105]. However, commercially available chitosan is of high molecular weight

(100–400 kDa) and as a result exhibits low solubility at physiological pH. In addition, chitosan solutions possess high viscosity at concentrations required for effective delivery. In an effort to improve the in vivo properties of chitosan in nucleic acid delivery applications, Koping-Hoggard et al. [106] utilized high-molecular-weight fully deacetylated chitosan and employed nitrous acid to depolymerize the polymer into two distinct fractions containing populations of degree of polymerization 25 and 18. The 18-unit fraction was further fractionated using gel filtration into fractions based on degree of polymerization: 10–14, 15–21, 22–35, and 36–50. These low-molecular-weight fractions of chitosan displayed comparable behavior to low-molecular-weight PEIs in that there was no correlation between in vitro and in vivo activity among the fractions at the optimal N : P ratio of 60 : 1. In this study, the fraction containing 15–21 monomer units displayed the highest transfection levels after intratracheal administration into mouse lungs, which gave particle sizes in the range of 35–120 nm as the concentration ranged from 25 to 500 μg/mL; however, the particles showed very little activity in vitro. The authors argue that the moderate polyplex stability afforded by this fraction compared to those of higher molecular weight are optimal for uptake by the epithelial lining of the airways, but not for cells in culture. In addition, the lower viscosity of the 15–21 fraction resulted in a more favorable aerosol droplet size.

The galactosylated complexes of Jiang et al. were targeted to normal liver and showed good efficiency [107]. The synthesis approach employed was unique in that it used chitosan as the backbone polymer and conjugated LBA and BPEI to it using NHS/EDC and periodate chemistry. First, chitosan and LBA were combined to form a chitosan–LBA conjugate and then BPEI (1800 Da) was grafted onto chitosan in the presence of potassium periodate. The final polymer, LBA–chitosan–BPEI, displayed high molecular weight (25 kDa) and contained 16 mol% BPEI. Complexation of plasmid DNA with LBA–chitosan–BPEI polymer reduced the DNA size to <100 nm and protected DNA from nuclease degradation. The cytotoxicity of this functionalized polymer was considerably lower than that of BPEI (25 kDa). The hepatocyte specificity of the delivery system was verified by comparing transfection activity in asialoglycoprotein receptor-positive and -negative cells. Intraperitoneal administration of ^{99m}Tc-labeled LBA–chitosan–BPEI/DNA complexes in mice gave higher gene uptake and transfection in liver compared to BPEI (25 kDa)/DNA complexes.

Cyclodextrins. Cyclodextrins are water-soluble oligosaccharides that form inclusion complexes with a wide variety of substances including lipid-soluble drugs, dyes, fragrances, and food stuffs [108]. The ability of cyclodextrins to form inclusion complexes with small hydrophobic compounds such as adamantane may be utilized to modify the surface of DNA-containing particles without interfering with the polymer–DNA binding interactions and overall particle shape [109]. These systems have been combined with both BPEI and LPEI by Pun et al. [110] to transfect mouse liver. BPEI and LPEI (25 kDa) were

grafted with 6-monotosyl-β-cyclodextrin in varying ratios. In order to utilize the adamantane-binding properties of β-cyclodextrin, adamantane−PEG (5000 Da) (AD−PEG) was generated by reacting the NHS-activated derivative of mPEG with 1-adamantanemethylamine. To form polyplexes with plasmid DNA, an aqueous PEI-β-cyclodextrin (PEI−CD) solution was mixed with an equal volume of an aqueous PEG−adamantane (PEG−AD) solution. This mixture was then combined with a solution of plasmid DNA at the appropriate ratios to give the desired N : P ratios. At an N : P ratio of 10 : 1, BPEI−CD+PEG−AD gave particles of less than 150 nm in diameter, which did not aggregate in the presence of high salt concentrations. LPEI−CD+PEG−AD also resisted aggregation and resulted in even smaller particles ($<$100 nm). In each case, the PEG−AD component dramatically reduced aggregation compared to PEI−CD alone. The CD-grafted LPEI and BPEIs were investigated as in vitro and in vivo gene delivery agents. The in vitro toxicity and transfection efficiency were sensitive to the level of CD grafting. PEGylated LPEI-CD-based particles give in vitro gene expression equal to or greater than LPEI as measured by the percentage of EGFP-expressing cells. The authors reported decreased transfection ability with increasing CD grafting, which is attributed to the reduced endosomal release efficiencies caused by the consumption of available amines with grafted CD units and their subsequent effect on the pK_a profiles of neighboring secondary amines. Tail vein injections into mice of 120 μg of plasmid DNA formulated with CD−LPEI and PEG−AD did not reveal observable toxicities, and both nucleic acid accumulation and expression were observed in the liver.

15.3 SYNTHETIC NONCONDENSING POLYMERS

15.3.1 Poloxamers

Nucleic acid delivery to skeletal muscle is a potentially valuable therapeutic application. However, traditional condensing cationic polymer−DNA systems have shown poor activity, primarily due to poor diffusion and uptake at the site of administration [111]. As a result, alternative noncondensing delivery systems have been developed that offer a moderate amount of complexation and protection for DNA in vivo, and which also afford increased diffusivity of the formulation at the site of administration. The most prominent example is a group of amphiphilic copolymers termed poloxamers, which are composed of PEG and its more hydrophobic derivative, poly(propylene glycol) (PPG). A large number of poloxamers are commercially available under the trade name Pluronics, and which vary in the ratio of PEG : PPG blocks in the PEG−PPG−PEG triblock copolymer configuration. Poloxamers assume a weak micelle-type arrangement in aqueous solution and associate through hydrophobic/hydrophilic interactions with a variety of therapeutic molecules including plasmid DNA.

Poloxamer CRL1005, formulated with a small amount of the cationic surfactant benzalkonium (BAK) chloride, was developed to deliver a plasmid DNA vaccine for the treatment of CMV-associated disease and is currently being tested in a phase II clinical trial [112]. To create the formulations, poloxamer and plasmid DNA were dissolved in separate PBS solutions and combined at appropriate ratios, after which BAK was added. The poloxamer suspension spontaneously assembled into particles, and the authors suggested the multiple small micelles likely fused to form micron-sized particles with variable diameters and slightly negative surface charges. This behavior is attributed to the high surface concentration of electronegative oxygen atoms within the PEG blocks of the poloxamer chains. Formulations containing the cationic surfactant (BAK) produced more uniform-sized particles with a mean hydrodynamic diameter of ~200 nm, presumably due to anchoring of BAK on poloxamer surface yielding a positive charge preventing particle fusion due to repulsion. Studies in rhesus macaques showed CRL1005–BAK/DNA formulated vaccines enhanced the antigen-specific cellular and humoral immune response, presumably by improving DNA delivery.

Roques et al. [113] demonstrated reduction in PEI–DNA complex toxicity after intrapericardial administration by formulating the PEI complexes in a poloxamer 407-based thermosensitive gel. A ternary solution was formed, first by the combination of plasmid DNA and BPEI (25 kDa), then by the addition of an aqueous suspension of poloxamer 407. Characterization of the size and zeta potential of the complexes suggested interactions between the polyplexes and the poloxamer gel increased the polyplex size and afforded shielding of the BPEI surface charges. However, in vivo evaluation revealed a moderate degree of toxicity toward the myocardium, likely due to the rapid unmasking of the positively charged BPEI–DNA particles as a result of their loose association with the poloxamer. Despite this observation, feasibility of intrapericardial injection of poloxamer-based formulations as well as their decreased toxicity was established.

Poloxamer 188 has also been used to deliver plasmid encoding the extracellular matrix protein Del-1 gene to promote neovascularization in ischemic muscle [114]. Intramuscular injection into mouse tibialis muscle of Del-1 or VEGF plasmid formulated in 5% (w/v) poloxamer 188 resulted in significant transgene expression in the injected muscle measured 7 days after the treatment. In a mouse model of hind-limb ischemia, both formulations induced formation of new blood vessels and restored hind-limb function. The capillary/myofiber ratio in the treated muscle was approximately 1.7-fold greater than in control-treated muscles. Similar results were obtained in a rabbit model of hind-limb ischemia.

15.3.2 Encapsulating Systems

Poly(lactic acid-*co*-glycolic acid) (PLGA) is a degradable polyester that has found wide utility in small-molecule drug and protein delivery. Traditional PLGA particles have sizes in the micron range and are synthesized using standard emulsion techniques. PLGA delivery systems are often used in slow-release applications, which may be advantageous for nucleic acid delivery for

several reasons, including (1) sustained and predictable release, (2) protection from tissue degradation before release, (3) site-specific delivery by local implants, (4) low injection frequency, and (5) improved patient compliance [115]. Achieving high incorporation efficiencies and control over release kinetics are significant challenges in encapsulating hydrophilic molecules such as DNA within submicron particles fabricated from PLGA.

PLGA-based systems, like poloxamer-based systems, are noncondensing, and due to the ester linkages between monomer units are sensitive to hydrolytic degradation. Chang et al. [116] have developed the PLGA equivalent of poloxamer for the delivery of plasmid DNA to rat skeletal muscle. PEG_{13}–$PLGA_{10}$–PEG_{13} (lactic : glycolic = 3 : 1) (total molecular weight 3750 Da) was synthesized by combining PEG (550 Da) and stannous 2-ethylhexanoate-copolymerized DL-lactide and DL-glycolide (3 : 1 molar ratio) [117]. Polymer/plasmid DNA working solutions were prepared by mixing various amounts of PEG_{13}–$PLGA_{10}$–PEG_{13} stock solutions with plasmid DNA saline solution. At a lower weight ratio of 1 : 1 (PEG_{13}–$PLGA_{10}$–PEG_{13} : plasmid DNA), the supercoiled morphology of the DNA was retained and the overall zeta potential of the particles was −85 mV. The loose association indicative of nonionic amphiphilic polymers such as poloxamers and triblock copolymers of this type was apparent when the weight ratio was increased to 25 : 1. Based on atomic force microscopy (AFM) observations, the morphology of the DNA was compacted slightly and the zeta potential increased to −60 mV. However, the authors confirmed that this loose association was not sufficient to retard the mobility of plasmid DNA in gel electrophoresis experiments. Although no degradation studies were reported, reference was made to a previous report of similar triblock copolymers [118]. However, when cell viability was examined over time (9–24 h) in comparison to Pluronic P85, the fraction of live cells remained relatively constant (70 to 66%) while those in contact with the poloxamers decreased markedly (55 to 21%). The higher viability is attributed to the degradability afforded the PLGA-based polymer at the ester linkages, as such degradable linkages are absent in the poloxamer structure and can lead to increased toxicity. Intramuscular injection of VEGF or luciferase plasmid DNA formulated in a 0.25% PEG_{13}–$PLGA_{10}$–PEG_{13} solution produced gene expression in muscle that was 2–3 times higher than that from naked plasmid DNA. In addition, injection of fluorescence-labeled plasmid DNA showed greater dispersion of PEG_{13}–$PLGA_{10}$–PEG_{13}/DNA compared to BPEI (25 kDa)/DNA.

Blum et al. [119] attempted to improve PLGA for nucleic acid applications by conjugating PLL to PLGA (PLGA–PLL) to create an electrostatic interaction between the carrier material and DNA. Conjugation proceeded through the carboxylic acid end groups to amine-terminated side chains of PLL via DCC coupling. PLGA–PLL/PLGA particles were prepared using standard double-emulsion techniques and then loaded with plasmid DNA. Particles fabricated with higher weight percentages of PLGA–PLL displayed remarkably increased loading (>90%), as well as a reduction in "burst" release kinetics of DNA. The shift to a more gradual release pattern compared to PLGA-only particles could

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be the result of PLGA ester aminolysis by side-chain-terminal primary amine groups of the PLL component. In addition, the authors used homogenization and sonication to improve encapsulation efficiency and release kinetics over PLGA nanoparticles. Particles prepared with homogenization expressed higher encapsulation efficiency and linear release profile. By comparison, sonication produced low encapsulation efficiency and a burst release profile.

In addition to grafting polymers to PLGA, applicability of physical blends of other noncondensing polymers with PLGA have been explored. Csaba et al. [120] reported blends of PLGA and poloxamer derivatives, which exhibit the capacity to associate and release plasmid DNA in a controlled manner for intranasal delivery. The poloxamer derivative used was poloxamine, a 4-arm branched poloxamer containing a bis-tertiary amine core that provides a small amount of cationic nature to the polymer. Nanoparticles were formed using a modified oil-in-water emulsion technique containing poloxamer or poloxamine. Particle size was similar for the poloxamer and poloxamine-containing formulations; however, the slightly cationic poloxamine-containing nanoparticles displayed an increase in DNA encapsulation efficiency. The ability of these nanoparticles to overcome cellular and mucosal barriers was studied in vitro and in vivo. Fluorescent labels showed nanoparticles entered the cells and transported the associated DNA molecule across the cell membrane. The poloxamer-containing nanoparticles elicited a fast and strong immune response, significantly more pronounced than that corresponding to both the poloxamine-containing nanoparticles and naked plasmid DNA for up to 6 weeks. In intranasal applications, the more hydrophobic poloxamer derivative confered increased uptake by the nasal-associated lymphoid tissues and, consequentially, delivery to the antigen presenting cells.

Bhavsar et al. [121] prepared a novel nanoparticle-in-microsphere oral system (NiMOS) for gene delivery to the gastrointestinal (GI) tract. Plasmid DNA, encoding for β-galactosidase or green fluorescent protein, was encapsulated in type B gelatin nanoparticles. NiMOS was prepared by further protecting the DNA-loaded nanoparticles in a PCL matrix to form microspheres < 5 μm in diameter. Lipase (to degrade PCL) and protease (to degrade gelatin) were found to enhance the release of DNA from the particles, with protease inducing full payload release after 5 h. Biodistribution studies following oral administration in rats showed that while gelatin nanoparticles traversed through the GI tract quickly with more than 85% of the administered dose per gram localizing in the large intestine within the first hour, NiMOS resided in the stomach and small intestine for a relatively longer duration. Transgene expression was observed in the small and large intestines of rats. NiMOS shows significant potential as a novel gene delivery vehicle for therapeutic and vaccination purposes.

15.4 siRNA DELIVERY POLYMERS

The dramatic increase in interest in small inhibitory RNA (siRNA)-based therapeutics has resulted in the extension of PEI as a carrier. Initial reports

have suggested that paradigms developed for the in vivo delivery of plasmid DNA do not necessarily apply for siRNA. The much smaller size (19–25 base pairs compared to the average 5000–10,000 base pairs for plasmid DNA) has proven to require different properties in delivery systems. The unique beneficial properties of PEI (polycationic structure, proton sponge ability, readily functionalizable) allow its continued development for delivery of siRNA therapeutics, however, its lack of degradability remains an issue. Other polymers traditionally used for plasmid delivery such as polysaccharides and polypeptides have been modified to fit siRNA applications. In addition, the small size of siRNAs allows the introduction of direct conjugation techniques that are not available with large plasmids.

Grzelinski et al. [122] and Urban-Klei et al. [123] have reported two studies in which a commercially available form of low molecular weight LPEI, jetPEI, was used for intraperitoneal and systemic delivery of siRNA formulations targeting pleiotrophin (PTN) and the c-erbB2/neu (HER-2) receptor, respectively. Physical characterization of the PEI/siRNA complexes was performed with AFM at an N : P ratio of 10 : 1 where the particles showed a mean complex diameter of 42 nm with no free siRNA visible in the field. In addition, RNase cleavage of the siRNA in the presence of fetal calf serum (FCS) was not observed. After IP administration in murine models, subcutaneous xenograft tumor growth was reduced compared to naked siRNA and nonsilencing controls, which the authors attribute to siRNA-mediated downregulation of the targets after tumor cell uptake. In addition, no IFN-α or TNF-α response was observed as a result of siRNA-induced off-target effects.

In an effort to utilize high-molecular-weight BPEI for siRNA delivery, Schiffelers et al. [124] reported the development of a BPEI–PEG–RGD system for the systemic delivery of siRNA to solid tumors. A heterobifunctional PEG (NHS–PEG–VS) (3400 Da) was used as a macrolinker between BPEI (25 kDa) and RGD. RGD was first attached to PEG via the NHS group, then the RGD–PEG–VS coupled to BPEI through the vinyl sulfone group. The degree of RGD–PEG conjugation to BPEI was found to be 7% of the primary amines, or an average of about 40 RGD–PEG molecules attached to each BPEI molecule. Over a range of N : P ratios, RGD–PEG–BPEI/siRNA complexes displayed particle sizes distributed around 100 nm and zeta potentials of 5 mV. Intravenous injection into tumor-bearing mice in luciferase plasmid DNA-cotransfection experiments resulted in ligand-dependent reduction in luciferase signal. RGD–PEG–BPEI was used to deliver siRNA targeting VEGF receptor 2, and although the authors were not able to report a reduction in VEGF receptor 2 messenger RNA (mRNA) or protein levels, tumor vascular was reduced compared to naked siRNA, which suggests that the observed efficacy was a result of low target inhibition or a nonspecific effect of the siRNA delivery.

Heidel et al. [125–127] employed β-cyclodextrin derivatives to target siRNA-containing nanoparticles via a Tf peptide ligand to tumors after intravenous injection in nonhuman primates. The β-cyclodextrin (CDP) derivative employed in this study contained Tf–PEG–adamantane units that

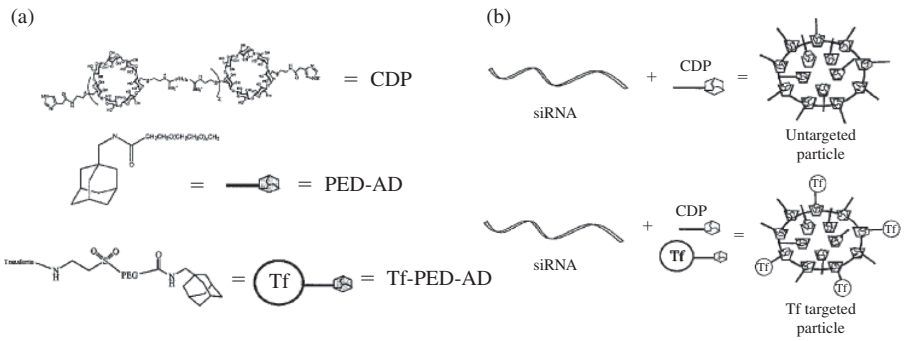

FIGURE 15.3 Configuration of CDP-AD-PEG-Tf delivery system [126].

form inclusion complexes within the β-cyclodextrin structure (CDP–AD–PEG–Tf). The cationic nature of the β-cyclodextrin units allowed the complexation of the nucleic acids (Figure 15.3). The CDP–AD–PEG–Tf/siRNA particle sizes were ~70 nm and were shown to be stable in physiologic fluid and demonstrated ability to protect the nucleic acid from nuclease degradation for up to 72 h. SiRNA targeting the M2 subunit of ribonucleotide reductase was formulated with CDP–AD–PEG–Tf and at a dose as high as 27 mg/kg produced only small diet-related toxicity responses. In addition, repeated doses over 18 days did result in an antibody response to the Tf ligand. In a separate study, CDP–AD–PEG–Tf was able to target Tf-receptor–expressing tumor cells after tail-vein administration in mice [109, 127].

Several groups have developed a method to eliminate the need for cationic polymers to bind anionic nucleic acids for delivery. An elegant approach was taken by Rozema et al. [128] to conjugate the siRNA molecule directly to the polymer via a degradable linkage in order to target apolipoprotein B (*apo*B) to hepatocytes. The structure of the polymeric system contained a latent endosomalytic segment that was designed to be triggered within the endosomes' acidic environment. The backbone polymer was synthesized by the polymerization of 2-vinyloxyethylphthalamide and either methyl vinyl ether, ethyl vinyl ether, propyl vinyl ether, or butyl vinyl ether. Among the vinyl polymers synthesized, the butyl derivative (PBAVE) produced the highest in vitro transfection levels and was therefore used for further modification [129]. The backbone polymer contained primary amine-terminated side chains that were first modified by the addition of 4-succinimidyloxycarbonyl-α-methyl-α-[2-pyridyldithio]toluene (SMPT) to give protected disulfide-terminated side chains. Amine-terminated siRNA was activated by the addition of SATA to give a protected thiol group. PBAVE–SMTP and siRNA–SATP were added to a glucose solution containing TAPS buffer, which served to cleave the thioacetyl group from SATP, thereby revealing free thiol-terminated siRNA for disulfide exchange with the SMTP group of the polymer. On average, 70–90% of the siRNA added to the mixture was successfully conjugated to the polymer. Two additional

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components of the delivery system were then added that contained the triggered endosomalytic mechanism, carboxydimethyl maleic anhydride (CDM), *N*-acetyl galactosamine (NAG), and PEG (450 Da). The NAG–CDM and PEG–CDM were synthesized from the acid chloride generated by adding oxalyl chloride [130]. The resulting polymer–siRNA conjugate, termed dynamic polyconjugate (DPC), contained an average of one siRNA molecule, and as a result of the side-chain-terminal primary amines being "muted" by the addition of either NAG–CDM or PEG–CDM, the overall charge of the 10-nm particles was negative. The NAG ligand served to target the asialoglycoprotein receptor displayed on the surface of hepatocytes. The CDM linkage was reversible, cleaved at acidic pH, which then induced the proton sponge effect by revealing primary amines and also by dramatically increasing the overall molecule number within the endosome (1 molecular conjugate became 1 polymer, 1 siRNA, several PEG, and several NAG). SiRNA conjugated to DPC targeting *apo*B and delivered via tail-vain injection resulted in sequence-specific knockdown of *apo*B mRNA by 87% and in a reduction of total serum cholesterol levels by 42%. The reduced levels of *apo*B persisted for 10 days and returned to normal levels in 2 weeks. Using fluorescence staining, the activity of the DPCs was demonstrated to be confined to hepatocytes rather than Kupffur cells, thereby validating the effectiveness of liver targeting with the NAG ligand.

Kim et al. [131] have also developed a delivery system that involves the covalent modification of siRNA, with the added step of formulation with PEI. VEGF siRNA was synthesized bearing a 3′-terminal hexylamine group on the sense strand, which was capped with *N*-succinimidyl-3-(2-pyridylthio)propionate (SPDP), a protected disulfide terminated with an activated linker for conjugation to primary amines. The siRNA was coupled via disulfide exchange to mPEG-SH (5000 Da) to give siRNA-S-S-PEGm. The micelles were obtained by the addition of BPEI (25 kDa) to a concentrated solution of siRNA-S-S-PEGm. The siRNA conjugates were shown to be cleaved by 10 mM glutathione, however, the entire complex retained its integrity over 48 h in serum-conditioned medium [132]. The micelles displayed a spherical morphology with a size of <80 nm based on AFM measurements. Interestingly, the hydrodynamic diameters determined by light scattering of the same particles gave values of $\sim$20 nm larger. Intratumoral injection of the siRNA–PEG/BPEI micelles resulted in significant reduction in VEGF mRNA and protein levels within the treated tumors compared to nonsilencing and BPEI-only controls. In addition, IFN-α levels were largely unchanged by the administration, suggesting the siRNA was effectively shielded from immune system surveillance through PEG conjugation.

15.5 CLINICAL DEVELOPMENT OF POLYMERIC DELIVERY SYSTEMS

Clinical development of polymeric gene carriers has been sluggish. Only a small number of polymers have advanced into the clinic. An overview of the clinical

advancement of polymeric gene carriers by disease indications is provided in the following sections. The general synthetic methods, formulations, and gene transfection properties of these polymeric carriers have been described in earlier sections.

15.5.1 Oncology

Cancer represents a group of diseases that result from an uncontrolled proliferation of cells. Current cancer therapies are far from ideal as many of them have low response rates and serious side effects. There is an urgent need for safer and more effective therapies for cancer. Modulation of gene expression by gene therapy or RNAi therapy offers a new class of potential treatments that are distinct from conventional therapeutics. Cancer gene therapy approaches that utilize polymeric carriers are described in this chapter.

LPEI (22 kDa) was evaluated for local gene delivery of diphtheria toxin antigen H19 in patients with refractory superficial transitional cell carcinoma of the bladder [30]. Intravesicular administration of H19 gene plasmid formulated with LPEI into two bladder cancer patients was safe. Interestingly, there was no evidence of treatment-related local or systemic toxicity previously reported with the use of large molecular weight PEIs in animal studies. The antigen DNA was detected in urine samples but not in blood samples that demonstrated that the plasmid DNA did not distribute into the systemic circulation. Analysis of bladder tissue showed significant uptake of the toxin plasmid by tumor tissue. Video-cystoscopy performed 6 weeks after treatment showed reduction in tumor size by 75% compared to pretreatment size. This study demonstrates that gene transfer with high-molecular-weight PEI is safe if administered locally.

A low-molecular-weight BPEI (1800 Da) functionalized by covalent attachment of cholesterol and PEG (550 Da) (PEG−PEI−cholesterol) (PPC) is in clinical development for local gene delivery of IL-12 into women with recurrent ovarian cancer. IL-12 is one of the most potent anticancer cytokines that works by activation of the natural and acquired immune systems against cancer and inhibition of tumor angiogenesis. A phase I trial to assess the safety and tolerability of human IL-12 plasmid (phIL-12) formulated with PPC polymer was conducted in women with chemotherapy-resistant recurrent ovarian cancer. A total of 13 patients were enrolled in four dose-escalating cohorts and treated with 0.6, 3, 12, or 24 mg/m^2 of the formulated plasmid once a week for 4 weeks. The phIL-12/PPC delivery was generally safe and well tolerated. Common side effects included low-grade fever and abdominal pain. High concentrations of IL-12 plasmid were detected in peritoneal fluid samples while approximately 1000-fold lower concentrations were detected in blood samples. The biological activity of IL-12 was also confined to peritoneal cavity as little activity was found in serum. These data demonstrate that IL-12 gene delivery with a functionalized low-molecular-weight BPEI is safe and offers therapeutic benefits to ovarian cancer patients. The safety and activity of PPC

formulated IL-12 plasmid is now being examined in conjunction with platinum/carboplatin/docetaxel in women with chemotherapy-sensitive ovarian cancer. The preliminary results have shown that the addition of IL-12/PPC to standard chemotherapy for treatment of ovarian cancer is safe. Based on promising phase I results the IL-12/PPC product is ready for advancement into phase II testing in ovarian cancer patients [42, 133, 134].

15.5.2 HIV–AIDS

A functionalized cationic polymer based on a mannose-conjugated PEI is being developed for the delivery of a DNA vaccine against HIV after a successful preclinical testing in macaques [70, 135]. The antigen DNA is formulated to mannose–LPEI nanoparticles to target antigen-presenting cells and to protect the DNA from intracellular degradation. In SIV-infected macaques, topically administered PEI-mannose-formulated DNA antigen successfully transfected dendritic cells and induced an immune response against the virus. This vaccine product is currently under clinical evaluation for safety and immunogenicity as a single agent or in combination with antiviral therapy in phase I and phase II human studies. To our knowledge, the clinical results from these trials have not been published.

15.5.3 Cardiovascular Disease

Peripheral vascular disease is a prevalent and disabling disease affecting over 10 million people in the United States. The disease is characterized by intermittent claudication resulting from arterial atherosclerosis of peripheral blood vessel, impairing blood flow to the lower limbs. Growth factors, such as vascular endothelial growth factor (VEGF-A), have been shown to promote angiogenesis and improve blood flow to the lower limbs in animal models. Gene therapy is an attractive approach to peripheral vascular disease since it can provide expression of a vascular growth factor for a long period of time in the affected limb. The use of naked DNA for this indication has its limitations due to low transfection efficiency. Noncondensing polymers such as poloxamers have been shown to produce good efficacy results in preclinical models of peripheral vascular disease. In a phase II clinical trial a plasmid encoding an angiomatrix protein Del-1 was formulated with poloxamer 188 and administered by intramuscular injection into patients with moderate to severe peripheral arterial disease [136]. One-hundred five patients randomized to treatment and control groups received Del-1 plasmid formulation with poloxamer 188 or poloxamer 188 alone as 21 intramuscular injections to each lower extremity. The treatment was safe and produced a significant increase in the mean peak walking time, claudication onset time, and ankle brachial index compared to baseline values in both treatment and control groups. Both groups also demonstrated significantly improved quality of life at follow-up compared to baseline. None of the serious adverse events were determined to be treatment

related. Interestingly, both efficacy and adverse events were not significantly different between the treatment and control group, which suggests that the delivery polymer by itself has biological activity useful for treatment of peripheral arterial disease. In another human study, poloxamer 407 was used to deliver plasmid DNA encoding a zinc finger protein transcription factor engineered to enhance the VEGF expression in skeletal muscle of patients with intermittent claudication [137]. The results from this randomized, double-blind, dose-escalation, placebo-controlled study, designed to determine the safety, preliminary efficacy, and biological activity of the poloxamer-formulated plasmid, have not been published.

Wloch et al. [138] used a poloxamer-based formulation to deliver a viral vaccine in human subjects. VCL-CB01, a test cytomegalovirus (CMV) DNA vaccine that contains plasmids encoding CMV phosphoprotein 65 (pp65) and glycoprotein B (gB) was formulated with poloxamer CRL1005 and a cationic surfactant BAK to induce cellular and humoral immune responses in 44 healthy adult subjects. Thirty-two subjects received 1- or 5-mg doses of vaccine on a 0-, 2-, and 8-week schedule, and 12 subjects received 5-mg doses of vaccine on a 0-, 3-, 7-, and 28-day schedule. The vaccine was generally well tolerated, with no serious adverse events. Common adverse events included mild to moderate injection site pain and tenderness, induration, erythema, mild to moderate malaise, and myalgia. Evidence of immunogenicity including IFN-γ and T-cell responses were observed in several of the treated patients.

15.5.4 Cystic Fibrosis

Cystic fibrosis remains one of the very few genetic diseases that have been targeted by gene therapy. A genetic mutation results in abnormal fluid and electrolyte conductance across the bronchial airways leading to the clinical manifestation of this fatal disease. Previous gene therapy approaches have utilized viral vectors or cationic liposomes to achieve gene transfer of the cystic fibrosis gene with limited success. A polymeric gene carrier, PEG–PLL, has been evaluated for safe and efficient delivery of the cystic fibrosis gene in patients with cystic fibrosis after successful evaluation in animal models [98]. In a double-blind study, a single intranasal administration of escalating doses of cystic fibrosis gene plasmid (0.8, 2.67, and 8.0 mg DNA) formulated with PEG–PLL in 12 cystic fibrosis (CF) patients was safe and well tolerated. There was no evidence of a significant increase in inflammatory mediators in serum or nasal washing. Approximately 0.58 copies of the cystic fibrosis gene were quantified per cell in the nasal scrapings. Partial to complete biological response was achieved in eight subjects with some evidence of a dose trend. The corrections generally persisted for up to 6 days after gene transfer. This study shows gene transfer to nasal epithelium with PEG–PLL is safe and effective. It would be interesting to see if the duration of the observed effects can be extended by repeated delivery since the effect of single dosing lasted for about one week.

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1. Bloomfield, V. Condensation of DNA by multivalent cations: Considerations on mechanism. *Biopolymers* 1991;**31**:1471–1481.
2. Bloomfield, V. DNA condensation. *Curr. Opin. Struct. Biol.* 1996;**6**:334–341.
3. Wolfert, M. and L. Seymour. Atomic force microscopic analysis of the influence of the molecular weight of poly(L)lysine on the size of polyelectrolyte complexes formed with DNA. *Gene Ther.* 1996;**3**:269–273.
4. Adami, R., W. Collard, S. Gupta, K. Kwok, J. Bonadio, and K. Rice. Stability of peptide-condensed plasmid DNA formulations. *J. Pharm. Sci.* 1998;**87**:678–683.
5. Park, S., D. Harries, and W. Gelbart. Topological defects and the optimum size of DNA condensates. *Biophys. J.* 1998;**75**:714–720.
6. Lechardeur D. and G. Lukacs. Intracellular barriers to non-viral gene transfer. *Curr. Gene Ther.* 2002;**2**:183–194.
7. De Smedt, S., J. Demeester, and W. Hennink. Cationic polymer based gene delivery systems. *Pharm. Res.* 2000;**17**:113–126.
8. Wagner, E., M. Cotton, R. Foisner, and M. Birnstiel. Transferrin-polycation-DNA complexes: The effect of polycations on the structure of the complex and DNA delivery to cells. *Proc. Natl. Acad. Sci. U.S.A.* 1991;**88**:4255–4259.
9. Ogris, M., P. Steinlein, M. Kursa, K. Mechtler, R. Kircheis, and E. Wagner. The size of DNA/transferrin-PEI complexes is an important factor for gene expression in cultured cells. *Gene Ther.* 1998;**5**:1425–1433.
10. Pouton, C., P. Lucas, B. Thomas, A. Uduehi, D. Milroy, and S. Moss. Polycation-DNA complexes for gene delivery: A comparison of the biopharmaceutical properties of cationic polypeptides and cationic lipids. *J. Control. Release.* 1998;**53**:289–299.
11. Tang M. and F. Szoka. The influence of polymer structure on the interactions of cationic polymers with DNA and morphology of the resulting complexes. *Gene Ther.* 1997;**4**:823–832.
12. Bielinska, A., J. Kukowska-Latallo, and J. Baker. The interaction of plasmid DNA with polyamidoamine dendrimers: Mechanism of complex formation and analysis of alterations induced in nuclease sensitivity and transcriptional activity of the complexed DNA. *Biochim. Biophys. Acta* 1997;**1353**:180–190.
13. Haensler, J. and F. Szoka. Polyamidoamine cascade polymers mediate efficient transfection of cells in culture. *Bioconjug. Chem.* 1993;**4**:372–379.
14. Ruponen, M., S. Yla-Herttuala, and A. Urtti. Interactions of polymeric and liposomal gene delivery systems with extracellular glycosaminoglycans: Physico-chemical and transfection studies. *Biochim. Biophys. Acta* 1999;**1415**:331–341.
15. Kabanov, V. and A. Kabanov. Interpolyelectrolyte and block ionomer complexes for gene delivery: Physico-chemical aspects. *Adv. Drug Deliv. Rev.* 1998;**30**:49–60.
16. Smith, L., J. Duguid, M. Wadhwa, M. Logan, C. Tung, V. Edwards, and J. Sparrow. Synthetic peptide-based DNA complexes for nonviral gene delivery. *Adv. Drug Deliv. Rev.* 1998;**30**:115–131.
17. Izumrudov, V., M. Zhiryakova, S. Kargov, A. Zezin, and V. Kabanov. Competitive reactions in solutions of DNA-containing polyelectrolyte complexes. *Macromol. Symp.* 1996;**106**:179–192.

18. Boussif, O., F. Lezoualc'h, M. Zanta, M. Mergny, D. Scherman, B. Demeneix, and J. A. Behr. Versatile vector for gene and oligonucleotide transfer into cells in culture and in vivo : Polyethyleneimine. *Proc. Natl. Acad. Sci. U.S.A.* 1995;**92**:7297–7301.
19. Garnett, M. Gene delivery systems using cationic polymers. *Crit. Rev. Ther. Drug Carrier Syst.* 1999;**15**:147–207.
20. Godbey, W., K. Wu, and A. Mikos. Size matters: Molecular weight affects the efficacy of poly(ethylenimine) as a gene delivery vehicle. *J. Biomed. Mater. Res.* 1999;**45**:268–275.
21. Behr, J. Gene transfer with synthetic cationic amphiphiles: Prospects for gene therapy. *Bioconjug. Chem.* 1994;**5**:382–389.
22. Kunath K., A. von Harpe, D. Fisher, H. Peterson, U. Bickel, K. Voigt, and T. Kissel. Low-molecular-weight polyethylenimine as a non-viral vector for DNA delivery: comparison of physicochemical properties, transfection efficiency and in vivo distribution with high-molecular-weight polyethylenimine. *J. Control. Release* 2003;**89**:113–125.
23. Abdallah, B., A. Hassan, C. Benoist, D. Goula, J. P. Behr, and A. Mazabraud. A powerful nonviral vector for in vivo gene transfer into the adult mammalian brain: Polyethylenimine. *Hum. Gene Ther.* 1996;**7**:1947–1954.
24. Boletta, A., A. Benigni, J. Lutz, G. Remuzzi, M. Soria, and L. Monaco. Nonviral gene delivery to the rat kidney with polyethyleneimine. *Hum. Gene Ther.* 1997;**8**:1243–1251.
25. Furgeson, D. and S. Kim. Linear PEI-cholesterol conjugates for the LDL-R pathway. *Mol. Ther.* 2003;**7**:S372.
26. Gharwan, H., L. Wightman, R. Kercheis, R. Wagner, and K. Zatloukal. Nonviral gene transfer into fetal mouse livers (a comparison between the cationic polymer PEI and naked DNA). *Gene Ther.* 2003;**10**:810–817.
27. Wightman, L., R. Kircheis, V. Rossler, S. Carotta, R. Ruzicka, M. Kursa, and E. Wagner. Different behavior of branched and linear polyethylenimine for gene delivery in vitro and in vivo. *J. Gene Med.* 2001;**3**:362–372.
28. Burke, R. and S. Pun. Extracellular barriers to in vivo PEI and PEGylated PEI polyplex-mediated gene delivery to the liver. *Bioconj. Chem.* 2008;**19**:693–704.
29. Chollet, P., M. Favrot, A. Hurbin, and J. Coll. Side-effects of a systemic injection of linear polyethyleneimine-DNA complexes. *J. Gene Med.* 2002;**4**:84–91.
30. Ohana, P., O. Gofrit, S. Ayesh, W. Al-Sharef, A. Mizrahi, T. Birman, T. Schneider, I. Matouk, N. deGroot, E. Tavdy, A. Ami Sidi, and A. Hochberg. Regulatory sequences of the H19 gene in DNA based therapy of bladder cancer. *Gene Ther. Mol. Biol.* 2004;**8**:181–192.
31. Harris, J. M. *PEG: Chemistry and Biological Applications*. American Chemical Society: Washington, DC, 1997.
32. Kakizawa, Y. and K. Kataoka. Block copolymer micelles for delivery of gene and related compounds. *Adv. Drug Deliv. Rev.* 2002;**54**:203–222.
33. Oupicky, D., K. Howard, C. Konak, P. Dash, K. Ulbrich, and L. Seymour. Steric stabilization of poly-L-Lysine/DNA complexes by the covalent attachment of semitelechelic poly[N-(2-hydroxypropyl)methacrylamide]. *Bioconjug. Chem.* 2000; **11**:492–501.

34. Ogris, M., S. Brunner, S. Schuller, R. Kircheis, and E. Wagner. PEGylated DNA/transferrin-PEI complexes: Reduced interaction with blood components, extended circulation in blood and potential for systemic gene delivery. *Gene Ther*. 1999;**6**:595–605.

35. Katayose, S. and K. Kataoka. Remarkable increase in nuclease resistance of plasmid DNA through supramolecular assembly with poly(ethylene glycol)-poly (L-lysine) block copolymer. *J. Pharm. Sci*. 1998;**87**:160–163.

36. Lim, D., Y. Yeom, and T. Park. Poly(DMAEMA-NVP)-*b*-PEG-galactose as gene delivery vector for hepatocytes. *Bioconjug. Chem*. 2000;**11**:688–695.

37. Kwok, K., D. Mckenzie, D. Evers, and K. Rice. Formulation of highly soluble poly (ethylene glycol)-peptide DNA condensates. *J. Pharm. Sci*. 1999;**88**:996–1003.

AQ10 38. Merdan et al.

39. Hong, J., J. Park, K. Huh, H. Chung, I. Kwon, and S. Jeong. PEGylated polyethyleneimine for in vivo local gene delivery based on lipiodolized emulsion system. *J. Control. Release* 2004;**99**:167–176.

40. Neu, M., J. Sitterberg, U. Bakowsky, and T. Kissel. Stabilized nanocarriers for plasmids based upon cross-linked poly(ethyleneimine). *Biomacromolecules* 2006;**7**:3428–3438.

41. Neu, M., O. Germershaus, M. Behe, and T. Kissel. Bioreversably crosslinked polyplexes of PEI and high molecular weight PEG show extended circulation times in vivo. *J. Control. Release* 2007;**124**:69–80.

AQ11 42. Fewell, J., M. Matar, Slobodkin, Han, S., J. Rice, B. Hovanes, D. Lewis, and K. Anwer. Synthesis and characterization of a non-viral gene delivery system for immunogene therapy of cancer. *J. Control. Release* 2005;**109**:288–298.

43. Fewell, J., J. Rice, M. Matar, and K. Anwer. Safety and toxicity following intraperitoneal injection of murine interleukin-12 plasmid formulated with a novel polymeric delivery system. *Mol. Ther*. 2006;**13**:S109(#287).

44. Brunhoeber, E., M. Matar, K. Anwer, and J. Fewell. Biodistribution and clearance following intraperitoneal injection of murine interleukin-12 plasmid formulated with a novel polymeric delivery system. *Mol. Ther*. 2006;**13**:S109(#286).

45. Sonabend, A., S. Velicu, I. Ulasov, Y. Han, B. Tyler, H. Brem, M. Matar, J. Fewell, K. Anwer, and M. Lesniak. A safety and efficacy study of local delivery of IL-12 transgene by PPC polymer in a model of experimental glioma. *Anticancer Drug* 2008;**19**:133–142.

46. Tang, G., H. Guo, F. Alexis, X. Wang, S. Zeng, T. Lim, J. Ding, Y. Yang, and S. Wang. Low molecular weight polyethyeneimines linked by -cyclodextrin for gene transfer into the nervous system. *J. Gene Med*. 2006;**8**:736–744.

47. Wong, K., G. Sun, X. Zhang, H. Dai, Y. Liu, C. He, and K. Leong. PEI-*g*-chitosan, a novel gene delivery system with transfection efficiency comparable to polyethyleneimine in vitro and after liver administration in vivo. *Bioconj. Chem*. 2006;**17**:152–158.

48. Gosselin, M., W. Guo, and R. Lee. Efficient gene transfer using reversibly cross-linked low-molecular-weight polyethyleneimine. *Bioconj. Chem*. 2001;**12**:989–994.

49. Thomas, M., Q. Ge, J. Lu, J. Chen, and A. Klibanov. Cross-linked small polyethyleneimines: While still nontoxic, deliver DNA efficiently to mammalian cells in vitro and in vivo. *Pharm. Res*. 2005;**22**:373–380.

50. Matar, M., G. Slobodkin, A. Rea-Ramesy, E. Brunhoeber, J. Skoyen, J. Fewell, D. Lewis, and K. Anwer. Synthesis and characterization of low-molecular-weight linear polyethyleneimines for gene delivery. *J. Biomed. Nanotech*. 2006;**2**:53–61.

AQ10 51. Ahn, 2002.

52. Neu, M., O. Germershaus, S. Mao, K. Voigt, M. Behe, and T. Kissel. Crosslinked nanocarriers based upon poly(ethylene imine) for systemic plasmid delivery: In vitro characterization and in vivo studies in mice. *J. Control. Release* 2007;**118**,: 370–380.

53. Park., M., H. Kim, C. Hwang, K. Han, Y. Choi, S. Song, M. Cho, and C. Cho. Highly efficient gene transfer with degradable poly(ester amine) based on poly (ethylene glycol) diacrylate and polyethyleneimine in vitro and in vivo. *J. Gene Med*. 2008;**10**:198–207.

54. Hart, S., R. Harbottle, R. Cooper, A. Miller, R. Williamson, and C. Coutelle. Gene delivery and expression mediated by an integrin-binding peptide. *Gene Ther*. 1995;**2**:552–554.

55. Sakae, M., T. Ito, C. Yoshihara, N. Iida-Tanaka, H. Yanagie, M. Eriguchi, and Y. Koyama. Highly efficient in vivo gene transfection by plasmid/PEI complexes coated by anionic PEG derivatives bearing carboxyl groups and RGD peptide. *Biomed. Pharmacother*. 2008;**62**:448–453.

56. Yockman, J., W. Kim, C. Chang, and S. Kim. Non-viral delivery of interleukin-2 and soluble Flk-1 inhibits metastatic and primary tumor growth in renal cell carcinoma. *Gene Ther*. 2007;**14**:1399–1405.

57. Suh, W., S. Han, L. Yu, and S. Kim. An angiogenic, endothelial-cell-targeted polymeric gene carrier. *Mol. Ther*. 2002;**6**:664–672.

58. Kim, W., J. Yockman, J. Jeong, J. Christensen, M. Lee, Y. Kim, and S. Kim. Anti-angiogenic inhibition of tumor growth by systemic delivery of PEI-*g*-PEG-RGD/ pCMV-sFlt-1 complexes in tumor-bearing mice. *J. Control. Release* 2006;**114**:381–388.

59. Kleeman, E., M. Neu, N. Jekel, L. Fink, T. Schmehl, T. Gessler, W. Seeger, and T. Kissel. Nanocarriers for DNA delivery to the lung base upon a TAT-derived peptide covalenly coupled to PEG-PEI. *J. Control. Release* 2005;**109**:299–316.

60. Zeng, J., X. Wang, and S. Wang. Self-assembled ternary complexes of plasmid DNA, low molecular weight polyethyleneimine and targeting peptide for nonviral gene delivery into neurons. *Biomaterials* 2007;**28**:1443–1451.

61. Rudolph, C., N. Sieverling, U. Schillinger, E. Lesina, C. Plank, A. Thunemann, H. Schonberger, and J. Rosenecker. Thyroid hormone (T3)-modification of poly-ethyleneglycol (PEG)–polyethyleneimine (PEI) graft copolymers for improved gene delivery to hepatocytes. *Biomaterials* 2007;**28**:1900–1911.

62. Elfinger, M., C. Maucksch, and C. Rudolph. Characterization of lactoferrin as a targeting ligand for nonviral gene delivery to airway epithelial cells. *Biomaterials* 2007;**28**:3448–3455.

63. Moffatt, S., C. Papasakelariou, S. Wiehle, and R. Cristiano. Successful in vivo tumor targeting of prostate-specific membrane antigen with a highly efficient J591/ PEI/DNA molecular conjugate. *Gene Ther*. 2006;**13**:761–772.

64. Moffatt, S., S. Wiehle, and R. Cristiano. Tumor-specific gene delivery mediated by a novel peptide-polyethyleneimine-DNA polyplex targeting aminopeptidase N/ CD13. *Human Gene Ther*. 2005;**15**:57–67.

65. Kim, E., H. Jeong, I. Park, C. Cho, H. Moon, D. Yu, H. Bom, M. Sohn, and I. Oh. Asialoglycoprotein receptor targeted gene delivery using galactosylated polyethyleneimine-*graft*-poly(ethylene glycol): In vitro and in vivo studies. *J. Control. Release* 2005;**108**:557–567.

AQ10 66. Cook 2005.

67. Fajac, I., G. Thevenot, L. Bedouet, C. Daniel, M. Riquet, M. Merten, C. Figarella, J. Dall'Ava-Santucci, M. Monsigny, and P. Briand. Uptake of plasmid/glycosylated polymer complexes and gene transfer efficiency in differentiated airway epithelial cells. *J. Gene Med.* 2003;**5**:38–48.

68. Grosse, S., G. Thevenot, Y. Aron, E. Duverger, M. Abdelkarim, A. Roche, M. Monsigny, and I. Fajac. In vivo gene delivery in the mouse lung with lactosylated polyethyleneimine, questioning the relevance of in vitro experiments. *J. Control. Release* 2008;**132**:105–112.

AQ10 69. Lisziewicz et al. 2004.

70. Lisziewicz, J., J. Trocio, L. Whitman, G. Varga, J. Xu, N. Bakare, P. Erbacher, C. Fox, R. Woodward, P. Markham, S. Arya, J. Behr, and F. Lori. DermaVir: A novel topical vaccine for HIV/AIDS. *J. Invest. Dermatol.* 2005;**124**:160–169.

71. Nguyen, D., S. Raghavan, L. Tashima, E. Lin, S. Fredette, R. Langer, and C. Wang. Enhancement of poly(orthoester) microspheres for DNA vaccine delivery by blending with poly(ethyleneimine). *Biomaterials* 2008;**29**:2783–2793.

72. Turk, M., S. Dincer, and E. Piskin. Smart and cationic poly(NIPA)/PEI block copolymers as nonviral vectors: In vitro and in vivo transfection studies. *J. Tissue Eng. Reg. Med.* 2007;**1**:377–388.

73. Lei, Y. and T. Segura. DNA delivery from matrix metalloprotease degradable poly (ethylene glycol) hydrogels to mouse mesenchymal stem cells. *Biomaterials* 2009;**30**:254–265.

74. Arote, R., T. Kim, Y. Kim, S. Hwang, H. Jiang, H. Song, J. Nah, M. Cho, and C. Cho. A biodegradable poly(ester amine) based on polycaprolactone and polyethyleneimine as a gene carrier. *Biomaterials* 2007;**28**:735–744.

AQ10 75. Langer et al.

76. Lynn, D., D. Anderson, D. Putnam, and R. Langer. Accelerated discovery of synthetic transfection vectors: Parallel synthesis and screening of a degradable polymer library. *J. Am. Chem. Soc.* 2001;**123**:8155–8166.

77. Lynn, D. and R. Langer. Degradable poly(β-amino esters): Synthesis, characterization and self-assembly with plasmid DNA. *J. Am. Chem. Soc.* 2000;**122**: 10761–10768.

78. Green, J., G. Zugates, N. Tedford, Y. Huang, L. Griffith, D. Lauffenburger, J. Sawicki, R. Langer, and D. Anderson. Combinatorial modification of degradable polymers enables transfection of human cells comparable to adenovirus. *Adv. Mater.* 2007;**19**:2836–2842.

79. Tomalia, D., A. Naylor, and W. Goddard. Starburst dendrimers: molecular-level control of size, shape, surface chemistry, topology, and flexibility from atoms to macroscopic matter. *Angew. Chem. Int. Ed. Engl.* 1990;**29**:138–175.

80. Tang, M., C. Redemann, and F. Szoka. In vitro gene delivery by degraded polyamidoamine dendrimers. *Bioconj. Chem.* 1996;**7**:703–714.

81. Mamede et al.

82. Malik, R., R. Wiwattanapatapee, R. Klopsch, K. Lorenz, H. Frey, J. Weener, E. Meijer, W. Paulus, and R. Duncan. Dendrimers: Relationship between structure and biocompatibility in vitro, and preliminary studies on the biodistribution of ^{125}I-labelled polyamidoamine dendrimers in vivo. *J. Control. Release* 2000;**65**:133–148.

83. Huang, R., W. Ke, Y. Liu, C. Jiang, and Y. Pei. The use of lactoferrin as a ligand for targeting the polyamidoamine-based gene delivery system to the brain. *Biomaterials* 2008;**29**:238–246.

84. Huang, R., W. Ke, Y. Qu, J. Zhu, Y. Pei, and C. Jiang. Characterization of lactoferrin receptor in brain endothelial capillary cells and mouse brain. *J. Biomed. Sci.* 2007;**14**:121–128.

85. Okuda, T., S. Kawakami, N. Akimoto, T. Niidome, F. Yamashita, and M. Hashida. PEGylated lysine dendrimers for tumor-selective targeting after intravenous injection in tumor-bearing mice. *J. Control. Release* 2006;**116**:330–336.

AQ10 86. Ribeiro et al. 2007.

87. Russ, V., H. Elfberg, J. Kloeckner, M. Ogris, and E. Wagner. Novel degradable oligoethyleneimine acrylate ester-based pseudodendrimers for in vitro and in vivo gene transfer. *Gene Ther*. 2008;**15**:18–29.

AQ10 88. Schatzlein et al.

89. Ward, C., M. Pechar, D. Oupicky, K. Ulbrich, and L. Seymour. Modification of pLL/DNA complexes with a multivalent hydrophilic polymer permits folate-mediated targeting in vitro and prolonged plasma circulation in vivo. *J. Gene Med.* 2002;**4**:536–547.

90. Mannisto, P., S. Vanderkerken, V. Toncheva, M. Elomaa, M. Ruponen, E. Schacht, and A. Urtii. Structure-activity relationships of poly(L-lysines): Effects of pegylation and molecular shape on physiochemical and biological properties in gene delivery. *J. Control. Release* 2002;**83**:169–182.

91. Nishikawa, M., Y. Takemura, and M. Hashida. Targeted delivery of plasmid DNA to hepatocytes in vivo: Optimization of the pharmacokinetics of plasmid DNA/galactosylated poly(L-lysine) complexes by controlling their physiochemical properties. *J. Pharmacol. Exp. Ther*. 1998;**287**:408–415.

92. Brown, M., A. Schatzlein, A. Brownlie, V. Jack, W. Wand, L. Tetley, A. Gray, and I. Uchegbu. Preliminary characterization of novel amino acid based polymeric vesicles as gene and drug delivery agents. *Bioconj. Chem*. 2000;**11**:880–891.

93. Harada-Shiba, M., K. Yamauchi, A. Harada, I. Takamisawa, K. Shimokado, and K. Kataoka. Polyion complex micelles as vectors in gene therapy pharmacokinetics and in vivo gene transfer. *Gene Ther*. 2002;**9**:407–414.

94. Lee, M., S. Han, K. Ko, J. Koh, J. Park, J. Yoon, and S. Kim. Repression of GAD autoantigen expression in pancreas cells by delivery of antisense plasmid/PEG-*g*-PLL complex. *Mol. Ther*. 2001;**4**:339–346.

95. Unnamalai, N., B. Kang, and W. Lee. Cationic oligopeptide-mediated delivery of dsRNA for post-transcriptional gene silencing in plant cells. *FEBS Let* 2004; **566**:307–310.

96. Moriguchi, R., K. Kogure, H. Akita, S. Futaki, M. Miyagishi, K. Taira, and H. Harashima. A multifunctional envelope-type nanodevice for novel gene delivery of siRNA plasmids. *Pharm. Nanotech*. 2005;**301**:277–285.

97. Hatefi, A., Z. Megeed, and H. Ghandehari. Recombinant polymer-protein fusion: A promising approach towards efficient and targeted gene delivery. *J. Gene Med.* 2006;**8**:468–476.

98. Konstan, M., P. Davis, J. Wagener, K. Hilliard, R. Stern, L. Milgram, T. Kowalczyk, S. Hyatt, T. Fink, C. Gedeon, S. Oette, J. Payne, O. Muhammad, A. Ziady, R. Moen, and M. Cooper. Compacted DNA nanoparticles administered to the nasal mucosa of cystic fibrosis subjects are safe and demonstrate partial to complete cystic fibrosis transmembrane regulator reconstitution. *Hum. Gene Ther.* 2004;**15**:1255–1269.

99. Liu, G., D. Li, M. Pasumarthy, T. Kowalczyk, C. Gedeon, S. Hyatt, J. Payne, T. Miller, P. Brunovskis, T. Fink, O. Muhammad, R. Moen, R. Hanson, and M. Cooper. Nanoparticles of compacted DNA transfect postmitotic cells. *J. Biol. Chem.* 2003;**278**:32578–32586.

100. Ziady, A., C. Gedeon, T. Miller, W. Quan, J. Payne, S. Hyatt, T. Fink, O. Muhammad, S. Oette, T. Kowalczyk, M. Pasumarthy, R. Moen, M. Cooper, and P. Davis. Transfection of airway epithelium by stable PEGylated poly-L-lysine DNA nanoparticles in vivo. *Mol. Ther.* 2003;**8**:936–947.

101. Ziady, A., C. Gedeon, O. Muhammad, V. Stillwell, S. Oette, T. Fink, W. Quan, T. Kowalczyk, S. Hyatt, J. Payne, A. Peischl, J. Seng, R. Moen, M. Cooper, and P. Davis. Minimal toxicity of stabilized compacted DNA nanoparticles in the murine lung. *Mol. Ther.* 2003;**8**:948–956.

102. Itaka, K., S. Ohba, K. Miyata, H. Kawaguchi, K. Nakamura, T. Takato, U. Chung, and K. Kataoka. Bone regeneration by regulated in vivo gene transfer using biocompatible polyplex nanomicelles. *Mol. Ther.* 2007;**15**:1655–1662.

103. Kanayama, N., S. Fukeshima, N. Nishiyama, K. Itaka, W. Jang, K. Miyata, Y. Yamasaki, U. Chung, and K. Kataoka. A PEG-based biocompatible block catiomer with high buffering capacity for the construction of polyplex micelles showing efficient gene transfer toward primary cells. *Chem. Med. Chem.* 2006;**1**:439–444.

104. Mumper, R., J. Wang, J. Claspell, and A. Rolland. Novel polymeric condensing carriers for gene delivery. *Proc. Int. Symp. Control. Release Bioactive Mater.* 1995;**22**:178–179.

105. MacLaughlin, F., R. Mumper, J. Wang, J. Tagliaferri, I. Gill, M. Hinchcliffe, and A. Rolland. Chitosan and depolymerized chitosan oligomers as condensing carriers for in vivo plasmid delivery. *J. Control. Release* 1998;**56**:259–272.

106. Koping-Hoggard, M., K. Varum, M. Issa, S. Danielson, B. Christiensen, B. Stokke, and P. Artursson. Improved chitosan-mediated gene delivery based on easily dissociated chitosan polyplexes of highly defined chitosan oligomers. *Gene Ther.* 2004;**11**:1441–1452.

107. Jiang, H., J. Kwon, Y. Kim, E. Kim, R. Arote, H. Jeong, J. Nah, Y. Choi, T. Akaike, M. Cho, and C. Cho. Galactosylated chitosan-*graft*-polyethyleneimine as a gene carrier for hepatocyte targeting. *Gene Ther.* 2007;**14**:1389–1398.

108. Li, S. and W. Purdy. Cyclodextrins and their applications in analytical chemistry. *Chem. Rev.* 1992;**92**:1457–1470.

109. Davis, M., S. Pun, N. Bellocq, T. Reineke, S. Popielarski, S. Mishra, and J. Heidel. Self assembling nucleic acid delivery vehicles via linear, water soluble, cyclodextrin containing polymers. *Curr. Med. Chem.* 2004;**11**:179–197.

110. Pun, S., N. Bellocq, A. Liu, G. Jensen, T. Machemer, E. Quijano, T. Schluep, S. Wen, H. Engler, J. Heidel, and M. Davis. Cyclodextrin-modified polyethyleneimine polymers for gene delivery. *Bioconj. Chem*. 2004;**15**:831–840.

111. Rolland, A. and R. Mumper. Plasmid delivery to muscle: Recent advances in polymer delivery systems. *Ad. Drug Deliv. Rev*. 1998;**30**:151–172.

112. Hartikka, J., A. Geall, V. Bozoukova, D. Kurniadi, D. Rusalov, J. Enas, J. Yi, A. Nanci, and A. Rolland. Physical characterization and in vivo evaluation of poloxamer-based DNA vaccine formulations. *J. Gene Med*. 2008;**10**:770–782.

AQ10 113. Roques et al. 2007.

114. Zhong, J., B. Eliceiri, D. Stupack, K. Penta, G. Sakamoto, T. Quertermous, M. Coleman, N. Boudrou, and J. Varner. Neovascularization of ischemic tissues by gene delivery of the extracellular matrix protein Del-1. *J. Clin. Invest*. 2003;**112**:30–41.

115. Anwer, K., B. Rhee, and S. Mendiratta. Recent progress in polymeric gene delivery systems. *Crit. Rev. Ther. Drug*. 2003;**20**:249–293.

116. Chang, C., D. Choi, W. Kim, J. Yockman, L. Christensen, Y. Kim, and S. Kim. Non-ionic amphiphilic biodegradable PEG-PLGA-PEG copolymer enhances gene delivery efficiency in rat skeletal muscle. *J. Control. Release* 2007;**118**:245–253.

117. Zentner, G., R. Rathi, C. Shih, J. McRea, M. Seo, H. Oh, B. Rhee, J. Mestecky, Z. Moldoveanu, M. Morgan, and S. Weitman. Biodegradable block copolymers for delivery of proteins and water-insoluble drugs. *J. Control. Release*. 2001;**72**: 203–215.

118. Ronneberger, B., W. Kao, J. Anderson, and T. Kissel. In vivo biocompatibility study of ABA triblock copolyemers consisting of poly(*L*-lactic-*co*-glycolic acid) A blocks attached to central poly(oxyethylene) B blocks. *J. Biomed. Mater. Res*. 1996;**30**:31–40.

119. Blum et al. 2008.

AQ10 120. Csaba et al. 2006.

AQ10 121. Bhavsar et al. 2007.

122. Grzelinski, M., B. Urban-Klein, T. Martens, K. Lamszus, U. Bakowsky, S. Hobel, F. Czubayko, and A. Aigner. RNA interference-mediated gene silencing of pleiotrophin through polyethyleneimine-complexed small interfering RNAs in vivo exerts antitumoral effects in glioblastoma xenografts. *Hum. Gene Ther*. 2006;**17**:751–766.

123. Urban-Klein, B., S. Werth, S. Abuharbeid, F. Czubayko, and A. Aigner. RNAi-mediated gene-targeting through systemic application of polyethyleneimine (PEI)-complexes siRNA in vivo. *Gene Ther*. 2005;**12**:461–466.

124. Schiffelers, R., A. Ansari, J. Xu, Q. Zhou, Q. Tang, G. Storm, G. Molema, P. Lu, P. Scaria, and M. Woodle. Cancer siRNA therapy by tumor selective delivery with ligand-targeted sterically stabilized nanoparticle. *Nucleic Acids Res*. 2004;**32**:e149.

125. Heidel, J., Z. Yu, J. Liu, S. Rele, Y. Liang, R. Zeidan, D. Kornbrust, and M. Davis. Administration in non-human primates of escalating intravenous doses of targeted nanoparticles containing ribonucleotide reductase subunit M2 siRNA. *Proc. Natl. Acad. Sci. U.S.A*. 2007;**104**:5715–5721.

126. Hu-Licskovan, S., J. Heidel, D. Bartlett, M. Davis, and T. Triche. Sequence specific knockdown of EWS-FLI1 by targeted, nonviral delivery of small

interfering RNA inhibits tumor growth in a murine model of metastatic Ewing's sarcoma. *Cancer Res*. 2005;**69**:8584–8992.

127. Pun, S. and M. Davis. Development of a nonviral gene delivery vehicle for systemic application. *Bioconj. Chem*. 2002;**13**:630–639.
128. Rozema, D., D. Lewis, D. Wakefield, S. Wong, J. Klein, P. Roesch, S. Bertin, T. Reppen, Q. Chu, A. Blokhin, J. Hagstrom, and J. Wolff. Dynamic polyconjugates for targeted in vivo delvivery of siRNA to hepatocytes. *Proc. Natl. Acad. Sci. U.S.A*. 2007;**104**:12982–12987.
129. Wakefield, D., J. Klein, J. Wolff, and D. Rozema. Membrane activity and transfection ability of amphipathic polycations as a function of alkyl group size. *Bioconj. Chem*. 2005;**16**:1204–1208.
130. Rozema, D., K. Ekena, D. Lewis, A. Loomis, and J. Wolff. Endosomalysis by masking of a membrane-active agent (EMMA) for cytoplasmic release of macromolecules. *Bioconj. Chem*. 2003;**14**:51–57.
131. Kim, S., J. Jeong, S. Lee, S. Kim, and T. Park. Local and systemic delivery of VEGF siRNA using polyelectrolyte compex micelles for effective treatment of cancer. *J. Control. Release* 2008;**129**:107–116.
132. Kim, S., J. Jeong, S. Lee, S. Kim, and T. Park. PEG conjugated VEGF siRNA for anti-angiogenic gene therapy. *J. Control. Release* 2006;**116**:123–129.
133. Matthews, K., J. Kendrick, M. Straughn, M. Barnes, S. Makhija, J. Fewell, K. Anwer, and R. Alvarez.A phase I trial of intraperitoneal EGEN-001, a novel IL-12 gene therapeutic, administered alone or in combination with chemotherapy in patients with recurrent ovarian cancer. *J. Clin. Oncol*. 2008;**26**(May 20 suppl; Abstract 5572).
134. Alvarez, R., M. Barnes, M. Anderson, S. Saddekni, S. Makhija, I. Maya, J. Kendrick, J. Fewell, D. Lewis, and K. Anwer. Progress in clinical development of a novel IL-12 gene therapeutic for the treatment of recurrent ovarian cancer. *Mol. Ther*. 2008;**16**:S63(Abstract 167).
135. Lisziewicz, J., J. Trocio, J. Xu, L. Whitman, A. Ryder, N. Bakare, M. Lewis, W. Wagner, A. Pistorio, S. Arya, and F. Lori. Control of viral rebound through therapeutic immunization with DermaVir. *AIDS* 2005;**19**:35–43.
136. Grossman, P., F. Mendelsohn, T. Henry, J. Hermiller, M. Litt, J. Saucedo, R. Weiss, D. Kandzari, N. Kleiman, R. Anderson, D. Gottlieb, R. Karlsberg, J. Snell, and K. Rocha-Singh. Results from a phase II multicenter, double-blind placebo-controlled study of Del-1 (VLTS-589) for intermittent claudication in subjects with peripheral arterial disease. *Am. Heart J*. 2007;**153**:874–880.
137. Dai, Q., J. Huang, and B. Klitzman. Engineered zinc finger-activating vascular endothelial growth factor transcription factor plasmid DNA induces therapeutic angiogenesis in rabbits with hindlimb ischemia. *Circulation* 2004;**110**:2467–2475.

AQ10 138. Wloch et al. 2008.

BIBLIOGRAPHY

Chemin, I., D. Moradpour, S. Wieland, W. Offensperger, E. Walter, J. Behr, and H. Blum. Liver-directed gene transfer: A linear polyethyleneimine derivative mediates

highly efficient DNA delivery to primary hepatocytes in vitro and in vivo. *J. Viral Hepatitis* 1998;**5**:369–375.

Choi, Y., F. Liu, J. Choi, S. Kim, and J. Park. Characterization of a targeted gene carrier, lactose-polyethylene glycol-grafted poly-L-lysine and its complex with plasmid DNA. *Hum. Gene Ther*. 1999;**10**:2657–2665.

Choi, Y., F. Liu, J. Park, and S. Kim. Lactose-poly(ethylene glycol)-grafted poly-L-lysine as hepatoma cell-targeted gene carrier. *Bioconj. Chem*. 1998;**9**:708–718.

Hofland, H., C. Masson, S. Iginnla, I. Osetinsky, J. Reddy, C. Lemon, D. Scherman, M. Bessodes, and P. Wils. Folate-targeted gene transfer in vivo. *Mol. Ther*. 2002;**5**:739–744.

Janat-Amsbury, M., J. Yockman, M. Lee, S. Kern, D. Furgeson, M. Bikram, and S. Kim. Combination of local, nonviral IL12 gene therapy and systemic paclitaxel treatment in a metastatic breast cancer model. *Mol. Ther*. 2004;**9**:829–836.

Lim, Y., S. Han, H. Kong, Y. Lee, J. Park, B. Jeong, and S. Kim. Biodegradable polyester, poly[-(4-aminobutyl)-L-glycolic acid], as a non-toxic gene carrier. *Pharm. Res*. 2000;**17**:811–816.

Park, I., T. Kim, Y. Park, B. Shin, E. Choi, E. Chowdhury, T. Akaike, and C. Cho. Galactosylated chitosan-*graft*-poly(ethylene glycol) as hepatocyte-targeting gene carrier. *J. Control. Release*. 2001;**76**:349–362.

Ross, J., P. Chaudhuri, and M. Ratnam. Differential regulation of folate receptor isoforms in normal and malignant tissues in vivo and in established cell lines. Physiologic and clinical implications. *Cancer* 1994;**73**:2432–2443.

Slobodkin, G., M. Matar, J. Fewell, and K. Anwer. U.S. Patent Application, US 2006/
AQ12 0093674 A1.

Wilson, J., M. Grossman, C. Wu, N. Chowdhury, G. Wu, and J. Chowdhury. Hepatocyte-directed gene transfer in vivo leads to transient improvement of hypercholesterolemia in low density lipoprotein receptor-deficient rabbits. *J. Biol. Chem*. 1992;**267**:963–967.

Wu, G., J. Wilson, F. Shalaby, M. Grossman, D. Shafritz, and C. Wu. Receptor-mediated gene delivery in vivo. Partial correction of genetic analbuminemia in Nagase rats. *J. Biol. Chem*. 1991;**266**:14338–14342.

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