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Polymeric Vectors for Strategic Delivery of Nucleic Acids

Andrew Dunn*,‡ and Donglu Shi*,†,§
*Department of Mechanical and Materials Engineering
University of Cincinnati, Cincinnati, OH 45221, USA

†Shanghai East Hospital
The Institute for Biomedical Engineering & Nano Science
Tongji University School of Medicine
Shanghai 200120, P. R. China
‡dunnaw@mail.uc.edu
§shid@ucmail.uc.edu

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Genomic modification through nucleic acid delivery is a frequently applied method in fundamental biological studies and offers a potent therapeutic strategy for disease treatment and biological research. Delivery of nucleic sequences is therefore an attractive facet of biological nanotechnology as highly specific, efficient, and nonantagonistic delivery is necessary for *in vivo* and clinical use. Previous vectors have suffered from immunogenic responses, serum dependent inactivation, and cytotoxicity, hindering their translational applicability. Current research in polymeric-based nucleotide delivery strives to offer a highly biocompatible, broad use vector through the utilization of polypeptide and polyamine conjugation that can be easily tailored for specific targeting or wide dissemination. Cross-linking low molecular weight polyamines and lipophilic derivatization for amphiphile creation has lead to improved biocompatibility and transfection efficiency compared to higher molecular weight polyamines. Derivatization of hyperbranched and dendritic polyamido- and polyamines has allowed for the formation of efficient *in vivo* transfection vectors; ring opening synthesis of N-carboxyanhydride amino acids have led to controlled peptide architectures for improved transfection while simultaneously providing convenient primary amines useful in functionalization. Polymer libraries of poly(ß-amino esters) have provided insights into useful architectures for *in vitro* and *in vivo* gene delivery. Grafting small molecules to polyamines, such as folate and galactose, for enhanced interaction with cell surface receptors for selective targeting of specific cell types has proven to be encouraging and remains a prominent aspect in biological nanotechnology.

Keywords: Polymer nanotechnology; gene delivery; non-viral; therapeutics.

1. Introduction — A Brief History Behind the Interest in Polymeric Vectors

Polymeric vectors for nucleic acid delivery are garnering high interest in the field of biological nanotechnology for research and medical applications. However, the first vector that saw a rise to prominence was not polymeric, but rather viral.[1] The virus of choice was the icosahedral, nonenveloped adenovirus. With over 50 serotypes consisting of a double stranded DNA genome of approximately 36 kilobases (kb), the human adenovirus has been observed to possess a wide range of tissue and organ specificity.[2,3] The adenovirus genome consists of four early gene transcripts (E1-E4) and five late gene transcripts (L1-L5) with the early gene transcripts primarily controlling viral replication (E1), late gene transcriptional activation (E2), immune system evasion and viral release (E3), and functional regulation of the cell cycle gene transcription (E4).[4–7]

A problem that arose, however, was an inherent immunogenicity of the adenovirus vector (AdV). To combat this, excision of E1 and E1/E3 in first generation AdV led to replication incompetent AdV.[5] This caused the propagation of the AdV to require helper cells *in vitro* which would provide the E1 function *in trans*, providing the potential for the reacquisition of the E1 region in AdV by homologous recombination with the helper cell's genome creating replication-competent AdV.[8] Second and third generation AdV saw the further removal of E2 and E4 or all viral genes, creating gutless vectors, which inhibited the production of replication-competent AdV as well as reduced adaptive immunity against delivered AdV, albeit with some reduction in transduction efficiency as well as the need for helper viruses for replication.[9–12] Combined with easy genomic insertion and highly efficient transduction, the adenovirus found its way onto the clinical stage.[5,13]

However, a pertinent problem persisted. Delivery of AdV antagonized the innate immune system independently of viral transcription with inflammatory responses observed in a dose-dependent manner, mediated by the adenovirus capsid. This inflammatory injury was the cause of death for one patient during a clinical trial.[14–17] With AdV plagued by innate immunogenicity, nonviral vectors for genetic transfection began to gain increased interest.

Early polymeric vectors (polyplexes) for transfection consisted of polyamidoamine (PAMAM) dendrimers and polyethylenimine (PEI), polymers containing a high density of primary (1°), secondary (2°), and tertiary (3°) amines.[18–21] Extensive amination leads to a highly protonated structure at physiological pH. The polymers can therefore efficiently condense DNA through noncovalent binding of the cationic charges on the protonated amines with the anionic charges on the phosphate backbone.[21] Research began to reveal dependencies of transfection efficiency, cytotoxicity, and polyplex stability based on physical properties such as molecular weight and branching architecture. The hunt was on to understand the physical mechanisms behind polyplex interaction with physiological systems to improve overall transfection performance through new synthesis and conjugation schemes.

2. Challenges

Polymeric vectors possess substantially reduced immunogenicity and may deliver large gene constructs which would be otherwise challenging to incorporate into the viral vector as transgene capacity in AdV is space limited by the included viral genes.[5,22] However, there is a very distinct opportunity cost for using polymeric, nonviral vectors. These vectors traditionally do not possess the high level of tissue tropism and transfection efficiency observed in viral vectors. Variables dictating transfection efficiency of polymeric vectors also becomes more convoluted as the degree of complexation, frequently quantified through the ratio of the molar number of nitrogens in the polymer to the molar number of phosphates in the nucleic acid chain (N/P) or polymer to nucleic acid mass ratio (w/w), polymer molecular weight, and chain morphology have been observed to significantly impact transfection efficiency.[21,23,24] Difficulty is further compounded when delivering *in vivo* with protein binding leading to rapid clearance by the mononuclear phagocyte system (MPS) through the binding of opsonins and through clearance by the liver and spleen.[22,25,26] To combat this clearance and provide prolonged circulation *in vivo* as well as reducing toxicity, poly(ethylene glycol) (PEG) surface modification is frequently used.[27–30]

Transfection requires the successful delivery of intact DNA/RNA sequences into the intracellular space with subsequent release from the vector and

escape of compartmentalization. Cellular entry for cationic polymeric vectors is generally mediated by caveolae or clathrin-mediated endocytosis with endosomal and lysosomal compartmentalization following uptake.[25] This compartmentalization not only hinders translocation, but also threatens nucleotide sequence integrity through degradation. Efficiency cellular targeting, uptake, and escape from these membrane bound vesicles provide a few of the last major challenges for translational application.

3. Polymers for Gene Transfection

Cationic polymers offer a versatile nonviral system for gene therapy. Polyplex formation in solution spontaneously occurs due to the charge interaction between the cationic polymer and deliverable nucleic acids.[31,32] Condensation frequently forms toroidal structures with exceptionally large DNA chains forming spherical globules.[32] The ratio of polymer to nucleotides is frequently represented as a mass ratio (w_p/w_n) where (w_p) is the mass of polymer and (w_n) is the mass of nucleotides, or a molar ratio (N/P) which represents the number of moles of amines in solution (N) relative to the molar number of phosphate groups on the nucleotide backbone (P).[33–35] Condensation by the cationic polymer further forms a protective barrier to DNase.[36] Terminal amines on cationic polymers further provide convenient nucleophilic groups for functionalization and polymerization initiation.

3.1. *Polyethylenimine (PEI)*

PEI contains a repeating group of R(NR-CH_2-CH_2), where R may be a carbon or hydrogen. Primary, secondary, or tertiary amines are denoted by one, two, or three carbons bonded to the nitrogen, respectively. Terminal amines are primary amines and provide convenient nucleophilic sites. Linear PEI can be formed through ring opening synthesis of 2-oxazolines, synthesized using an array of amino alcohols or amides,[37,38] followed by hydrolysis of acid labile pendant groups.[38–41] Branched PEI can be easily synthesized through the ring opening of ethylene imine (aziridine) under acidic conditions,[21,42–45] with the aziridine precursor created through the cyclization of ethanolamine (Wenker Synthesis).[42,43,46]

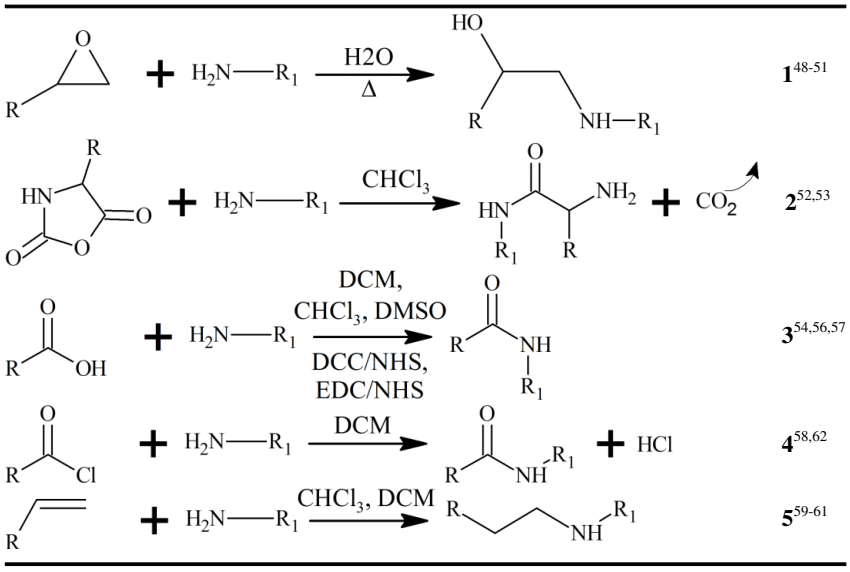
Transfection experiments were carried out by Godby *et al.* using an array of PEIs with varying molecular weights. Generally higher molecular weight PEIs offered greater transfection efficiency.[21,23] However, the elicitation of cytotoxicity of higher molecular weight PEI has been shown to be correlated with reduced transfection juxtaposed with less toxic, lower molecular weight PEI.[44,45] PEI forms a condensed colloid upon mixing with nucleic acids. These polyplexes spontaneously form at physiological pH due to the high density of cationic charges on the polymer associating with the negatively charged phosphate backbone.

Hyperbranched PEI and linear PEI have been observed to possess different behavior in salt solutions. Wightman *et al.*[24] examined branched PEI, MW = 25 kDa, against linear PEI, MW = 22 kDa, and observed significant aggregation of polyplexes formed at a N/P ratio of 6 using linear PEI in a solution of 75 mM NaCl, 20 mM (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) (HEPES), 2.5% glucose, whereas the polyplexes formed using 22 kDa branced PEI were resistant to aggregation under these conditions. Furthermore, it was found that *in vitro* transfection of luciferase reporter pCMVluc in Neuro 2a, MCA-38, and C26 by linear PEI was significantly greater than branched PEI when polyplexes were formed in a sodium chloride containing solution as well as greater than linear PEI when polyplexes were formed in a sodium chloride free, 5% glucose solution. Interestingly, *in vivo* injections showed opposite results. Injections performed with linear PEI in a sodium chloride free, 5% glucose bolus achieved greater luciferase expression in the lung, heart, kidney, spleen, and liver than injections of linear PEI polplexes formed in a sodium chloride solution.[24] Moreover, Zou *et al.*[47] observed the highest transfection efficiency *in vivo* using a 5% glucose, linear PEI polyplex bolus, with serum inclusion reducing efficiency. Jeong *et al.*[40] performed transfection *in vitro* using linear PEI and noted a molecular weight dependence on the transfection efficiency in 3T3 mouse fibroblasts as well as a dependence of polyplex size on the N/P ratio with large aggregates appearing near the N/P ratio used by Wightman *et al. in vivo*.

3.1.1. *Modified PEI and polypropylenimine*

The formation of PEI-based nanoparticles relies on the establishment of an amphiphilic molecule. This

Table 1. Common coupling reactions for modification of polyethylenimine.



may be easily done through directly grafting lipophilic molecules or polymers onto the PEI chain as PEI provides convenient nucleophilic primary amines on terminal branches. From a multitude of schemes, commonly used reactions include functionalization through ring opening synthesis,[48–53] amidation using carboxylate groups activated by 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide/N-hydroxysuccinimide (EDC/NHS) or N,N′-dicyclohexylcarbodiimide (DCC)[54–57] or acid chlorides (Schotten-Baumann),[58] and Micheal Addition.[59–61] Table 1 details commonly found reaction schemes for the conjugation of substituents onto PEI and polypropylenimine for nucleic acid delivery. It is important to note that these commonly found conjugation schemes may be applied to an array of polymers, polyamines and small molecules possessing primary amines.

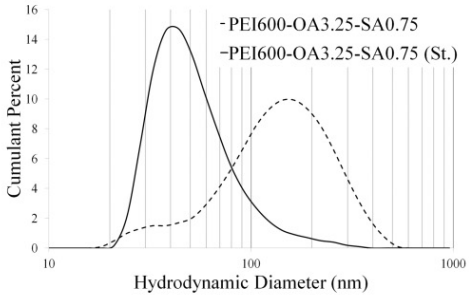
Recent literature has examined the ability for low molecular weight hyperbranched PEI to deliver nucleic acids following the functionalization of long alkane tails.[34,50,59,63,64] This modification alters the colloidal nature of the PEI-based nanoparticles, or pseudo-lipids, and is dependent on factors such as the degree of grafting, tail length, and inclusion of sp^2 carbon bonds in the alkane tail. Figure 1(a) shows the colloidal characteristics of low molecular weight PEI, Mn = 600 (PEI_{600}), grafted with various biological fatty acids through EDC/NHS coupling. Transfection experiments using the TurboGFP reporter plasmid pCMV-TurboGFP show that, by flow cytometry, the expression TurboGFP following PEI_{600} mediated transfection is nominally zero, whereas the alkane modified PEI_{600} can achieve significantly greater expression, as shown in Fig. 1(c).

Schroeder *et al.*[48] examined the ability for PEI_{600} grafted with saturated 18 carbon tails to deliver short interference ribonucleic acids (siRNA) against luciferase into expressing HeLa cells. Alkane tails were grafted onto PEI through epoxide ring opening in water at elevated temperature. A significant dependence of colloidal size as well as knockdown efficiency on the molar degree of grafting was found. Love *et al.* and Khan *et al.* further found a significant variance in gene knockdown efficiency *in vivo* and *in vitro*, dependent on the alkane tail length of polyamine colloids grafted through epoxide ring opening.[49,50] Khan *et al.*[49] demonstrated the ability for alkane-grafted polypropylamine to selectively associate with and deliver siRNA into endothelial cells in wild type murine lung after tail vein injection. The dependency of delivery efficiency on degree of grafting of lipophilic tails was also highlighted by Guo *et al.*[59] during luciferase knockdown by octylacrylamide modified PEI_{600}. Moreover, the importance of nucleic acid stability within the polyplex is highlighted by the significant changes in knockdown efficiency achieved through varying the N/P ratio of individual polyplex formulations. A detraction of PEI is the observed poor biodegradability.[65]

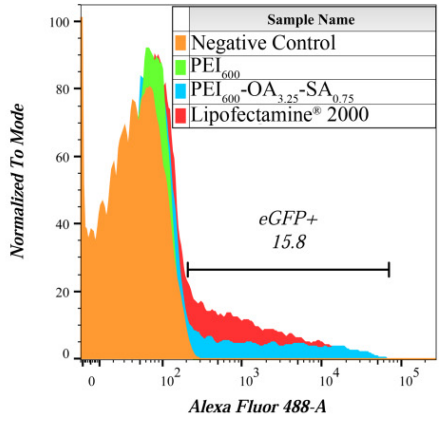
Stability of the spontaneously forming colloids of alkane modified low-molecular weight PEI was

	Size (d. nm)	*PDI*
PEI_{600}-$EDD_{2.0}$	89	*0.368*
PEI_{600}-$OA_{1.5}$	124	*0.183*
PEI_{600}-$OA_{1.5}$-$SA_{0.5}$	125	*0.420*
PEI_{600}-$OA_{3.25}$-$SA_{0.75}$	113	*0.273*
PEI_{600}-$AD_{3.0}$	126	*0.403*

(a)



(b)



(c)

Fig. 1. (a) *Z*-average size and polydispersity of colloids created by grafting alkane tails onto low molecular weight PEI_{600}. 1,2-epoxydodecane (EDD), Oleic acid (OA), Stearic Acid (SA), Arachidonic acid (AD). Subscript numbers following alkane tail abbreviation indicates molar degree of grafting. (b) Dynamic light scattering intensity plot of unstabilized and stabilized (St.) pseudo-lipid colloid using cholesterol incorporated through solvent diffusion and microfluidic mixing. (c) Expression of TurboGFP in HEK-293T cultures 24 h post transfection examined by flow cytometry on isolated singlet cells.

achieved by Khan *et al.*[49] using cholesterol incorporation through microfluidic mixing. Dynamic light scattering confirmed a reduction in both size polydispersity and mean hydrodynamic diameter. Figure 1(b) shows a comparison of colloidal sizes of an alkane-modified 600 dalton branched PEI stabilized with cholesterol in 10 mM (3-(*N*-morpholino)propanesulfonic acid (MOPS) buffered to pH 7.4.

3.2. *Poly(amido amine) (PAMAM)*

PAMAMs fall into the class of polymers known as dendrimers, continually branching polymers. An initiator begins dendrimer branching with each successive generation giving rise to a greater number of branches. PAMAM dendrimers are commonly synthesized starting with a multivalent initiator reacting with methyl acrylate through a Micheal Addition followed by amidation with diethylamine, doubling the number of surface primary amines with each generation.[66] Even numbered generations ($G1$, $G2$, etc.) are capped with diethylamine while half generations ($G0.5$, $G1.5$, etc.) are capped with methyl acrylate, giving a terminal methoxy.

Table 2. Number of terminal functional groups for PAMAM dendrimer using diethylamine/methylacrylate.

Generation G = (0, 1, 2, 3, 4, etc.) Amine (G), Methoxy (G + 0.5)	
Trivalent Initiator	3, 6, 12, 24, 48
Tetravalent Initiator	4, 8, 16, 32, 64

The number of terminal functional groups (N_g), shown in Table 2, can be calculated through Eq. (1) using the initiator valency (N_i), branching multiplicity (N_b), and generation (G). The number of branching amines within the core (N_b) can be calculated through Eq. (2).[66]

$$N_g = N_i N_b^G, \tag{1}$$

$$N_b = N_i \left[\frac{N_b^G - 1}{N_b - 1}\right]. \tag{2}$$

With the multiplicative increase in terminal functional groups, successive generations begin to require longer reaction times as steric hinderance begins to reduce the availability of terminal groups; this also affects the ability for small molecule conjugation.[67,68]

PAMAM dendrimers have improved transfection efficiency at higher generations but due to the high density of amines, also begin to display greater cytotoxicity.[69] Biodegradability may be improved by inclusion ester linkages in the backbone. Liu *et al.*[70] investigated the ability for cross-linked low generation PAMAM to transfect HEK293 cells *in vitro* in an investigation to attenuate the cytotoxicity of larger PAMAMs by using a cleavable cross-linker. Generation 2 PAMAMs were cross-linked

with a bifunctional NHS activated disulfide containing cross-linker and tested against unmodified *G*2 and *G*5 PAMAM. A significant increase in cytotoxicity was observed for *G*5 in HeLa and HEK293 cultures compared to *G*2 and cross-linked *G*2 (*G*2DSP). *G*2DSP improved transfection efficiency over both *G*2 and *G*5 delivering both enhanced green fluorescent protein (eGFP) and luciferase plasmids. Tang *et al.*[71] was able to achieve significantly reduced *in vitro* cytotoxicity of *G*5 and *G*6 PAMAM in Cos-7 cultures through direct conjugation of 5 kDa methoxy terminated PEG. The PEG-PAMAM conjugates significantly improved eGFP transfection in 293A cultures over nonfunctionalized PAMAM and were able to achieve transient knockdown in GFP transgenic mice after intramuscular injection.

Huang *et al.*[72] delivered a plasmid encoding tumor necrosis factor related apoptosis–inducing ligand (TRAIL) to C6 gliomas established in the striatum of nude mice using angiopep-2 functionalized PEG conjugated PAMAM injected through the tail vein. Mouse survival significantly improved using the aniopep-2 targeting ligand for enhancing crossing of the blood brain barrier compared to untargeted PEG-PAMAM.

A class of arginine rich cell-penetrating peptides has been found to efficiently mediate membrane translocation.[73–75] Surface grafting and inclusion of arginine has therefore been a topic of investigation for mediation of gene delivery. Liu *et al.*[76] grafted arginine onto low geneartion PAMAM through Fmoc conjugation synthesis. Heat shock protein 27 (Hsp27) was significantly down regulated in human prostate cancer PC-3 *in vitro* cultures by siRNA delivered by arginine-terminated G4 compared to unmodified G4. Arginine-terminated G4 significantly reduced Hsp27 expression *in vivo* through siRNA delivery by direct injection into PC-3 tumors established in five weeks old nude mice compared with siRNA alone or scrambled siRNA/arginine-G4.

3.3. *Chitosan and chitosan-based*

Chitosan is a polymer made from a glucose-based backbone that is the deacetylated form of chitin, the polysaccharide commonly found in the exoskeleton of arthropods. The degree of deacetylation directly corresponds to the percentage of primary amines along the polysaccharide backbone with each *mer* unit as either the *N*-acetyl-D-glucosamine (acetylated) or deacetylated D-glucosamine presenting the pendent primary amine. This primary amine, along the exceptional biocompatibility, is the basis behind utilizing chitosan as a transfection agent.[77,78]

Höggård *et al.*[79] demonstrated the ability for pure chitosan to transfect cells *in vitro* and *in vivo*. Expression of chloramphenicol acetyltransferase (CAT) in HEK-293 cultures was achieved using ultrapure chitosan with a high degree of amination at a similar level to 800 kDa PEI. β-glactosidase (β-gal) expression after intratracheal administration of polyplexes formed from ultrapure chitosan was compared against PEI and free β-gal encoding plasmid. Expected of the delivery route, expression was observed along the epithelial cells lining bronchial airways using both chitosan and PEI with no expression from free plasmid by immunohistochemistry.

Similar to most cationic polymers used for gene transfection, chitosan does not possess innate targeting specificity. Endocytosis commonly occurs through nonspecific association from the positively charged chitosan interacting with the negatively charged cell membrane.[78] Galactosylated chitosan has been used to improve targeting in hepatocytes expressing receptor specific for galatose terminated glycoproteins. Gao *et al.*[80] demonstrated the increased ability for galatose grafted low-molecular weight chitosan to transfect HepG2 cells expressing asialoglycoprotein receptors (ASGR, galatose terminated glycoprotein receptor). Galatosylation was able to significantly improved transfection in HepG2 cultures while galatosylation did not effect transfection efficiency in nonASGR expressing HeLa cells *in vitro*. Jiang *et al.*[81] grafted galatose through PEG linkages to chitosan with pendant PEI. Transfection efficiency was significantly improved in HepG2 over HeLa cultures *in vitro*. Furthermore, free galatose was found to significantly reduce transfection efficiency over nongalatose supplemented medium, indicating that receptor, mediated endocytosis was a likely mechanism for internalization. Yang *et al.*[82] significantly improved knockdown efficiency of GADPH and COX-2 using folate conjugated chitosan in RAW 264.7 cultures *in vitro* as well as improving nanoparticle uptake in activated macrophages.

3.4. *Polypeptides*

Versatile polypeptides can be created through controlled opening of N-carboxyanhydride (NCA) rings.

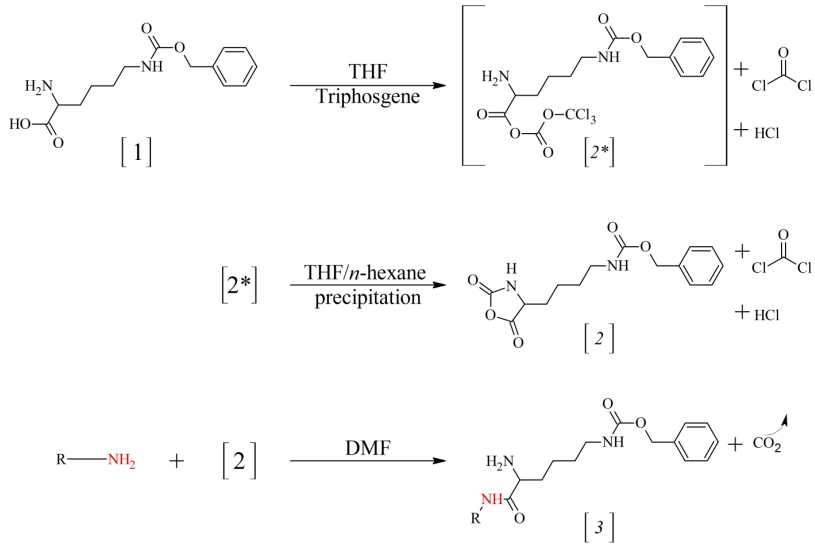


Fig. 2. Scheme of common NCA-Lys(Z) formation and polymerization.

A common methodology for the formation of peptides for gene delivery uses amine terminated poly(ethylene glycol) (PEG), PEI, or a small molecule such as benzylamine. While Polylysine complexes DNA, it has been observed to possess poor transfection efficiency. It was hypothesized that this observation was due to the inability for polylysine-based polyplexes to escape compartmentalization, eventually leading to the degradation of the gene cargo.[22,83]

Formation of the NCA ring can be completed through the Fuchs-Farthing method using triphosgene when using unprotected *N*-terminal amino acids or by using thionyl chloride or phosphorous pentachloride when using benzyloxycarbonyl (Cbz or Z) or *tert*-butyloxycarbonyl (Boc) protected *N*-terminal amino acids, respectively. In the case of triphosgene, two molecules of phosgene are liberated during cyclization and may subsequently cyclize unreacted amino acid, allowing one mole of triphosgene to theoretically cyclize three moles of amino acid. Common molar ratios in literature are found around 1:2.5, triphosgene:amino acid. Figure 2 shows the general scheme used during cyclization of N_ε-Z-L-lysine. Polymerization of NCAs is frequently performed in DMF using a primary amine initiator such as a small molecule or amine terminated polymer.

Lysosomal/endosomal escape can be completed through a few different methodologies. Physical membrane disruption through pore formation or rupture by photochemical degradation or membrane swelling by pH buffering as well as membrane fusion are currently studied strategies for improving delivery efficiency.[84] Membrane fusion and pore formation are commonly found mechanisms in natural nano-bio agents (such as viruses and viral toxins), whereas lysosomal/endosomal buffering is frequently the mechanism behind polyplex delivery.[84] Conveniently, the amino acid histidine contains an imidazole ring and has been successfully used to improve the buffering capacity and transfection efficiency of polypeptide chains upon its inclusion.[33,85,86] Figure 3 shows a general schematic of lysosomal/endosomal rupture through pH buffering.

Midoux *et al.*[87] investigated the effect of an endosomal proton pump inhibitor, bafilomycin A_1, on the delivery efficiency of the pSV2LUC plasmid encoding firefly luciferase. A significant reduction in luciferase expression was found upon cellular incubation with bafilomycin A_1. Furthermore, a significant improvement to poly(L-lysine) (PLL) buffering and subsequent PLL-based polyplex transfection through histidine grafting investigated by Hwang *et al.*[86] or by random inclusion during polypeptide polymerization investigated by Zhu *et al.*[33] was found.

PEI has been used as a macroinitiator for PLL synthesis through NCA ring opening, creating PLL multi-armed polymers[52] as well as providing pendant groups during aminolysis of poly(γ-benzyl L-glutamate) for highly efficient *in vitro* transfection.[65,88–90] Gao *et al.*[52] delivered a direct anti-microRNA-21 (α-miR-21) oligonucleotide inhibitor

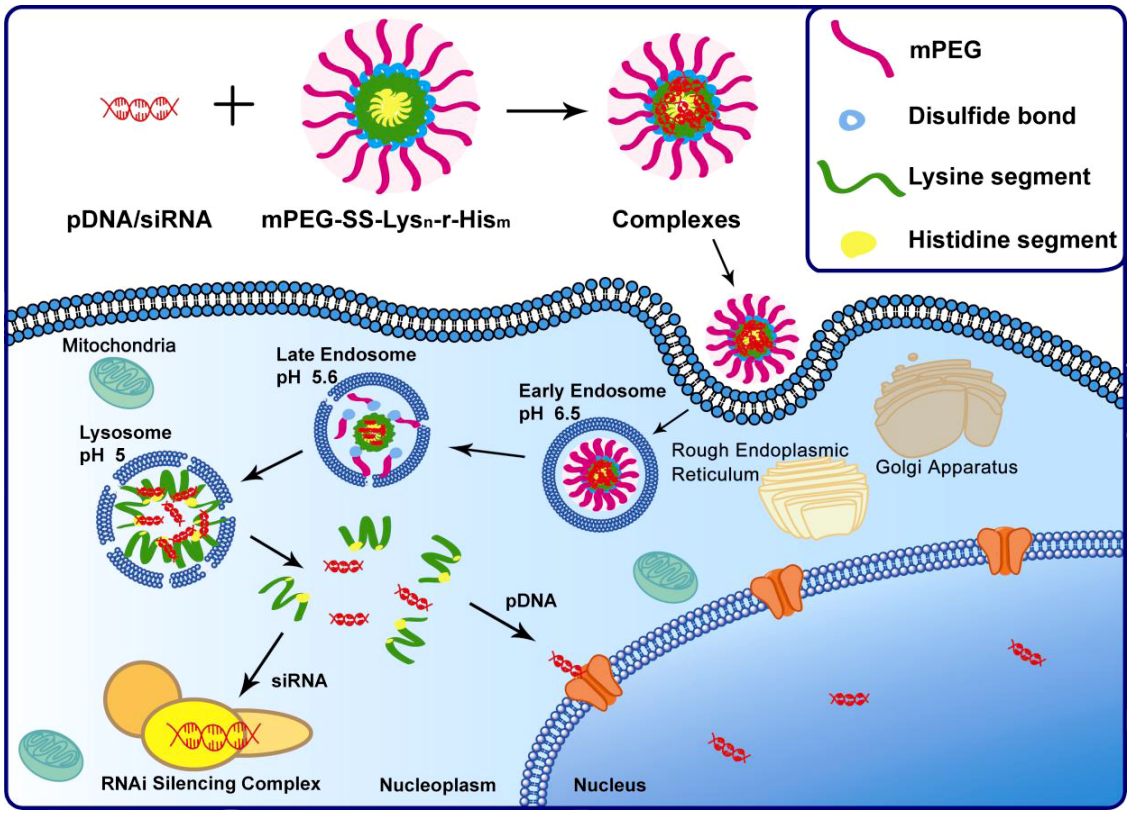


Fig. 3. Schematic of polyplex uptake by endocytosis and subsequent lysosomal escape through pH buffering mediated by histidine incorporation.[33] Reproduced with permission from American Chemical Society Applied Materials & Interfaces (ACS Publications).

as well as a plasmid encoding an α-miR-21 oligonucleotide inhibitor by polyplex formation with PEG-PLL. Both vectors successfully inhibited expression of miR-21 in MCF-7 cells *in vitro* 48 h post transfection by qRT-PCR. Li *et al.*[56] investigated the delivery of siRNA targeting the gene encoding X-linked inhibitor of apoptosis protein (XIAP) *in vivo* through PEI grafted PEG-*b*-PLL. Alexa Fluor® 750 tagged PEG-*b*-PLL-*g*-PEI showed preferential accumulation in the tumor site. Both untargeted (only acting through the enhanced permeability and retention effect) as well as direct targeted PEG-*b*-PLL-*g*-PEI through Her2 targeting by Herceptin conjugation showed significant tumor volume reduction compared to a scrambled siRNA control. Significant reduction in tumor volume was also mirrored with improved survival rates.

3.5. *Poly(ß-amino esters)*

Poly(ß-amino esters) (PBAEs) encompass a wide range of polymer architectures created generally though the Michael Addition of a primary or secondary amine to a diacrylate. PBAEs provide the opportunity for biodegradation by hydrolytic cleavage ester backbone to generate respective diacids and diols[91] with a reported half-life of a few hours in PBS at 37°C for a selection of PBAEs synthesized from 4-amino-1-butanol, 4-amino-1-pentanol, and 1,4-butanediol diacrylate, 1,4-hexanediol diacrylate.[92] Furthermore, the backbone of PBAEs can easily be created under solvent-free conditions by mixing the diacrylate with the amine at 90–100°C The fantastic flexibility of reagents allows for large libraries of varying backbone architectures to be compiled and tested for efficacy in successfully delivering genes. Transfection is highly dependent upon polymer architecture and molecular weight (controlled through amine/acrylate ratio) with sensitivity to end-group design,[92–96] and smaller particle sizes generally allow for higher transfection.[95] Through this method, polymers can be easily synthesized with competitive, even greater, transfection efficiency than commercially available nonviral transfection standards.[96]

Zugates *et al.*[94] achieved a significant improvement of luciferase expression of intraperitoneal injected luciferase expressing plasmid *in vivo* by end capping a previously unmodified, acrylate terminal PBAE synthesized from 1,4-butanediol diacrylate and 1-amino-5-pentanol. Significant changes to the *in vitro* transfection achieved by Zugates *et al.* by simply changing the capping group further highlights the dependence of PBAE transfection on the terminal group.

Mangraviti *et al.*[96] designed a PBAE using 1,4-butanediol diacrylate and 4-amino-1-butanol capped with 1-(3-aminopropyl)-4-methylpiperazine that significantly improved F344 rat survivability in a L9 glioblastoma model by the delivery of a plasmid expressing herpes simplex virus type 1 thymidine kinase (HSVtk) paired with ganciclovir treatment compared with ganciclovir treatment alone. PBAE-based transfection has further been shown to benefit from co-incubation with acetic acid modified polyhistidine. Gu *et al.*[97] co-incubated poly(l-histidine) modified by iodoacetic acid with PBAE synthesized from 1,4-butanediol diacrylate and 5-amino-1-pentanol in a 1.2:1 amine:diacrylate ratio which allowed for a significant improvement in transfection over PBAE/DNA polyplexes *in vitro* and *in vivo*. Tang *et al.*[98] demonstrated the ability for PBAEs to co-deliver doxorubicin with a short hairpin RNA against survivin in a MCF-7/ADR tumor-bearing mouse model.

3.6. *Reversible addition-fragmentation chain transfer (RAFT) polymerization*

RAFT polymerization is an extremely flexible, promising polymerization technique for therapeutic application which falls under the category of degenerative transfer polymerization.[99] A radical initiator interacts with a thioketone group on the RAFT agent which can subsequently generate a reinitiating radical for polymerization propagation. RAFT polymerization may be used to generate controlled architectures with stimuli responsiveness to conditions such as pH and temperature.[100]

The amine containing methacrylate-based polymer poly(2-dimethylaminoethyl methacrylate) (pDMAEMA). A block copolymer of pDMAEMA, poly(propylacrylic acid) (pPAA), and poly(butyl methacylate) (pBMA) was able to significantly reduce glyceraldehyde 3-phosphate dehydrogenase (GAPDH) by the delivery of a siRNA to GAPDH near positive control levels. pBMA inclusion was found to significantly improve transfection efficiency and act as an endosomolytic agent.[101] This architecture was able to significantly increase vascular endothelial growth factor (VEGF) and fibroblast growth factor 2 (FGF-2) by silencing prolyl hydroxylase domain protein 2 (PHD2) in subcutaneously implanted porous polyester urethane resulting in improved vascular incorporation and vessel size.[102] The copolymer of PEG terminated PDAEMA-co-pBMA improved circulation half-life and serum stability allowing for a significant reduction in cyclophilin B expression by siRNA delivery *in vivo* in the liver, kidney, and spleen compared to PDAEMA-mediated delivery without the inclusion of pBMA.[103]

4. Conclusions

	Pros	Cons
PEI	– Commercially available – Inexpensive – Easily modified	– Cytotoxic – Poor biodegradability
PAMAM	– Commercially available – Cytotoxicity of higher generations can be overcome by using bio-responsive, cleavable linked low generations – Easily modified	– Expensive – Time consuming and tedious synthesis for high generation dendrimers
Chitosan	– Highly biocompatible – Commercially available – Easily modifiable	– Lower innate transfection compared to alternatives
Polypeptides	– Extremely flexible in design – Biocompatible	– Only a few backbones are commercially available
Poly(ß-amino esters)	– Extremely flexible in design – Fast synthesis and purification – Can provide superb transfection efficiency	– Highly subject to hydrolytic cleavage – Not commercially available
RAFT	– Minimal cytotoxicity – Highly controllable architecture – Flexible in design	– Monomers and RAFT agents are not widely available and must be synthesized from precursors

Dependence of transfection efficiency, cytotoxicity, and colloid stability on branching structure, molecular weight, and terminal groups has been observed for a wide range of polyamines. Grafting of small hydrophobic molecules can significantly change colloidal behavior and nucleotide delivery efficiency; targeting ligands can similarly be grafted for improving cellular association for specific lineages. Library testing of functionalized polyamines and PBAEs has identified a collection of precursors that have been successfully used *in vitro* and *in vivo* with significant results.

Conveniently, perhaps, the amines responsible for forming a stable complex with the phosphate backbone of DNA/RNA may be used in a wide range of functionalization reactions using a myriad of reagents. With greater understanding of colloidal properties and alteration of cellular interaction and transfection *in vitro* and *in vivo* by polymer architecture and functionalization, polyamines will become promising tools in nucleotide delivery for therapeutics.

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References

1. R. G. Crystal, *Hum. Gene Ther.* **25**, 3 (2014).
2. N. Tatsis and H. C. J. Ertl, *Mol. Ther.* **10**, 616 (2004).
3. H. Liu, L. Jin, S. B. Koh, I. Atanasov, S. Schein, L. Wu and Z. H. Zhou, *Science* **329**, 1038 (2010).
4. J. Alonso-Padilla, T. Papp, G. L. Kaján, M. Benkő, M. Havenga, A. Lemckert, B. Harrach and A. H. Baker, *Mol. Ther.* **24**, 6 (2016).
5. X. Danthinne and M. J. Imperiale, *Gene Ther.* **7**, 1707 (2000).
6. K. N. Leppard, *J. Gen. Virol.* **78**, 2131 (1997).
7. M. Moore, N. Horikoshi and T. Shenk, *Proc. Natl. Acad. Sci. U.S.A.* **93**, 11295 (1996).
8. F. J. Fallaux, A. J. van der Eb and R. C. Hoeben, *Gene Ther.* **6**, 709 (1999).
9. A. Amalfitano, M. A. Hauser, H. Hu, D. Serra, C. R. Begy and J. S. Chamberlain, *J. Virol.* **72**, 926 (1998).
10. R. Alba, A. Bosch and M. Chillon, *Gene Ther.* **12**, S18 (2005).
11. M. A. Kay, J. C. Glorioso and L. Naldini, *Nat. Med.* **7**, 33 (2001).
12. D. A. Muruve, M. J. Cotter, A. K. Zaiss, L. R. White, Q. Liu, T. Chan, S. A. Clark, P. J. Ross, R. A. Meulenbroek, G. M. Maelandsmo and R. J. Parks, *J. Virol.* **78**, 5966 (2004).
13. R. G. Crystal, N. G. McElvaney, M. A. Rosenfeld, C.-S. Chu, A. Mastrangeli, J. G. Hay, S. L. Brody, H. A. Jaffe, N. T. Eissa and C. Danel, *Nat. Genet.* **8**, 42 (1994).
14. D. A. Muruve, *Hum. Gene Ther.* **15**, 1157 (2004).
15. D. A. Muruve, M. J. Barnes, I. E. Stillman and T. A. Libermann, *Hum. Gene Ther.* **10**, 965 (1999).
16. Y. Zhang, N. Chirmule, G.-P. Gao, R. Qian, M. Croyle, B. Joshi, J. Tazelaar and J. M. Wilson, *Mol. Ther.* **3**, 697 (2001).
17. S. E. Raper, N. Chirmule, F. S. Lee, N. A. Wivel, A. Bagg, G.-P. Gao, J. M. Wilson and M. L. Batshaw, *Mol. Genet. Metab.* **80**, 148 (2003).
18. A. Bielinska, J. F. KukowskaLatallo, J. Johnson, D. A. Tomalia and J. R. Baker, *Nucleic Acids Res.* **24**, 2176 (1996).
19. J. F. KukowskaLatallo, A. U. Bielinska, J. Johnson, R. Spindler, D. A. Tomalia and J. R. Baker, *Proc. Natl. Acad. Sci. U.S.A.* **93**, 4897 (1996).
20. O. Boussif, F. Lezoualch, M. A. Zanta, M. D. Mergny, D. Scherman, B. Demeneix and J. P. Behr, *Proc. Natl. Acad. Sci. U.S.A.* **92**, 7297 (1995).
21. W. T. Godbey, K. K. Wu and A. G. Mikos, *J. Control. Release* **60**, 149 (1999).
22. H. Yin, R. L. Kanasty, A. A. Eltoukhy, A. J. Vegas, J. R. Dorkin and D. G. Anderson, *Nat. Rev. Gene.* **15**, 541 (2014).
23. W. T. Godbey, K. K. Wu and A. G. Mikos, *J. Biomed. Mater. Res.* **45**, 268 (1999).
24. L. Wightman, R. Kircheis, V. Rossler, S. Carotta, R. Ruzicka, M. Kursa and E. Wagner, *J. Gene Med.* **3**, 362 (2001).
25. M. Morille, C. Passirani, A. Vonarbourg, A. Clavreul and J.-P. Benoit, *Biomaterials* **29**, 3477 (2008).
26. D. W. Pack, A. S. Hoffman, S. Pun and P. S. Stayton, *Nat. Rev. Drug Discov.* **4**, 581 (2005).
27. R. Gref, M. Luck, P. Quellec, M. Marchand, E. Dellacherie, S. Harnisch, T. Blunk and R. H. Muller, *Colloids Surf. B, Biointerfaces* **18**, 301 (2000).
28. S. M. Moghimi, A. C. Hunter and J. C. Murray, *Pharmacol. Rev.* **53**, 283 (2001).
29. B. Romberg, W. E. Hennink and G. Storm, *Pharm. Res.* **25**, 55 (2008).
30. R. A. Petros and J. M. DeSimone, *Nat. Rev. Drug Discove.* **9**, 615 (2010).
31. M. Neu, D. Fischer and T. Kissel, *J. Gene Med.* **7**, 992 (2005).

32. S. C. De Smedt, J. Demeester and W. E. Hennink, *Pharm. Res.* **17**, 113 (2000).
33. H. Zhu, C. Dong, H. Dong, T. Ren, X. Wen, J. Su and Y. Li, *ACS Appl. Mater. Interfaces* **6**, 10393 (2014).
34. M. L. Forrest, J. T. Koerber and D. W. Pack, *Bioconjug. Chem.* **14**, 934 (2003).
35. H. M. Wu, S. R. Pan, M. W. Chen, Y. Wu, C. Wang, Y. T. Wen, X. Zeng and C. B. Wu, *Biomaterials* **32**, 1619 (2011).
36. H. G. Abdelhady, S. Allen, M. C. Davies, C. J. Roberts, S. J. Tendler and P. M. Williams, *Nucleic Acids Res.* **31**, 4001 (2003).
37. J. A. Frump, *Chem. Rev.* **71**, 483 (1971).
38. K. Aoi and M. Okada, *Prog. Polym. Sci.* **21**, 151 (1996).
39. H. M. L. Lambermont-Thijs, van der Woerdt, F. S. A. Baumgaertel, L. Bonami, F. E. Du Prez, U. S. Schubert and R. Hoogenboom, *Macromolecules* **43**, 927 (2009).
40. J. H. Jeong, S. H. Song, D. W. Lim, H. Lee and T. G. Park, *J. Control. Release* **73**, 391 (2001).
41. B. Brissault, A. Kichler, C. Guis, C. Leborgne, O. Danos and H. Cheradame, *Bioconjug. Chem.* **14**, 581 (2003).
42. A. von Harpe, H. Petersen, Y. Li and T. Kissel, *J. Control. Release* **69**, 309 (2000).
43. J. B. Sweeney, *Chem. Soc. Rev.* **31**, 247 (2002).
44. D. Fischer, T. Bieber, Y. X. Li, H. P. Elsasser and T. Kissel, *Pharm. Res.* **16**, 1273 (1999).
45. K. Kunath, A. von Harpe, D. Fischer, H. Peterson, U. Bickel, K. Voigt and T. Kissel, *J. Control. Release* **89**, 113 (2003).
46. X. Li, N. Chen and J. Xu, *Synthesis* **2010**, 3423 (2010).
47. S.-M. Zou, P. Erbacher, J.-S. Remy and J.-P. Behr, *J. Gene Med.* **2**, 128 (2000).
48. A. Schroeder, J. E. Dahlman, G. Sahay, K. T. Love, S. Jiang, A. A. Eltoukhy, C. G. Levins, Y. Wang and D. G. Anderson, *J. Control. Release* **160**, 172 (2012).
49. O. F. Khan, E. W. Zaia, S. Jhunjhunwala, W. Xue, W. Cai, D. S. Yun, C. M. Barnes, J. E. Dahlman, Y. Dong and J. M. Pelet, *Nano Lett.* **15**, 3008 (2015).
50. K. T. Love, K. P. Mahon, C. G. Levins, K. A. Whitehead, W. Querbes, J. R. Dorkin, J. Qin, W. Cantley, L. L. Qin, T. Racie, M. Frank-Kamenetsky, K. N. Yip, R. Alvarez, D. W. Sah, A. de Fougerolles, K. Fitzgerald, V. Koteliansky, A. Akinc, R. Langer and D. G. Anderson, *Proc. Natl. Acad. Sci. USA* **107**, 1864 (2010).
51. P. Y. Teo, C. Yang, J. L. Hedrick, A. C. Engler, D. J. Coady, S. Ghaem-Maghami, A. J. T. George and Y. Y. Yang, *Biomaterials* **34**, 7971 (2013).
52. S. Gao, H. Tian, Y. Guo, Y. Li, Z. Guo, X. Zhu and X. Chen, *Acta Biomater.* **25**, 184 (2015).
53. H. Tian, L. Lin, Z. Jiao, Z. Guo, J. Chen, S. Gao, X. Zhu and X. Chen, *J. Control. Release* **172**, 410 (2013).
54. M. Zheng, Y. Zhong, F. Meng, R. Peng and Z. Zhong, *Mol. Pharm.* **8**, 2434 (2011).
55. G. Navarro, S. Essex, R. R. Sawant, S. Biswas, D. Nagesha, S. Sridhar, C. T. de ILarduya and V. P. Torchilin, *Nanomed. Nanotechnol. Biol. Med.* **10**, 411 (2014).
56. J. Li, D. Cheng, T. Yin, W. Chen, Y. Lin, J. Chen, R. Li and X. Shuai, *Nanoscale* **6**, 1732 (2014).
57. J. Hu, S. Miura, K. Na and Y. Han Bae, *J. Control. Release* **172**, 69 (2013).
58. A. Masotti, F. Moretti, F. Mancini, G. Russo, N. D. Lauro, P. Checchia, C. Marianecci, M. Carafa, E. Santucci and G. Ortaggi, *Bioorg. Med. Chem.* **15**, 1504 (2007).
59. G. Guo, L. Zhou, Z. Chen, W. Chi, X. Yang, W. Wang and B. Zhang, *Int. J. Pharm.* **450**, 44 (2013).
60. L. Liu, M. Zheng, T. Renette and T. Kissel, *Bioconjug. Chem.* **23**, 1211 (2012).
61. A. Zintchenko, A. Philipp, A. Dehshahri and E. Wagner, *Bioconjug. Chem.* **19**, 1448 (2008).
62. C.-J. Chen, J.-C. Wang, E.-Y. Zhao, L.-Y. Gao, Q. Feng, X.-Y. Liu, Z.-X. Zhao, X.-F. Ma, W.-J. Hou and L.-R. Zhang, *Biomaterials* **34**, 5303 (2013).
63. A. A. Eltoukhy, D. Chen, C. A. Alabi, R. Langer and D. G. Anderson, *Adv. Mater.* **25**, 1487 (2013).
64. R. M. Schiffelers, A. Ansari, J. Xu, Q. Zhou, Q. Tang, G. Storm, G. Molema, P. Y. Lu, P. V. Scaria and M. C. Woodle, *Nucl. Acids Res.* **32**, e149 (2004).
65. Y. Wen, S. Pan, X. Luo, X. Zhang, W. Zhang and M. Feng, *Bioconjug. Chem.* **20**, 322 (2009).
66. R. Esfand and D. A. Tomalia, *Drug Discov. Today* **6**, 427 (2001).
67. J. Khandare, P. Kolhe, O. Pillai, S. Kannan, M. Lieh-Lai and R. M. Kannan, *Bioconjug. Chem.* **16**, 330 (2005).
68. C. C. Lee, J. A. MacKay, J. M. J. Fréchet and F. C. Szoka, *Nat. Biotechnol.* **23**, 1517 (2005).
69. L. Jin, X. Zeng, M. Liu, Y. Deng and N. He, *Theranostics* **4**, 240 (2014).
70. H. Liu, H. Wang, W. Yang and Y. Cheng, *J. Am. Chem. Soc.* **134**, 17680 (2012).
71. Y. Tang, Y.-B. Li, B. Wang, R.-Y. Lin, M. van Dongen, D. M. Zurcher, X.-Y. Gu, M. M. B. Holl, G. Liu and R. Qi, *Mol. Pharm.* **9**, 1812 (2012).
72. S. Huang, J. Li, L. Han, S. Liu, H. Ma, R. Huang and C. Jiang, *Biomaterials* **32**, 6832 (2011).
73. S. Futaki, T. Suzuki, W. Ohashi, T. Yagami, S. Tanaka, K. Ueda and Y. Sugiura, *J. Biol. Chem.* **276**, 5836 (2001).

74. H. D. Herce, A. E. Garcia, J. Litt, R. S. Kane, P. Martin, N. Enrique, A. Rebolledo and V. Milesi, *Biophys. J.* **97**, 1917 (2009).
75. S. Futaki, *Adv. Drug Deliv. Rev.* **57**, 547 (2005).
76. C. Liu, X. Liu, P. Rocchi, F. Qu, J. L. Iovanna and L. Peng, *Bioconjug. Chem.* **25**, 521 (2014).
77. S. Mansouri, P. Lavigne, K. Corsi, M. Benderdour, E. Beaumont and J. C. Fernandes, *Eur. J. Pharm. Biopharm.* **57**, 1 (2004).
78. S. Mao, W. Sun and T. Kissel, *Adv. Drug Deliv. Rev.* **62**, 12 (2010).
79. M. Koping-Hoggard, I. Tubulekas, H. Guan, K. Edwards, M. Nilsson, K. M. Varum and P. Artursson, *Gene Ther.* **8**, 1108 (2001).
80. S. Gao, J. Chen, X. Xu, Z. Ding, Y.-H. Yang, Z. Hua and J. Zhang, *Int. J. Pharm.* **255**, 57 (2003).
81. H.-L. Jiang, J.-T. Kwon, E.-M. Kim, Y.-K. Kim, R. Arote, D. Jere, H.-J. Jeong, M.-K. Jang, J.-W. Nah and C.-X. Xu, *J. Control. Release* **131**, 150 (2008).
82. C. Yang, S. Gao and J. Kjems, *J. Mater. Chem. B* **2**, 8608 (2014).
83. M. A. Mintzer and E. E. Simanek, *Chem. Rev.* **109**, 259 (2008).
84. A. K. Varkouhi, M. Scholte, G. Storm and H. J. Haisma, *J. Control. Release* **151**, 220 (2011).
85. X. Cai, H. Zhu, H. Dong, Y. Li, J. Su and D. Shi, *Adv. Healthc. Mater.* **3**, 1818 (2014).
86. H. S. Hwang, J. Hu, K. Na and Y. H. Bae, *Biomacromolecules* **15**, 3577 (2014).
87. P. Midoux and M. Monsigny, *Bioconjug. Chem.* **10**, 406 (1999).
88. J. Deng, Y. Wen, C. Wang, S. Pan, H. Gu, X. Zeng, L. Han, Y. Zhao, M. Feng and C. Wu, *Pharm. Res.* **28**, 812 (2011).
89. X. Zeng, S. Pan, J. Li, C. Wang, Y. Wen, H. Wu, C. Wang, C. Wu and M. Feng, *Nanotechnology* **22**, 375102 (2011).
90. S. Pan, D. Cao, R. Fang, W. Yi, H. Huang, S. Tian and M. Feng, *J. Mater. Chem. B* **1**, 5114 (2013).
91. D. M. Lynn and R. Langer, *J. Am. Chem. Soc.* **122**, 10761 (2000).
92. J. C. Sunshine, D. Y. Peng and J. J. Green, *Mol. Pharm.* **9**, 3375 (2012).
93. J. C. Sunshine, S. B. Sunshine, I. Bhutto, J. T. Handa and J. J. Green, *PLoS One* **7**, e37543 (2012).
94. G. T. Zugates, W. Peng, A. Zumbuehl, S. Jhunjhunwala, Y.-H. Huang, R. Langer, J. A. Sawicki and D. G. Anderson, *Mol. Ther.* **15**, 1306 (2007).
95. D. G. Anderson, A. Akinc, N. Hossain and R. Langer, *Mol. Ther.* **11**, 426 (2005).
96. A. Mangraviti, S. Y. Tzeng, K. L. Kozielski, Y. Wang, Y. Jin, D. Gullotti, M. Pedone, N. Buaron, A. Liu and D. R. Wilson, *ACS Nano* **9**, 1236 (2015).
97. J. Gu, X. Wang, X. Jiang, Y. Chen, L. Chen, X. Fang and X. Sha, *Biomaterials* **33**, 644 (2012).
98. S. Tang, Q. Yin, Z. Zhang, W. Gu, L. Chen, H. Yu, Y. Huang, X. Chen, M. Xu and Y. Li, *Biomaterials* **35**, 6047 (2014).
99. C. Boyer, V. Bulmus, T. P. Davis, V. Ladmiral, J. Liu and S. Perrier, *Chem. Rev.* **109**, 5402 (2009).
100. A. W. York, S. E. Kirkland and C. L. McCormick, *Adv. Drug Deliv. Rev.* **60**, 1018 (2008).
101. A. J. Convertine, D. S. W. Benoit, C. L. Duvall, A. S. Hoffman and P. S. Stayton, *J. Control. Release* **133**, 221 (2009).
102. C. E. Nelson, A. J. Kim, E. J. Adolph, M. K. Gupta, F. Yu, K. M. Hocking, J. M. Davidson, S. A. Guelcher and C. L. Duvall, *Adv. Mater.* **26**, 607 (2014).
103. C. E. Nelson, J. R. Kintzing, A. Hanna, J. M. Shannon, M. K. Gupta and C. L. Duvall, *ACS Nano* **7**, 8870 (2013).