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Exploring the role of polymer structure on intracellular nucleic acid delivery via polymeric nanoparticles

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Abstract

Intracellular nucleic acid delivery has the potential to treat many genetically-based diseases, however, gene delivery safety and efficacy remains a challenging obstacle. One promising approach is the use of polymers to form polymeric nanoparticles with nucleic acids that have led to exciting advances in non-viral gene delivery. Understanding the successes and failures of gene delivery polymers and structures is key to engineering optimal polymers for gene delivery in the future. This article discusses the polymer structural features that enable effective intracellular delivery of DNA and RNA, including protection of nucleic acid cargo, cellular uptake, endosomal escape, vector unpacking, and delivery to the intracellular site of activity. The chemical properties that aid in each step of intracellular nucleic acid delivery are described and specific structures of note are highlighted. Understanding the chemical design parameters of polymeric nucleic acid delivery nanoparticles is important to achieving the goal of safe and effective non-viral genetic nanomedicine.

Graphical abstract

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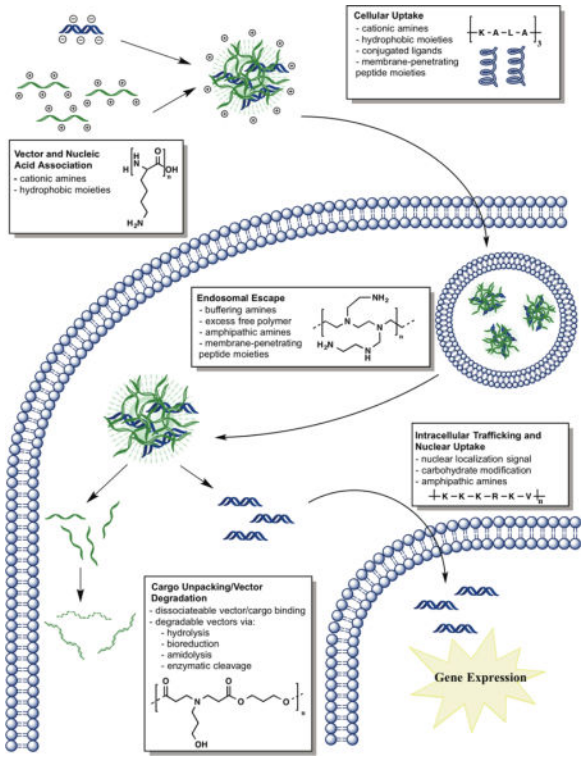

Introduction

Because aberrations in the genetic code are the root cause of many inheritable diseases (i.e., cystic fibrosis, hematological disorders, severe combined immunodeficiency disorder) [1] and acquired diseases such as cancer, gene therapy, if fully realized, could provide treatments, and potentially even cures [2]. Although gene therapy is promising, realizing the potential of gene therapy has proven to be a tremendous challenge. Despite more than 2,000 clinical trials worldwide [3], to date only two gene therapies have reached approval by regulatory bodies; namely the Chinese Food and Drug Administration (2003; adenovirus vector delivering the P53 gene for a head and neck squamous cell carcinoma application) and the European Medicines Agency (2012; associated-adenovirus vector delivering the lipoprotein lipase gene for a lipoprotein lipase deficiency application). There have not been any U.S. FDA approvals, underscoring the need for safer and more effective gene delivery vectors.

Because viral vectors have evolved to be highly efficient, many of the past and ongoing clinical trials have focused on viral methods. However, viral methods are known to be associated with insertional mutagenesis, can cause deleterious side effects, and are immunogenic which raise safety concerns. There are a host of attractive non-viral alternatives which enable transient non-integrating gene transfer and mitigate safety concerns. Non-viral methods do not have cargo size restrictions that viruses do, enabling larger genes to be delivered and combinations of genes. Non-viral biomaterials are also able to be manufactured on a larger scale more easily than viruses and are able to be more readily chemically modified for enhanced function as well.

Although non-viral vectors are typically considered safer than viral vectors, further optimization of non-viral vectors is necessary for clinical translation. As biomaterial and nanoparticle structure-function relationships are more thoroughly characterized, rational design principles can be implemented to engineer superior polymeric vectors, aiding the clinical translation of gene therapies.

This review article discusses how structural elements of natural (i.e., peptides such as poly(L-lysine) (PLL) and carbohydrates, such as chitosan) and synthetic (i.e., poly(β-amino ester)s (PBAE), poly(ethyleneimine) (PEI), polyurethane, disulfide-containing poly(amido amine)s, alkyl-amine containing polymers, imine-containing polyamines, and polyorthoesters) polymers are used to aid the transport of nucleic acid cargo (i.e., short hairpin RNA (shRNA), plasmid DNA (pDNA), short interfering RNA (siRNA), micro RNA (miRNA), and messenger RNA (mRNA) [4]) to overcome intracellular barriers (Table 1). These barriers include: vector and nucleic acid cargo association for protection of nucleic acid from degradation; cellular uptake; endosomal escape to the cytoplasm; nucleic acid unpacking from the vector carrier; vector degradation to minimize toxicity; and lastly, nuclear uptake in the case of DNA (Figure 1) [4].

Vector and nucleic acid cargo association

Nucleic acid molecules are difficult to deliver intracellularly due in large part to the ease in which enzymes can degrade them and the difficulty of transporting large negatively charged biomacromolecules across cellular membranes. Therefore, the first step required from a polymer to be used as a non-viral polymeric gene delivery vector is nucleic acid binding, complexation, and/or encapsulation. Typically anionic nucleic acids are ionically complexed to cationic polymer vectors through electrostatic interactions via amine functional groups capable of forming nanoparticles (also referred to as polyplexes) on the order of 25–300 nm [6, 7]. Cationic amine-containing vectors have included natural polymers of amino acids [8] and sugars such as histone proteins [9], peptides [10], and chitosan [1], as well as synthetic polymers such as linear and branched poly(ethyleneimine) (PEI) [11], dendrimers [12], poly(β-amino ester)s (PBAE) [13], peptide nucleic acid (PNA) [14], and many others. Structures of commonly used polymers that form nanoparticles with nucleic acids for intracellular delivery are shown in Figure 2 [1, 13, 15–17]. A number of the effective polymers (i.e., PBAEs, epoxide-containing block copolymers) for gene delivery have been discovered via combinatorial high throughput methods [18–20].

Due to charge neutralization by cationic materials, large anionic biomacromolecules such as plasmid DNA can be condensed into a small sized particles. This is critical to protect nucleic acids from nuclease degradation [22] extracellularly and intracellularly. The half-life of uncomplexed nucleic acid extracellularly is on the order of 10 minutes [23] and intracellularly on the order of 1 hour [24]. Therefore, protection of nucleic acid within polymeric nanoparticles is important for successful nucleic acid delivery. It has been shown for example that a polymer complexed single-stranded nucleic acid’s half-life can be improved 3- to 6-fold during a 5 hour incubation in serum and more than 90% of polymer complexed pDNA remained full length in 1.5 and 3 hour incubations in serum and culture medium (10% serum) [25].

In addition, polymer/DNA polyplex complexation is critical to enable nucleic acids to be effectively internalized through a cell's anionic phospholipid bilayer. Typically zeta potentials of polymer/DNA polyplexes are neutral to positively charged, which allows for better interaction of the polyplex with the anionic cell membrane. Binding of a polymer to a nucleic acid is multivalent due to the many positive charges of amines on a polymer (primarily the primary and secondary amines) associating with the many negatively charged phosphate groups on a nucleic acid. Several groups have quantified these polymer/DNA binding interactions for various cationic polymers and peptides [10, 11, 13, 26]. A generalized finding observed between different polymer structures and molecular weights in these studies is that binding affinity between cationic polymers and DNA is often biphasic, with binding affinity that is too low or too high contributing to poorer transfection efficacy than intermediate binding affinities. For example, with PBAEs it was found that polymer/DNA binding affinity increased with increasing PBAE molecular weight, with decreasing numbers of carbon atoms in the backbone or sidechain of the constitutive monomers, and with the incorporation of amine-containing polymer terminal groups [13]. The transfection levels were biphasic with binding affinity in two evaluated human cell lines, with optimal transfection activity with this class of polymers occurring with binding constants per amine in the range of $1–6\times10^4$ M^{-1}. The importance of complexation has also been demonstrated through microfluidics assisted confinement experiments where polyplexes with 40–50% smaller diameters are formulated compared to bulk mixing approaches [27]. These more compact polyplexes were found to transfect 6–31% more cells and have 1.9–6.8-fold higher total exogenous gene expression [27].

Natural cationic peptides such as PLL were among of the first materials used as polymeric transfection agents due to their availability and their ability to form nanoparticles with DNA. PLL's strong positive charge allowed it to bind to and charge-invert DNA [28–30]. Because histones naturally complex DNA through charged residues, histone proteins, namely H2A, have been used to complex DNA [9]. This approach has shown many fold higher transfection compared to other non-viral methods such as PLL, poly(L-arginine), and liposomes (phosphatidylcholine/serine, 7:3 molar ratio) [9]. The type of histone protein is critical as in this study there was little transfection observed using H1, H2B, H3, and H4. Synthetic polymers useful for binding DNA took their bio-inspired cues from biological DNA binding proteins that have high cationic charge. PEI, for example, has an extremely high cationic density as, throughout its structure, for every two carbon atoms there is an amine group.

Nucleic acid binding can be particularly challenging in the case of shorter therapeutic nucleic acids such as siRNA and miRNA compared to plasmid DNA. These nucleic acids are important for engineered gene regulation as both siRNA and miRNA can induce sequence-specific gene knockdown [31]. However, both RNAs are ~21–25 bp in length, approximately 200 times shorter than the length of plasmids typically used for gene delivery. Double stranded RNA is also stiffer, as a macromolecule, than double stranded DNA [32, 33]. Shorter nucleic acid length reduces the multivalency in the charge interaction between anionic nucleic acids and cationic polymers, and stiffness can prevent RNA from conforming into shapes favorable for polymer binding. To increase polymer/RNA binding and subsequent intracellular delivery, researchers have engineered siRNAs to be longer by

using covalent linkages [34], sticky RNA overhangs [35], or have formed much larger structures such as siRNA microsponges [36]. Strategies that focus on polymer engineering have included chemically modifying polymers to be more cationic or hydrophobic. For example, poly(amido amine)s (PAAs) have been modified using ethylene diamine or triethylenetetraamine to increase polymer positive charge and increase siRNA binding [37, 38]. For PBAEs, increasing polymer hydrophobicity [39, 40] or molecular weight [40] led to successful siRNA binding and delivery. Additionally, increasing the PBAE to siRNA weight ratio (wt/wt) of polyplexes to 100:1 or greater has led to successful siRNA binding and intracellular delivery, while PBAE:DNA wt/wt is typically in a lower range from 25–75 wt/wt [41].

Chemically controlling siRNA binding for optimal delivery has also been employed by modifying poly(acrylic acid) polymers with amine-containing molecules. By functionalizing acrylic acid size chains with hydrophobic amine-containing agmatine (Agm) or hydrophilic amine-containing monosaccharide D-galactosamine (Gal), Pelet *et al* [42]. were able to fine tune siRNA binding to enable enhanced delivery. Polymers containing higher Agm content bound siRNA tightly and effectively knocked down protein expression, but were increasingly toxic as Agm content increased, likely due to the hydrophobicity of Agm [42].

Siegwart et al., via high throughput modular robotic synthesis, investigated 1,536 epoxide-functionalized, amine-containing block polymeric nanoparticle formulations which form hairy core-shells (Figure 3) [20]. The authors designed these materials to have a cationic core for siRNA entrapment and also found that optimal cross-linkers contained tertiary dimethylamine or piperazine, capable of aiding the proton sponge effect through endosomal buffering. Utilizing these materials, the authors were able to deliver both siRNA and pDNA *in vitro* as well as to murine hepatocytes *in vivo* [20].

A small compact nanoparticle size is also important for systemic biodistribution of nanoparticles administered *in vivo*, particularly as cancer therapeutics. Tumor neovasculature is heterogeneous and more permissive to the transport of nanostructures from the blood than healthy vasculature [43]. Thus, polymers with suitable binding affinity have the potential to bind nucleic acid to form a small and stable nanoparticle that is above the glomerular filtration cutoff (> 10 nm) and below the cutoff for permeability in leaky tumor vasculature (< 200 nm preferably) [44, 45] to pass into the extravascular space. Once into the tumor space, the nanoparticles are more likely to be retained there due to compromised lymphatic drainage (enhanced permeability and retention effect) [43]. For a cancer therapeutic, this is an important nanomedicine feature.

A common structural feature among gene delivery polymers to satisfy the design requirements of complexing nucleic acid is cationic amines. Although primary amines have stronger binding affinity with nucleic acids than secondary or tertiary amines, the presence of primary amines is not a strict requirement for successful condensation and transfection. Generally, the overall charge of polyplex nanoparticles post-nucleic acid complexation is cationic, aiding the interaction with the anionic phospholipid bilayer membrane.

Cellular Uptake

Cellular uptake of polymeric nanoparticles is often through adsorptive endocytosis [46]. Analysis of the cell membrane's interaction with amphiphilic materials has demonstrated that adsorption of hydrophobic moieties can cause disruption or deformation of the outer layer of the cell membrane [47], and that this disruption can induce endosomal uptake (Figure 4A) [48]. Therefore, promoting cellular uptake is often accomplished using polymers designed to increase the interaction between nanoparticles and the cell membrane.

Cationic materials are employed to electrostatically interact with the negative charge of the phospholipid bilayer membrane [49]. In order to accomplish this, it is necessary to cause the nucleic acid to charge-invert through the use of adequate cationic polymer. As previously mentioned, polymers like PLL can bind to anionic DNA to form a cationic polyplex that can then associate with the anionic cell surface [28–30]. It should be noted, however, that very strong positive charges could lead to cytotoxicity, likely due to electrostatic interactions that can deform the cell membrane to an unstable state [50]. It is therefore important to balance cationic polymer structure to optimize uptake without inducing cytotoxicity.

Hydrophobic polymers can also induce cell membrane adsorption, and have been shown to enhance gene delivery [51]. Moreover, cationic polymers that are more hydrophobic require less polymer mass to achieve charge-inversion of DNA, thus enabling uptake [30]. Functionalizing conventional polymers with hydrophobic moieties has promoted enhanced cell uptake. For example, PEI and PLL modified with palmitic acid promoted increased binding to the cell surface, and binding increased as the degree of substitution increased [52]. PAAs modified with alkyl chains have shown increased uptake as the number or length of alkyl modifications increased [12]. Tzeng *et al.* screened a library of PBAEs for siRNA delivery and found that only the more hydrophobic polymers tested were capable of any measureable gene knockdown [40].

Li, et al. determined which cationic lipid-like structures or lipidoids amongst 200 were able to efficiently deliver pDNA and siRNA (comparable or superior to Lipofectamine 2000 in HEK293T cells; approximately 2% of library) using a high throughput screening technique which involved a single-step alkylation of amines. They found they were able to enhance transfection by combining single-chain with double-chain lipidoids [53].

For optimization of siRNA delivery, bioreducible PBAE monomers have been copolymerized with hydrophobic monomers at varying ratios. Gene knockdown using these polymers demonstrated a biphasic response in which moderate hydrophobicity optimized gene delivery but increasing hydrophobicity led to cytotoxicity [39]. This demonstrates that polymer hydrophobicity, as with positive charge, must be balanced for optimal gene delivery without cytotoxicity.

A principal component analysis of PBAEs elucidated the degree that 24 physicochemical properties drove transfection, cellular uptake, and viability in human glioblastoma cells [54]; Hydrophobicity, as measured by the LogP partition coefficient and by the number of carbons in a polymer's repeat unit, were both key drivers of cellular uptake and transfection [54]. While hydrophobicity drove principal component 1, molecular weight was found to

drive principal component 2, with increased molecular weight being the most critical for successful transfection of the most hydrophilic polymers. Broadly among polymeric gene delivery nanoparticles, a common structural feature varied to optimize cellular uptake is the length of alkyl chains in the backbones or sidechains (i.e., PBAE, epoxide-containing block co-polymers, alkyl-amine-containing polymers, and poly(amido amine)s).

Cell-penetrating peptides (CPPs), a component of certain gene delivery polymers, often employ electrostatic or hydrophobic interactions to interact with the cell membrane. Amphipathic polypeptide GALA, consisting of 4 Glu-Ala-Leu-Ala repeats, is designed to interact with cell membrane lipid bilayers [55]. Peptide KALA, containing 3 Lys-Ala-Leu-Ala repeats, interacts similarly with the cell membrane but is cationic at slightly acidic to neutral pH, enabling nucleic acid binding [56]. Trans-activating transcriptional activator (TAT) peptide derived from HIV also penetrates lipid bilayers and can be functionalized onto polymeric nanoparticles to promote uptake [57, 58]. Manickam *et al.* designed multimeric TAT peptide linked with disulfide bonds as a gene delivery vehicle designed to enhance uptake [59]. Arginine-rich CPPs have also been employed for DNA delivery via modification of poly(methacrylate) polyplexes [60].

Small changes to polymer structure can dramatically change the efficacy and mechanism of cellular uptake. PBAE polymers end-capped with either amine-containing or acrylate-containing small molecules of similar size, DNA binding strength, and buffering capacity were used to form nanoparticles that were measured to have similar sizes and zeta potentials. Although every measured physical property showed the nanoparticles to be similar, uptake and transfection were negligible in acrylate-terminated polymers while amine-terminated polymers enabled successful uptake and transfection [61].

Small molecule changes to polymer structure can also direct uptake to specific endocytotic pathways (Figure 4B and 4C) [48]. Cellular uptake pathways relevant to polymeric gene delivery include macropinocytosis, clathrin-mediated endocytosis, and caveolae-mediated endocytosis. Non-specific, actin-driven uptake via macropinocytosis envaginates the cell membrane, engulfing the surrounding extracellular fluid [62]. Macropinocytosis has been implicated in nanoparticle uptake but often results in reduced transfection due to endosomal recycling [63]. Clathrin-mediated endocytosis, in which ~100–150 nm pits form via clathrin macromolecular organization, can be targeted by modification of nanoparticles with MC1SP-peptide or transferrin [64]. Unmodified liposomes and lipid-based materials have demonstrated clathrin-mediated endocytosis as their major mechanism of uptake [65]. Caveolae-mediated endocytosis involves ~50–100 nm flask-shaped pits which form within the cell membrane. These pits can be targeted via conjugation of folic acid to nanoparticles [66]. Caveolae-mediated endocytosis has been shown to be the major uptake pathway for several polymeric gene delivery nanoparticles [5, 67, 68]. Caveolae-mediated endocytosis has also been demonstrated to be the major uptake pathway of PBAE nanoparticles to human breast cancer cells, but the pathway which most determined transfection efficacy varied with polymer structure and molecular weight. Overall in PBAE-based studies, clathrin-mediated endocytosis enabled the most efficient transfection, while certain particles uptaken via caveolae encountered inefficient intracellular delivery that generally did not enable transfection [5].

It is important to note that although cellular uptake is a necessary step in successful transfection, uptake does not always lead to transfection. In work by Guerrero-Cazares *et al.*, DNA delivery via PBAE nanoparticles was significantly more effective in primary human brain cancer cells versus human neural progenitor cells, while cell uptake was similar *in vitro*. Delivery of the same particles to brain cancer cells *in vivo* demonstrated selective transfection of cancer versus healthy tissue, even though nanoparticle uptake was distributed amongst both tissues [69]. However, in other cases, cell uptake is the key step in cell-type specificity. Modifying amine-containing molecules with glucopyranose block copolymers enabled Wu *et al.* to selectively target human hepatocellular carcinoma over human cervix adenocarcinoma, while removing the sugar modifications had the opposite targeting effect [70].

To increase the cellular uptake of polymeric nanoparticles in target cells, a ligand may also be conjugated to the nanoparticles that targets an overexpressed receptor of interest. For example, Zhang, et al, and Ogris, et al. have conjugated various ligands to their polymeric gene delivery nanoparticles such as folic acid [71] and epidermal growth factor [72]. In addition, polymeric nanoparticle affinity for a particular receptor may also trigger other biological responses. For example, a viologen-based dendrimer has been known to have affinity for CXC receptor 4, functioning as an antagonist of cancer cell metastasis [73]. Understanding the molecular pathways involved in cellular uptake and intracellular nanoparticle trafficking is an important consideration for future polymer structure design and may enable polymeric nanoparticles to target specific routes of uptake in target cells for enhanced transfection in specific cell types.

Endosomal escape

Following cellular uptake via adsorptive endocytosis, polymeric nanoparticles must escape the endosomal compartment in order for nucleic acids to reach their subcellular targets in the cytosol or nucleus. For amine-containing or titratable polymers, a mechanism that has been well reported in the literature [74, 75], but also still actively debated [76], is known as the “proton sponge” hypothesis. In this mechanism, protons pumped into the endosome do not decrease the endosomal pH as they normally would as this potential pH is buffered by reversibly-protonable polymeric moieties such as tertiary amines. A buildup of positive charge causes an influx of chloride counter-ions within the endosomes. The resulting hypertonic environment causes osmosis and an influx of water triggers endosome lysis and the nanoparticles are released into the cytosol. This process also protects the nucleic acids from damage due to the acidification of the endosome and leads to higher binding affinity between the polymer and nucleic acid as the polymer becomes further protonated. The proton-sponge effect was introduced to explain the gene delivery efficacy of PEI, which contains a high proportion of buffering tertiary and secondary amines, versus PLL which contains relatively more non-buffering primary amines [74]. Although the proton-sponge mechanism is only a theory and has been challenged [76], it remains the most widely accepted explanation for the endosomal escape of polymeric materials [75, 77]. While PLL has generally been shown incapable of endosomal release on its own [78, 79], it can be modified to allow buffering through covalent addition of chemical moieties with titratable amines, such as histidine and arginine, or via coencapsulation of amphipathic amines such as

chloroquine [80, 81]. Many PAAs contain chemical groups that are able to be protonated at pH 5.1–7.4 which makes them capable of proton buffering and endosomal lysis [82]. PBAEs also contain titratable tertiary and secondary amines. Although the buffering capacities of common PBAEs are not as high as PEI on a per-mass basis, (1.4–4.6 mmol of H^+/g vs. 6.2 mmol of H^+/g, respectively) the PBAEs are less cytotoxic and can be used at higher wt/wt ratio to enable nanoparticle concentrations with approximately 5–fold higher total buffering capacity than PEI [61].

Lachelt, et al. were able to fine-tune proton sponge activity using a library of oligo(ethanamino)amides, containing oligoethanamino acids and histidines. This system has the ability to be protonated and has the pH profile need to rupture endosomes [83] which is critical for gene transfer. Furthermore, modification of poly(glycoamidoamine)s with moieties with higher buffering capacities has shown an increase in transfection based on the proton sponge theory [84].

It has been shown that without free polymer in solution, there is relatively very little transfection [85], that supplementation with free polymer restores gene transfection, and also that the physical states of polyplexes are not affected by free polymer [86–88]. Because polyplexes have an overall positive charge they can interact with the negatively charged cell membranes [89]. The excess polymer has been shown to facilitate uptake, decrease inhibitory polyplex interaction with glycosaminoglycans, and to play a role in the buffering of the endosome for the proton sponge effect [89]. Researchers have also shown that cellular uptake of polyplexes may not be dependent on the presence of free polymer and that free polymer may aid mostly with downstream delivery steps. It was shown in CT 26 cells that transfection was highest when free PEI was added 4 hours post plasmid incubation. As a result, the authors concluded that free polymer likely played a larger role in endosomal release or another mechanism downstream from cellular uptake. Figure 5 shows polyplexes (red Cy3-DNA in Figures 5A and 5D) and free linear PEI (green Cy5 in Figures 5B and 5E) in live cells 8 hours post transfection. Figure 5C and 5F show combined fluorescence channels with overlaid differential interference contrast (DIC). The yellow color indicates co-localization of free linear PEI and Cy3-conjugated plasmid. Figures 5A, 5B, and 5C are cells that were transfected simultaneously with free linear PEI whereas Figures 5D, 5E, and 5F are cells that were transfected and had the free linear PEI added 4 hours after transfection [86]. Because there was no difference in the intracellular distribution of Cy3-DNA (Figures 5C and 5F), it was demonstrated that the majority of free Cy5 PEI was colocalized with polyplexes 8 hours after transfection whether or not the free Cy5 PEI was added simultaneously or 4 hours after transfection, which was after when the polyplexes were completely internalized. This suggests that the free polymer did not play a role initially during the cellular uptake of the polyplexes.

Perhaps the most common structural feature of gene delivery polymers to enable endosomal escape is amine groups capable of buffering in physiologically relevant pH (4.5–7.4). Yet, endosomal escape strategies also extend beyond buffering-induced endosomal lysis, often using materials engineered to directly interact with and disrupt the endosomal membrane. For example, anionic polymers have been employed to induce membrane disruption at moderately acidic endosomal pH [90]. CPPs designed to interact with and disrupt the cell

membrane can also be engineered to disrupt the endosome. GALA and KALA peptides have been added to nanoparticles to induce endosomal escape [29, 91, 92]. KALA peptide is particularly good at disrupting late endosomes as it is alpha-helical at lower pH (4.5), and has been shown to induce 100% endosomal leakage over the pH range 4.5–8 [56]. The same arginine-rich CPPs that can enhance cell uptake have also been employed to facilitate endosomal escape [60]. In sum, there are multiple polymer strategies to protect nucleic acids from degradation and facilitate their release to the cytoplasm.

Cargo unpacking/vector degradation to minimize toxicity

Nucleic acid unpackaging from the nanoparticle is an imperative step in successful delivery. Research with PAAs and PEI-based polymers has demonstrated that DNA must be unbound from its delivery material in order for transfection to take place [93, 94]. Similarly, siRNA functionalized to gold nanoparticles has been shown to be unable to induce RNA interference (RNAi) when a siRNA release mechanism is not in place [95], but similar nanoparticles that enable release also enable RNAi [96]. As stated earlier, nucleic acid binding must be balanced to promote strong nanoparticle complexation while eventually allowing for cargo release, as transfection efficacy has been shown to have a biphasic relationship with nucleic acid binding affinity [13]. This can be achieved by balancing cationic character with hydrophobicity. Forrest *et al.* demonstrated that acetylation of primary amines in PEI could increase transfection as much as 21-fold over branched PEI when 43% of primary amines were acetylated [97]. This was later directly correlated with decreased polymer-DNA binding strength [98]. The most common mechanism of nucleic acid release is thermodynamic unbinding of the polymer and nucleic acid. The most common approach of increasing the rate of nucleic acid release is via polymer degradation, which enables high affinity nucleic acid binding to occur during polyplex formation and then this binding can be weakened over time or in a specific targeted intracellular environment. Polymer degradation has the added benefit of reducing cytotoxicity, as the molecular weight of polycations has been positively correlated with cytotoxicity [99]. Polymer molecular weight of conventional DNA delivery polymers such as linear PEI-PEG block copolymers has been shown to correlate positively with cytotoxicity. While increasing PEG chain length can reduce some of this toxicity, a PEG content higher than 50% was shown to also reduce transfection efficacy [100].

Hydrolytic polymer degradation via the addition of ester groups into a polymer’s structure can impart biodegradability to conventional polymers, which are normally non-degradable. Poly[alpha-(4-aminobutyl)-L-glycolic acid] (PAGA) is an analog of PLL with amide (peptide) bonds replaced with esters. DNA delivery using PAGA has shown enhanced transfection over PLL with reduced cytotoxicity [101]. Short, linear 800 kDa PEI linked with ester-containing diacrylate monomers has been shown to enable the same nanoparticle size, charge, DNA binding strength, and polymer MW as commercially-available 25 kDa branched PEI, but also enables hydrolytic degradation, and consequently,16-fold higher transfection and less toxicity [102].

Ester-containing monomers can be used to engineer polymers that are biodegradable without the need of subsequent polymer modification. Solid poly(lactic-co-glycolic acid) PLGA

nanoparticles, as opposed to cationic polyplexes, can encapsulate DNA and siRNA via a double emulsion method or by grafting polyamines onto the PLGA backbone. PLGA degrades hydrolytically and its cargo release rate can be easily tuned by adjusting polymer properties such as monomer ratios and chain length [103–105]. PBAEs contain hydrolytically degradable esters whose half-lives are on the order of hours at pH 7.4 at 37°C, but this rate slows at moderately acidic pH and can protect the cargo in endosomes [40].

Hydrolytic biodegradation is also possible using urethanes, imines, and orthoesters. Yang *et al.* demonstrated DNA delivery using amine-containing polyurethanes [17]. For acid-labile hydrolysis, such as in the acidic tumor microenvironment, imine bonds have been employed to link short PEI chains [106]. Polyorthoesters can also be used for acid-labile hydrolysis as they degrade at pH 5 but not 7 [16, 107].

Disulfide linkages can enable environmentally-triggered release in the cytosol, which is approximately 1000-fold more reducing than the extracellular space [109]. This is particularly beneficial for delivery of mRNA, miRNA, and siRNA, whose site of action is in the cytoplasm [31]. It should be noted that disulfide bioreduction of DNA-carrying nanoparticles has in some cases decreased the expression of exogenously delivered DNA, which is likely due to reducing the amount of intact DNA that ultimately reaches the nucleus [41]. As with esters, addition of disulfides to conventionally nondegradable polymers can enhance nucleic acid delivery while reducing cytotoxicity. Disulfide-linked linear chains of PEI has shown comparable siRNA delivery efficacy versus branched PEI while significantly reducing cytotoxicity [110]. PLL modified with cysteines or with thiol-modified lysines allow for oxidation-induced crosslinking of nanoparticles and enable delivery of DNA or siRNA [111–113]. A comparison was made between linear, dendritic and hyperbranched (randomly branched; one-step preparation on a large scale) PLL structures [114]. The authors of the study found that transcription and translation were proportional to molecular weight, and that at similar molecular weights, the hyperbranched PLL analogs were superior. Disulfide-containing PAAs synthesized from diacrylamide monomers with disulfide linkers have been extensively studied for both DNA and siRNA delivery (Figure 6) [108, 115] and show improved intracellular nucleic acid release leading in many cases to enhanced efficacy. As discussed previously, PAA binding of siRNA can be too weak to form stable nanoparticles, but this can be overcome by modifying the polymers to be more cationic [37, 38]. KALA peptides covalently linked to cysteines have been used to form fusogenic nanoparticles capable of binding siRNA, using peptide conformation to induce cellular uptake and endosomal escape, and then releasing siRNA upon entry to the cytosol [116]. PBAEs containing disulfide bonds in the polymer endcaps [40, 117] or along the polymer backbone [39, 118] have enabled successful siRNA delivery to mesenchymal stem cells and brain cancer cells. Bioreducible PBAEs have demonstrated complete siRNA release within minutes of exposure to cytosolic redox conditions, which is likely what enables them to be used at higher (>100) wt/wts without significant cytotoxicity.

Other modes of degradation can enable more specific spatial control of nucleic acid release. Kim *et al.* used MMP-cleavable peptide linkages to enable tumor-targeted DNA release [119]. External triggers can also be used for spatial control with specific polymer structures. For example, light-responsive polymers enable user-controlled spatial triggering of nucleic

acid release [120]. As shown in Table 1, the most common polymeric gene delivery mechanisms utilized for unpacking nucleic acid cargo and mitigating toxicity from the polymer are amidolysis (amide bonds), hydrolysis (ester bonds), and reduction (disulfide bonds).

Intracellular trafficking and nuclear uptake

While the intracellular target for nucleic acids such as mRNA, siRNA, and miRNA is in the cytoplasm, the intracellular target for DNA is the nucleus. For successful gene expression, exogenous DNA must be translocated to the nucleus for transcription and translation to proceed within a relatively quick time frame as the half-life of DNA plasmids in different cells types has been shown to be on the order of 1 hour [121]. The cytoplasm is viscous and molecularly congested at approximately 100 mg/mL of protein, which greatly inhibits transport via diffusion, making the process of transport to the nucleus highly inefficient [122–124]. It has been shown that plasmids >2 kilobases in length were >100-fold slower in the cytoplasm than in water and were essentially immobile in cytoplasm for the study duration [122].

There is typically higher transfection in dividing cells as the nuclear membrane breaks down and plasmids in the cytoplasm can be stochastically encapsulated by the reformation of the nuclear envelope post-mitosis [124, 125]. To further increase nuclear uptake, nuclear localization signals (NLS) have been utilizes. NLSs are structural elements that can be associated with the DNA cargo either directly through conjugation or indirectly, such as through the binding of transcription factors. For example, plasmids can contain nucleotide sequences such as a DNA-targeted sequence (DTS) of a 77 base pair structural element from the simian virus (SV40): 5′-AACCAGCTGT GGAATGTGTG TCAGTTAGGG TGTGGAAAGT CCCCAGGCTC CCCAGCAGGC AGAAGTATGC AAAGCAT-3′ [126]. The DTS is a binding site for an endogenous transcription factor, which contains an embedded NLS. After DNA is released from a polyplex, the transcription factor can bind it. Following a morphological change that can reveal a hidden NLS, the NLS peptide handle can shuttle the DNA cargo into the nucleus via karyopherins. In one approach to incorporate an NLS into a polyplex system, FMOC is used to combine PNA to an SV40 NLS using a hydrophilic linker (PKKKRKV-linker-GCGCTCGGCCCTTCC; linker Fmoc-NC6O3H11-OH) [14]. The PNA conjugated to the NLS can be hybridized to a plasmid and then further ionically complexed with PEI to form polyplexes. The authors of this work were able to achieve 8-fold greater transfection with the NLS containing polyplexes than without the NLS [14].

Research has also revealed how polymer complexation affects pDNA import into nuclei and how polymer structures including linear PEI (JetPEI™) and two other carbohydrate-based poly(glycoamidoamine)s (one containing meso-galactarate (G4) and the other L-tartarate (T4)) affect nuclear import [127]. The authors found JetPEI and G4 exhibited higher transfection and were also able to permeabilize the nuclear envelop more efficiently than T4 [127]. They found that the carbohydrate moieties in the absence of plasmid were able to cross the nuclear membrane as well, suggesting key polymeric structures for rational design [127].

Other bioinspired methods for improved nuclear import have also been utilized. For example, researchers have used trans-cyclohexane-1,2-diol, which is an amphipathic alcohol, to emulate a hypothesized activity of importins at dissolving a hydrophobic phase within nuclear pores to cause the nuclear pores to become more permeable [128]. Using this approach, the authors showed high molecular weight dextrans and pDNA passed through the nuclear envelope without toxicity and enhanced transfection efficacy [128]. Thus, either the DNA plasmid or the polymer can incorporate structural elements to improve nuclear uptake.

Conclusions/Future Perspectives

Gene delivery represents a promising strategy for the treatment of many inheritable and acquired diseases, as it can enable the delivery of therapeutic genes or the knockdown of harmful or detrimental genes. Understanding the chemical properties and function of gene delivery polymers, which facilitate intracellular delivery of DNA and RNA, enables the design of safer and more effective gene therapies. Nucleic acid binding, cellular uptake, endosomal escape, cargo release, intracellular trafficking and nuclear uptake are all necessary steps to transfection. Therefore, engineering gene delivery polymers requires consideration of each obstacle simultaneously.

It should be noted that design of gene delivery vectors discussed herein focused on the optimization of intracellular delivery and trafficking of nucleic acids. Effective gene therapy nanoparticles will also need to address tissue-scale and systemic concerns. These include but are not limited to nanoparticle clearance by the mononuclear phagocytic system, colloidal stability in physiological salt, nanoparticle aggregation in blood serum, and controlled tissue targeting [129–131]. Nonetheless, effective gene delivery strategies must be optimized to carry nucleic acids and direct them to their site of action within the cell. Understanding the polymer chemistry that enables succesful delivery of nucleic acids is a cruicial step in the engineering of optimal gene therapy nanomedicines.

It is interesting to note structural similarities between polymers often used for gene delivery. Chitosan, the epoxide-containing block co-polymers, histone proteins, PNAs, linear and branched poly(amido amine)s, poly(L-lysine), end-modified PBAEs, linear and branched PEI, and poly(L-arginine) all have primary and secondary amines. Most of these structures also contain tertiary amines, with certain naturally derived materials such as chitosan, poly-L-lysine, and poly(L-arginine) being exceptions and in many cases, less effective for gene delivery as a result. While endosomal buffering following the proton sponge hypothesis remains a commonly used polymer design principle for gene delivery, other methods of endosomal disruption, such as incorporation of cell-penetrating peptides, can also be effective. Most gene delivery polymers are chosen from naturally degradable materials (i.e. enzymatically degradable peptides) or are designed to contain linkages that are degradable under physiological conditions (i.e. hydrolytic degradation of esters or reduction of disulfides). One exception to this rule is PEI, which was one of the first off-the-shelf commercial polymers shown effective for polymeric gene delivery even though it also exhibited dose dependent cytotoxicity. Since then, PEI analogs that contain hydrolytic or reducible linkages have shown improved efficacy and reduced cytotoxicity, demonstrating an approach to engineer synthetic gene delivery polymers for improved performance and

safety. In some cases, polymeric gene delivery nanoparticle degradability can be engineered to be triggered by a particular microenvironment, such as through the use of matrix metalloproteinase-cleavable polymers which enzymatically degrade in pathologic tissues.

One of the advantages of polymeric gene delivery vectors is that polymers are amenable to high throughput synthesis strategies where a library of polymeric structures can be analyzed in parallel for gene delivery efficacy. Many of the chemically versatile polymer synthesis platforms, such as PBAEs and epoxide containing-block co-polymers, are able to vary hydrophobicity easily by variation in the number of carbons of constituent monomer units and able to vary molecular weight by simple variation to reaction conditions. These library approaches have allowed investigators to probe parameters linked to successful transfection. For the future development of next-generation polymeric gene delivery nanoparticles, it is useful for investigators to know quantitatively how differential changes to polymer structure tune nanoparticle properties and biological efficacy. This will enable rational design of polymers with specific attributes including hydrophobicity, molecular weight, amine content, and degradation kinetics. Critically, research suggests that these attributes may not be universal, but instead significantly dependent on the cargo type (ie DNA vs. siRNA) and the cell type of interest for each application.

A trend in the chemical properties discussed herein is the need for moderation and balance when designing polymers for gene delivery. Increasing polymer positive charge enhances nucleic acid binding while electrostatic interactions that are too strong will prevent cargo release and transfection. Hydrophobic polymers will bind nucleic acids and promote uptake, but hydrophobic interactions with the cell membrane that are excessively destabilizing can lead to toxicity. Biodegradable materials can improve nucleic acid release and reduce cytotoxicity, but may also reduce particle stability, which is particularly important for therapeutic applications. Several biodegradable cationic polymers have been identified as leading candidates due to their delivery efficacy, biodegradability, and safety. Although there are currently no U.S. FDA-approved polymeric nanoparticle systems for DNA or siRNA delivery, there are many ongoing clinical trials [132–135]. Through greater understanding of the underlying mechanisms to control and optimize polymer structure for gene delivery, enhanced therapeutic polymers for intracellular delivery of nucleic acids can be created.

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Abbreviations

CPP	cell-penetrating peptides
DTS	DNA-targeted sequence
FDA	Food and Drug Administration

miRNA	micro RNA
mRNA	messenger RNA
NLS	nuclear localization signal
PAA	poly(amido amine)
PBAE	poly(β-amino ester)
pDNA	plasmid DNA
PEI	polyethyleneimine
PLGA	poly(lactic-co-glycolic acid)
PLL	poly-L-lysine
PNA	peptide nucleic acid
shRNA	short hairpin RNA
siRNA	short interfering RNA
TAT	trans-activating transcriptional activator
wt/wt	weight/weight

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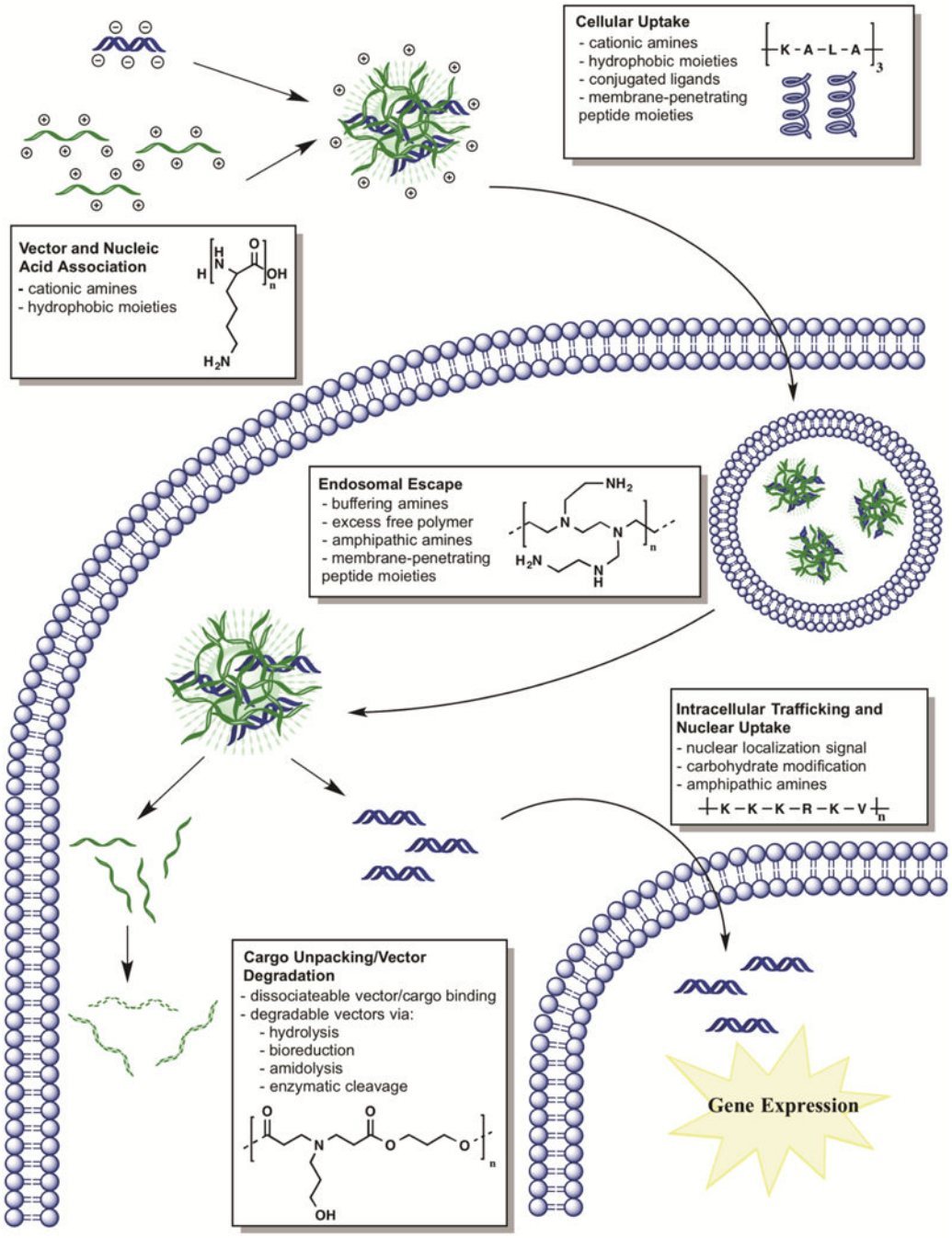

Figure 1.

Intracellular nucleic acid delivery steps and polymer design strategies. These steps include: 1) Vector and nucleic acid association; 2) Cellular uptake via a variety of mechanisms (i.e., clathrin-mediated, caveolae, and macropinocytosis) [5]; 3) Endosomal escape to circumvent the lysosomal degradation pathway; 4) Nucleic acid cargo unpacking, in some cases due to degradation of the nucleic acid vector, minimizing toxicity; 5) Intracellular trafficking and nuclear import in the case of DNA.

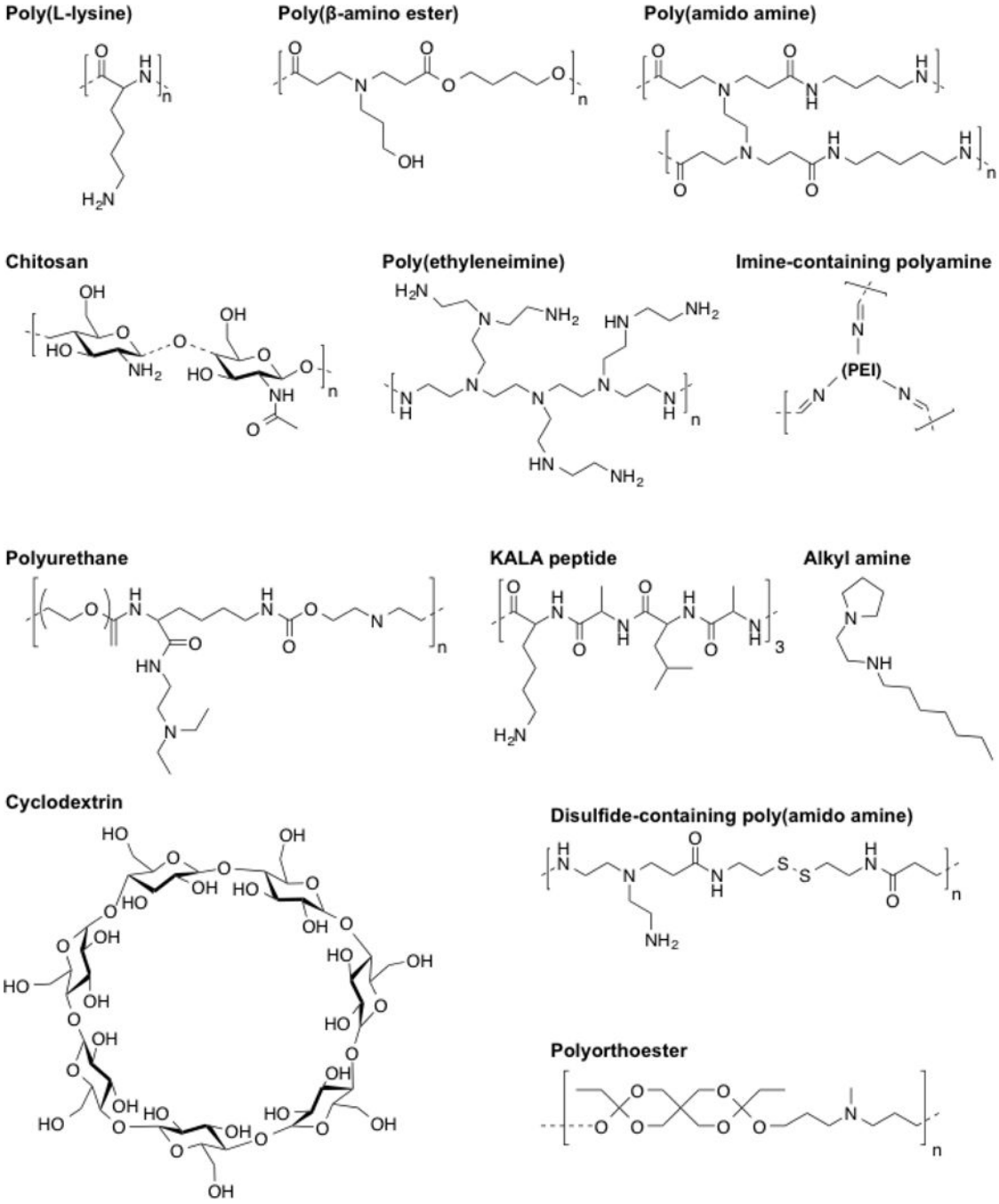

Figure 2.
Chemical structures of select polymers employed for gene delivery [1, 13, 15–17, 21].

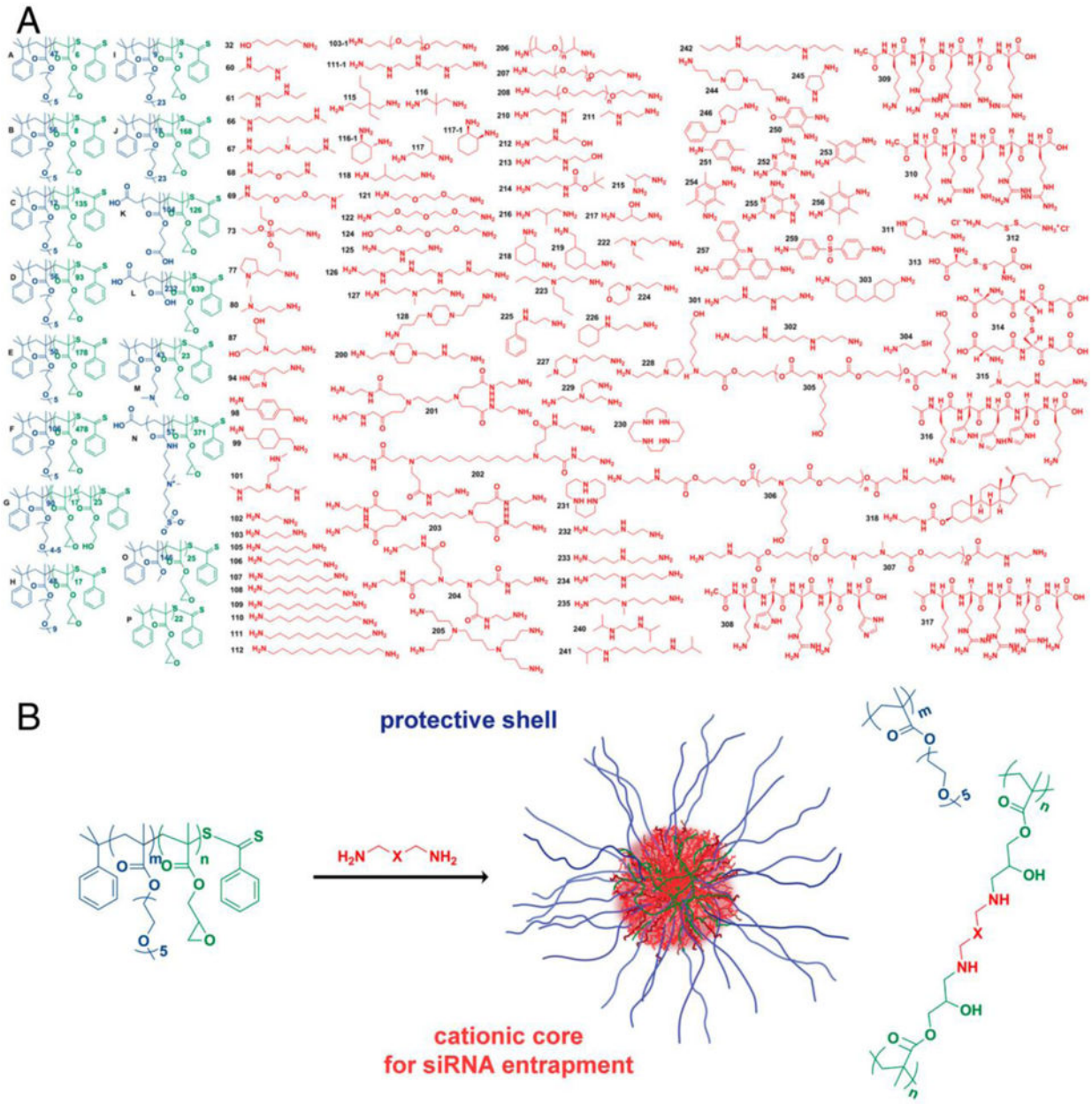

Figure 3.

Library used for the formation of hairy core-shell nanoparticles. A) Epoxide-containing block copolymers and the amine-containing molecules used; B) The reaction proceeds via the ring-opening reaction with the amines which form β-hydroxyl groups. Reproduced with permission from Siegwart, et al. *PNAS* 108(32):12996–3001 [20]. Copyright (2011) National Academy of Sciences, USA.

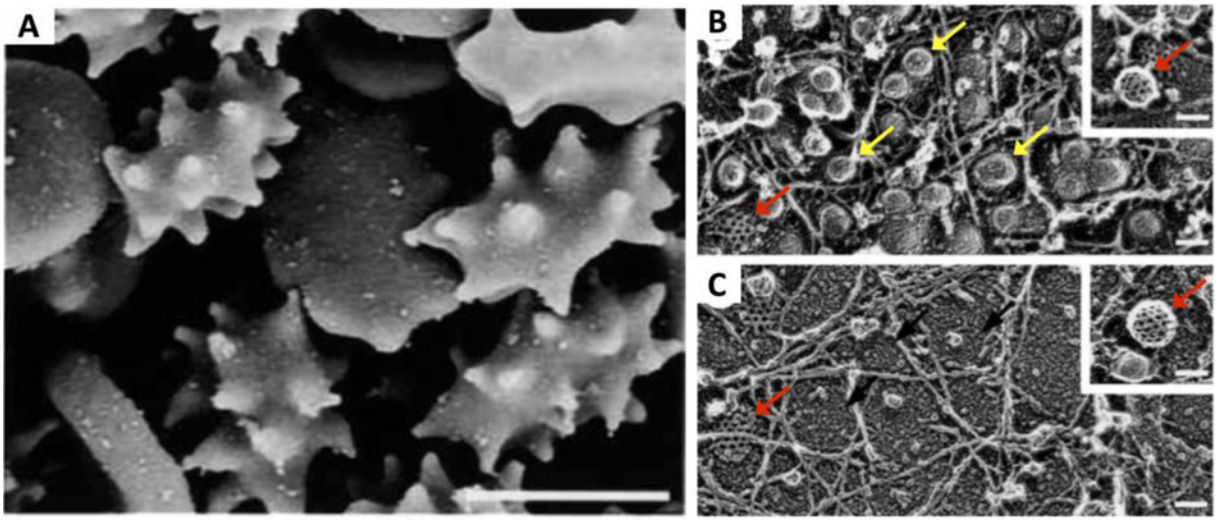

Figure 4.

(A) Amphiphilic PEG-cholesterol induces erythrocyte membrane stress and causes protrusions along the cell membrane. **(B)** Increasing concentrations of amphiphilic polymer changes the primary mode of endosomal uptake in A431 human squamous carcinoma cells from combined caveolae-mediated and clathrin-mediated endocytosis to **(C)** primarily clathrin-mediated endocytosis. This can be observed by the decreased prevalence of smooth membrane protrusions indicative of caveolae (yellow arrows) in **(B)** versus **(C)**, whereas the polyhedral structure typical of clathrin membrane protrusions (red arrows) can be seen in both images. Polymer/nucleic acid nanoparticles can likewise be engineered to preferentially enter the cell via one or both of these pathways. Scale bars: panel A: 5μm; all others: 100 nm. Reproduced with permission from [48]. Copyright John Wiley and Sons 2001.

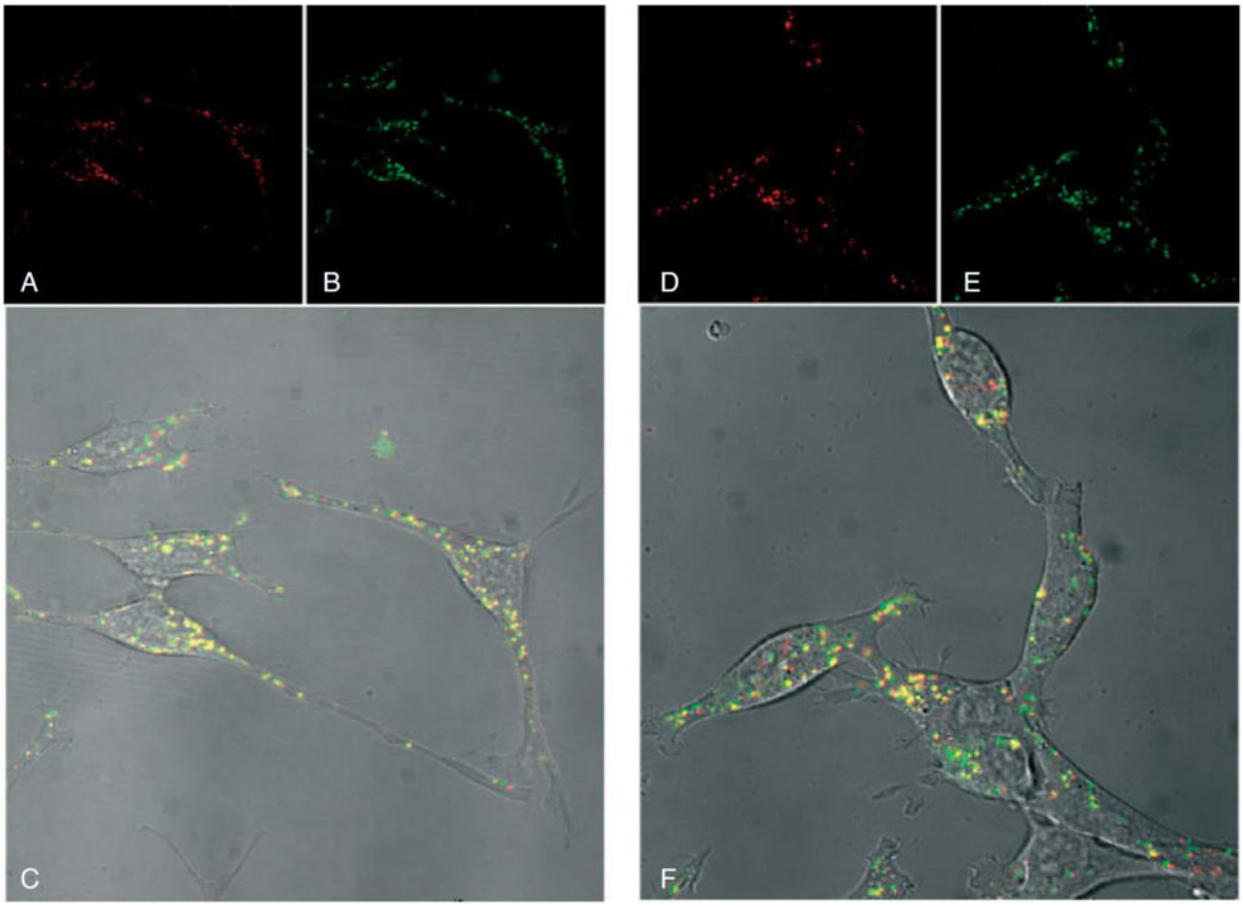

Figure 5.

(A and D) CT 26 cells transfected with Cy3 (red) -labeled plasmid and **(B and E)** uncomplexed Cy5 (green) –labeled linear PEI, which was added either simultaneously (**A, B, and C**) or 4 hours post-transfection (**D, E, and F**). Similar intracellular delivery (yellow colocalization) is observed when PEI is added either (**C**) simultaneously with DNA or (**F**) 4 hrs later. Reproduced with permission from [86]. Copyright John Wiley and Sons 2004.

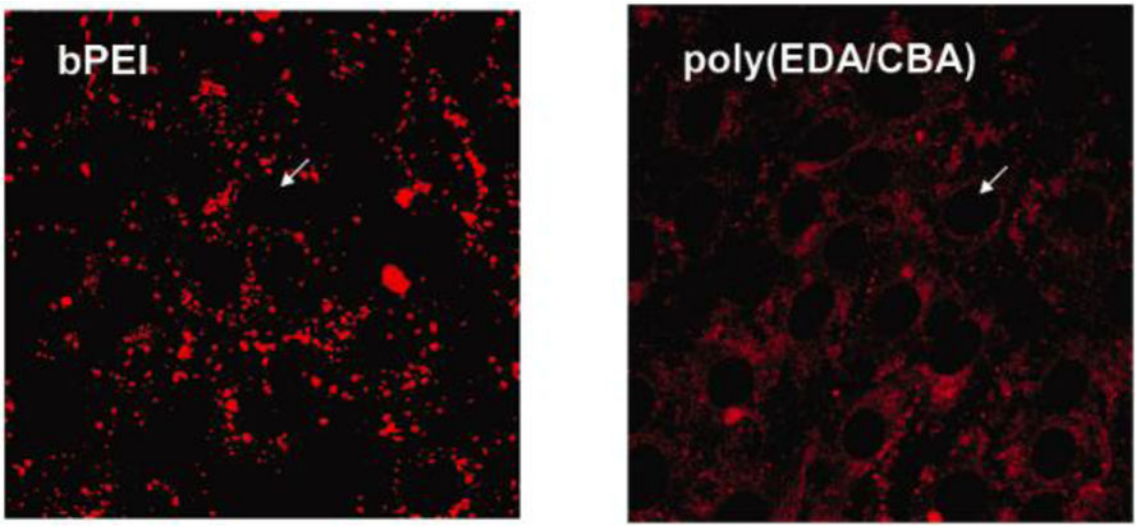

Figure 6.

Confocal microscope images of NIH 3T3 bovine aortic endothelial cells 6 h following transfection using either branched PEI (bPEI) or a disulfide-containing poly(amido amine) (poly(EDA/CBA)). Fluorophore-labeled DNA is dispersed throughout the cell when poly(EDA/CBA) was used as the transfection agent, but not bPEI, suggesting that polymer bioreduction enabled improved DNA release. Reprinted (adapted) with permission from [108]. Copyright 2006 American Chemical Society.

Table 1

Select polymeric structural features to overcome intracellular gene delivery barriers.

Gene Delivery Barrier	Polymer Structural Features
Vector and Nucleic Acid Cargo Association	Electrostatic Binding via Cationic Amine Charges (1°>2°>3°)
Cellular Uptake	Upregulated Ligand-Conjugation (i.e., Folic Acid) Site-specific Triggered Release to Target Cell Type (i.e., MMP) Cationic Carrier and Anionic Membrane Affiliation Membrane Adsorption via Lipophilicity/Hydrophobicity (i.e., Akyl Addition) Membrane Penetration via Cell Penetrating Peptides (i.e., KALA)
Endosomal Escape	Amine Buffering Moieties Leading to Endosomolysis (3°>2°>1°) Buffering via Excess Free Polymer Disruption via Amphipathic Amines or Penetratable Entities (i.e., CPP)
Cargo Unpacking/Vector Degradation	Modifying Binding Strength (i.e., Molecular Weight, Amine Density) Polymer Degradation (i.e., Amidolysis, Hydrolysis, Reduction (siRNA), MMP-cleavable)
Intracellular Trafficking and Nuclear Uptake	Optimal Polymer Degradation Kinetics to Minimize Nucleic Acid Time in Free State (Nucleic Acid in Free State is More Prone to Nuclease Degradation) Nuclear Localization Signal Conjugation (i.e., PKKKRKV) Incorporating a DNA-Targeted Sequence into the Cargo Carbohydrate Shuttling through Nuclear Membrane Nuclear Pore Permeabilization via Amphipathic Properties (Emulating Importin Activity)