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Enhancing the In Vitro and In Vivo Stabilities of Polymeric Nucleic Acid Delivery Nanosystems

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Abstract

Gene therapy holds great promise for various medical and biomedical applications. Nonviral gene delivery systems formed by cationic polymer and nucleic acids (e.g., polyplexes) have been extensively investigated for targeted gene therapy; however, their in vitro and in vivo stability is affected by both their intrinsic properties such as chemical compositions (e.g., polymer molecular weight and structure, and N/P ratio) and a number of environmental factors (e.g., shear stress during circulation in the bloodstream, interaction with the serum proteins, and physiological ionic strength). In this review, we surveyed the effects of a number of important intrinsic and environmental factors on the stability of polymeric gene delivery systems, and discussed various strategies to enhance the stability of polymeric gene delivery systems, thereby enabling efficient gene delivery into target cells. Future opportunities and challenges of polymeric nucleic acid delivery nanosystems were also briefly discussed.

Graphical Abstract

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INTRODUCTION

Gene therapy has gained considerable attention over the past three decades as it holds great promise for the treatment of many diseases including cancers, genetic disorders, cardiovascular diseases, infectious diseases, and neurological diseases.^{1–10} Nucleic acid-based genetic materials, including DNA, messenger RNA (mRNA), small interfering RNA (siRNA), microRNA (miRNA), and antisense oligonucleotides (ASOs), are highly negatively charged macromolecules that are vulnerable to chemical/enzymatic degradation in vitro and in vivo.^{11–13} Thus, vectors are needed to protect the nucleic acids and efficiently deliver them into target cells. There have been extensive efforts in developing safe and efficient nonviral gene delivery systems suitable for both in vitro and in vivo applications.^{11,14,15} In particular, cationic polymers with desirable features such as chemical versatility, biological safety, low cost, and high reproducibility have been intensively investigated for highly efficient nonviral-based gene delivery systems such as polyplexes.^{16–19} However, additional challenges such as a relatively low transfection efficiency, cytotoxicity associated with certain polycationic polymers, and short therapeutic duration may limit clinical translation.²⁰

Conventional nonviral polymeric gene-delivery nanoparticles (NPs) such as polyplexes are formed mainly via the relatively weak electrostatic interactions between the cationic polymers and positively charged nucleic acids. Thus, a key property that can significantly affect the transfection efficiency of the polymeric gene delivery NPs in target tissues and/or cells is their in vitro and in vivo stability.^{21–23} The stability of the polyplexes is affected by both their intrinsic properties such as chemical compositions including polymer molecular weight and structure (e.g., linear vs branched), and N/P ratio, as well as environmental factors including shear stress in the bloodstream, interactions with the serum proteins, and physiological ionic strength.²⁴ Furthermore, while gene delivery NPs need to exhibit superior stability during circulation in the bloodstream and before being taken up by the

target cells, genetic materials can only function in the target cell after they are released from the delivery vehicles. Thus, excessive stability that hinders intracellular payload release may also impair efficient transfection. Therefore, a judiciously designed gene delivery nanosystem should demonstrate sufficient stability to maintain their morphology and structure during the transport process (e.g., during circulation in the bloodstream) and before being taken up by the target cells, while being capable of rapid release of the payload inside of the target cell. This requirement is often achieved via stimuli-controlled release mechanisms²⁴ (Figure 1). In this review, the various intrinsic and environmental factors that may affect the in vitro and in vivo stability of polymeric gene-delivery systems will be discussed, and current strategies for overcoming these destabilizing factors will be illustrated.

FACTORS THAT AFFECT THE IN VITRO AND IN VIVO STABILITY OF POLYMERIC GENE DELIVERY NPS (E.G., POLYPLEXES)

Intrinsic Properties of the Polymeric Gene Delivery NPs.

Polymer Molecular Weight.—The molecular weight of the cationic polymers can greatly affect the NP stability and transfection efficiency.²⁵ Since every material has its own unique features including charge density and hydrophilicity, the range of the most desirable molecular weight used to form polyplexes differs. For instance, polyethylenimine (PEI), at a molecular weight of 25 kDa, is reported to have the highest transfection efficiency and acts as a commercially available “gold standard” across multiple studies.^{26,27} However, several reports claimed that for in vitro applications, PEI with a lower molecular weight (e.g., 1.8 kDa or 5.4 kDa) had a higher transfection efficiency.^{28,29} Since polyplexes formed by low-molecular-weight cationic polymers exhibited poor stability, they had a tendency to form micron-sized (e.g., 0.7–1.3 μm) aggregates.²⁹ Ogris et al.³⁰ speculated that larger polyplex aggregates could facilitate cell attachment and subsequent uptake, resulting in improved transfection efficiency. Chitosan with a molecular weight of around 45 kDa exhibited superior transfection efficiency as compared to chitosan with other molecular weights (e.g., 20, 200, and 460 kDa), although chitosan with larger molecular weights provided higher stability.³¹ For dendrimers such as poly(amidoamine) (PAMAM), generations 0–4 revealed more than 1000 times lower transfection efficiency compared with higher generations (i.e., generations 6–9).³² This phenomenon may be attributed to a higher amount of surface amines present on higher generations of PAMAM as well as the change in PAMAM morphology. Lower generations of PAMAM have a planar, elliptical shape. However, by generation 5, PAMAM exhibits a more spheroidal structure, which is beneficial for DNA packaging and condensing.³³ Interestingly, the effects of molecular weight on the morphology of the NPs may differ for different polymers. PEI, with its extremely high charge density, forms smaller polyplexes as its molecular weight increases, which may help to better condense DNA.²⁸ On the other hand, higher molecular weight chitosan (e.g., 32 to 540 kDa) tends to form larger NPs than lower molecular weight chitosan, likely due to the reduced aqueous solubility associated with high molecular weight chitosan.^{25,34}

Typically, cationic polymers with higher molecular weights have stronger interactions with DNA, which enhance the stability of polyplexes and help with the protection of nucleic

acids, but also inhibit the intracellular release of its payload. Fischer et al. compared PEI 11.9 kDa with 1616 kDa and found that polyplexes formed by low-molecular-weight polymer induced a 20-fold higher luciferase expression as compared to those formed by high-molecular-weight polymer.³⁵ PEI with an excessively high molecular weight hampered DNA release and wrought serious toxicity.³⁶ On the other hand, polymers with too low of a molecular weight may have lower stability and fail in DNA protection. Godbey and co-workers tested a series of PEI polymers from 600 Da to 70 kDa, and they found that the transfection efficiency dropped about 60% when the molecular weight of PEI was reduced from 70 kDa to 10 kDa. Furthermore, PEI with a molecular weight ranging from 600 to 1800 Da barely showed any transfection efficiency.³⁷ Dash et al. also linked the stability of poly(L-lysine) (PLL)/DNA polyplexes with the molecular weight of PLL and found that lower molecular weight PLL (e.g., 1–4 kDa) afforded poor DNA protection from enzyme degradation.³⁸ Therefore, the molecular weight of the cationic polymers needs to be carefully optimized in order to delicately balance the need for nucleic acid protection and release.

N/P Ratio.—The N/P ratio is defined as the ratio between the moles of the amine groups (N) of cationic polymers over the moles of the negatively charged phosphorus (P) in the nucleic acid.^{39,40} For those polymers with other positively charged groups such as sulfonium, there would be an S/P ratio to represent the same concept.⁴¹ The transfection efficiency of polyplexes highly depends on the N/P ratio. A higher N/P ratio generally indicates a higher NP zeta potential, which helps to better condense nucleic acids into smaller-sized NPs and enhances the polyplex stability and cellular uptake.^{24,42,43} In addition, the free or loosely bound cationic polymers at high N/P ratios are known to play an important role in enhancing nucleic acid transfection by avoiding NP aggregation and enhancing NP endosomal escape.^{44–46} Every polymer, with its unique features, has a different optimal N/P ratio, and these ratios may vary by several orders of magnitude.⁴⁷ Typically, a polymer with a higher charge density and molecular weight tends to be more stable and requires a lower N/P ratio for efficient gene delivery.

Polymer Structure.—The stability of polyplexes is also strongly affected by polymer structure. For instance, NPs composed of linear PEI (LPEI) revealed inferior stability with exposure to polyanions and salt solutions as compared to their branched counterpart (BPEI). They also required a higher N/P ratio to condense the nucleic acids into nanosized polyplexes.⁴⁸ It is believed that the flexible hyperbranched structure of BPEI provides the nucleic acids more three-dimensional folding options that help to stabilize the structure of polyplexes.⁴⁹

It is worth noting that different cationic polymers may be required to achieve optimal NP stability and maximal transfection efficiency for different nucleic acid payloads. A well-studied example is DNA and siRNA. DNA, which has a much larger molecular weight compared to siRNA, can induce stronger interactions with polycations, thus resulting in a higher NP stability. Zintchenko et al. reported that the BPEI-DNA polyplexes exhibited a 2-fold higher resistance to salt dissociation than BPEI-siRNA.⁵⁰ Both LPEI and BPEI were used to deliver DNA and siRNA. LPEI-DNA exhibited a higher transfection efficiency than

BPEI-DNA; however, only BPEI could efficiently deliver siRNA.^{48,50,51} The branched 3-dimensional structure of BPEI is more beneficial for nucleic acid binding and packaging, thereby allowing for more stable NP formation than LPEI.^{48,52} Kwok and colleagues reported that BPEI-DNA and BPEI-siRNA showed higher stabilities when exposed to heparin than LPEI-DNA and LPEI-siRNA, respectively. Furthermore, LPEI-DNA and BPEI-siRNA exhibited higher transfection efficiencies than their corresponding counterparts.⁴⁸ The insufficient stability of LPEI-siRNA and the excessive stability of BPEI-DNA may have hindered efficient transfection, demonstrating the importance of an optimal NP stability.⁴⁸

Environmental Factors.

Ionic Strength.—The stability of polyplexes can be affected by ionic strength.⁵³ Generally, polyplexes possess higher stability in lower ionic strength solutions, since enhancing the salt level weakens interactions between vector polymers and nucleic acids, as well as impairs electrostatic repulsion, which is an important force for preventing aggregation.²⁴ For many systems, physiological saline or PBS solution may destabilize the NPs after it has been prepared in low ionic strength solution.^{54,55}

Picola et al. studied the effect of ionic strength on the stability of chitosan–DNA polyplexes with different molecular weights.⁵⁶ The polyplexes' stability decreased drastically when the ionic strength increased from 10 mM to 500 mM, causing the formation of large spherical aggregates, toroids, and rods. For NPs made by lower molecular weight chitosan (5–29 kDa), the particle size increased gradually after enhancing the ionic strength, and reached micron size within an hour. For NPs prepared with 150 kDa chitosan, the particle size increased instantly from 210 to 280 nm when the ionic strength increased from 10 mM to 500 mM. However, after that, their hydrodynamic diameters remained constant for at least 3 h. Meanwhile, the ionic strength also affected the surface charge of the particles.⁵⁷ With increasing salt levels, the zeta potentials of the NPs dropped significantly.⁵⁸

An increase in ionic strength also affects the stability of other gene-delivery nanosystems made of lipids and peptide nucleic acids (PNAs). Costa and co-worker developed a series of PNAs with either positive, neutral, or negative charge in its backbone. They found the positively and negatively charged PNAs exhibited different stability in solutions with different ionic strength. At low salt concentrations, positively charged PNA exhibited stronger interactions with DNA; however, at medium and high salt concentrations, negatively charged PNA showed stronger interactions with DNA.⁵⁹

Competing Polyanions.—For polyplexes formed via electrostatic interactions between nucleic acids and cationic polymers, polyanions that have similar charge properties to nucleic acids can competitively bind cationic polymers and destabilize the polyplexes.⁶⁰ Proteins (e.g., serum albumin) and polysaccharides (e.g., heparan sulfate and hyaluronic acid) are representative competing polyanions that are abundant in physiological environments. Polyplexes exhibiting insufficient stability in the presence of competing polyanions can be a major hurdle for intravenously (i.v.) administered NPs as the interactions between the polyplexes and serum proteins (e.g., albumin) can quickly destabilize the polyplexes before they reach the targeted tissues and cells. PEI, for instance,

is a frequently used vector that attains highly efficient transfection in serum-free media, but is significantly attenuated by 2 orders of magnitude in serum-containing media.⁶¹ Polyanions with higher charge densities (e.g., heparin) are prone to having a higher binding affinity with cationic polymers. Therefore, high density polyanions can easily destabilize polyplexes and, hence, are widely applied as a benchmark to test the stability of polyplexes.^{58,62}

Despite the fact that competing polyanions can destabilize the polyplexes, judiciously designed polyanion coatings on polyplexes can reduce the zeta potential and enhance the biocompatibility of the positively charged polyplex core.⁶³ Ito et al. used a spermine-modified hyaluronic acid (HA) coating on a PEI/plasmid core that enhanced stability and transfection efficiency in serum-containing media.⁶⁴ This strategy was further improved by He et al. by introducing reduction-sensitive disulfide bonds onto HA. Such chemically modified HA coating can shield the surface charges of the polyplexes and also induce GSH-responsive dissociation when in the cytosol.⁶⁵

Shear Stress.—For intravenously administered NPs, shear stress in the bloodstream is another critical factor that can destabilize the NPs during circulation.^{66,67} Although polyplexes can be shielded by a stealth polymer layer or lipid,⁶⁸ shear stress could still gradually strip polymer strands from the polyplexes, impair its resistance to serum proteins, and finally cause the whole particle to disintegrate.⁶⁷

It should be noted that high polyplex stability alone does not warrant better transfection efficiency since successful delivery of nucleic acids by polyplexes depends on more than just the stability of the delivery vehicle. It also relies on a number of other key factors, such as NP chemical composition, morphology, and surface characteristics such as surface charge and surface modification (e.g., various targeting ligands and/or cellular uptake-enhancing ligands).^{69,70} Furthermore, once the NPs are inside the target cells, rapid release of the payload from the NPs is also an important factor. As discussed earlier, polyplexes with “excessive” stability can reduce transfection efficiency.⁴⁹

A better understanding of the various intrinsic and environmental factors that may affect the in vitro and in vivo stability of the polymeric gene delivery nanosystems can enable researchers to devise proper strategies that ensure excellent NP stability before reaching the target cells while allowing efficient release of the payload once inside of the target cells.

STRATEGIES TO ENHANCE THE STABILITY OF POLYMERIC GENE DELIVERY NANOSYSTEMS

Surface shielding by poly(ethylene glycol) (PEG) or polyzwitterion can increase the NP stability by reducing nonspecific interactions with serum proteins during circulation,^{66,71,72} thereby increasing the circulation time and NP accumulation at the target site (e.g., tumors).^{73,74} Previous studies found that PEG chain length and graft density can greatly affect the stability of the polyplexes.^{75,76} Moreover, without additional stabilizing strategy, polycation-PEG block copolymer molecules can continuously detach from self-assembled polyplexes formed via electrostatic interactions alone, leading to disassociation of the NPs.^{66,67}

Therefore, more reliable alternative strategies, including unimolecular NPs, chemical and/or physical cross-linking, are required in order to yield polymeric gene delivery NPs with superior stability during transport while capable of rapid release of the payload once inside of the cells (Figure 2). Table 1 summarizes the strategies that can enhance the stability of polymeric gene delivery nanosystems.

Unimolecular NPs.

Unimolecular NP is typically formed by a single/individual dendritic/hyperbranched multiarm block copolymer molecule, thus it only contains covalent bonds.¹²³ Due to its covalent nature, the various environmental factors described earlier can hardly affect the stability of unimolecular NP and thus unimolecular NP possesses excellent stability.^{124,125} Unimolecular NPs are widely used for the delivery of drugs, peptides, and nucleic acids.^{78,126–129}

Unimolecular NPs suitable for gene delivery typically exhibit a core-shell structure with the core made of polycationic polymer segments grafted to a central dendritic/hyperbranched/brush-like polymer (e.g., PAMAM and Boltorn H40). The shell of unimolecular NP is made of charge-neutral hydrophilic polymer (e.g., PEG or polyzwitterion) (Figure 3). The polycationic core of unimolecular NPs is complexed with negatively charged nucleic acids via electrostatic interactions.

Jiang et al. reported a brush-like polymer-based unimolecular NP (i.e., a polyelectrolyte brush (PFNBr)).⁷⁷ The unimolecular NP was composed of a polyfluorene backbone with positively charged poly(2-(dimethyl-amino)ethyl methacrylate) (PDMAEMA) side chains. This unimolecular NP can complex with siRNA via electrostatic interactions to obtain excellent stability against nuclease. In addition, this siRNA-loaded unimolecular NP remained intact at high NaCl concentration (up to 0.1 M), indicating good stability against high ionic strength.

We reported a pH and redox dual-responsive unimolecular NP for the delivery of siRNA.⁷⁸ The unimolecular NP was formed by a multiarm star block copolymer with a pH and redox dual-responsive H40-poly(aspartic acid-(2-aminoethyl disulfide)-(4-imidazolecarboxylic acid)) (H40-P(Asp-AED-ICA)) core and a PEG shell (Figure 4).⁷⁸ This type of unimolecular NP exhibits enhanced stability and stimuli-responsive siRNA release once it enters cells, making it a potentially effective siRNA delivery system in vivo.

Despite its advantages in maintaining NP integrity and stabilizing the nucleic acid payload, unimolecular NPs typically can only be used to deliver relatively small nucleic acids (e.g., siRNA and miRNA) due to their confined structure. Thus, for the delivery of large nucleic acids (e.g., DNA and mRNA), multimolecular polymer-nucleic acid complexes (i.e., polyplexes) are needed.

Chemical Cross-Linking.

Integration of stimuli-responsive chemical cross-links into polyplexes formed between cationic polymer molecules and nucleic acids via electrostatic interactions can substantially stabilize the polyplexes both during circulation in the bloodstream and in extracellular

spaces.^{83,130–132} Stimuli-responsive cross-links can also enable controlled intracellular release of the payload. A well-established strategy is to reversibly cross-link the polyplexes with disulfide bonds.^{67,79,82} Disulfide cross-links can keep the integrity of the polyplex structure by avoiding undesirable disassembly during circulation and in extracellular spaces, but can be efficiently cleaved to quickly release the payload in cytosol where it is reductive due to the presence of high concentration of GSH (i.e., 2–10 mM).^{80,133,134} A disulfide-cross-linked, poly-(hydroxypropyl methacrylamide-*co*-*N*[2-(2-pyridyldithio)]-ethylmethacrylamide-*co*-*N*(3-aminopropyl) methacrylamide)-*b*-poly(ethylene glycol) (p(HPMA-*co*-PDTEMA-*co*-APMA)-*b*-PEG)-based polyplex system was reported by Novo et al. Disulfide cross-linking is formed among PDTEMA in the polyplexes, exhibiting excellent stability in human plasma for 48 h.⁷⁹ Also, a poly(ethylene glycol)-poly(L-lysine) (PEG–PLL)-based polyplex system with 50% amines modified with thiol groups for disulfide cross-linking was reported to have excellent stability against shear stress up to 100 dyn/cm,² mimicking the shear stress in large blood vessels.⁶⁷

In vivo studies also indicated that the introduction of disulfide cross-links in the polyplexes improved circulation kinetics.^{67,80,81} Oupicky et al. reported that disulfide-cross-linked, DNA-loaded PEG–PLL polyplexes exhibited a 10-fold increase in blood NP concentration 30 min post-intravenous administration as compared to non-cross-linked polyplexes.⁸⁰ Meanwhile, Vachutinsky et al. reported that disulfide-cross-linked, DNA-loaded PEG–PLL polyplexes exhibited a 2.5- to 3-fold increase in blood DNA concentration as compared to non-cross-linked polyplexes 15 min after intravenous injection, thus indicating a prolonged blood circulation time enabled by the chemical cross-linking strategy.⁸¹ The cross-linked, therapeutic, DNA-loaded PEG–PLL polyplexes exhibited in vivo tumor growth inhibition via intravenous administration.⁸¹ Similar findings were also reported by Takeda et al.⁶⁷ For non-cross-linked, DNA-loaded, PEG–PLL polyplexes, only 2% of the DNA payload remained in the blood 60 min after intravenous injection, while 23% remained in the blood for cross-linked polyplexes.⁶⁷ Furthermore, cross-linked DNA polyplexes exhibited a circulation half-life of 45.6 min, 2.5 times longer than non-cross-linked polyplexes. Loaded with DNA encoding soluble fms-like tyrosine kinase 1 (sFlt-1), the cross-linked polyplexes exhibited more significant tumor growth inhibition efficacy than non-cross-linked polyplexes, thus indicating that chemical cross-linking is a promising strategy for efficient systemic gene delivery.⁶⁷

Besides disulfide cross-linking, other stimuli-responsive cross-linkers have also been introduced into polyplexes. For instance, Yoshinaga et al. reported a phenylboronate-diol cross-linked polyplex system for DNA delivery.⁸³ The backbone of the two types of cationic polymers used to form the polyplexes cross-linked with the reversible covalent dynamic phenylboronic acid-diol ester bonds was poly(*N'*-(*N*-(2-aminoethyl)-2-amino-ethyl)-aspartamide) (PAsp(DET)), which was conjugated with either 4-carboxy-3-fluorophenylboronic acid (FPBA) or diol-containing D-gluconamide (GlcAm) moieties (Figure 5). This type of DNA-loaded polyplex showed extraordinary stability against competing polyanions, with up to 9-fold higher negative charge than the DNA payload. A similar cross-linking strategy was also reported by Kim et al.⁸⁴ The phenylboronate-diol cross-links exhibit ATP and pH dual-responsive capabilities, thus facilitating the ultimate release of the payload in the cytosol.

Another strategy to achieve a stable polymeric nucleic acid delivery system is to form a cross-linked polymeric shell (aka nanocapsule) surrounding the nucleic acid via in situ polymerization^{85,86} (Figure 6). Typically, positively charged monomers, neutral monomers, and stimuli-responsive cross-linkers are enriched around negatively charged nucleic acid payload. Then a cross-linked polymer network encapsulating the payload is formed by in situ polymerization. This strategy enables single payload-encapsulation that can form small NPs (i.e., less than 30 nm with siRNA or miRNA as the payload), with in vitro stability against high-serum-concentration media.⁸⁶

PHYSICAL INTERACTIONS TO ENHANCE POLYPLEX STABILITY

Hydrophobic Modifications.

Introducing hydrophobic moieties into polycations is a very common and effective method of improving the properties of polyplexes. It provides hydrophobic interactions beyond basic electrostatic interactions, thereby significantly enhancing polyplex stability.¹³⁵ Various hydrophobic modification methods have been applied leading to enhanced stability (Figure 7).

The most straightforward way of hydrophobic modification is integrating hydrophobic small molecules into polycations.^{87,88} Since the 1990s, hydrophobic segments—including aliphatic chains and fatty acids with different lengths and saturations,^{89–91} cholesterol and its derivatives,^{92–94} deoxycholic acid and its derivatives,^{95,96} vitamins (e.g., vitamin E, folic acid),^{97–99} phthalocyanine,¹⁰⁰ and dexamethasone¹⁰¹—have all been conjugated onto PEI, PLL, chitosan, and PAMAM to achieve higher stability and other desired properties, such as cancer targeting¹⁰² and nuclear translocation.¹⁰³

Hydrophobic modifications can significantly enhance transfection efficiencies, and as high as a 58-fold increase in comparison with that of unmodified polymers was reported.¹³⁶ The degree of modification and the amine types to be modified are both important factors that determine transfection. Acetylation of 25% to 57% primary amines was proven to be optimistic for PEI modification, while beyond this point, the transfection efficiency dropped because of the deteriorated buffering capacity.^{91,136} Furthermore, the modification of tertiary amines which are more likely distributed at the interior of the hyperbranched PEI helped form polyplexes with better stability and, thereafter, higher transfection efficiencies, compared to the alkylation of peripheral primary amines.^{88,135}

Compared to small molecules, the integration of polymers with more functionalities and a higher tendency toward self-assembly has shown advantages in gene delivery. Beyond grafting regular hydrophobic polymers, such as polycaprolactone (PCL) or poly(γ -benzyl L-glutamate) (PBLG), onto the polycation backbone for better stability,^{104,105} introducing hydrophobic moieties into the main chain of polycations is another promising way to improve the system. Eltoukhy et al.¹⁰⁷ constructed a small library of poly(β -amino ester) (PBAE) polymers and found that the transfection efficiency was generally positively correlated with increasing hydrophobicity. Among these polymers in the library, those with bisphenol in their main chains exhibited superior stability and transfection efficiency. This finding was verified by Yan and co-workers with a different library of poly(alkylene maleate

mercaptamine) (PAMA) polymers.¹⁰⁸ They found that it was more efficient to improve nucleic acid delivery efficiency by enhancing the hydrophobicity of the main chain than the side chains. A polymer with phenylene in its main chain that possessed moderate hydrophobicity showed the best efficacy among nearly 100 of its counterparts.

Hydrophobic modification of polymeric nucleic acid delivery nanosystems can achieve a higher transfection efficiency in vivo.^{18,137} Nelson et al. reported an siRNA delivery system formed by poly[(ethylene glycol)-*b*-[(2-(dimethylamino)ethyl methacrylate)-*co*-(butyl methacrylate)] (i.e., PEG-(DMAEMA-*co*-BMA)), a diblock polymer containing a hydrophobic BMA segment.¹³⁷ When injected intravenously, the siRNA-loaded NP formed by PEG-(DMAEMA-*co*-BMA) with 50:50 mol % of DMAEMA:BMA (cationic:hydrophobic) exhibited a 3-fold higher blood circulation half-life and a 2.3-fold higher gene silencing efficiency, compared to NPs formed by PEGDMAEMA, due to the improved stability and a reduced rate of renal clearance.¹³⁷

In addition to enhancing stability, functional polymers conjugated to polycations can also allow for controlled DNA release. Poly(*N*-isopropylacrylamide) (PNIPAm) is a typical thermoresponsive polymer that provides hydrophobic interactions at temperature above its lower critical solution temperature (LCST) during polyplex preparation. It can also change its conformation and facilitate DNA release intracellularly under a “cold-shock” at 32.5 °C, resulting in an improved transfection efficiency compared with jetPEI, a commercially available reagent.^{104–106} Pu et al. developed a near-infrared (NIR)-controlled gene-delivery system based on a hydrophobic semiconducting polymer grafted with PAMAM dendrons.¹⁰⁹ The hydrophobic semiconducting polymer backbone not only helped condense the DNA that resulted in smaller polyplexes (38 nm), it also permitted the conversion of NIR light into thermal energy. With the integration of a heat-inducible promoter, this system achieved light-controlled gene expression with a 25-fold transfection efficiency enhancement.

Host–Guest Interactions.

Supramolecular host–guest interactions have broad applications in the biomedical field. Specifically, they have been recognized as an important approach in the design of gene-delivery systems.^{113,138} Formation of the host–guest complex requires a host moiety with a cavity and a guest moiety that can be encapsulated into the cavity via noncovalent interactions (e.g., hydrophobic interactions, hydrogen bonding, or π – π stacking).^{139–141} Similar to hydrophobic interactions as mentioned above, host–guest interactions coupled with electrostatic interactions can enhance the stability of the polyplexes.

The host–guest interactions between β -cyclodextrin (β -CD) and adamantane (AD) have been explored for a number of polymeric gene-delivery systems.^{110,112–115} Liu et al. incorporated AD into low molecular PEI. The AD-modified PEI can complex with nucleic acids (i.e., DNA and siRNA) and be cross-linked by poly(β -cyclodextrin) (PCD). This gene-delivery system, which was strengthened by β -CD-AD host–guest interactions, exhibited increased stability against salt and serum albumin.¹¹⁰ We also developed cross-linked redox-responsive polyplexes for the delivery of multiple types of nucleic acid payloads (Figure 8).¹¹¹ A redox-responsive polycationic poly(*N,N'*-bis(acryloyl)cystamine-*co*-triethylenetetramine) (p(BAC-TET)) polymer—a type of poly(*N,N'*-bis(acryloyl)cystamine-

poly(aminoalkyl)) (PBAP) polymer—was synthesized to overcome the potential poor stability of the non-cross-linked polyplex. AD and β -CD were conjugated to the polymer backbone and cross-linked during the polyplex formation process. The resulting cross-linked PBAP polyplex exhibited excellent stability in both FBS-containing cell culture media and high-concentration BSA solution (i.e., 40 mg/mL) for at least 48 h, while non-cross-linked PBAP polyplexes formed large aggregates in high-concentration BSA solution (i.e., 40 mg/mL), demonstrating the effectiveness of the host–guest interactions on enhancing polyplex stability.

Judiciously designed host–guest interactions can be used in stimuli-responsive gene-delivery systems. Azobenzene, another guest molecule of β -CD, is widely used as a light-responsive moiety. The trans–cis isomerization of azobenzene after UV irradiation can trigger the dissociation of the polyplex, as only trans-azobenzene (i.e., not cis-azobenzene) can form host–guest complexes with β -CD.^{142,143} This strategy was used for designing light-responsive nucleic acid delivery systems.^{116,117}

Other Stabilizing Strategies via Physical Interactions.

Fluorination of polycations is another stabilizing strategy. Cheng et al. found that G5-PAMAM modified with 68 heptafluorobutyric acids revealed extremely high transfection efficiency, excellent serum resistance, and reduced cytotoxicity.¹¹⁸ Fluorination also performed well on other polymers such as poly(propylene imine) (PPI)¹¹⁹ and PEI.¹²⁰ The improved performance was attributed to the unique amphiphobic properties of the fluorinated moiety that helped to stabilize the polyplex and resist serum protein, yet also increased cellular uptake and facilitated endosomal escape as well as intracellular DNA disassociation.¹²¹

The stability of polyplexes can also be enhanced by the incorporation of aromatic moieties that can form π – π stacking. Since aromatic moieties are usually hydrophobic, both hydrophobic interactions and the π – π stacking effect can stabilize polyplexes in addition to electrostatic interactions.^{100,122} These cross-linking strategies, as well as other chemical/physical cross-linking strategies, can be introduced into polymeric nucleic acid-delivery systems to develop more stable and efficient gene-delivery vectors.

CONCLUSIONS AND FUTURE PERSPECTIVES

We have given an overview of the various factors that can potentially compromise the integrity of polymeric gene-delivery systems, both in vitro and in vivo, including the intrinsic properties (e.g., chemical compositions) of the NPs and external environmental factors. To overcome these issues, unimolecular NPs, as well as chemical and/or physical cross-linking strategies, can be applied to achieve in vivo and in vitro stability and enhanced gene-delivery efficacy.

Despite the advantages of chemical and physical cross-linking strategies to stabilize the polymeric gene delivery nanosystems, challenges still remain. While unimolecular NPs and chemical cross-linking strategies can offer excellent stability and also stimuli-responsive release of the nucleic acid payloads, the preparation of these types of NPs may involve more

complicated synthesis processes (e.g., the protection–deprotection of functional groups, and/or delicate in situ polymerization),^{83,85} and thus can create more challenges for scaling-up. Physical cross-linking strategies are relatively more straightforward, and the polyplex formation processes are usually simple. However, a critical self-assembly concentration still exists due to weak, noncovalent interactions.

As discussed earlier, while gene-delivery NPs with superior stability during transport are highly desired, rapid release of the payloads inside the target cell is also important. This can often be achieved using stimuli-responsive polymeric systems that utilize either a physiological stimulus (e.g., lower pH in the endosomal compartments, high GSH concentration in the cytosol, or elevated ATP and ROS levels inside the cells) or an external stimulus (e.g., light, ultrasound, heat, or magnetic field).

Clinical translation of polymeric nucleic acid delivery nanosystems is still at an early stage. Most clinical trials on polymeric nucleic acid delivery NPs are at Phase I or II.^{16,144} Recent developments on stable and stimuli-responsive polymeric gene delivery nanosystems will likely energize the field of gene therapy. Besides NP stability, a number of other pharmacokinetic and pharmacodynamic properties need to be investigated and optimized in order to facilitate the clinical translation of gene delivery systems.

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Figure 1. Schematic illustration of successful gene delivery to target cells and possible factors that cause premature payload release.

Figure 2.
Strategies to stabilize polymeric gene delivery systems. PEG: poly(ethylene glycol).

Figure 3. Illustration of a representative unimolecular NP for gene delivery. PAMAM: poly(amidoamine).

Figure 4. pH/redox dual-sensitive unimolecular NP for siRNA delivery and its subcellular trafficking for siRNA in the cytosol. Reproduced with permission from ref 78.

Figure 5.

Phenylboronate-diol-based chemical cross-linking strategy for stabilizing polyplex micelles (PMs) and further enhancing gene-delivery efficiency via the cumulative process of pH and ATP dual-responsive pDNA release. Reproduced with permission from ref 83. PM: polyplex micelle; FPBA: fluorophenylboronic acid; GlcAm: D-gluconamide.

Figure 6.

Schematic illustration of in situ cross-linked nanocapsule synthesis for gene delivery. (I) Enrichment of the monomers and cross-linkers around the nucleic acid. (II) Formation of cross-linked nanocapsules by in situ polymerization of a thin polymer shell. (III) Intracellular delivery of nanocapsules. (IV) Release of payload from the nanocapsules into the cytosol upon degradation of the nanocapsule shell. Reproduced with permission from ref 85.

Figure 7.
Various strategies to stabilize the polyplexes with hydrophobic interactions.

Figure 8.

Illustration of a cross-linked PBAP polyplex for the delivery of various negatively charged payloads, and their proposed intracellular trafficking pathways. Reproduced with permission from ref 111. RNP: Cas9-sgRNA ribonucleoprotein; S1mplex: RNP-donor DNA complex; p(BAC-TET): poly(*N,N'*-bis(acryloyl)cystamine-*co*-triethylenetetramine; Im:imidazole; GSH: glutathione.

Table 1.

Representative Stabilizing Strategies for Polymeric Nucleic Acid Delivery Nanosystems

stabilization strategy	chemical composition	nucleic acid payloads	refs
Unimolecular NPs	PDMAEMA side chains conjugated to a polyfluorene backbone	siRNA	77
	H40-P(Asp-AED-ICA)-PEG	siRNA	78
Chemical cross-linking	p(HPMA-co-PDTEMA-co-APMA)- <i>b</i> -PEG	DNA	79
	PEG-PLL with thiol groups for disulfide bond formation	DNA	67, 80–82
	PAsp(DET) conjugated with 4-carboxy-3-fluorophenylboronic acid (FPBA) or D-gluconamide (GlcAm) moieties	DNA	83
	BPEI conjugated with FPBA or GlcAm moieties	DNA	84
	Enrichment of monomers and degradable cross-linkers around nucleic acid followed by in situ polymerization	siRNA, miRNA	85, 86
Physical cross-linking	Modifying cationic polymer with hydrophobic small molecules	DNA, siRNA, antisense oligonucleotide (ASO)	87–103
	Grafting hydrophobic polymers onto cationic polymer main chain	DNA, siRNA	104–106
	Integrating hydrophobic segments in cationic polymer main chain	DNA	107–109
	Adamantane (AD) and β -cyclodextrin (β -CD)-modified cationic polymers	DNA, mRNA, siRNA	110–115
Host–guest interaction	Azobenzene and β -CD-modified cationic polymers	siRNA, DNA	116, 117
Other physical interaction	Polymer fluorination	DNA	118–121
	Integration of aromatic moieties into cationic polymers	DNA	100, 122