VIP DNA Nanoparticles

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Dual Responsive, Stabilized Nanoparticles for Efficient In Vivo Plasmid Delivery**

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Nucleic acid-based therapeutics, including plasmid DNA (pDNA) and small interfering RNA (siRNA), have been considered highly promising strategies to treat a gamut of diseases.[1] Successful nucleic acid delivery relies on the development of safe and efficient delivery vectors. Viral vectors have dominated as the delivery vehicles used in clinical trials but their progress has been hampered by their immunogenicity, safety risks, and high manufacturing cost.[2] Therefore, the use of non-viral vectors, such as cationic polymer-based vectors that offer advantages over viral vectors in these aspects, has attracted broad attention.[3,4]

Although diverse polymeric vectors have been developed so far,[5,6] the adaptation of in vitro optimized materials for successful in vivo use remains difficult. One challenge is to prevent particle flocculation in physiological salt concentrations. Modification of polyplexes (polymer/nucleic acid complexes) with a poly(ethylene glycol) (PEG) coating is a leading strategy to achieve colloidal stability. However, the PEG coating negatively affects cellular uptake and intracellular trafficking.[7] Polyplexes that detach the PEG shielding layer in response to signals in the biological microenvironments are an elegant solution to these issues.[8–11]

Biodistribution studies with PEGylated polyplexes have revealed an additional complication: PEGylated polyplexes can exhibit poor stability and premature unpackaging in vivo, possibly because of interactions with serum proteins and extracellular matrix components.[12–14] Hydrophobic modification of polycations has been explored as a potential solution.[15–17] For example, hydrophobized polyethylenimine (PEI) derivatives have improved blood circulation time and higher transfection efficacy compared to parent PEI.[18–21] Hydrophobic modification of polycations with poly(lactide) (PLA) or poly(ε-caprolactone) (PCL) reduce the cytotoxicity of polycations while promoting the overall biodegradability of gene carriers.[22–25]

Another important consideration in the clinical translation of non-viral vectors is the controlled synthesis of well-defined materials. The rapid advance in controlled radical living polymerization (CRLP), such as reversible addition–fragmentation chain transfer (RAFT) polymerization, and atom-transfer radical polymerization (ATRP), has enabled the synthesis of well-defined cationic polymers with preselected composition and narrowly distributed molecular weight, parameters that have been shown to affect both transfection efficiency and cytotoxicity.[26–30]

Herein we report the development of a new block copolymer designed specifically for in vivo gene transfer and demonstrate its effectiveness in plasmid delivery to the mouse brain. Ternary, amphiphilic block copolymers (Scheme 1) containing a sheddable hydrophobic PCL block, pH-sensitive oligoamine tetraethylenepentamine (TEPA)-decorated poly(glycidyl methacrylate) (PGMA) block, and a hydrophilic oligo(ethylene glycol) monomethyl ether methacrylate (OEGMA) segment were synthesized by integrated ring-opening polymerization (ROP) and ATRP.[31] The sheddable hydrophobic PCL block was synthesized by ROP using a reducible double-head initiator HO-SS-*i*BuBr[32] and then used as a macro-initiator for subsequent ATRP. The block-statistical copolymer (that is, a copolymer with a block and a statistical region) was synthesized by a one-pot ATRP of GMA and OEGMA with the macro-initiator. Two triblock copolymers with a central or terminal OEGMA block were synthesized by a consecutive two-step ATRP with the macro-initiator. Finally, GMA units were decorated with TEPA to generate pH-sensitive polycations (Supporting Information, Scheme S1). Two control diblock copolymers, PCL_{40}-*b*-P-$(GMA\text{-}TEPA)_{57}$ (non-reducible diblock) and PCL_{40}-SS-P-$(GMA\text{-}TEPA)_{52}$ (reducible diblock) were also synthesized.

Endosomal escape is a critical step in successful gene transfer.[33] A main advantage of branched PEI (bPEI) is its buffering capacity, which facilitates its endosomal escape.[34] To assess the proton buffering capacity (BC, μmol H^+/mg polymer required to decrease the pH value of a 0.2 mg mL^{-1} polymer solution from 7.4 to 5.0) of the various PCL-based copolymers, acid–base titration was performed (Figure S3). The reducible diblock copolymer (BC = 2.8) and block-statistical copolymer (BC = 2.8) have a similar buffering capacity to that of the parent $P(GMA\text{-}TEPA)_{50}$ homopolycation (BC = 2.9). However, both triblock copolymers have a significantly lower buffering capacity (BC = 2.0), possibly because of shielding by the OEGMA block.

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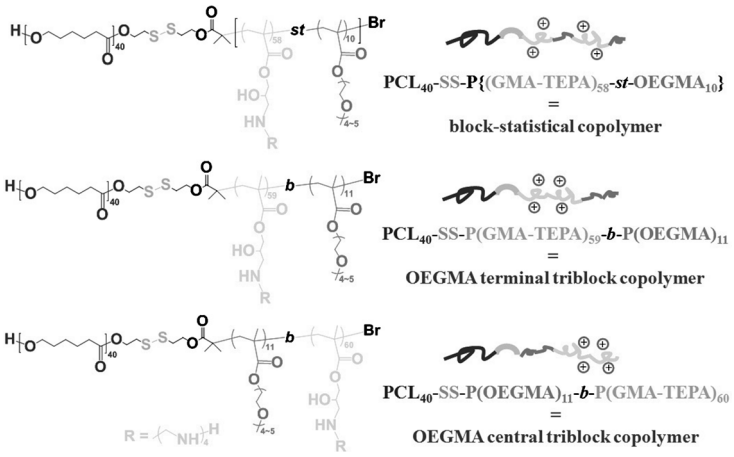


Scheme 1. Structural formula and schematic illustration of the three ternary cationic copolymers.

All the polymers have an effective DNA binding ability (Figure S4). The spherical (diameter < 100 nm) morphology of polyplexes formed by the ternary copolymers was confirmed by TEM (Figure 1 A). The stability of polyplexes in physiological salt concentrations was determined by dynamic light scattering (DLS). Control diblock polymers without OEGMA were unable to condense DNA into colloidally stable, sub-200 nm polyplexes in the salt medium (Figure 1 B). Incorporation of the hydrophilic OEGMA shielding segment significantly enhances the salt stability of the polyplexes formed from each ternary copolymers by providing steric stabilization.[35,36]

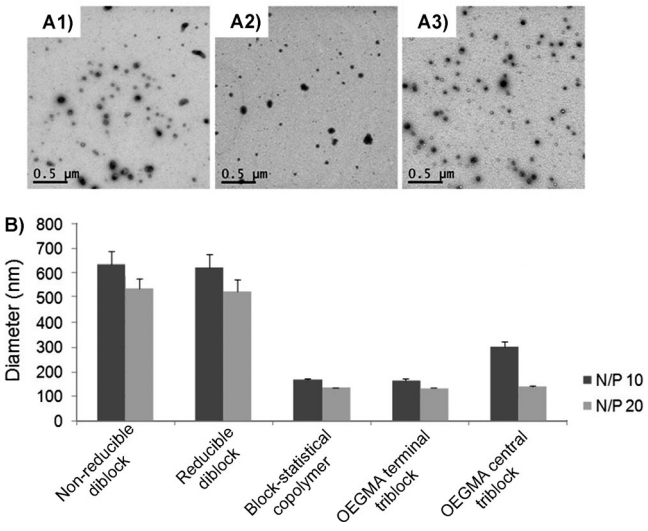


Figure 1. TEM images of polyplexes formed by A1) non-reducible diblock, A2) reducible diblock, and A3) block-statistical copolymer with plasmid DNA at an N/P ratio of 10 (that is, the mole ratio of the amine groups of cationic polymers and the phosphate groups of DNA; scale bar: 500 nm). B) Average hydrodynamic diameter of various polyplexes formed at different N/P ratios in 150 mM PBS.

The Kissel group has demonstrated that modification of PEG-PEI with PCL results in polyplexes that are more stable in the systemic circulation.[21,22] We hypothesized that polycations made more hydrophobic by PCL modification with a disulfide bond might form polyplexes that have improved extracellular stability in in vivo environments but that destabilize in intracellular environments after reduction-triggered PCL release. The entrapment of OEG chains in the polyplexes has been theorized to lead to more thermodynamic destabilization compared to unmodified polycations.[12,13] Therefore, incorporation of OEGMA in the polycations would provide not only extracellular colloidal stability (through steric stabilization) but help to facilitate intracellular polyplex unpackaging after PCL removal (owing to weaker DNA binding compared to non-OEG containing polycations).

We confirmed the release of the PCL blocks from ternary copolymers in the presence of reducing agent tris(2-carboxyethyl)phosphine hydrochloride (TCEP·HCl; Figure S5). Next, we tested the stability of polyplexes formed from ternary copolymers in both non-reducing and reducing environments. YOYO-1 is a DNA intercalating dye whose fluorescence is self-quenched when DNA is in a condensed complex. Polyplexes formed in the absence of serum show nearly complete fluorescence quenching of YOYO-labeled pDNA (Figure S6). In the presence of serum, some polyplex loosening was observed in non-reducing environments (Figure 2). DLS measurements showed that the average diameters increase by approximately 50 nm (data not shown), thus suggesting that polyplexes are not unpackaging but instead swelling which allows for increases in YOYO-1 fluorescence.

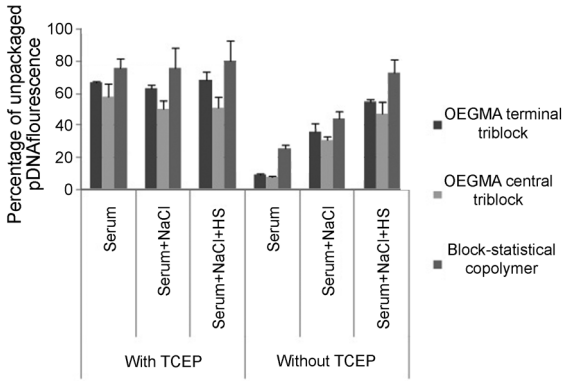


Figure 2. Unpackaging study of polyplexes (N/P = 15) formed by block-statistical and triblock copolymers in serum. Polyplexes were treated with a reducing agent (TCEP, 10 mM) and with 1 M NaCl and heparan sulfate (HS) to promote polyplex destabilization. Each sample was tested with a sample size (n) = 3.

In contrast, all the polyplexes are significantly destabilized in reducing environments in the presence of serum, that is, compared to pDNA, the fluorescence in non-reducing

versus reducing environments is 9% versus 67% for OEGMA terminal triblock, 8% versus 58% for OEGMA central triblock, and 30% versus 76% for block-statistical copolymer formulations. The block-statistical formulation unpackaged more than the triblock copolymers, with almost complete release of pDNA, demonstrating the architecture of OEGMA significantly affects the unpackaging of pDNA. In some samples, heparan sulfate (HS) and/or NaCl was added to promote pDNA release further. Again, polyplex unpackaging is consistently higher in polyplexes exposed to reducing environments. These results confirm that PCL incorporation increases the stability of polyplexes formed from the ternary copolymers, but that site-specific intracellular release of nucleic acid cargo can be triggered through reduction of the disulfide bond and release of PCL blocks.

The effect of polymer architecture on the transfection efficacy was evaluated in both serum-free (Figure S7) and serum conditions (Figure 3) by delivery of the luciferase

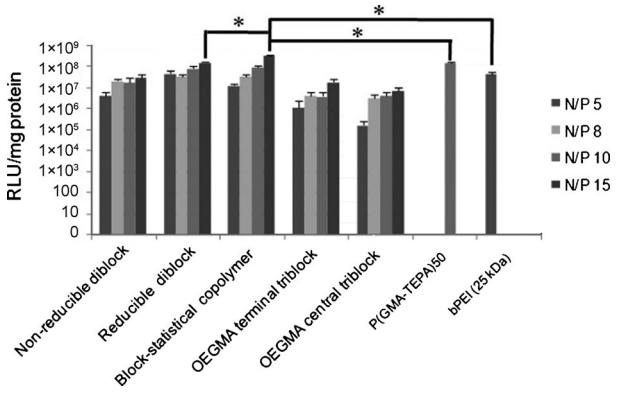


Figure 3. Transfection efficiency of polyplexes in serum to HeLa cells at various N/P ratios. Data are shown as mean ± SD ($n = 4$; student's t test, $*p < 0.001$).

reporter plasmid to HeLa cells. Several observations are worth noting. First, the transfection efficiency in the presence of serum of select PCL-based copolymers approached and even superseded (by ca. eightfold) that of PEI. Low cytotoxicity was observed from all copolymers, with over 80% cell viability (Figure S8). We also tested the effect of polymer backbone degradability (Figure S7), PCL block length (Figure S9), and P(GMA-TEPA) content (Figure S10), and found that 1) reduction-sensitive polyplexes displayed significantly higher transfection efficacy than reduction-insensitive analogues at all the tested N/P ratios, 2) reversibly hydrophobized polyplexes with longer PCL chain length (degree of polymeration (DP) of 62) were less efficient and 3) lower P(GMA-TEPA) incorporation (DP 41) resulted in reduced transfection efficiency. Thus, the block-statistical copolymer was the best performing polymer tested.

Second, polyplexes formed from PCL-based copolymers were insensitive to serum, likely a result of increased particle stability. In contrast, the transfection efficiencies of P(GMA-TEPA)$_{50}$ and bPEI were decreased by approximately fivefold and 20-fold,[37] respectively, in the presence of serum. In addition, steric stabilization by OEGMA contributes to serum stability. While optimal transfection efficiency of PCL-based diblocks decreased slightly by around 4.3-fold and around 2.1-fold (for non-reducible and reducible diblocks, respectively) because of serum addition, the optimal transfection efficiency of the ternary copolymers generally remained unchanged. The ternary block-statistical copolymer is especially effective at gene delivery in serum-containing media, displaying higher delivery efficiency than the parent reducible diblock copolymer.

Last, the sequence of monomers in the ternary copolymers critically affects transfection efficiency. The block-statistical copolymer mediates significantly higher transfection efficacy than both triblock copolymers at all the tested N/P ratios in both serum-free and serum conditions. This trade-off between particle stabilization and transfection efficiency resulting from the introduction of hydrophilic OEGMA blocks has been reported for several other polycations used for gene delivery.[30,38]

The panel of PCL-based copolymers was also tested as siRNA delivery agents by packaging a siRNA against the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene (Figure 4). A siRNA sequence against the luciferase gene was

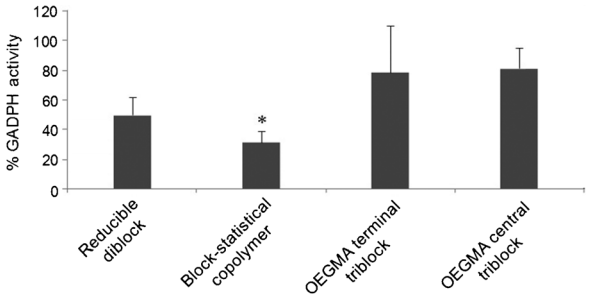


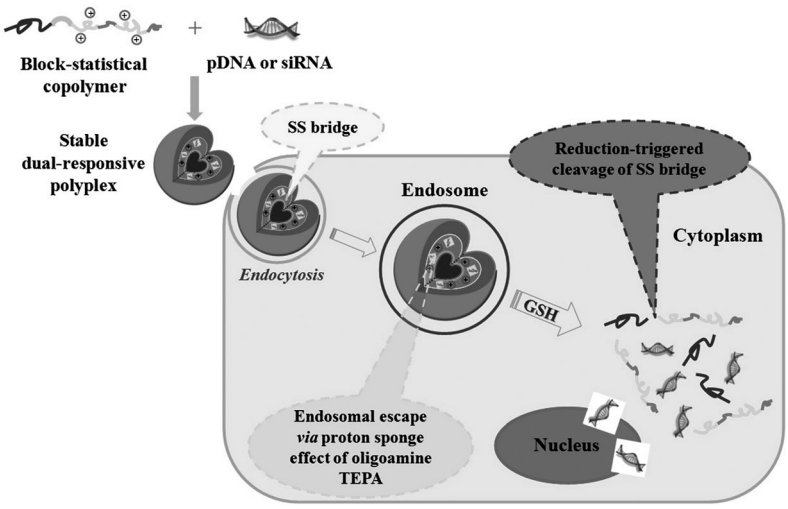
Figure 4. siRNA delivery study in HeLa cells. GAPDH activity levels in cells treated with GAPDH siRNA normalized with cells treated with control, luciferase siRNA using various PCL-based complexes at an N/P ratio of 15. [siRNA] = 100 nM, Data are shown as mean ± SD ($n = 3$; student's t test, $*p < 0.02$).

used for control complexes. Polymeric vectors that are effective for plasmid delivery often have to be modified to efficiently deliver siRNA.[39–41] In this case, the ternary block-statistical copolymer that showed highest gene transfer was also most effective at siRNA delivery without the need for additional modifications. The triblock copolymers in contrast display an insignificant silencing effect. The combination of reversible hydrophobization and statistical hydrophilization is therefore also advantageous for siRNA delivery.

The effectiveness of the PCL$_{40}$-SS-P{(GMA-TEPA)$_{58}$-*st*-OEGMA$_{10}$} block-statistical copolymer-based polyplex is due to its unique features that satisfy two seemingly contradictory requirements essential for efficient gene transfer. On one hand, the incorporation of both hydrophobic PCL and hydrophilic OEGMA segments provides superior stability of the polyplex and adequate DNA protection in the extracellular milieu. On the other hand, the reduction-

triggered removal of the hydrophobic segment and statistically incorporated hydrophilic units facilitate efficient release of DNA under the intracellular environments. The balance of DNA protection and release have remained challenging for non-viral gene transfection.[42–44] Our study reveals that the combining reversible hydrophobization and statistical hydrophilization of cationic polymers addresses this dilemma successfully.

Based on these studies, we present the proposed intracellular trafficking route for nucleic acid delivery using the PCL-SS-P{(GMA-TEPA)-*st*-OEGMA} block-statistical copolymer shown in Scheme 2. The block-statistical copolymer is expected to transfect cells by condensing DNA efficiently to form stable dual-responsive polyplexes. Once internalized into endocytic vesicles, the protonatable amines in TEPA (pH-responsiveness) facilitate endosomal escape by the proton sponge effect, and reducing agents, such as glutathione (GSH) in the cytosol degrade the disulfide bridges, leading to detachment of the hydrophobic PCL core. The resulting destabilized P{(GMA-TEPA)-*st*-OEGMA}/pDNA complex facilitates nucleic acid release. It is interesting to note that the block-statistical copolymer is more easily displaced than the triblock copolymers (Figure 2), which may lead to more efficient intracellular cargo release.



Scheme 2. Formation of dual-responsive stable polyplexes, and proposed intracellular delivery route of the polyplexes formed from the block-statistical ternary copolymer. The desired cellular compartment for siRNA and plasmid DNA are cytosol and nucleus, respectively.

Based on the high in vitro transfection efficiency obtained using the block-statistical copolymer, we selected this formulation along with the two control diblock copolymers to investigate for in vivo gene transfection. We have an interest in developing materials for gene delivery to the central nervous system (CNS) for potential treatment of neurodegenerative diseases.[11,45,46] Therefore, these three polyplexes containing the luciferase reporter gene were injected into the right lateral ventricle of mice. All the formulations were well tolerated and no significant gross morbidities or mortalities were observed as a result of polyplex injection.

The in vivo delivery efficiencies correlate well with the in vitro serum-conditioned delivery efficiencies, that is, the polyplexes based on block-statistical copolymer combining reversible hydrophobization and statistical hydrophilization displayed approximately 3.0- and 15.6-fold higher luciferase activity compared to the polyplexes of reducible and non-reducible diblock copolymers, respectively, and the reducible diblock formulation mediated 5.1-fold higher transfection activity than the non-reducible diblock analogue (Figure 5).

In summary, we present a novel block-statistical copolymer, PCL_{40}-SS-P{$(GMA\text{-}TEPA)_{58}$-*st*-$OEGMA_{10}$}, that provides one solution to the in vivo stability versus high transfection efficiency dilemma. This material combines reversible hydrophobization for enhanced polyplex stability in extracellular environments and statistical hydrophilization for efficient plasmid and siRNA release inside cells. The block-statistical formulation mediated high transfection efficacy in vitro in addition to effective delivery to the mouse brain in vivo.

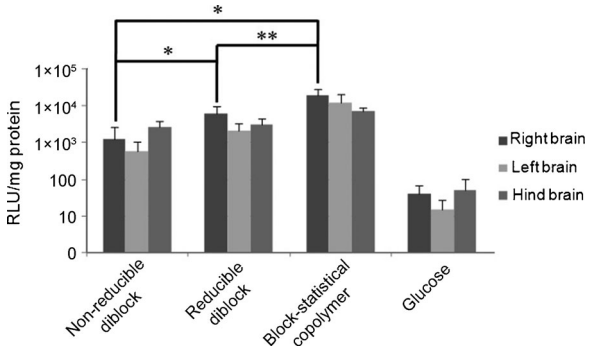


Figure 5. Transfection efficacy in mouse brains two days after intraventricular injection using glucose control, and luciferase plasmid complexes formed from non-reducible diblock, reducible diblock, and block-statistical copolymers at an N/P ratio of 15. Data are shown as mean ± SD ($n = 6$; student's t test, $*p < 0.01$, $**p \approx 0.01$).

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