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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*, likely due to 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 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 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 pre-selected composition and narrowly-distributed molecular weight, parameters that have been shown to affect both transfection efficiency and cytotoxicity.^[26-30]

Here 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 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-iBuBr^[32] and then used as a macro-initiator for subsequent ATRP. The block-statistical copolymer 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 by TEPA to generate pH-sensitive polycations. (Scheme S1). Two control diblock copolymers, PCL₄₀-*b*-P(GMA-TEPA)₅₇ (non-reducible diblock) and PCL₄₀-SS-P(GMA-TEPA)₅₂ (reducible diblock) were also synthesized.

Endosomal escape is a critical step in successful gene transfer.^[33] A main advantage of bPEI is its buffering capacity, which facilitates its endosomal escape.^[34] To assess the proton buffering capacity (BC, $\mu\text{mol H}^+/\text{mg}$ polymer required to decrease the pH of a 0.2 mg/ml 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) possess similar buffering capacity to that of the parent P(GMA-TEPA)₅₀ homopolymer (BC=2.9). However, both triblock copolymers have significantly reduced buffering capacity (BC=2.0), possibly due to shielding by the OEGMA block.

All polymers exhibited effective DNA binding ability (Figure S4). The spherical (diameter < 100 nm) morphology of polyplexes formed by the ternary copolymers was confirmed by TEM (Figure 1A). 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 colloidal stable, sub-200 nm polyplexes in the salt medium (Figure 1B). 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]

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 hydrophobized by PCL modification *via* 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 (due to steric stabilization) but help to facilitate intracellular polyplex unpacking after PCL removal (due to weaker DNA binding compared to non-OEG containing polycations).

We first 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 ~50 nm (data not shown), thus suggesting that polyplexes are not unpacking but instead swelling which allows for increases in YOYO-1 fluorescence.

In contrast, all polyplexes are significantly destabilized in reducing environments in the presence of serum *i.e.*, 9% vs 67%, 8% vs 58%, and 30% vs 76% fluorescence compared to pDNA for OEGMA terminal triblock, OEGMA central triblock, and block-statistical copolymer formulations, respectively, in non-reducing versus reducing environments. The block-statistical formulation unpacked more than the triblock copolymers, with almost complete release of pDNA, demonstrating the architecture of OEGMA significantly affects the unpacking of pDNA. In some samples, heparan sulfate (HS) and/or NaCl was added to promote pDNA release further. Again, polyplex unpacking 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 disulfide bond reduction 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 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 ~8-fold) that of PEI. Low cytotoxicity was observed from all copolymers, with >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 (DP 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 due to increased particle stability (Figure 1B). In contrast, the transfection efficiencies of P(GMA-TEPA)₅₀ and bPEI were decreased by ~5-fold 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 ~4.3-fold and ~2.1-fold (for non-reducible and reducible diblocks, respectively) due to serum addition, optimal transfection efficiency of the ternary copolymers generally remained unchanged. The ternary block-statistical copolymer is especially effective at gene delivery in complete 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 previously 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 used for control complexes. Polymeric vectors that are effective for plasmid delivery often have to be modified in order 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 PCL₄₀-SS-P{(GMA-TEPA)₅₈-*st*-OEGMA₁₀} block-statistical copolymer-based polyplex is likely 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 via 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.

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 a long-standing 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 due to polyplex injection.

The *in vivo* delivery efficiencies correlate well with the *in vitro* serum-conditioned delivery efficiencies, *i.e.*, the polyplexes based on block-statistical copolymer combining reversible hydrophobization and statistical hydrophilization displayed ~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 here a novel block-statistical copolymer, PCL₄₀-SS-P{(GMA-TEPA)₅₈-*st*-OEGMA₁₀}, 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*.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

References

- [1]. Zuber G, Dauty E, Nothisen M, Belguise P, Behr JP. Adv. Drug Deliv. Rev. 2001; 52:245–253. [PubMed: 11718949]
- [2]. Mintzer MA, Simanek EE. Chem Rev. 2008; 109:259–302. [PubMed: 19053809]
- [3]. Lynn DM, Langer R. J. Am. Chem. Soc. 2000; 122:10761–10768.
- [4]. Kakizawa Y, Kataoka K. Adv. Drug Deliv. Rev. 2002; 54:203–222. [PubMed: 11897146]
- [5]. Park TG, Jeong JH, Kim SW. Adv. Drug Deliv. Rev. 2006; 58:467–486. [PubMed: 16781003]
- [6]. Pack DW, Hoffman AS, Pun SH, Stayton PS. Nat. Rev. Drug Discov. 2005; 4:581–593. [PubMed: 16052241]
- [7]. Hatakeyama H, Akita H, Kogure K, Oishi M, Nagasaki Y, Kihira Y, Ueno M, Kobayashi H, Kikuchi H, Harashima H. Gene Ther. 2007; 14:68–77. [PubMed: 16915290]

- [8]. Takae S, Miyata K, Oba M, Ishii T, Nishiyama N, Itaka K, Yamasaki Y, Koyama H, Kataoka K. *J. Am. Chem. Soc.* 2008; 130:6001–6009. [PubMed: 18396871]
- [9]. Walker GF, Fella C, Pelisek J, Fahrmeir J, Boeckle S, Ogris M, Wagner E. *Mol. Ther.* 2005; 11:418–425. [PubMed: 15727938]
- [10]. Yang XZ, Du JZ, Dou S, Mao CQ, Long HY, Wang J. *ACS Nano.* 2012; 6:771–781. [PubMed: 22136582]
- [11]. Wei H, Schellinger JG, Chu DSH, Pun SH. *J. Am. Chem. Soc.* 2012; 134:16554–16557. [PubMed: 23013485]
- [12]. Merdan T, Kunath K, Petersen H, Bakowsky U, Voigt KH, Kopecek J, Kissel T. *Bioconjugate Chem.* 2005; 16:785–792.
- [13]. Merkel OM, Librizzi D, Pfestroff A, Schurrat T, Buyens K, Sanders NN, De Smedt SC, Behe M, Kissel T. *J. Control. Release.* 2011; 138:148–159. [PubMed: 19463870]
- [14]. Burke RS, Pun SH. *Bioconjugate Chem.* 2008; 19:693–704.
- [15]. Sharma R, Lee JS, Bettencourt RC, Xiao C, Konieczny SF, Won YY. *Biomacromolecules.* 2008; 9:3294–3307. [PubMed: 18942877]
- [16]. Anderson DG, Lynn DM, Langer R. *Angew Chem Int. Ed.* 2003; 42:3153–3158.
- [17]. Xiong XB, Uludag H, Lavasanifar A. *Biomaterials.* 2009; 30:242–253. [PubMed: 18838158]
- [18]. Thomas M, Klibanov AM. *Proc. Natl. Acad. Sci. USA.* 2002; 99:14640–14645. [PubMed: 12403826]
- [19]. Neamark A, Suwantong O, Bahadur KCR, Hsu CYM, Supaphol P, Uludag H. *Mol. Pharm.* 2009; 6:1798–1815. [PubMed: 19719326]
- [20]. Dehshahri A, Oskuee RK, Shier WT, Hatefi A, Ramezani M. *Biomaterials.* 2009; 30:4187–4194. [PubMed: 19464054]
- [21]. Zheng MY, Librizzi D, Kiliç A, Liu Y, Renz H, Merkel OM, Kissel T. *Biomaterials.* 2012; 33:6551–6558. [PubMed: 22710127]
- [22]. Shuai XT, Merdan T, Unger F, Wittmar M, Kissel T. *Macromolecules.* 2003; 36:5751–5759.
- [23]. Zhu CH, Jung S, Luo SB, Meng FH, Zhu XL, Park TG, Zhong ZY. *Biomaterials.* 2010; 31:2408–2416. [PubMed: 19963269]
- [24]. Yue XY, Qiao Y, Qiao N, Guo ST, Xing JF, Deng LD, Xu JQ, Dong AJ. *Biomacromolecules.* 2010; 11:2306–2312. [PubMed: 20666510]
- [25]. Zheng MY, Librizzi D, Kiliç A, Liu Y, Renz H, Merkel OM, Kissel T. *Biomaterials.* 2012; 33:6551–6558. [PubMed: 22710127]
- [26]. Georgiou TK, Vamvakaki M, Patrickios CS, Yamasaki EN, Phylactou LA. *Biomacromolecules.* 2004; 5:2221–2229. [PubMed: 15530036]
- [27]. Nemoto Y, Borovkov A, Zhou YM, Takewa Y, Tatsumi E, Nakayama Y. *Bioconjugate Chem.* 2009; 20:2293–2299.
- [28]. Chu DSH, Schellinger JG, Shi J, Convertine AJ, Stayton PS, Pun SH. *Acc. Chem. Res.* 2012; 45:1089–1099. [PubMed: 22242774]
- [29]. Ahmed M, Narain R. R. *Prog. Polym. Sci.* 2013 <http://dx.doi.org/10.1016/j.progpolymsci.2012.09.008>.
- [30]. Wei H, Pahang JA, Pun SH. *Biomacromolecules.* 2013; 14:275–284. [PubMed: 23240866]
- [31]. Zhang WL, He JL, Liu Z, Ni PH, Zhu XL. *J. Polym. Sci. Part A: Polym. Chem.* 2010; 48:1079–1091.
- [32]. Sourkahi BK, Cunningham A, Zhang Q, Oh JK. *Biomacromolecules.* 2011; 12:3819–3825. [PubMed: 21879701]
- [33]. Suk JS, Suh J, Lai SK, Hanes J. *Exp. Biol. Med.* 2007; 232:461–469.
- [34]. Behr JP. *Chimia.* 1997; 51:34–36.
- [35]. Zhulina EB, Borisov OV, Priamitsyn VA. *J. Colloid Interface Sci.* 1990; 137:495–511.
- [36]. Milner ST. *Science.* 1991; 251:905–914. [PubMed: 17847384]
- [37]. Forrest ML, Meister GE, Koerber JT, Pack DW. *Pharm. Res.* 2004; 21:365–371. [PubMed: 15032320]

- [38]. Georgiou TK, Phylactou LA, Patrickios CS. *Biomacromolecules*. 2006; 7:3505–3512. [PubMed: 17154481]
- [39]. Zintchenko A, Philipp A, Dehshahri A, Wagner E. *Bioconjugate Chem*. 2008; 19:1448–1455.
- [40]. Biswas S, Deshpande PP, Navarro G, Dodwadkar NS, Torchilin VP. *Biomaterials*. 2013; 34:1289–1301. [PubMed: 23137395]
- [41]. Creusat G, Rinaldi AS, Weiss E, Elbaghdadi R, Remy JS, Mulherkar R, Zuber G. *Bioconjugate Chem*. 2010; 21:994–1002.
- [42]. Schaffert D, Wagner E. *Gene Ther*. 2008; 15:1131–1138. [PubMed: 18528432]
- [43]. Grigsby CL, Leong KW. *J. Roy Soc. Interface*. 2010; 7:S67–S82. [PubMed: 19734186]
- [44]. Liu ZZ, Zheng M, Meng FH, Zhong ZY. *Biomaterials*. 2011; 32:9109–9119. [PubMed: 21890198]
- [45]. Schellinger JG, Pahang JA, Johnson RN, Chu DSH, Sellers DL, Maris DO, Convertine AJ, Stayton PS, Horner PJ, Pun SH. *Biomaterials*. 2013; 34:2318–2326. [PubMed: 23261217]
- [46]. Kwon EJ, Lasienne J, Jacobson B, Park IK, Horner PJ, Pun SH. *Biomaterials*. 2010; 31:2417–2424. [PubMed: 20004466]

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 (scale bar: 500 nm). (B) Average hydrodynamic diameter of various polyplexes formed at different N/P ratios in 150 mM PBS.

Figure 2.

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

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

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 $\pm$ SD (n = 3; student's t test, * $p < 0.02$).

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 $\pm$ SD (n = 6; student's t test, * p < 0.01, ** p ~ 0.01).

Scheme 1.

Chemical structure and schematic illustration of the three ternary cationic copolymers.

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.