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Neuron-Targeted Copolymers with Sheddable Shielding Blocks Synthesized Using a Reducible, RAFT-ATRP Double-Head Agent

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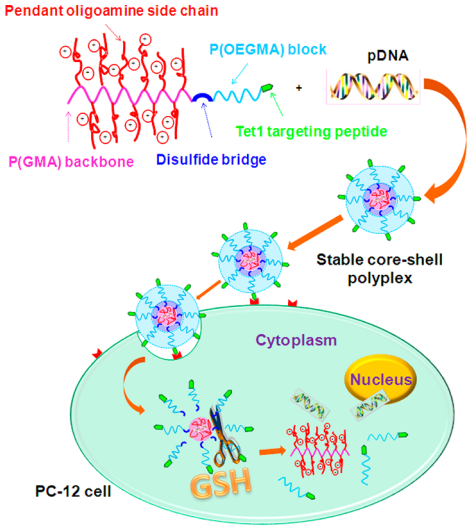
Supporting Information

ABSTRACT: Adaptation of *in vitro* optimized polymeric gene delivery systems for *in vivo* use remains a significant challenge. Most *in vivo* applications require particles that are sterically stabilized, which significantly compromises transfection efficiency of materials shown to be effective *in vitro*. We present a multifunctional well-defined block copolymer that forms particles useful for cell targeting, reversible shielding, endosomal release, and DNA condensation. We show that targeted and stabilized particles retain transfection efficiencies comparable to the nonstabilized formulations. A novel, double-head agent that combines a reversible addition−fragmentation chain transfer agent and an atom transfer radical polymerization initiator through a disulfide linkage is used to synthesize a well-defined cationic block copolymer containing a hydrophilic oligoethyleneglycol and a tetraethylenepentamine-grafted polycation. This material effectively condenses plasmid DNA into salt-stable particles that deshield under intracellular reducing conditions. *In vitro* transfection studies show that the reversibly shielded polyplexes afford up to 10-fold higher transfection efficiencies than the analogous stably shielded polymer in four different mammalian cell lines. To compensate for reduced cell uptake caused by the hydrophilic particle shell, a neuron-targeting peptide is further conjugated to the terminus of the block copolymer. Transfection of neuron-like, differentiated PC-12 cells demonstrates that combining both targeting and deshielding in stabilized particles yields formulations that are suitable for *in vivo* delivery without compromising *in vitro* transfection efficiency and are thus promising carriers for *in vivo* gene delivery applications.

Polycations are attractive for gene delivery because they self-assemble with and condense nucleic acids, can be synthesized at large scale, and offer flexible chemistries for functionalization.[1] Many polymer compositions and architectures have been synthesized, and *in vitro* screening of these polymers has yielded many materials that efficiently transfect cultured mammalian cells, [2] but only a small subset of these materials is suitable for *in vivo* use due to additional extracellular barriers. Polyplexes—complexes of polycations and nucleic acids—are colloids typically unstable in physiological conditions.[3] They are prone to protein adsorption and aggregation, which can lead to inflammation and mortality. Furthermore, when used *in vivo*, they must preferentially transfect the target cell type rather than the vast majority of other cells present.

Scheme 1. Polymer Structure, DNA Condensation, Cell Binding, Endocytosis, and Proposed Route for Subsequent Reduction-Triggered Intracellular Gene Release



To address the aggregation issue, a hydrophilic polymer shell, e.g. poly(ethylene glycol) (PEG) or *N*-(2-hydroxypropyl)-methacrylamide (HPMA), is incorporated into most polyplexes designed for *in vivo* use.[4] However, polyplexes shielded against protein adsorption and aggregation are also poorly recognized and internalized by cells, compromising transfection efficiencies. Formulations with reversible deshielding properties that are triggered by acid conditions (found in tumor microenvironments or in endosomes after cellular uptake) or reducing conditions (found in the cell cytosol) are generally more efficient than formulations with stable polymer shields. Notable examples have been tested for *in vivo* delivery: Wagner's ternary complexes containing targeting ligand conjugated to one polycation, PEG conjugated to a second polycation via an acid-labile hydrazone, and plasmids; Wang's ternary complexes of polyplexes coated with a charge-reversing polymer that deshield in mildly acidic environments; and Kataoka's block copolymers of PEG and poly[Asp(DET)] that deshield in reducing conditions.[5] However, to our knowledge, the formulations reported to date

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do not incorporate targeting ability and/or require multiple polymer components in the final structures, complicating scale-up and manufacturing.

The major advancement reported in this work is the development of a polymeric nucleic acid carrier that incorporates cell targeting, reversible colloidal stability, and efficient intracellular delivery into a single well-defined material (Scheme 1). The key to this is a novel, reducible double-head agent consisting of both a reversible addition−fragmentation chain transfer (RAFT) agent and an atom transfer radical polymerization (ATRP) initiator connected by a disulfide bond (Figure 1a).

We used this double-head agent to synthesize an optimized reduction-responsive cationic block copolymer, P(OEGMA)-SS-P(GMA-TEPA), by a combination of RAFT polymerization of oligo(ethylene glycol) monomethyl ether methacrylate (OEGMA) and ATRP of glycidyl methacrylate (GMA), followed by postpolymerization decoration of reactive epoxy groups in the P(GMA) block by tetraethylenepentamine (TEPA) (Figure 1b). Diblock copolymers synthesized using this double-head agent possess several notable qualities: (1) They have well-controlled composition with narrowly distributed molecular weight. (2) They can be easily modified with a targeting ligand at the outer corona. (3) The "*in vivo* ready" diblock formulation shows transfection efficiency similar to that of the *in vitro* optimized polycation segment due to the reversible shielding combined with targeting ability. The neuron targeting peptide Tet1-conjugated $P(OEGMA)_{15}$-SS-$P(GMA\text{-}TEPA)_{50}$ is expected to transfect cells by condensing DNA efficiently to form core−shell type polyplexes with the P(GMA-TEPA)/DNA electrostatic complex as the core and the P(OEGMA) block as the shell. Once internalized, the polyplexes become localized within the endocytic vesicles. The protonatable amines in TEPA were included to facilitate endosomal escape through the proton sponge effect,[6] and glutathiones in the intracellular environment are expected to degrade the disulfide links, leading to detachment of the hydrophilic P(OEGMA) coating and release of DNA (Scheme 1).

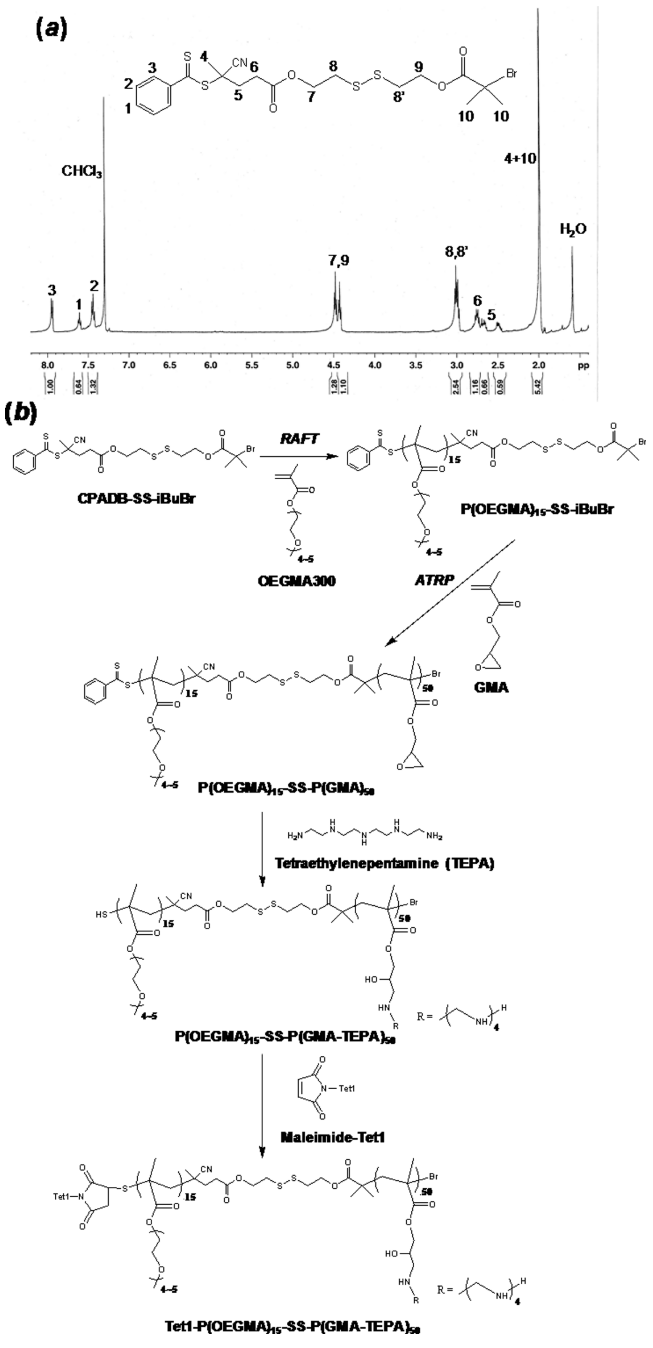

Figure 1. (a) ^{1}H NMR spectrum of the double-head agent (CPADB-SS-iBuBr). (b) Synthesis of Tet1-$P(OEGMA)_{15}$-SS-$P(GMA\text{-}TEPA)_{50}$.

The double-head agent CPADB-SS-iBuBr was synthesized by *N*,*N*′-dicyclohexylcarbodiimide coupling between the RAFT chain transfer agent (CTA) 4-cyanopentanoic acid dithiobenzoate (CPADB)[7] and 2-hydroxyethyl-2′-(bromoisobutyryl)ethyl disulfide initiator (OH-SS-iBuBr)[8] in the presence of 4-(dimethylamino)pyridine as a catalyst. After purification by column chromatography, CPADB-SS-iBuBr was successfully obtained with 43.1% yield (Figure 1a).

The most commonly used hydrophilic shell employed in polyplex stabilization is PEG, which offers facile incorporation by various methods, e.g. using a PEG-based macroinitiator,[5a,9] macro-CTA,[10] or a coupling reaction.[11] Coupling through disulfide-thiol exchange usually requires synthesis of both a mercapto-functionalized and a pyridyl disulfide-functionalized polymer;[11] thus it involves reaction between large macromolecules and extra separation steps to remove the excess homopolymers from desired block copolymers. In addition to PEG, OEGMA and HPMA are biocompatible, hydrophilic, shell-building units of nanocarriers.[12,13] However, there are no published reports thus far on the synthesis of well-defined copolymers containing sheddable OEGMA and HPMA blocks by controlled living radical polymerization (CLRP). Chain extension using different monomers by consecutive CLRP processes always leads to block copolymers with nondegradable C−C links in the block junctions. Oh et al. reported the synthesis of poly(lactide)-SS-polymethacrylate amphiphilic block copolymers with SS linkages positioned at the block junction by ring-opening polymerization and ATRP,[8] but these methods are limited to cyclic monomers.

The CPADB-SS-iBuBr double-head agent developed herein offers a simple and versatile means to prepare block copolymers based on diverse hydrophilic and hydrophobic monomers with cleavable links in the block junctions for various applications. The design principle of the double-head agent integrates RAFT and ATRP techniques, taking advantage of their different mechanisms for orthogonal synthetic strategy. The resulting double-head agent was first used as a RAFT CTA to polymerize both OEGMA ($M_n \approx 300$) and HPMA. The RAFT kinetics of OEGMA (Table S1) and HPMA (Table S2) polymerization

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using CPADB-SS-iBuBr was monitored by ^{1}H NMR and SEC-MALLS. Both monomers were polymerized with first-order kinetics (Figure S2a–c) and low PDI (<1.3), demonstrating excellent synthesis control via RAFT polymerization.

The hydrophilic OEGMA and HPMA blocks were then used as macroinitiators for ATRP of GMA. To minimize the possibility of concurrent RAFT-ATRP,[14] GMA, which is polymerized with fast kinetics by ATRP but with slower kinetics by RAFT, was polymerized with short reaction time. We demonstrate by NMR and gel permeation chromatography that polymerization of GMA occurs predominantly by ATRP (see Supporting Information (SI)). Block copolymers of P(OEGMA)-SS-P(GMA) were characterized by ^{1}H NMR to verify successful polymerization and to determine the degree of polymerization (DP) (Figure S1b). P(GMA) contains pendant reactive epoxy groups that were further functionalized by TEPA to generate the polycation block. The final diblock copolymer was characterized by ^{1}H NMR (Figure S1c) and SEC-MALLS (Figure S2d). In earlier work we optimized the polycation block, screening various oligoamine and lengths of polymer backbone for optimal cell transfection efficiency (data not shown). From this initial screen, we identified P(GMA) of DP 50 grafted with TEPA, P(GMA-TEPA)$_{50}$ (M_n = 22.5 kDa, PDI = 1.11, dn/dc = 0.216), to be the most effective carrier. Its transfection efficiencies were comparable to that of branched poly(ethylenimine) (bPEI, 25 kDa). We further tested diblocks of P(GMA-TEPA)$_{50}$ with P(OEGMA) and P(HPMA) of various lengths and identified an optimal material, P(OEGMA)$_{15}$-SS-P(GMA-TEPA)$_{50}$ (M_n = 31.5 kDa, PDI = 1.29, dn/dc = 0.202). As a control, the reduction-insensitive P(OEGMA)$_{15}$-*b*-P(GMA-TEPA)$_{50}$ copolymer (M_n = 30.1 kDa, PDI = 1.28, dn/dc = 0.201) was also synthesized by consecutive RAFT polymerizations using CPADB as a CTA. To incorporate cell targeting, we selected the neuron targeting peptide Tet1, which we previously have shown to facilitate targeted transfection both *in vitro* and *in vivo* when grafted to PEI.[15] An N-terminus maleimide-functionalized Tet1 was conjugated by Michael-type addition to the terminal free thiols of P(OEGMA)$_{15}$-SS-P(GMA-TEPA)$_{50}$ and P(OEGMA)$_{15}$-*b*-P(GMA-TEPA)$_{50}$ that were generated by aminolysis of the dithioester end group during TEPA functionalization. Tet1 functionalization efficiency was ~33%, likely due to competing thiolactone formation[16] (see SI).

DNA binding of polymers was investigated by agarose gel retardation assay. The results (Figure S5) indicate that all the polymers exhibit similar DNA condensation above an N/P (amino to phosphate) ratio of 4–5, and Tet1 peptide conjugation does not significantly affect DNA binding of block copolymers. The morphologies of the five polyplex formulations were visualized by TEM (Figure 2a) at an N/P of 5. All materials condensed plasmid DNA into compact particles with diameter <50 nm. Polyplexes formed using block copolymers were more compact than those formed by the P(GMA-TEPA)$_{50}$ homopolymer, and polyplexes containing Tet1 modification were more polydisperse. The uniformity of polyplex morphology and size might be improved in future work by controlling formulation, e.g. by slow mixing or microfluidics-facilitated mixing[17] as opposed to the bulk mixing used here.

Poor salt stability has been a significant obstacle for *in vivo* application of unshielded polyplexes.[18,19] The salt stability of polymer/DNA complexes was studied in both PBS (150 mM, pH 7.4) and Opti-MEM using dynamic light scattering (DLS, Figure 2b; see Figure S6 for full kinetics). The larger size measured by DLS compared to TEM might be attributed to

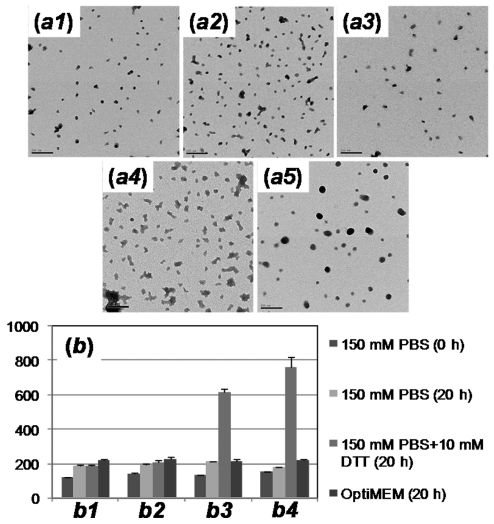

Figure 2. (a) TEM images of polyplexes formed by (a1) P(OEGMA)$_{15}$-*b*-P(GMA-TEPA)$_{50}$, (a2) Tet1-P(OEGMA)$_{15}$-*b*-P(GMA-TEPA)$_{50}$, (a3) P(OEGMA)$_{15}$-SS-P(GMA-TEPA)$_{50}$, (a4) Tet1-P(OEGMA)$_{15}$-SS-P(GMA-TEPA)$_{50}$, and (a5) P(GMA-TEPA)$_{50}$ at an N/P ratio of 5 (scale bar: 200 nm). (b) Change in the size of various polyplexes as measured by DLS at 37 °C in the presence of 150 mM PBS, 150 mM PBS + 10 mM DTT, and OptiMEM: (b1) P(OEGMA)$_{15}$-*b*-P(GMA-TEPA)$_{50}$, (b2) Tet1-P(OEGMA)$_{15}$-*b*-P(GMA-TEPA)$_{50}$, (b3) P(OEGMA)$_{15}$-SS-P(GMA-TEPA)$_{50}$, (b4) Tet1-P(OEGMA)$_{15}$-SS-P(GMA-TEPA)$_{50}$. All the polyplexes were prepared at an N/P of 5.

minority populations of larger particles that skew the average diameters measured by light scattering and also to measurement in salt medium versus water. Only a slight increase in particle size was observed for the polyplexes formed with the block copolymers over 20 h following the addition of physiological levels salt or Opti-MEM media, demonstrating excellent colloidal stability of formed polyplexes. In contrast, polyplexes of P(GMA-TEPA)$_{50}$ homopolymer formed large aggregates with diameter >1000 nm within 1 h under the same conditions (Figure S6c). The overall results confirm that the 4.5 kDa P(OEGMA) block provides sufficient extracellular colloidal stability for P(GMA-TEPA)$_{50}$ polyplexes. To investigate whether the polyplexes formed using reducible block copolymers would be deshielded in the cytosol to facilitate DNA release in the intracellular reducing environment, the particle sizes of polyplexes in the presence of 10 mM dithiothreitol (DTT) were monitored over time. Polyplexes of Tet1-P(OEGMA)$_{15}$-SS-P(GMA-TEPA)$_{50}$ and P(OEGMA)$_{15}$-SS-P(GMA-TEPA)$_{50}$ were found to increase in size due to reduction-triggered deshielding, whereas polyplexes formed from the nonreducible polymers were stable in size. Hence, polyplexes of P(OEGMA)$_{15}$-SS-P(GMA-TEPA)$_{50}$ may have excellent colloidal stability in the circulation and still be readily destabilized in the cell cytosol, facilitating DNA release.

The *in vitro* transfection efficiency of reducible and nonreducible polyplexes was evaluated in four different cell lines—HeLa, HEK293T, HepG2, and 2-day differentiated PC-12 cells by luciferase assay—using P(GMA-TEPA)$_{50}$ as a control. Figure 3a summarizes the transfection data for these cell lines. The reversibly shielded polyplexes formed by P(OEGMA)$_{15}$-SS-P(GMA-TEPA)$_{50}$ mediated significantly higher transfection efficiency in all four cell lines compared to the stably shielded analogue under identical conditions, affording up to 10 times higher transfection efficiency depending on the cell type. The results confirm that the reducible disulfide bond can improve

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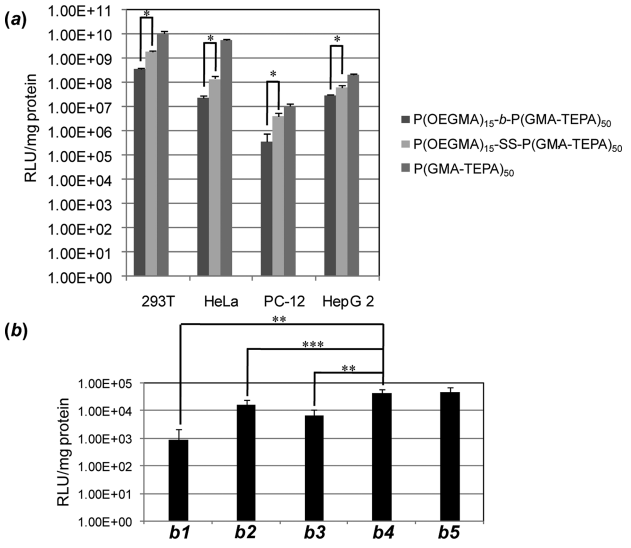

Figure 3. (a) Transfection efficiency of polyplexes based on $P(OEGMA)_{15}$-*b*-$P(GMA\text{-}TEPA)_{50}$, $P(OEGMA)_{15}$-SS-$P(GMA\text{-}TEPA)_{50}$, and $P(GMA\text{-}TEPA)_{50}$ in HEK293T, HeLa, 2-day differentiated PC-12, and HepG2 cells at an N/P ratio of 10. Data are shown as mean ± SD (n = 3; student's t test, $^{*}p < 0.05$). (b) Transfection efficiency of polyplexes formed by (b1) $P(OEGMA)_{15}$-*b*-$P(GMA\text{-}TEPA)_{50}$, (b2) Tet1-$P(OEGMA)_{15}$-*b*-$P(GMA\text{-}TEPA)_{50}$, (b3) $P(OEGMA)_{15}$-SS-$P(GMA\text{-}TEPA)_{50}$, (b4) Tet1-$P(OEGMA)_{15}$-SS-$P(GMA\text{-}TEPA)_{50}$, and (b5) $P(GMA\text{-}TEPA)_{50}$ in 6-day differentiated PC-12 cells at an N/P ratio of 5. Data are shown as mean ± SD (n = 6; student's t test, $^{**}p < 0.01$, $^{***}p < 0.02$).

transfection activity. However, the reducible polymers are less efficient than the *in vitro* optimized homopolycation $P(GMA\text{-}TEPA)_{50}$. This is expected since the hydrophilic shielding layers have been shown to inhibit polyplex uptake.[20]

To address decreased polyplex uptake, the targeted transfection efficacy of Tet1-conjugated polyplexes was further assessed in 6-day differentiated PC-12 cells. Differentiated PC-12 cells display a neuron-like phenotype that includes increased binding of the Tet1 peptide.[15b] The results (Figure 3b) clearly show that conjugation of the Tet1 targeting peptide significantly enhances transfection compared to corresponding polymer lacking Tet1. Of all the block copolymers, the Tet1-$P(OEGMA)_{15}$-SS-$P(GMA\text{-}TEPA)_{50}$ displays the highest transfection efficacy. Its transfection efficacy is 50-fold higher than that of nonreducible, nontargeted complexes, 6.1-fold higher than nontargeted, reducible complexes, and 2.6-fold higher than nonreducible targeted complexes. Most importantly, polyplexes that include both targeting ligand and releasable shielding coronas transfect target cells with efficiencies similar to the homopolycation.

In summary, we have successfully developed a versatile method to prepare functionalizable reduction-sensitive block copolymers by integrated RAFT and ATRP techniques using a novel, reducible double-head agent. Here, we prepared a neuron-targeted copolymer for nucleic acid delivery applications. We further showed that the resulting materials form particles that are salt stable but, due to the combined properties of targeting and shielding, still retain high transfection efficiencies comparable to the analogous homopolycation vectors for targeted gene delivery. The approach developed herein provides a versatile means for preparing various types of multifunctional drug and gene delivery vehicles.

■ ASSOCIATED CONTENT

Supporting Information

Experimental details and characterization data. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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