

Reversibly Shielded DNA Polyplexes Based on Bioreducible PDMAEMA-SS-PEG-SS-PDMAEMA Triblock Copolymers Mediate Markedly Enhanced Nonviral Gene Transfection

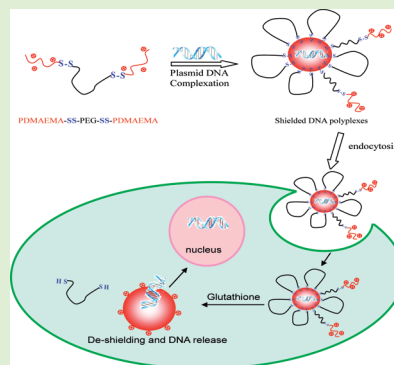
Caihong Zhu,[†] Meng Zheng,[†] Fenghua Meng,[†] Frauke Martina Mickler,[‡] Nadia Ruthardt,[‡] Xiulin Zhu,[†] and Zhiyuan Zhong^{*,†}

[†]Biomedical Polymers Laboratory, and Jiangsu Key Laboratory of Advanced Functional Polymer Design and Application, Department of Polymer Science and Engineering, College of Chemistry, Chemical Engineering and Materials Science, Soochow University, Suzhou, 215123, People's Republic of China

[‡]Department of Chemistry and Biochemistry and Center for NanoScience (CeNS), Ludwig-Maximilians-Universität, München, Butenandstr. 5-13, D-81377 München, Germany

S Supporting Information

ABSTRACT: Reversibly shielded DNA polyplexes based on bioreducible poly-(dimethylaminoethyl methacrylate)-SS-poly(ethylene glycol)-SS-poly-(dimethylaminoethyl methacrylate) (PDMAEMA-SS-PEG-SS-PDMAEMA) triblock copolymers were designed, prepared and investigated for in vitro gene transfection. Two PDMAEMA-SS-PEG-SS-PDMAEMA copolymers with controlled compositions, 6.6–6–6.6 and 13–6–13 kDa, were obtained by reversible addition–fragmentation chain transfer (RAFT) polymerization of dimethylaminoethyl methacrylate (DMAEMA) using CPADN-SS-PEG-SS-CPADN (CPADN: 4-cyanopentanoic acid dithionaphthalenoate; PEG: 6 kDa) as a macro-RAFT agent. Like their nonreducible PDMAEMA-PEG-PDMAEMA analogues, PDMAEMA-SS-PEG-SS-PDMAEMA triblock copolymers could effectively condense DNA into small particles with average diameters less than 120 nm and close to neutral zeta potentials (0 ~ +6 mV) at and above an N/P ratio of 3/1. The resulting polyplexes showed excellent colloidal stability against 150 mM NaCl, which contrasts with polyplexes of 20 kDa PDMAEMA homopolymer. In the presence of 10 mM dithiothreitol (DTT), however, polyplexes of PDMAEMA-SS-PEG-SS-PDMAEMA were rapidly deshielded and unpacked, as revealed by significant increase of positive surface charges as well as increase of particle sizes to over 1000 nm. Release of DNA in response to 10 mM DTT was further confirmed by gel retardation assays. These polyplexes, either stably or reversibly shielded, revealed a low cytotoxicity (over 80% cell viability) at and below an N/P ratio of 12/1. Notably, in vitro transfection studies showed that reversibly shielded polyplexes afforded up to 28 times higher transfection efficacy as compared to stably shielded control under otherwise the same conditions. Confocal laser scanning microscope (CLSM) studies revealed that reversibly shielded polyplexes efficiently delivered and released pDNA into the perinuclei region as well as nuclei of COS-7 cells. Hence, reduction-sensitive reversibly shielded DNA polyplexes based on PDMAEMA-SS-PEG-SS-PDMAEMA are highly promising for nonviral gene transfection.



INTRODUCTION

Gene therapy has been considered as one of the most promising future treatments of various human diseases such as cancers, cardiovascular diseases, genetic disorders, and viral infections.¹ The clinical applications of gene therapy, however, are restricted by a shortage of safe, efficient, and yet inexpensive gene delivery technology.² In the past decade, polymer-based vectors have emerged as the most versatile nonviral gene carriers.^{3–5} It should be noted, nevertheless, that positively charged polymer/DNA complexes (“polyplexes”) are frequently associated with acute systemic toxicity and low in vivo transfection activity.^{6–9} In the past years, shielded polyplexes have been developed to achieve prolonged circulation, targeted delivery, and decreased systemic toxicity.^{10–14} The shielded polyplexes, however, suffer from diminished cellular

interaction and uptake as well as inefficient intracellular release of DNA, which leads to largely reduced transfection activity.^{15,16} Linking of targeting ligands to stealthed polyplexes may partially restore their transfection activity due to receptor-mediated cellular uptake.^{17–20}

Recently, Wagner and Kataoka groups reported that deshielding of polyplexes and lipopolyplexes inside cells in response to endosomal pH^{21–25} or cytoplasmic redox potential²⁶ largely boost transfection efficiency as a result of enhanced intracellular release of DNA. We are, in particular, interested in reduction-responsive deshielding because disulfide bonds, though

Received: November 28, 2011

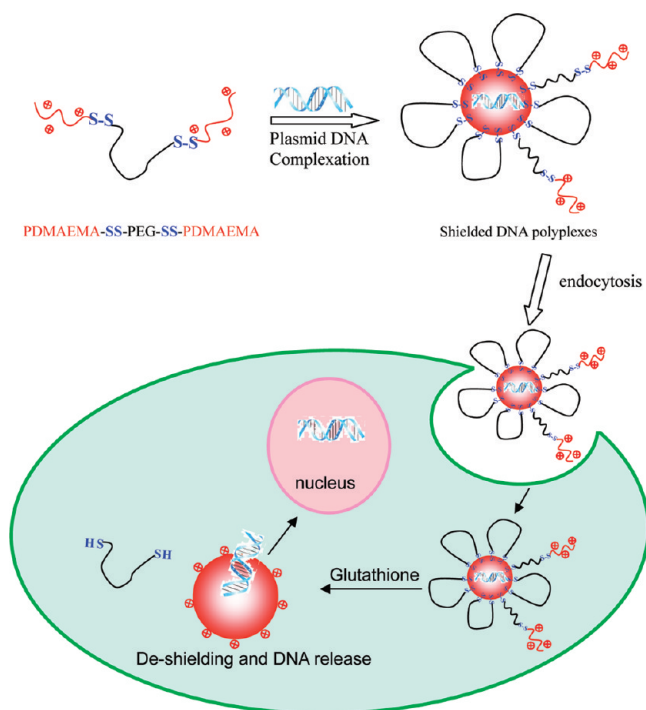
Revised: January 21, 2012

Published: January 26, 2012

sufficiently stable in the circulation and in the extracellular milieu, may be prone to rapid cleavage, at a time scale from minutes to hours, under a reductive environment present in intracellular compartments such as the cytoplasm and the cell nucleus.^{27,28} Inspired by this fascinating feature of disulfide bonds, bioresponsive micelles,²⁹ polymersomes,³⁰ nanoparticles,³¹ and polyplexes³² have been explored for intracellular drug, protein, and gene delivery. We and others have previously reported that ABA type triblock copolymers in which B is poly(ethylene glycol) (PEG) block and A is a short cationic polymer block such as linear polyethylenimine (PEI) and poly(amido amine) (PAMAM) dendrimer mediate improved gene transfection.^{33,34} The polyplexes of these triblock copolymers maintain good cellular uptake due to partial exposure of short polycations, but in the mean while exhibit excellent colloidal and serum stability and low toxicity.

In this paper, we report reversibly shielded DNA polyplexes based on structurally well-defined linear PDMAEMA-SS-PEG-SS-PDMAEMA triblock copolymers for enhanced intracellular gene delivery (Scheme 1). Unlike other cationic polymers,

Scheme 1. Illustration of Reversibly Shielded DNA Polyplexes Based on Bioreducible PDMAEMA-SS-PEG-SS-PDMAEMA Triblock Copolymer for Nonviral Transfection^a



^a(i) PDMAEMA-SS-PEG-SS-PDMAEMA effectively condenses DNA into partially shielded nano-sized polyplexes that show excellent colloidal stability; (ii) These polyplexes can be uptaken by cells due to presence of short PDMAEMA at the outer surface; (iii) The cleavage of disulfide bonds inside the cell results in rapid deshielding and DNA release into the cytoplasm and cell nucleus.

including PEI and PAMAM, PDMAEMA copolymers can be conveniently prepared with controlled macromolecular structures and compositions by living radical polymerization.^{35–37} High molecular weight PDMAEMA ($M_w > 300$ kDa) has received a lot of attention for gene delivery due to its good transfection activity in various types of cells.^{38–40} However, PDMAEMA is not biodegradable, which may render long-term

and acute toxicity when high molecular weight polymer is used. The decrease of PDMAEMA molecular weight though leading to lower toxicity results in reduced transfection activity.^{41,42} In the past years, bioreducible PDMAEMA polymers^{43–45} and biodegradable PDMAEMA copolymers^{46–50} have been developed to achieve reduced toxicity and enhanced transfection activity. As other cationic formulations, polyplexes of PDMAEMA also expose insufficient colloidal and serum stability, which restricts their applications in vivo. The PEGylation of PDMAEMA polyplexes though provide superior colloidal stability, prolonged circulation time, and tumor targeting in mice, is compromised by significantly reduced transfection activity.¹⁰ In this study, novel bioreducible PDMAEMA-SS-PEG-SS-PDMAEMA triblock copolymers were designed to combine reduction-triggered deshielding inside cells and features offered by ABA-type triblock copolymers. Their DNA complexation, unpacking and release of DNA in response to a reductive condition, as well as in vitro transfection activity, were studied and compared with the non-reducible PDMAEMA-PEG-PDMAEMA triblock copolymers.

EXPERIMENTAL SECTION

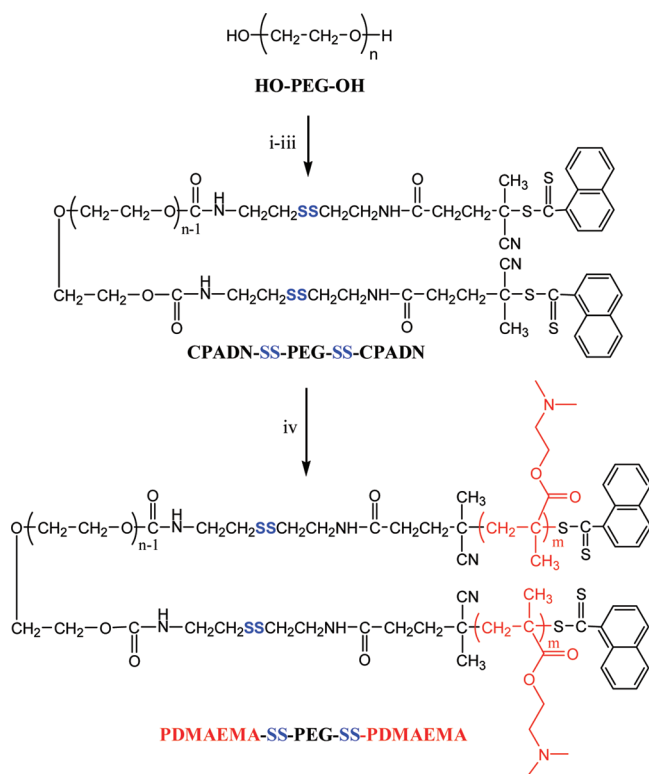
Materials. 2-*N,N*-Dimethylaminoethyl methacrylate (DMAEMA, 97%, Alfa Aesar) was purified by passing through a basic alumina column before use. Poly(ethylene glycol) (HO-PEG-OH, 6 kDa, Alfa Aesar) was dried by azeotropic distillation from toluene. 4-cyanopentanoic acid dithionaphthalenoate (CPADN) was synthesized according to the described procedure for 4-cyanopentanoic acid dithiobenzoate.⁵¹ Dichloromethane (DCM) and dimethyl sulfoxide (DMSO) were dried by refluxing over CaH_2 and distilled before use. *p*-Nitrophenyl chloroformate (*p*-NPC, 97%, Alfa Aesar), cystamine dihydrochloride (cystamine-2HCl, >98%, Alfa Aesar), pyridine (Py, 99.5%), triethylamine (Et_3N , 99%, Alfa Aesar), *N*-hydroxysuccinimide (NHS, 98%, Alfa Aesar), dicyclohexyl carbodiimide (DCC, 99%, Alfa Aesar), and dithiothreitol (DTT, 99%, Merck) were used as received.

Synthesis of Bioreducible PDMAEMA-SS-PEG-SS-PDMAEMA Triblock Copolymers. PDMAEMA-SS-PEG-SS-PDMAEMA triblock copolymers were prepared in four steps (Scheme 2).

(i). **Synthesis of *p*-NPC Activated Poly(ethylene glycol) (NPC-PEG-NPC).** Under a nitrogen atmosphere and vigorously stirring, to a solution of HO-PEG-OH (6.0 g, 1 mmol) and pyridine (0.8 g, 10 mmol) in 50 mL of anhydrous DCM at 0 °C was added dropwise a solution of *p*-NPC (1.6 g, 8 mmol) in 20 mL of DCM. The reaction mixture was then warmed to 30 °C and reacted for 20 h. The activated PEG was isolated by precipitation in cold diethyl ether, filtering, and drying in vacuo. Yield: 90.3%. ^1H NMR (400 MHz, CDCl_3): δ 3.64 (s, PEG $\text{OCH}_2\text{CH}_2\text{O}$), 4.44 (t, PEG- $\text{CH}_2\text{OC}(\text{O})$ -), 7.40/8.28 (d, phenyl).

(ii). **Synthesis of Cys-PEG-Cys.** To a solution of cystamine-2HCl (4.0 g, 18 mmol) and Et_3N (5 mL, 36 mmol) in 10 mL of anhydrous DMSO at room temperature was added dropwise a solution of NPC-PEG-NPC (5.4 g, 1.8 mmol activated hydroxyl group) in 30 mL DMSO. The reaction mixture was stirred for 27 h. The resulting product, Cys-PEG-Cys, was isolated by twice precipitation in diethyl ether, filtration, and drying in vacuo. Yield: 43.7%. ^1H NMR (400 MHz, CDCl_3): δ 3.66 (s, PEG $\text{-OCH}_2\text{CH}_2\text{O-}$), 4.23 (m, PEG- $\text{CH}_2\text{O-CO-NH-}$), 3.46 (m, $\text{-OCONHCH}_2\text{CH}_2\text{SS-}$), 3.16 (t, $\text{-SSCH}_2\text{CH}_2\text{NH}_2$), 3.03 (t, $\text{-OCONHCH}_2\text{CH}_2\text{SS-}$), 3.32 (t, $\text{-CH}_2\text{NH}_2$).

(iii). **Synthesis of CPADN-SS-PEG-SS-CPADN.** To a solution of CPADN (1.5 g, 4.6 mmol) and NHS (1.1 g, 9.4 mmol) in 25 mL of anhydrous DCM was added DCC (2.9 g, 14.1 mmol). The reaction was allowed to proceed in the dark at room temperature for 16 h. The reaction mixture was filtered to remove insoluble byproduct. The filtrate was concentrated and purified through a silica gel column with ethyl acetate/hexane (1/4, v/v) to yield NHS-CPADN. Cys-PEG-Cys

Scheme 2. Synthetic Pathway to Bio reducible PDMAEMA-SS-PEG-SS-PDMAEMA Triblock Copolymers^a

^aReagents and conditions: (i) p-NPC, toluene, 30 °C, 20 h; (ii) cystamine, DMSO, rt, 27 h; (iii) CPADN, NHS/DCC, DCM, rt, 48 h; (iv) RAFT polymerization, THF, 60 °C, 24 h.

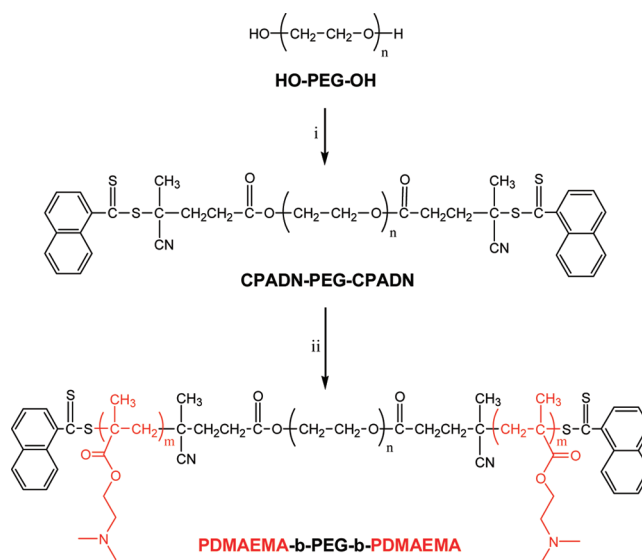
(1.5 g, 0.5 mmol amine groups) and NHS-CPADN (0.4 g, 1 mmol) were reacted in 30 mL of DCM in the dark at room temperature for 48 h. The resulting macro-RAFT agent, CPADN-SS-PEG-SS-CPADN, was isolated by twice precipitation in diethyl ether, filtration, and drying in vacuo. Yield: 85.6%. ¹H NMR (400 MHz, CDCl₃): δ 3.61 (s, PEG -OCH₂CH₂O-), 4.21 (m, PEG-CH₂OCONH-), 3.47 (m, -OCONHCH₂CH₂SSCH₂CH₂NHCO-), 2.81 (m, -CH₂SSCH₂-), 2.45–2.63 (m, -NHCOCH₂CH₂-), 2.00 (s, -CH₃), 7.52/7.90/8.15 (m, naphthalene).

(iv). **RAFT Polymerization.** Under a nitrogen atmosphere, AIBN (0.7 mg, 4.3 μmol), CPADN-SS-PEG-SS-CPADN (0.15 g, 22 μmol), PDMAEMA (0.31 g, 1.98 mmol) and 1.5 mL of THF were charged into a 10 mL Schlenk flask. The polymerization was performed at 60 °C for 24 h. The resulting copolymer was precipitated in cold hexane, filtered, and dried in vacuo. Yield: 79.9%. ¹H NMR (400 MHz, CDCl₃): δ 3.64 (PEG block); 1.04/0.88, 1.90, 2.28, 2.56, and 4.06 (PDMAEMA block).

Synthesis of Nonreducible PDMAEMA-*b*-PEG-*b*-PDMAEMA Triblock Copolymers. PDMAEMA-PEG-PDMAEMA triblock copolymers were prepared in two steps (Scheme 3).

(i). **Synthesis of CPADN-PEG-CPADN.** To a solution of HO-PEG-OH (3.0 g, 0.5 mmol), CPADN (0.66 g, 2 mmol), and a catalytic amount of DMAP (12 mg, 0.1 mmol) in 60 mL of DCM was added a solution of DCC (0.43 g, 2 mmol) in 20 mL of DCM. The reaction was allowed to proceed in the dark at room temperature for 48 h. The reaction mixture was filtered to remove insoluble byproduct. The filtrate was concentrated and precipitated in diethyl ether to yield CPADN-PEG-CPADN. Yield: 76.3%. ¹H NMR (400 MHz, CDCl₃): δ 3.64 (s, PEG -OCH₂CH₂O-), 4.27 (m, PEG-CH₂OCO-), 2.47–2.73 (m, -OCOCH₂CH₂-), 1.98 (s, -CH₃), 7.52/7.92/8.15 (m, naphthalene).

(ii). **RAFT Polymerization.** Under a nitrogen atmosphere, AIBN (0.7 mg, 4.3 μmol), CPADN-PEG-CPADN (0.15 g, 23 μmol), PDMAEMA

Scheme 3. Synthetic Pathway to Nonreducible PDMAEMA-*b*-PEG-*b*-PDMAEMA Triblock Copolymers^a

^aReagents and conditions: (i) CPADN, DMAP/DCC, DCM, rt, 48 h; (ii) RAFT polymerization, THF, 60 °C, 24 h.

(0.32 g, 2.07 mmol), and 1.5 mL of THF were charged into a 10 mL Schlenk flask. The polymerization was performed at 60 °C for 24 h. The resulting copolymer was precipitated in cold hexane, filtered, and dried in vacuo. Yield: 84.5%. ¹H NMR (400 MHz, CDCl₃, δ): 3.64 (PEG block); 1.04/0.88, 1.82, 2.28, 2.56, and 4.05 (PDMAEMA block).

Characterization. ¹H NMR spectra were recorded on an INOVA 400 MHz nuclear magnetic resonance instrument using deuterated chloroform (CDCl₃) as a solvent. The chemical shifts were calibrated against solvent signal of CDCl₃. The molecular weight and polydispersity (PDI) of the copolymers were determined with a Waters 1515 gel permeation chromatography (GPC) instrument equipped with HR1, HR3, and HR4 columns and a differential refractive index detector. The measurements were performed using THF as an eluent at a flow rate of 1.0 mL/min at 30 °C and a series of PMMA as standards.

Preparation and Characterization of DNA Polyplexes. The polyplexes were prepared by adding a HEPES buffer solution (600 μL, 20 mM, pH 7.4) of triblock copolymer with the desired concentration to a HEPES buffer solution (150 μL, 20 mM, pH 7.4) of plasmid DNA (37.5 μg/mL), which resulted in polyplexes with N/P ratios ranging from 3/1 to 18/1. The dispersions were vortexed for 5 s and incubated at room temperature for 30 min before ξ-potential and size measurements were carried out.

The ξ-potentials and hydrodynamic diameters of polyplexes in HEPES buffer (20 mM, pH 7.4) were determined at 25 °C using a Zetasizer Nano ZS instrument (Malvern) equipped with a standard capillary electrophoresis cell and dynamic light scattering (DLS, 10 mW He-Ne laser, 633 nm wavelength), respectively. The measurements were performed in triplicate.

Gel Retardation Assay. The DNA binding ability of reducible triblock copolymers was studied by agarose gel electrophoresis. The polymer/DNA complexes prepared at varying N/P ratios from 1/1 to 5/1 were electrophoresed through a 1% agarose gel containing ethidium bromide at 100 V in TAE buffer solution (40 mM Tris-HCl, 1 v/v % acetic acid, and 1 mM EDTA).

Colloidal Stability and Reduction-Triggered Deshielding and Unpacking of DNA Polyplexes. The colloidal stability of PDMAEMA-SS-PEG-SS-PDMAEMA polyplexes was studied using DLS at pH 7.4. The polyplexes were prepared as described above. Following addition of 150 mM NaCl, particle sizes of polyplexes were

monitored in time. PDMAEMA-*b*-PEG-*b*-PDMAEMA(6.4K) was used as a control.

Reduction-triggered deshielding and unpacking of DNA polyplexes was investigated by DLS and agarose gel retardation assays. Briefly, under a nitrogen flow, a predetermined amount of DTT was introduced into a cuvette containing 1 mL of polymer/DNA complexes solution (pH 7.4, 150 mM NaCl), to yield a final DTT concentration of 10 mM. The cuvette was sealed with a septum. ξ -potentials and particle sizes of polyplexes were monitored in time. For agarose gel retardation assays, 5 μ L of DTT solution in HBS (HEPES buffered saline, 20 mM, pH 7.4) was added to 20 μ L of polyplexes suspension in HBS to reach a final DTT concentration of 10 mM, and incubated for 1 h. Then, 5 μ L of dextran sodium sulfate (M_w = 500000) solution in HBS was added to give varying sodium sulfate/DNA phosphate ratios of 4, 8, and 12, respectively. Following incubation for 30 min, the polyplexes were electrophoresed through a 1% agarose gel containing ethidium bromide at 100 V in TAE buffer solution (40 mM Tris-HCl, 1 v/v % acetic acid, and 1 mM EDTA). PDMAEMA-*b*-PEG-*b*-PDMAEMA (6.4K) was used as a control.

In Vitro Transfection and Cell Viability Assays. Transfection experiments were performed in COS-7 cells using the plasmid pCMV-Luc as a reporter gene. Transfections were conducted using polyplexes at N/P ratios of 6/1 and 12/1. The cells were plated in a 12-well plate (cell density 1.5×10^5 cells/well) and maintained in DMEM supplemented with 10% FBS at 37 °C in a humidified atmosphere containing 5% CO₂ until 70% confluency. In a standard transfection experiment, the cells were rinsed with PBS and incubated with 100 μ L of polyplex dispersion (i.e., 1 μ g of plasmid DNA per well) and 400 μ L of culture medium containing 10% serum for 5 h at 37 °C. Next, the polyplexes were removed, 1 mL of fresh culture medium was added, and the cells were cultured for 43 h. Luciferase quantification was done using a commercial luciferase assay kit (Promega, Madison, WI) and a Lumat LB 9501 illuminometer (Berthold, Milbach, Germany). Transfection efficiency was expressed as relative light unit (RLU) per mg of protein. A 25 kDa bPEI/DNA formulation prepared at an optimal N/P ratio of 10/1 was used as a reference.

The cytotoxicity of polyplexes was evaluated with the same cell culture procedure as the transfection, followed by CCK assay. The cells were incubated with CCK-8 reagent solution for 3.5 h prior to measurement of absorption at 450 nm using a microplate reader (BIORAD, Model 550). The CCK value for the untreated cells (i.e., cells not exposed to transfection systems) was taken as 100% cell viability.

Confocal Microscopy. The cellular uptake and intracellular DNA release behaviors of PDMAEMA-SS-PEG-SS-PDMAEMA (6.6K) polyplexes at an N/P ratio of 12/1 were studied in COS-7 cells with CLSM using Cy5-labeled DNA. PDMAEMA-PEG-PDMAEMA (6.4K) polyplexes at an N/P ratio of 12/1 were used as a control. In brief, COS-7 cells were plated on coverslips in 24-well plates (cell density 6×10^4 cells/well) and maintained in DMEM supplemented with 10% FBS at 37 °C in a humidified atmosphere containing 5% CO₂ for 1 d. A 100 μ L aliquot of polyplex dispersions (1 μ g of Cy-5 labeled plasmid DNA per well) was added. The cells were cultured for 6 or 24 h at 37 °C. The polyplexes were removed and the cells were washed with PBS three times and fixed with 4% paraformaldehyde for 15 min. The nuclei were stained with 200 μ L of Hoechst 33342 (20 μ g/mL) for 15 min at rt. The cells following rinsing three times with PBS were observed with a confocal laser scanning microscope (TCS SPS Leica).

RESULTS AND DISCUSSION

Synthesis of Bioreducible PDMAEMA-SS-PEG-SS-PDMAEMA and Nonreducible PDMAEMA-PEG-PDMAEMA Triblock Copolymers. To synthesize bioreducible cationic PDMAEMA-SS-PEG-SS-PDMAEMA triblock copolymers, we adopted reversible addition-fragmentation chain-transfer (RAFT) polymerization of DMAEMA using a disulfide-linked PEG macro-RAFT agent, CPADN-SS-PEG-SS-CPADN (CPADN: 4-cyanopentanoic acid dithionaphthalenoate; Scheme 2).

RAFT polymerization, due to its “living” nature and tolerance to different functional groups, has recently generated a lot of interests in controlled synthesis of novel functional polymers and copolymers for biomedical applications.^{52,53} CPADN is a versatile RAFT agent through which we have obtained well-defined PDMAEMA-PCL-PDMAEMA and PEG-PCL-PDEAEMA triblock copolymers.^{47,54} CPADN-SS-PEG-SS-CPADN was prepared by the following three steps (Scheme 2). First, PEG (6.0 kDa) was treated with *p*-nitrophenyl chloroformate (p-NPC), which resulted in quantitative transformation of PEG hydroxyl terminal groups into *p*-nitrophenyl carbonate, as indicated by ¹H NMR. Second, p-NPC activated PEG was reacted with excess cystamine dihydrochloride in the presence of Et₃N. ¹H NMR spectrum (Figure 1A) showed besides signals of PEG main chain, resonances at δ 4.23 assignable to the methylene protons of PEG neighboring to the urethane bond and signals at δ 3.03–3.46 attributable to the protons of cystamine moieties, with an integral ratio close to the theoretical value of 1:4, indicating successful synthesis of Cys-PEG-Cys. Finally, CPADN was coupled to Cys-PEG-Cys by carbodiimide chemistry. As shown in Figure 1B, signals at δ 3.32 attributable to the methylene protons next to the primary amine group of cystamine moieties disappeared completely, and a new set of signals at δ 7.52–8.15 and 2.45–2.63 assignable to protons of CPADN were detected.

The polymerization of DMAEMA was carried out in the presence of AIBN in THF at 60 °C under nitrogen atmosphere for 24 h. The results of polymerization are shown in Table 1. ¹H NMR displayed clearly peaks characteristic of both PDMAEMA and PEG blocks (Figure 1C). The M_n values of PDMAEMA estimated by comparing the intensities of signals at δ 4.05 (methylene protons next to the ester bond of PDMAEMA) and δ 3.65 (methylene protons of PEG) were 6.6 k and 13.0 k for copolymers obtained at monomer-to-CPADN ratios of 45 and 90, respectively (Table 1, entries 1 and 2). The compositions of both triblock copolymers were close to the design. Gel permeation chromatography (GPC) showed a unimodal distribution with moderate polydispersities of 1.63–1.85 (Table 1, entries 1 and 2). These results corroborated successful synthesis of PDMAEMA-SS-PEG-SS-PDMAEMA triblock copolymers.

As nonreducible controls, PDMAEMA-PEG-PDMAEMA triblock copolymers were also prepared using CPADN-PEG-CPADN as a macro-RAFT agent (Scheme 3). CPADN-PEG-CPADN was readily synthesized by coupling CPADN to PEG in the presence of DCC and catalytic amount of DMAP. The structures of CPADN-PEG-CPADN and resulting PDMAEMA-PEG-PDMAEMA copolymers were confirmed by ¹H NMR (Figure S1). Under similar polymerization conditions, PDMAEMA-PEG-PDMAEMA triblock copolymers with comparable compositions to PDMAEMA-SS-PEG-SS-PDMAEMA were obtained. Furthermore, for comparison we have also synthesized PDMAEMA homopolymers with M_n of 6.3 and 12.0 k.

Biophysical Characterization of Polymer/DNA Complexes. The DNA complexation behaviors of PDMAEMA-SS-PEG-SS-PDMAEMA and PDMAEMA-PEG-PDMAEMA triblock copolymers were investigated by dynamic light scattering (DLS) and zeta-potential measurements. It is remarkable to note that all triblock copolymers, either with or without disulfide linkages, effectively condensed DNA into nanosized particles (average diameters ≤ 120 nm) at and above an N/P ratio of 3/1 (Figure 2A). The polydispersities (PDI) ranged from 0.12 to 0.32 (Figure S2). In contrast, 6.3 kDa

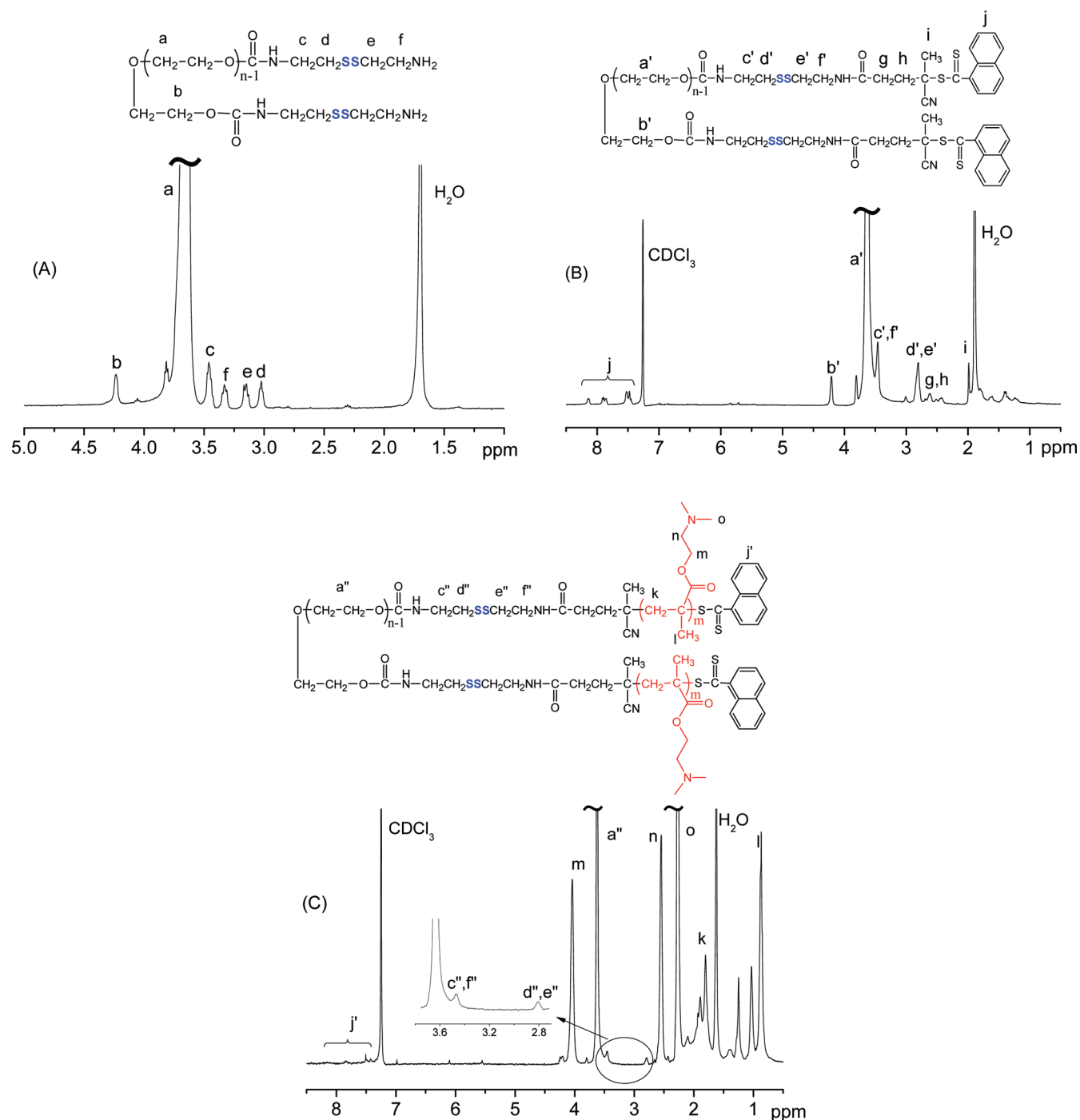


Figure 1. ^1H NMR spectra (400 MHz, CDCl_3) of Cys-PEG-Cys (A), macro-RAFT agent CPADN-SS-PEG-SS-CPADN (B), and PDMAEMA-SS-PEG-SS-PDMAEMA (6.6K) triblock copolymer (C).

Table 1. Synthesis of Bioreducible and Nonreducible PDMAEMA-PEG-PDMAEMA Triblock Copolymers

entry	polymer	M/CPADN ^a feed ratio	M_n /kDa (Design)	M_n^b /kDa (^1H NMR)	M_n^c /kDa (GPC)	PDI ^c	yield %
1	PDMAEMA-SS-PEG- SS-PDMAEMA(6.6K)	45/1	7–6–7	6.6–6–6.6	20.4	1.63	79.9
2	PDMAEMA-SS-PEG- SS-PDMAEMA(13K)	90/1	14–6–14	13–6–13	27.4	1.85	80.8
3	PDMAEMA-b-PEG-b- PDMAEMA(6.4K)	45/1	7–6–7	6.4–6.0–6.4	21.0	1.14	84.5
4	PDMAEMA-b-PEG-b- PDMAEMA(13K)	90/1	14–6–14	13.0–6.0–13.0	27.0	1.35	82.3

^aMolar feed ratio of DMAEMA monomer to CPADN of CPADN-PEG-CPADN or CPADN-SS-PEG-SS-CPADN macro-RAFT agents.

^bDetermined from ^1H NMR by comparing the integrals of signals at δ 4.06 and 3.64; ^cDetermined by GPC measurements (eluent: DMF containing 0.05 M LiBr; standards: PMMA; flow rate: 0.8 mL/min, 30 °C).

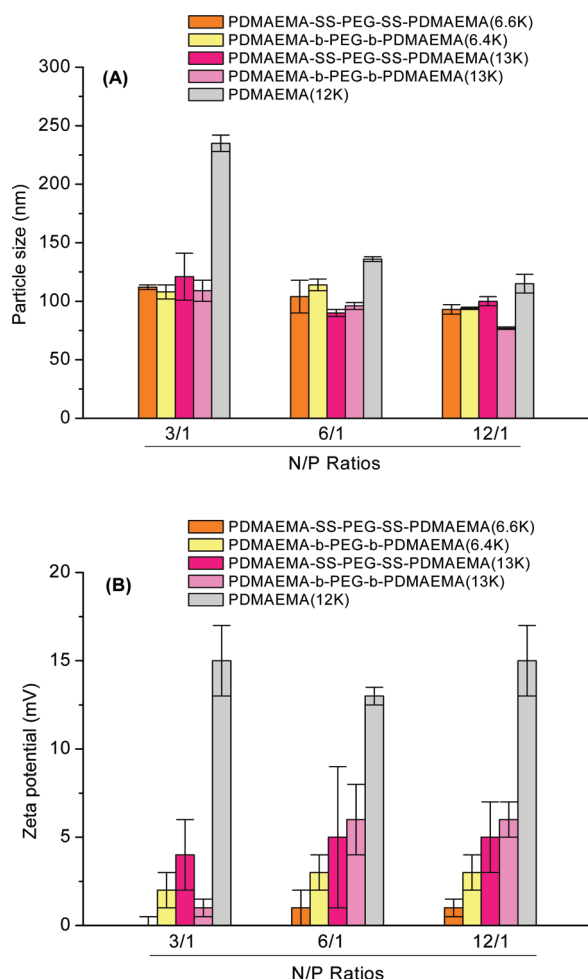


Figure 2. Average particle size (A) and zeta potential (B) of DNA polyplexes prepared at N/P ratios of 3/1, 6/1, and 12/1. Data are shown as mean \pm SD ($n = 3$).

PDMAEMA homopolymer was not able to effectively condense DNA, yielding large particles with an average size of about 740 nm at an N/P ratio of 12/1. Higher molecular weight PDMAEMA homopolymer (12 kDa) could condense DNA though resulted in somewhat larger particles (Figure 2A). The zeta potential measurements revealed that polyplexes based on these triblock copolymers had surface charges close to neutral ($0 \sim +6.0$ mV), which were significantly lower than those observed for PDMAEMA homopolymer (ca. +15 mV; Figure 2B). This reduced zeta potentials indicated effective shielding of the polyplexes by hydrophilic PEG chains. Notably, polyplexes of PDMAEMA-SS-PEG-SS-PDMAEMA (6.6K) showed better shielding of charge as compared to those of PDMAEMA-SS-PEG-SS-PDMAEMA (13K). The slightly positive surface charge was most likely due to that some triblock copolymers are only partly involved (i.e., via only one PDMAEMA block) in the DNA complexation, forming PEG shielded polyplexes with several short PDMAEMA chains extruding to the outer surface (Scheme 1). This modest positive surface charge is desirable for promoting cellular interactions and uptake of polyplexes. Gel retardation assays revealed that all triblock copolymers were capable of effectively complexing DNA at and above an N/P ratio of 2/1 (Figure 3). The reducible polyplexes showed slightly better DNA binding ability than the nonreducible counterparts likely due to presence of

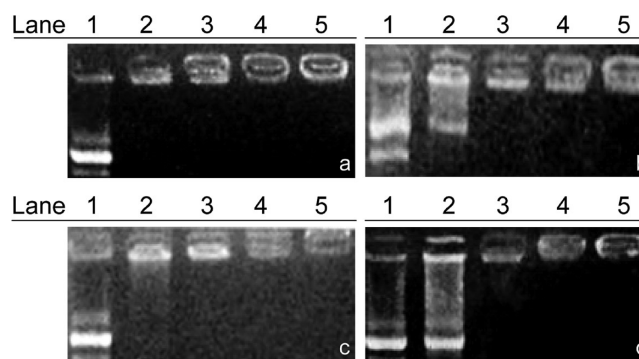


Figure 3. Agarose gel electrophoresis of polymer/DNA complexes prepared at different N/P ratios: (a) PDMAEMA-SS-PEG-SS-PDMAEMA (6.6K); (b) PDMAEMA-*b*-PEG-*b*-PDMAEMA (6.4K); (c) PDMAEMA-SS-PEG-SS-PDMAEMA (13K); (d) PDMAEMA-*b*-PEG-*b*-PDMAEMA (13K). Lane 1 is free DNA; lanes 2–5 correspond to N/P ratios of 1, 2, 3, and 5, respectively.

hydrophobic cystamine moieties in PDMAEMA-SS-PEG-SS-PDMAEMA copolymers. Hydrophobic modification has been reported to effectively enhance the DNA condensation ability of many cationic polymers.⁵⁵

Colloidal Stability and Reduction-Triggered Deshielding and Unpacking of DNA Complexes. Low colloidal and serum stability has been an obstacle for many cationic polymeric formulations including PEI to be applied in vivo.^{3,7} In the past decade, different strategies in particular PEG modifications have been explored to enhance the colloidal stability of polyplexes and to prolong their circulation time.¹³ The colloidal stability of PDMAEMA-SS-PEG-SS-PDMAEMA polyplexes prepared at an N/P ratio of 3/1 was studied in HEPES buffer (20 mM, pH 7.4) using DLS. Remarkably, no change in particle size was observed for polyplexes of PDMAEMA-SS-PEG-SS-PDMAEMA (6.6K) within 3 h following addition of 150 mM NaCl (Figure 4A). The polyplexes of PDMAEMA-SS-PEG-SS-PDMAEMA (13K) grew gradually from 110 nm to about 400 nm over a period of 10 h (Figure 4B). The polyplexes of PDMAEMA-SS-PEG-SS-PDMAEMA (6.6K) have enhanced colloidal stability compared to those of PDMAEMA-SS-PEG-SS-PDMAEMA (13K), likely due to their better shielding of charge. In contrast, polyplexes of 20 kDa PDMAEMA homopolymer formed large aggregates of over 1000 nm in less than 1 h under otherwise the same conditions (Figure 4), which is similar to the phenomenon reported for polyplexes of 22 kDa linear PEI.⁵⁶ Herewith, 20 kDa PDMAEMA homopolymer was used as a control in that lower molecular weight PDMAEMA homopolymers, i.e. 6.3 kDa and 12 kDa PDMAEMA, are not able to effectively condense DNA at an N/P ratio of 3/1 even in the absence of salt. It should be noted that polyplexes of PDMAEMA-*b*-PEG-*b*-PDMAEMA (6.4K) grew gradually to about 700 nm over a period of 1.5 h following addition of 150 mM NaCl (Figure S3). The higher colloidal stability of PDMAEMA-SS-PEG-SS-PDMAEMA (6.6K) polyplexes as compared to their nonreducible counterparts agrees well with their better DNA condensation ability as shown above.

However, overly stable polyplexes are not desirable either because for efficient transfection DNA has to be released into the nucleus.¹⁵ To investigate whether polyplexes based on PDMAEMA-SS-PEG-SS-PDMAEMA triblock copolymers would be deshielded and unpacked under an intracellular-mimicking reductive environment, particle sizes, and zeta

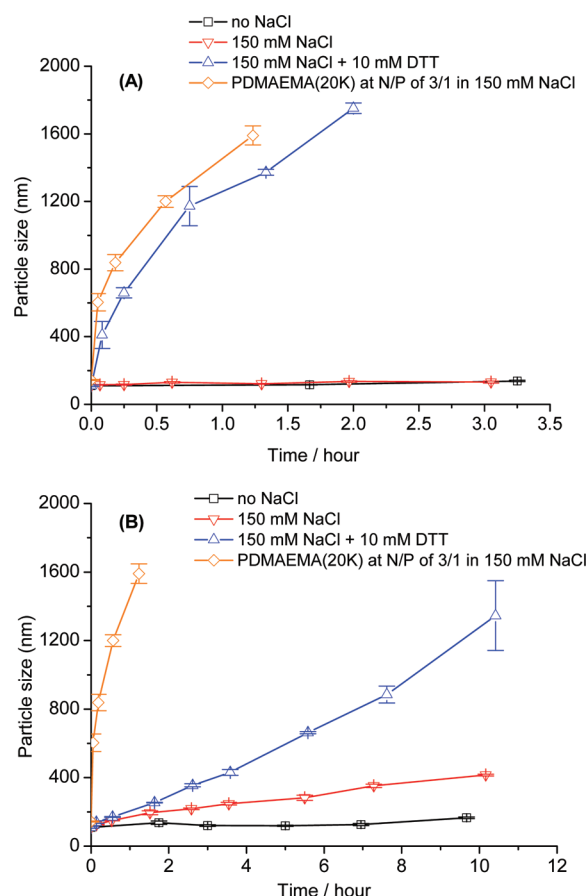


Figure 4. Influence of 150 mM NaCl and/or 10 mM DTT on sizes of polyplexes prepared from bio-reducible PDMAEMA-PEG-PDMAEMA triblock copolymers at an N/P ratio of 3/1: (A) PDMAEMA-SS-PEG-SS-PDMAEMA (6.6K); (B) PDMAEMA-SS-PEG-SS-PDMAEMA (13K). Data are shown as mean \pm SD ($n = 3$).

potentials of polyplexes in response to 10 mM DTT were monitored over time. It should be noted that sizes of PDMAEMA-SS-PEG-SS-PDMAEMA (6.6K) polyplexes rapidly increased to over 1000 nm in less than 1 h following addition of 10 mM DTT (Figure 4A). This is most likely due to cleavage of disulfide bond resulting in PDMAEMA homopolymer that is not able to condense DNA due to its low molecular weights. The deshielding effect was further corroborated by increase of polyplex zeta potentials from ca. +0.7 mV to +11 mV in 15 min after addition of DTT (Figure 5A). In comparison, particle sizes and zeta potentials of non-reducible PDMAEMA-*b*-PEG-*b*-PDMAEMA (6.4K) polyplexes were not altered by 10 mM DTT (Figures S3 and S4). Increase of particle sizes and zeta potentials in response to 10 mM DTT was also observed for those of PDMAEMA-SS-PEG-SS-PDMAEMA (13K; Figure 4B and 5B). It should be noted, however, that sizes of PDMAEMA-SS-PEG-SS-PDMAEMA (13K) polyplexes increase much more slowly compared to those of PDMAEMA-SS-PEG-SS-PDMAEMA (6.6K). This is in accordance with the observation that 13 kDa PDMAEMA homopolymer has better DNA condensation than 6.6 kDa PDMAEMA. Gel retardation assays showed that DNA polyplexes of PDMAEMA-SS-PEG-SS-PDMAEMA(6.6K) prepared at an N/P ratio of 6/1 were stable against exchange with excess of negative dextran sodium sulfates relative to DNA phosphate groups (Figure 6), further confirming that these

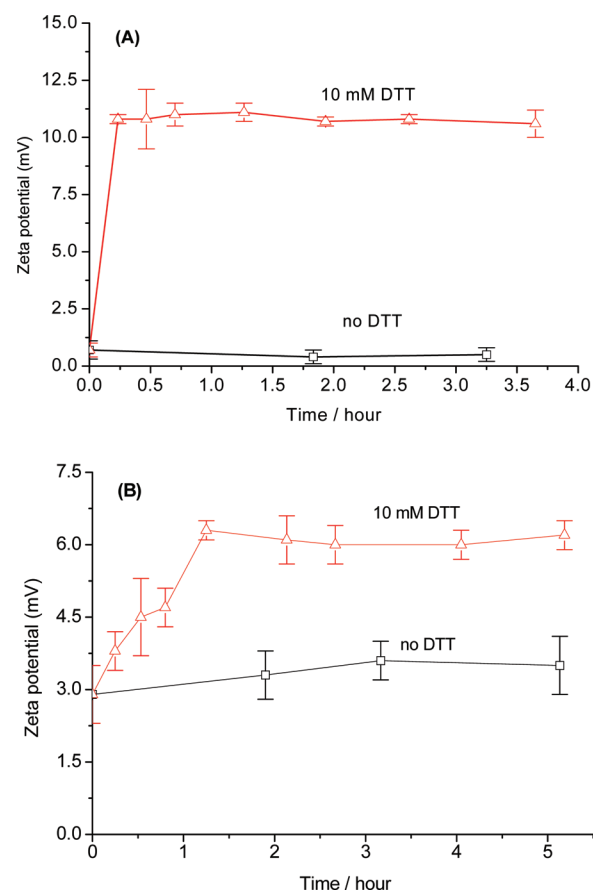


Figure 5. Influence of 10 mM DTT on zeta potentials of polyplexes prepared from bio-reducible PDMAEMA-PEG-PDMAEMA triblock copolymers at an N/P ratio of 3/1: (A) PDMAEMA-SS-PEG-SS-PDMAEMA (6.6K); (B) PDMAEMA-SS-PEG-SS-PDMAEMA (13K). Data are shown as mean \pm SD ($n = 3$).

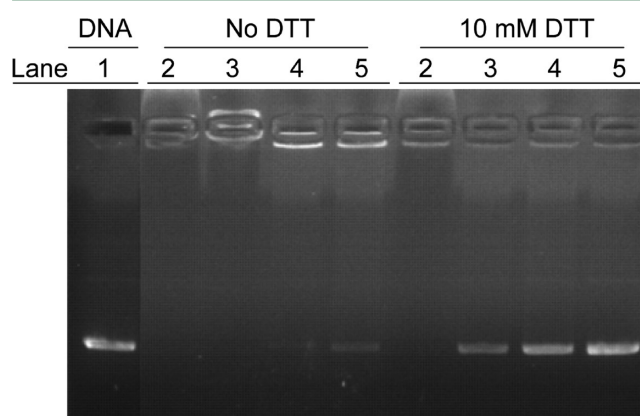


Figure 6. Agarose gel electrophoresis of PDMAEMA-SS-PEG-SS-PDMAEMA (6.6K)/DNA complexes prepared at an N/P ratio of 6/1 with or without 10 mM DTT. Lane 1 is free DNA; lane 2 is polyplexes formed at an N/P ratio of 6/1; and lanes 3–5 correspond to polyplexes formed at a N/P ratio of 6/1 with addition of dextran sodium sulfate at varying sodium sulfate/DNA phosphate ratios of 4, 8, and 12, respectively.

reversibly shielded polyplexes have excellent colloidal stability. Partial release of DNA was observed at a relatively high sodium sulfate/DNA phosphate ratio of 12/1 (Figure 6). The polyplex samples following treatment with 10 mM DTT, however, yielded DNA migration patterns identical to free DNA

(Figure 6), indicating effective unpacking and release of DNA as triggered by reduction. In contrast, DTT-triggered DNA release was not observed for PDMAEMA-*b*-PEG-*b*-PDMAEMA (6.4K) polyplexes (Figure S5). Hence, polyplexes of PDMAEMA-SS-PEG-SS-PDMAEMA triblock copolymers on one hand may have excellent colloidal stability in circulation and on the other hand may be rapidly decomplexed to release DNA inside cells to achieve superior transfection.

In Vitro Transfection and Cytotoxicity Studies. The cytotoxicity of triblock copolymer polyplexes was evaluated in COS-7 cells at varying N/P ratios from 6/1 to 18/1 using CCK cell viability assays. Interestingly, polyplexes of reducible and nonreducible triblock copolymers revealed similar cell viability (Figure 7). Moreover, molecular weight of PDMAEMA also

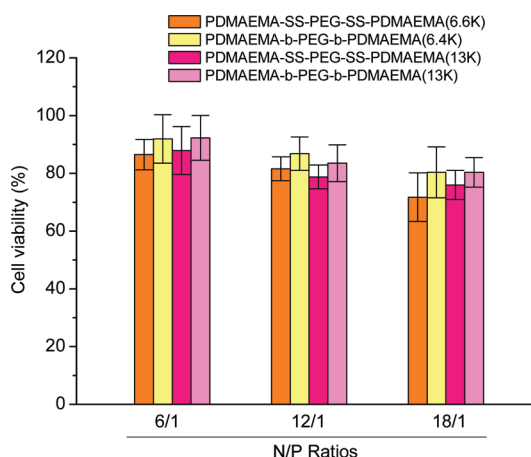


Figure 7. Cell viabilities of polymer/DNA complexes in COS-7 cells at N/P ratios of 6/1, 12/1, and 18/1. Data are shown as mean \pm SD ($n = 3$).

appeared to have little influence on cytotoxicity of polyplexes (Figure 7). In general, polyplexes of these triblock copolymers had a low toxicity (cell viability above 80%) at and below an N/P ratio of 12/1. In comparison, a cell viability of about 70% was observed for 25 kDa PEI polyplexes formed at an N/P ratio of 10/1.

The in vitro transfection activity of polyplexes based on reducible and nonreducible triblock copolymers was assessed in COS-7 cells by luciferase assay. The transfection experiments were performed in a 10% serum-containing medium at N/P ratios of 6/1 and 12/1. The results revealed that reversibly shielded polyplexes based on PDMAEMA-SS-PEG-SS-PDMAEMA mediated significantly more efficient transfection as compared to stably shielded polyplexes of PDMAEMA-PEG-PDMAEMA under otherwise the same conditions (Figure 8). For instance, polyplexes of PDMAEMA-SS-PEG-SS-PDMAEMA (6.6K) revealed approximately 28-fold higher transfection efficiencies than those of PDMAEMA-PEG-PDMAEMA (6.4K) at a N/P ratio of 6/1. In comparison, PDMAEMA-SS-PEG-SS-PDMAEMA (13K) yielded only several times higher transfection efficiency relative to the nonreducible control. The formulations of PDMAEMA-SS-PEG-SS-PDMAEMA (6.6K) are of particular interest for in vivo delivery of therapeutic DNA in that (i) they have small particle sizes (ca. 100 nm), close to neutral surface charges, and excellent colloidal stability under extracellular conditions, which contrast with many current gene delivery vectors including PEI, PAMAM dendrimer and chitosan; (ii) they show adequate transfection activity under serum conditions. The transfection

efficiency of PDMAEMA-SS-PEG-SS-PDMAEMA (6.6K) polyplexes at a N/P ratio of 12/1 was 4.3×10^6 RLU/mg protein, which was somewhat lower than 25 kDa PEI at its optimal formulation (Figure 8). It should be noted,

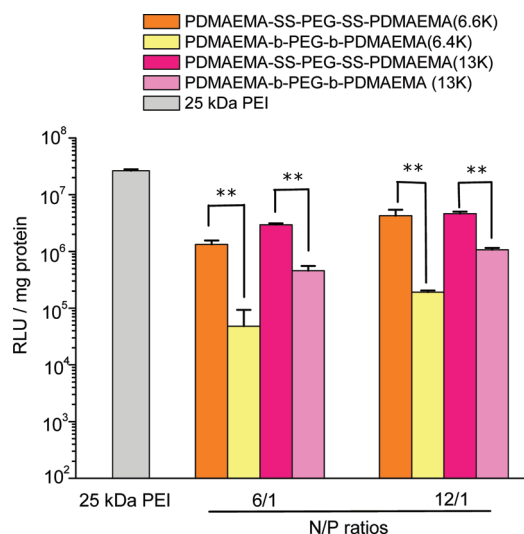


Figure 8. Transfection efficiencies of reversibly shielded and stably shielded polyplexes in COS-7 cells at N/P ratios of 6/1 and 12/1 in 10% serum condition. A 25 kDa PEI formulation at its optimal N/P ratio of 10/1 was used as a control. Data are shown as mean \pm SD ($n = 3$; student's *t* test, $**p < 0.01$).

nevertheless, that over 2 orders of magnitude reduction of transfection efficiency has been observed for 25 kDa PEI polyplexes after shielding with PEG;^{15,16} and (iii) they have low toxicity and furthermore are degradable into low molecular weight PDMAEMA and PEG that may circumvent possible acute and long-term toxicity encountered by high molecular weight polycations.

In the following, the cellular uptake and intracellular DNA release behaviors of reversibly shielded polyplexes were studied with live-cell imaging and CLSM using Cy5-labeled pDNA. Live-cell imaging experiments were performed on a single cell level with high spatial and temporal resolution.^{57,58} To evaluate the extent of polyplex internalization, HuH7 cancer cells expressing Rab9-GFP as a marker for endosomes were incubated with Cy5-labeled pDNA polyplexes. Z-stacks of single cells were recorded by spinning disk confocal microscopy in a time interval of 0–30 h following polyplex addition. Z-projections of the recorded image sequences were analyzed for colocalization of polyplexes with Rab9-GFP labeled endosomes. By comparing time-dependent colocalization degree, polyplex uptake kinetics could be determined. The results clearly showed that polyplexes of PDMAEMA-SS-PEG-SS-PDMAEMA (6.6K) and PDMAEMA-*b*-PEG-*b*-PDMAEMA (6.4K) had similar cellular uptake kinetics (Figure S6). Quantification of time-dependent colocalization degree with a custom-written analysis software revealed a linear increase of the colocalization degree in the first 25 h post polyplex application, reaching comparable levels for both reducible and nonreducible polyplexes (Figure S7). Though a broad spread of data points was observed, the mean colocalization values were similar for both polyplexes. These results indicate that disulfide bond had no influences on the cellular uptake of polyplexes. CLSM showed that considerable amount of pDNA (red) has been trans-

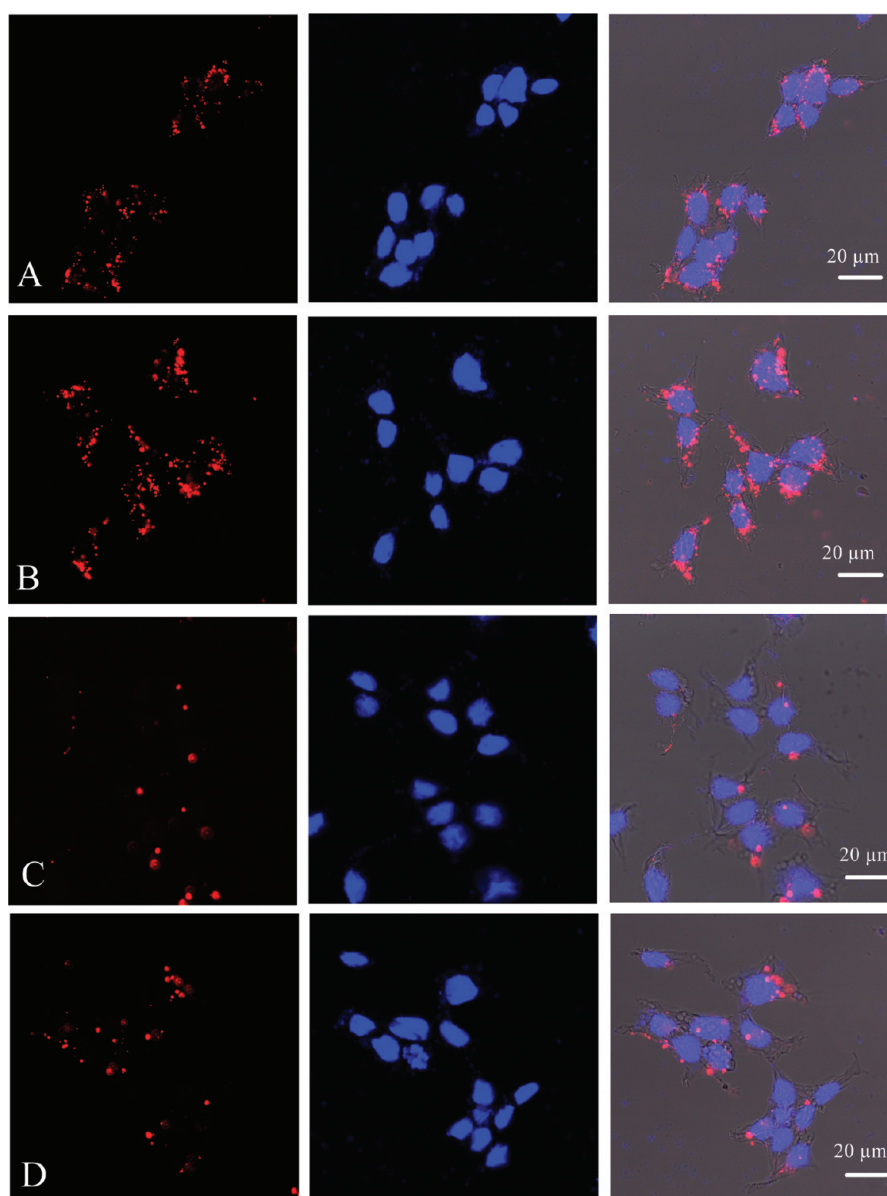


Figure 9. CLSM images of COS-7 cells transfected with Cy5-labeled pDNA polyplexes of PDMAEMA-SS-PEG-SS-PDMAEMA (6.6K) at an N/P ratio of 12/1 in the presence of 10% serum ($1 \mu\text{g}$ DNA/well). PDMAEMA-PEG-PDMAEMA (6.4K) at an N/P ratio of 12/1 was used as a control. Cells were incubated with polyplexes for 6 or 24 h. For each panel, images from left to right show Cy5-labeled pDNA, cell nuclei stained by Hoechst 33342 (blue), and overlays of both images. The bar represents $20 \mu\text{m}$. (A) PDMAEMA-SS-PEG-SS-PDMAEMA (6.6K), 6 h; (B) PDMAEMA-SS-PEG-SS-PDMAEMA (6.6K), 24 h; (C) PDMAEMA-PEG-PDMAEMA (6.4K), 6 h; (D) PDMAEMA-PEG-PDMAEMA (6.4K), 24 h.

ported to the perinuclei region of COS-7 cells following 6 h transfection with polyplexes of PDMAEMA-SS-PEG-SS-PDMAEMA (6.6K; Figure 9A). At a prolonged transfection time of 12 h, pDNA was delivered into the cell nuclei (Figure 9B). In contrast, significantly less pDNA was observed inside the cells transfected with the stably shielded polyplexes of PDMAEMA-PEG-PDMAEMA (6.4K) under otherwise the same conditions (Figure 9C,D). These results confirm that reduction-responsive reversibly shielded polyplexes mediate enhanced gene transfection as compared to stably shielded controls.

CONCLUSIONS

We have demonstrated that reversibly shielded DNA polyplexes based on low molecular weight bioreducible PDMAEMA-SS-PEG-SS-PDMAEMA triblock copolymers have excellent

colloidal stability under physiological salt conditions and mediate significantly enhanced transfection activity in the serum media compared to stably shielded polyplexes of PDMAEMA-PEG-PDMAEMA analogues. These triblock copolymers can be readily prepared with controlled molecular characteristics via RAFT polymerization. This study points to that reversible shielding may be an elegant approach to resolve the dilemma of in vivo applications of DNA polyplexes, that is, excellent stability in circulation but rapid deshielding and unpacking of polyplexes inside cells. These reversibly shielded polyplexes may be further developed for in vivo gene transfection.

ASSOCIATED CONTENT

Supporting Information

Live cell imaging experiment and results into ^1H NMR spectra of CPADN-PEG-CPADN and PDMAEMA-*b*-PEG-*b*-PDMAEMA

(6.4K), polyplex polydispersity (PDI) data, gel electrophoresis of PDMAEMA-*b*-PEG-*b*-PDMAEMA (6.4K)/DNA complexes, and colloidal stability of PDMAEMA-*b*-PEG-*b*-PDMAEMA (6.4K) polyplexes. This material is available free of charge via the Internet at <http://pubs.acs.org>.

AUTHOR INFORMATION

Corresponding Author

*Tel./Fax: +86-512-65880098. E-mail: zyzhong@suda.edu.cn.

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

This work is financially supported by research grants from the National Natural Science Foundation of China (NSFC 20874070, 50973078, 20974073, 51003070, and 51173126) and a Project Funded by the Priority Academic Program Development of Jiangsu Higher Education Institutions.

REFERENCES

- (1) Anderson, W. F. *Nature* **1998**, 392, 25–30.
- (2) Kaiser, J. *Science* **2007**, 317, 580–580.
- (3) Jeong, J. H.; Kim, S. W.; Park, T. G. *Prog. Polym. Sci.* **2007**, 32, 1239–1274.
- (4) Mastrobattista, E.; van der Aa, M.; Hennink, W. E.; Crommelin, D. J. A. *Nat. Rev. Drug Discovery* **2006**, 5, 115–121.
- (5) Mintzer, M. A.; Simanek, E. E. *Chem. Rev.* **2009**, 109, 259–302.
- (6) Morille, M.; Passirani, C.; Vonarbourg, A.; Clavreul, A.; Benoit, J. P. *Biomaterials* **2008**, 29, 3477–3496.
- (7) Schaffert, D.; Wagner, E. *Gene Ther.* **2008**, 15, 1131–1138.
- (8) Verbaan, F. J.; Bos, G. W.; Oussoren, C.; Woodle, M. C.; Hennink, W. E.; Storm, G. *J. Drug Delivery Sci. Technol.* **2004**, 14, 105–111.
- (9) Hunter, A. C. *Adv. Drug Delivery Rev.* **2006**, 58, 1523–1531.
- (10) Verbaan, F. J.; Oussoren, C.; Snel, C. J.; Crommelin, D. J. A.; Hennink, W. E.; Storm, G. *J. Gene Med.* **2004**, 6, 64–75.
- (11) Merdan, T.; Kunath, K.; Petersen, H.; Bakowsky, U.; Voigt, K. H.; Kopecek, J.; Kissel, T. *Bioconjugate Chem.* **2005**, 16, 785–792.
- (12) Oupicky, D.; Ogris, M.; Howard, K. A.; Dash, P. R.; Ulbrich, K.; Seymour, L. W. *Mol. Ther.* **2002**, 5, 463–472.
- (13) Lee, M.; Kim, S. W. *Pharm. Res.* **2005**, 22, 1–10.
- (14) Otsuka, H.; Nagasaki, Y.; Kataoka, K. *Adv. Drug Delivery Rev.* **2003**, 55, 403–419.
- (15) Mishra, S.; Webster, P.; Davis, M. E. *Eur. J. Cell Biol.* **2004**, 83, 97–111.
- (16) Petersen, H.; Fechner, P. M.; Martin, A. L.; Kunath, K.; Stolnik, S.; Roberts, C. J.; Fischer, D.; Davies, M. C.; Kissel, T. *Bioconjugate Chem.* **2002**, 13, 845–854.
- (17) Ogris, M.; Walker, G.; Blessing, T.; Kircheis, R.; Wolschek, M.; Wagner, E. *J. Controlled Release* **2003**, 91, 173–181.
- (18) Kichler, A. *J. Gene Med.* **2004**, 6, S3–S10.
- (19) Bennis, J. M.; Maheshwari, A.; Furgeson, D. Y.; Mahato, R. I.; Kim, S. W. *J. Drug Target.* **2001**, 9, 123–.
- (20) Itaka, K.; Kataoka, K. *Eur. J. Pharm. Biopharm.* **2009**, 71, 475–483.
- (21) Walker, G. F.; Fella, C.; Pelisek, J.; Fahrmeier, J.; Boeckle, S.; Ogris, M.; Wagner, E. *Mol. Ther.* **2005**, 11, 418–425.
- (22) Knorr, V.; Ogris, M.; Wagner, E. *Pharm. Res.* **2008**, 25, 2937–2945.
- (23) Knorr, V.; Allmendinger, L.; Walker, G. F.; Paintner, F. F.; Wagner, E. *Bioconjugate Chem.* **2007**, 18, 1218–1225.
- (24) Fella, C.; Walker, G. F.; Ogris, M.; Wagner, E. *Eur. J. Pharm. Sci.* **2008**, 34, 309–320.
- (25) Nie, Y.; Gunther, M.; Gu, Z.; Wagner, E. *Biomaterials* **2011**, 32, 858–869.
- (26) 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.
- (27) Meng, F. H.; Hennink, W. E.; Zhong, Z. Y. *Biomaterials* **2009**, 30, 2180–2188.
- (28) Cheng, R.; Feng, F.; Meng, F.; Deng, C.; Feijen, J.; Zhong, Z. *J. Controlled Release* **2011**, 152, 2–12.
- (29) Sun, H. L.; Guo, B. N.; Cheng, R.; Meng, F. H.; Liu, H. Y.; Zhong, Z. Y. *Biomaterials* **2009**, 30, 6358–6366.
- (30) Cheng, R.; Meng, F.; Ma, S.; Xu, H.; Liu, H.; Jing, X.; Zhong, Z. *J. Mater. Chem.* **2011**, 21, 19013–19020.
- (31) Li, Y. L.; Zhu, L.; Liu, Z. Z.; Cheng, R.; Meng, F. H.; Cui, J. H.; Ji, S. J.; Zhong, Z. Y. *Angew. Chem., Int. Ed.* **2009**, 48, 9914–9918.
- (32) Wang, Y.; Zheng, M.; Meng, F.; Zhang, J.; Peng, R.; Zhong, Z. *Biomacromolecules* **2011**, 12, 1032–1040.
- (33) Kim, T.; Seo, H. J.; Choi, J. S.; Jang, H. S.; Baek, J.; Kim, K.; Park, J. S. *Biomacromolecules* **2004**, 5, 2487–2492.
- (34) Zhong, Z. Y.; Feijen, J.; Lok, M. C.; Hennink, W. E.; Christensen, L. V.; Yockman, J. W.; Kim, Y. H.; Kim, S. W. *Biomacromolecules* **2005**, 6, 3440–3448.
- (35) Dubruel, P.; Schacht, E. *Macromol. Biosci.* **2006**, 6, 789–810.
- (36) Xu, F. J.; Li, H. Z.; Li, J.; Zhang, Z. X.; Kang, E. T.; Neoh, K. G. *Biomaterials* **2008**, 29, 3023–3033.
- (37) Zhu, C. H.; Jung, S.; Si, G. Y.; Cheng, R.; Meng, F. H.; Zhu, X. L.; Park, T. G.; Zhong, Z. Y. *J. Polym. Sci., Part A: Polym. Chem.* **2010**, 48, 2869–2877.
- (38) Verbaan, F. J.; Klouwenberg, P. K.; van Steenis, J. H.; Snel, C. J.; Boerman, O.; Hennink, W. E.; Storm, G. *Int. J. Pharm.* **2005**, 304, 185–192.
- (39) Cherng, J. Y.; vandeWetering, P.; Talsma, H.; Crommelin, D. J. A.; Hennink, W. E. *Pharm. Res.* **1996**, 13, 1038–1042.
- (40) vandeWetering, P.; Cherng, J. Y.; Talsma, H.; Hennink, W. E. *J. Controlled Release* **1997**, 49, 59–69.
- (41) van de Wetering, P.; Cherng, J. Y.; Talsma, H.; Crommelin, D. J. A.; Hennink, W. E. *J. Controlled Release* **1998**, 53, 145–153.
- (42) Layman, J. M.; Ramirez, S. M.; Green, M. D.; Long, T. E. *Biomacromolecules* **2009**, 10, 1244–1252.
- (43) You, Y. Z.; Manickam, D. S.; Zhou, Q. H.; Oupicky, D. *J. Controlled Release* **2007**, 122, 217–225.
- (44) You, Y. Z.; Zhou, Q. H.; Manickam, D. S.; Wan, L.; Mao, G. Z.; Oupicky, D. *Macromolecules* **2007**, 40, 8617–8624.
- (45) Dai, F.; Sun, P.; Liu, Y.; Liu, W. *Biomaterials* **2010**, 31, 559–569.
- (46) Jiang, X.; Lok, M. C.; Hennink, W. E. *Bioconjugate Chem.* **2007**, 18, 2077–2084.
- (47) Zhu, C. H.; Jung, S.; Luo, S. B.; Meng, F. H.; Zhu, X. L.; Park, T. G.; Zhong, Z. Y. *Biomaterials* **2010**, 31, 2408–2416.
- (48) Funhoff, A. M.; van Nostrum, C. F.; Janssen, A.; Fens, M.; Crommelin, D. J. A.; Hennink, W. E. *Pharm. Res.* **2004**, 21, 170–176.
- (49) Yue, X.; Qiao, Y.; Qiao, N.; Guo, S.; Xing, J.; Deng, L.; Xu, J.; Dong, A. *Biomacromolecules* **2010**, 11, 2306–2312.
- (50) Guo, S.; Huang, Y.; Wei, T.; Zhang, W.; Wang, W.; Lin, D.; Zhang, X.; Kumar, A.; Du, Q.; Xing, J.; Deng, L.; Liang, Z.; Wang, P. C.; Dong, A.; Liang, X.-J. *Biomaterials* **2011**, 32, 879–889.
- (51) Thang, S. H.; Chong, Y. K.; Mayadunne, R. T. A.; Moad, G.; Rizzardo, E. *Tetrahedron Lett.* **1999**, 40, 2435–2438.
- (52) Lowe, A. B.; McCormick, C. L. *Prog. Polym. Sci.* **2007**, 32, 283–351.
- (53) Boyer, C.; Bulmus, V.; Davis, T. P.; Ladmiraal, V.; Liu, J.; Perrier, S. *Chem. Rev.* **2009**, 109, 5402–5436.
- (54) Liu, G. J.; Ma, S. B.; Li, S. K.; Cheng, R.; Meng, F. H.; Liu, H. Y.; Zhong, Z. Y. *Biomaterials* **2010**, 31, 7575–7585.
- (55) Liu, Z. H.; Zhang, Z. Y.; Zhou, C. R.; Jiao, Y. P. *Prog. Polym. Sci.* **2010**, 35, 1144–1162.
- (56) Wightman, L.; Kircheis, R.; Rossler, V.; Carotta, S.; Ruzicka, R.; Kurs, M.; Wagner, E. *J. Gene Med.* **2001**, 3, 362–372.
- (57) Ruthardt, N.; Lamb, D. C.; Brauchle, C. *Mol. Ther.* **2011**, 19, 1199–1211.
- (58) Sauer, A. M.; Schlossbauer, A.; Ruthardt, N.; Cauda, V.; Bein, T.; Brauchle, C. *Nano Lett.* **2010**, 10, 3684–3691.