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Bioreducible Comb-Shaped Conjugates Composed of Secondary Amine and Hydroxyl Group-Containing Backbones and Disulfide-Linked Side Chains with Tertiary Amine Groups for Facilitating Gene Delivery

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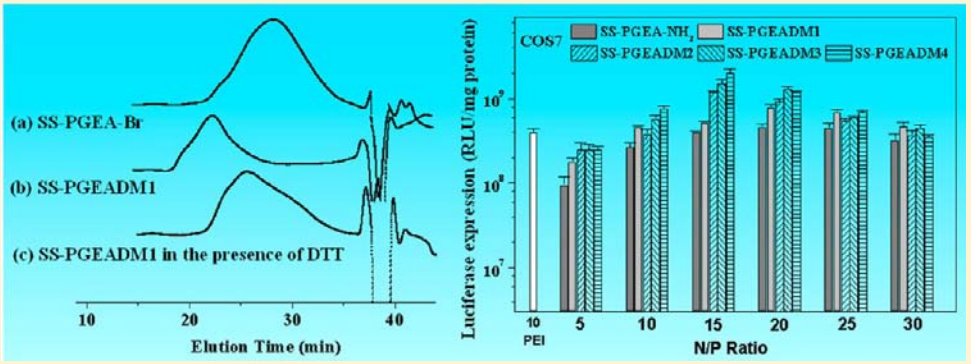

ABSTRACT: Comb-shaped polymeric vectors (SS-PGEADMs) consisting of ethanolamine/cystamine-functionalized poly(glycidyl methacrylate) (SS-PGEA-NH_2) backbones and bioreducible poly((2-dimethyl amino)ethyl methacrylate) (PDMEAMA) side chains were prepared by a combination of the ring-opening reaction and atom transfer radical polymerization (ATRP). The SS-PGEA-NH_2 backbones, which were prepared via the ring-opening reaction of the pendant epoxide groups of poly(glycidyl methacrylate) with the amine moieties of ethanolamine/cystamine, possess plentiful flanking secondary amine and hydroxyl groups and some flanking disulfide bond-containing cystamine derivatives. The primary amine groups of the cystamine derivatives were activated to produce bromoisobutylryl-terminated SS-PGEA (SS-PGEA-Br) as multifunctional initiators for subsequent ATRP of DMAEMA. The resultant disulfide-linked short PDMEAMA side chains possess pendant tertiary amine groups and are biocleavable. Such SS-PGEADMs can effectively condense pDNA. The cytotoxicity of SS-PGEADMs could be controlled by adjusting the grafting amount of PDMEAMA side chains. In comparison with the pristine SS-PGEA-NH_2, the moderate introduction of PDMEAMA side chains can further enhance the gene transfection efficiency in different cell lines. The present approach to well-defined comb-shaped vectors with multifunctional groups could provide a versatile means for tailoring the functional structures of advanced gene/drug vectors.

INTRODUCTION

Cationic polymers have been receiving considerable attention as the major type of nonviral drug delivery vectors, because of their low host immunogenicity, high flexibility, and easy preparation.[1−3] Under physiological conditions, polycations can spontaneously condense negatively charged DNA into compact nanocomplexes, reduce the electrostatic repulsion between DNA and cell surfaces, protect plasmid DNA from enzymatic degradation by nucleases, and facilitate cellular transfection. A great number of polycations, including polyethylenimine (PEI),[4] poly((2-dimethyl amino)ethyl methacrylate) (PDMEAMA),[5,6] poly(L-lysine),[7] and polyamidoamine,[8] have emerged as a leading class of transfection reagents. However, most of them suffer from either low gene transfection efficiency or significant toxicity. New cationic polymeric vectors will facilitate their applications in gene therapy. Reduction-sensitive polymers could be elegantly applied for intracellular triggered gene and drug delivery.[9] The design rationale of bioreducible polymers usually involves incorporation of easy intracellular reversible disulfide linkage(s). The bioreducible polymeric vectors containing disulfide bonds were already proven to be a highly advantageous feature for delivering a variety of nucleic acids.[10−12]

We recently found that ethanolamine (EA)-functionalized poly(glycidyl methacrylate) (PGMA), or PGEA with plentiful flanking secondary amine and hydroxyl groups, can produce

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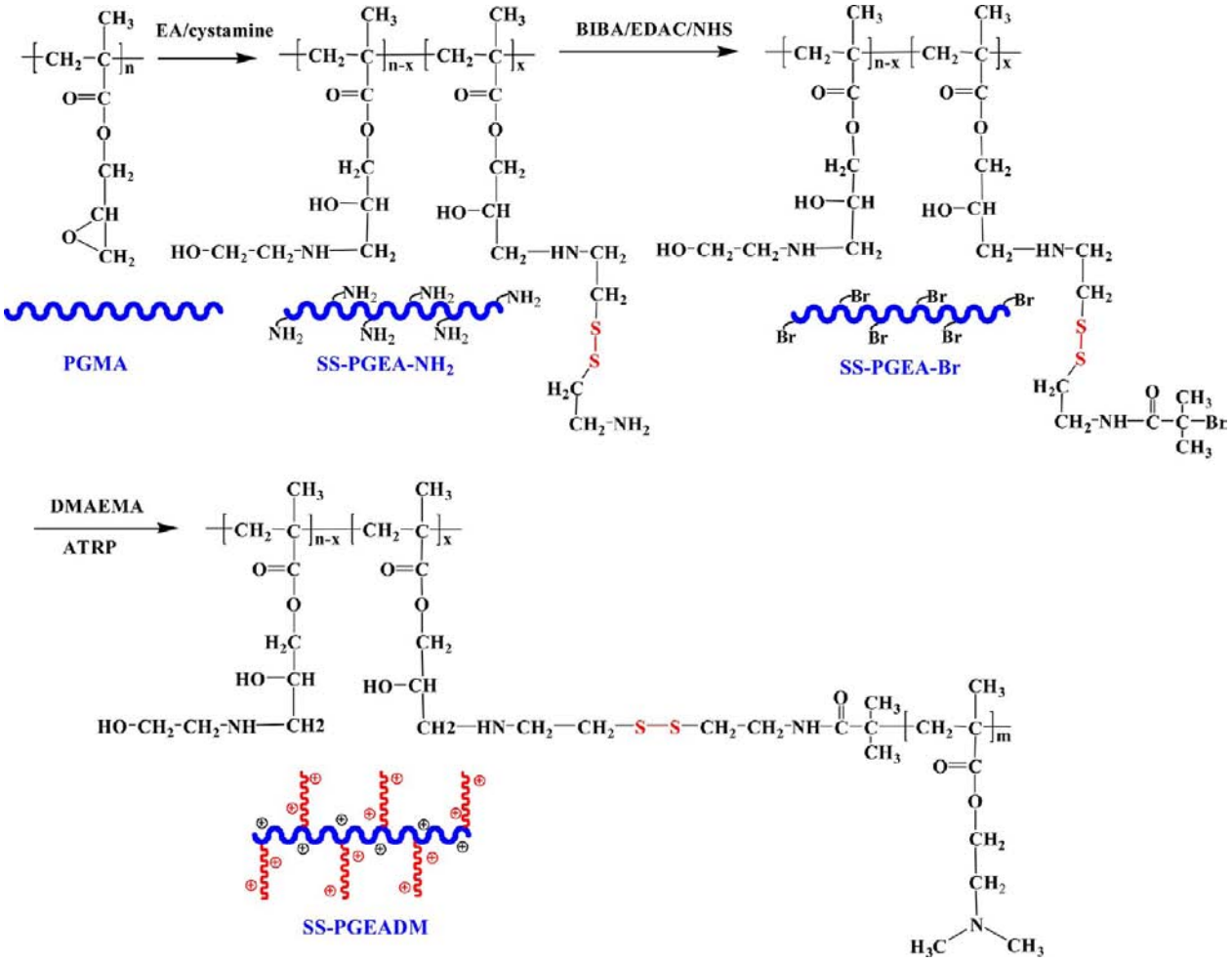

Figure 1. Schematic diagram illustrating the preparation processes of comb-shaped copolymers composed of secondary amine and hydroxyl group-containing backbones and bioreducible tertiary amine group-containing side chains.

excellent transfection efficiency in different cell lines, while exhibiting very low toxicity.[13] By taking advantage of PGEA derivatives and bioreducible polymers, in this work, the novel comb-shaped vectors (SS-PGEADMs) consisting of PGEA-derivative backbones (SS-PGEA-NH_2) and disulfide-linked PDMEAMA side chains were proposed by a combination of the ring-opening reaction and atom transfer radical polymerization (ATRP) (Figure 1). The SS-PGEA-NH_2 backbones from the ring-opening reaction of PGMA with ethanolamine/cystamine possess plentiful flanking secondary amine and hydroxyl groups and some flanking disulfide bond-containing cystamine derivatives. The disulfide-linked PDMEAMA side chains from ATRP possess pendant tertiary amine groups and could be biocleavable from the backbones. Such resultant SS-PGEADMs with flanking multifunctional groups can effectively interact with pDNA, exhibit good gene transfection efficiencies in different cell lines, and will be attractive as nonviral drug vectors for advanced biomedical applications.

■ EXPERIMENTAL PROCEDURES

Materials. Branched polyethylenimine (PEI, M_w ~25 000 Da), glycidyl methacrylate (GMA, 98%), 2-(dimethylamino)-ethyl methacrylate (DMAEMA, >98%), ethanolamine (EA, 98%), cystamine dihydrochloride (>98%), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDAC, 98%), *N*-hydroxysuccinimide (NHS, 98%), α-bromoisobutyric acid (BIBA, 98%), 2,2′-byridine (Bpy, 99%), and copper(I) bromide (CuBr, 99%) were obtained from Sigma-Aldrich Chemical Co., St. Louis, MO. GMA and DMEAMA were used after removal of the inhibitors in a ready-to-use disposable inhibitor-removal column (Sigma-Aldrich). 3-(4,5-Dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide (MTT), penicillin, and streptomycin were purchased from Sigma Chemical Co., St. Louis, MO. L929, COS7, and HEK293 cell lines were purchased from the American Type Culture Collection (ATCC, Rockville, MD).

Synthesis of Comb-Shaped SS-PGEADM Copolymers. As shown in Figure 1, the PGEA derivative backbones (SS-PGEA-NH_2) were first prepared by reacting well-defined poly(glycidyl methacrylate) (PGMA) (about 75 GMA units, M_n = 1.06 × 10^4 g/mol, PDI = 1.32) with the mixture of ethanolamine (EA) and cystamine. Details on the preparation of PGMA have been described earlier.[13] In order to obtain SS-PGEA-NH_2, 2.0 g of PGMA was dissolved in 14 mL of DMF. About 38.6 mL of the mixture consisting of EA (12.6 mL), cystamine dihydrochloride (3.4 g), and triethyleneamine (TEA, 6 mL) was then added, where the molar ratio of EA/cystamine was kept at about 5:1. The reaction mixture was degassed by bubbling argon for 10 min, sealed with a rubber stopper under an argon atmosphere, and stirred at 37 °C for 7 days to produce the functionalized PGMA backbones. The final reaction mixture was precipitated and washed with excess diethyl

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ether, prior to being redissolved in 30 mL of deionized water and dialyzed against deionized water (4 × 5 L) with dialysis membrane (MWCO, 3500 Da) at room temperature for 48 h. The final products were freeze−dried and about 1.6 g of SS-PGEA-NH_2 was obtained.

The bromoisobutylryl-terminated PGEA (SS-PGEA-Br, Figure 1) was synthesized via the reaction of primary groups of SS-PGEA-NH_2 with BIBA in the presence of EDAC and NHS. BIBA (0.26 g, 1.5 mmol), EDAC (0.41 g, 2.20 mmol), and NHS (0.26 g, 2.20 mmol) were dissolved in 5 mL of DMF. The mixture was stirred at 37 °C for 2 h, and mixed with 1.0 g of SS-PGEA-NH_2 dissolved in 5 mL of DMF. One milliliter of TEA was then added and the reaction mixture was stirred for 24 h at 37 °C. At the end of the reaction, the reaction mixture was precipitated with excess diethyl ether, prior to being redissolved in 20 mL of deionized water and dialyzed against deionized water (4 × 5 L) with dialysis membrane (MWCO, 3500 Da) at room temperature. The final products were freeze−dried and about 0.7 g of SS-PGEA-Br was obtained.

For the preparation of SS-PGEA-*g*-PDMEAMA comb-like polymers (named SS-PGEADMs) via ATRP, the molar feed ratio [DMAEMA (1 mL)]:[CuBr]:[Bpy] of 100:1:2.5 was used at room temperature in 5 mL of methanol/water (2/3, v/v) containing 0.10 g of SS-PGEA-Br. The reaction was conducted in a 25 mL flask equipped with a magnetic stirrer and under the typical conditions for ATRP.[5] DMAEMA, SS-PGEA-Br, and Bpy were introduced into the flask containing 5 mL of methanol/water, and the reaction mixture was degassed by bubbling argon for 10 min. Then, CuBr was added into the mixture under an argon atmosphere. The flask was then sealed with a rubber stopper under an argon atmosphere. The polymerization was allowed to proceed under continuous stirring at room temperature from 5 to 40 min. The reaction was stopped by diluting with water. The diluted reaction mixture was extensively dialyzed against DDW using a dialysis membrane (MWCO 3500) prior to lyophilization. The SS-PGEADM yields from ATRP time of 5, 10, 20, and 40 min are 0.13, 0.21, 0.28, and 0.45 g, respectively.

Polymer Characterization. The molecular weights of polymers were determined by gel permeation chromatography (GPC), chemical structure by nuclear magnetic resonance (NMR) spectroscopy, and chemical composition by X-ray photoelectron spectroscopy (XPS). GPC measurements were performed on a YL9100 GPC system equipped with a UV/vis detector and Waters Ultrahydrogel 250 and Ultrahydrogel Linear columns, where a pH 3.5 acetic buffer solution was used as the eluent at a low flow rate of 1.0 mL/min at 25 °C. Monodispersed poly(ethylene glycol) standards were used to obtain a calibration curve. ^{1}H NMR spectra were measured by accumulation of 1000 scans at a relaxation time of 2 s on a Bruker ARX 300 MHz spectrometer, using $CDCl_3$ (for PGMA) or D_2O (for PGEA) as the solvent. The XPS measurements were performed on a Kratos AXIS HSi spectrometer equipped with a monochromatized Al K_α X-ray source (1486.6 eV photons), using the same procedures as those described earlier.[5]

Characterization of Polymer/pDNA Complexes. The plasmid (encoding *Renilla* luciferase) used in this work was pRL-CMV (Promega Co., Cergy Pontoise, France). All polymer stock solutions were prepared at a nitrogen concentration of 10 mM in distilled water. Solutions were filtered via sterile membranes (0.2 μm) of average pore size and stored at 4 °C. Comb-like polymers to DNA ratios are expressed as molar ratios of nitrogen (N) in SS-PGEADMs to phosphate (P) in DNA (or as N/P ratios). All polymer/pDNA complexes were formed by mixing equal volumes of polymer and pDNA solutions to achieve the desired N/P ratio, where each mixture was vortexed and incubated for 30 min at room temperature.

Each cationic polymer was examined for its ability to bind pDNA through agarose gel electrophoresis in a Sub-Cell system (Bio-Rad Lab, Hercules, CA) using the similar procedures as those described earlier.[5] DNA bands were visualized and photographed by a UV transilluminator and BioDco-It imaging system (UVP Inc., Upland, CA). The particle sizes and zeta potentials of the polymer/pDNA complexes were measured using a Zetasizer Nano ZS (Malvern Instruments, Southborough, MA) and procedures similar to those described earlier.[5] The polymer/pDNA complexes were also observed with the transmission electron microscopy (TEM) (JEOL JEM 2010F), where the complex was observed at an acceleration voltage of 100 kV.

To evaluate the heparin-induced release of pDNA from cationic polymer/pDNA complexes in vitro, the procedures similar to those described earlier were used.[14] The complexes at the N/P ratio of 10 in 0.4 mL of PBS buffer containing 20 μg pDNA with or without heparin concentration of 100 μg/mL were incubated under the presence or absence of 10 mM DTT in a microcentrifuge tube at 37 °C shaking incubator. After 12 h, the samples were centrifuged at 14 000 rpm and the supernatant was collected, where the released pDNA was quantified by measuring absorbance at 260 nm with a UV-2450 spectrophotometer (Shimadzu, Japan).

Cell Viability and In Vitro Transfection Assay. The cytotoxicity of the comb-like polymers was evaluated using the MTT assay in HEK293, L929, and COS7 cell lines using procedures similar to those described earlier.[5] The cells were cultured in Dulbecco's modified eagle medium (DMEM), supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 units/mL of penicillin, and 100 μg/mL of streptomycin at 37 °C. The sterile-filtered MTT stock solution in PBS (5 mg/mL) was used. The absorbance was measured using a Bio-Rad Model 680 Microplate Reader (UK) at a wavelength of 570 nm. For each sample, the final absorbance was the average of those measured from six wells in parallel. The formula $[A]_{test}/[A]_{control} \times 100\%$ was used to calculate the cell viability (%), where $[A]_{test}$ and $[A]_{control}$ are defined as the absorbance values of the wells with the polycations and control ones (without the polycations), respectively.

Transfection assays were performed using plasmid pRL-CMV as the reporter gene in HEK293, L929, and COS7 cell lines in the presence of serum using the procedures as described earlier.[5] The cells were seeded in 24-well plates at a density of 5×10^4 cells in 500 μL of medium/well. The N/P ratios of the star-shaped polycation/pDNA complexes varied from 10 to 30. 1.0 μg of pDNA for one cell was used. After a total transfection time of 24 h, luciferase gene expression was quantified using a commercial kit (Promega Co., Cergy Pontoise, France) and a luminometer (Berthold Lumat LB 9507, Berthold Technologies GmbH. KG, Bad Wildbad, Germany). Gene expression results were expressed as relative light units (RLUs) per milligram of cell protein lysate (RLU/mg protein).

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RESULTS AND DISCUSSION

Synthesis and Characterization of SS-PGEADMs Composed of PGEA Backbones and Bioreducible Short PDMEAMA Side Chains. As shown in Figure 1, the comb-shaped SS-PGEADM copolymers consisting of EA/cystamine-functionalized PGMA (or SS-PGEA-NH_2) backbones and bioreducible low-molecular-weight PDMEAMA side chains were prepared by a combination of the ring-opening reaction and ATRP. The SS-PGEA-NH_2 backbones were prepared via the ring-opening reaction of the pendant epoxide groups of PGMA with the excess amine moieties of EA/cystamine. In this work, the starting PGMA with about 75 GMA units (number average molecular weight (M_n) = 1.06 × 10^4 g/mol, polydispersity index (PDI) = 1.32) was used and the molar ratio of EA/cystamine was kept at about 5:1. The obtained SS-PGEA-NH_2 possesses plentiful flanking secondary amine and hydroxyl groups and some flanking disulfide bond-containing cystamine derivatives. The primary amine groups of the flanking cystamine derivatives were activated to react with α-bromoisobutyric acid (BIBA) in the presence of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDAC) and *N*-hydroxysuccinimide (NHS). The carboxylic acid groups of BIBA were first converted into reactive esters (succinimidyl intermediates) in the presence of EDAC and NHS. The reactive esters underwent nucleophilic substitution reactions with the primary amine groups to form a stable amide linkage and produce the resultant bromoisobutyryl-terminated SS-PGEA (SS-PGEA-Br) as multifunctional initiators for subsequent ATRP.

The C 1s core-level X-ray photoelectron spectroscopy (XPS) spectra of the PGMA, SS-PGEA-NH_2, and SS-PGEA-Br were shown in Figure 2a,b,c, respectively. The C 1s core-level spectrum of PGMA can be curve-fitted into three peak components with binding energies (BE's) at about 284.6, 286.2, and 288.4 eV, attributable to the C—H/C—C, C—O, and O=C—O species, respectively,[13] in an approximate area ratio of 3:3:1, consistent with the chemical structure of PGMA. After the ring-opening reaction of PGMA, the spectrum of SS-PGEA-NH_2 can be curve-fitted by four peak components with BE's at about 284.6, 285.5, 286.2, and 288.4 eV, attributable to the C—H/C—C, C—N, C—O/C—S, and O=C—O species, respectively. Its corresponding S 2p core-level spectrum consisting of a single peak component with BE at about 164 eV was shown in Figure 2b′. The C 1s core-level spectrum (Figure 2c) of SS-PGEA-NH_2 can be curve-fitted by five peak components with BE's at about 284.6, 285.5, 286.2, 287.6, and 288.4 eV, attributable to the C—H/C—C, C—N, C—O/C—S/C—Br, O=C—NH, and O=C—O species, respectively. The new O=C—NH peak component is associated with the bromide-capped ester groups of SS-PGEA-Br. The corresponding Br 3d core-level spectrum is shown in Figure 2c′. The above XPS results clearly confirmed the successful preparation of SS-PGEA-NH_2 and SS-PGEA-Br.

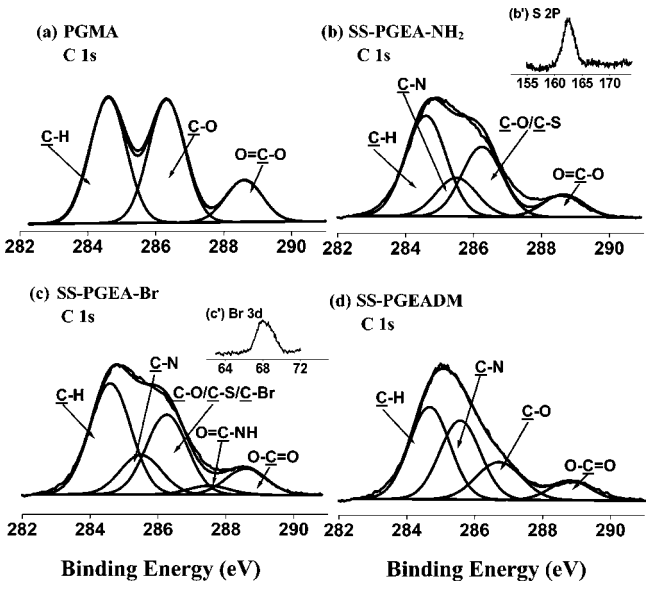

Figure 2. XPS C 1s spectra of (a) PGMA, (b) SS-PGEA-NH_2, (c) SS-PGEA-Br, and (d) SS-PGEADM1, (b′) S 2p core-level spectrum of SS-PGEA-NH_2, and (c′) Br 3d core-level spectrum of SS-PGEA-Br.

The representative structures of PGMA, SS-PGEA-NH_2, and SS-PGEA-Br were also characterized by ^{1}H NMR spectra as shown in Figure 3a,b,c, respectively. For PGMA, the signals at δ = 3.86 and 4.3 ppm correspond to the methylene protons adjacent to the oxygen moieties of the ester linkages (***a***, $\underline{CH_2}$−O−C=O). The peaks at δ = 3.2 ppm and δ = 2.63 and 2.84 ppm can be assigned to the $CH_2-\underline{CH}(O)-CH_2$ (***b***) methylidyne and $CH-\underline{CH}(O)-CH_2$ (***c***) methylene protons of the epoxide ring, respectively. The ratio of peak area of ***a***, ***b***, and ***c*** is about 2:1:2, indicating that the epoxy groups in the PGMA remained intact throughout ATRP. After the ring-opening reactions of PGMA with EA/cystamine (Figure 3b), the peaks (***b***,***c***) associated with the epoxy groups of PGMA have disappeared completely. The peaks (***a***, $\underline{CH_2}$−O−C=O) at δ = 3.86 and 4.3 ppm shifted to one position (***d***) at δ = 4.05 ppm. The new peak region located at chemical shifts of δ = 3.4−3.7 ppm is mainly associated with the resultant methylidyne and methylene protons adjacent to the hydroxyl groups (***e***, $\underline{CH}$−OH and $\underline{CH_2}$−OH). The signal at δ = 2.7 ppm is mainly attributable to the methylene protons adjacent to the amine groups (***f***, $\underline{CH_2}$−NH and $\underline{CH_2}$−NH_2). The signals at δ = 2.3−2.7 ppm correspond to the methylene protons adjacent to the disulfide bonds (***g***, $\underline{CH_2}$−S−S). For SS-PGEA-Br (Figure 3c), the chemical shift at δ = 1.92 ppm is associated with the methyl protons (***i***, C(Br)−$\underline{CH_3}$) of the 2-bromoisobutyryl groups. Thus, the above NMR results also indicated that the different functionalized PGMA vectors have been successfully prepared. When ATRP is carried out from a multifunctional backbone with a high local concentration of initiation sites, the radical−radical coupling of the propagating chains will occur and result in a gel. In order to avoid potential gelation and introduce some flexibility onto the comb-like cationic copolymers, the SS-PGEA-Br with moderate initiation sites is desired for subsequent comb-shaped copolymers. In this work, based on the ^{1}H NMR data of SS-PGEA-Br and the repeat units (about 75 GMA units) of the starting PGMA, it was calculated that about 6 functionalized GMA units of SS-PGEA-Br possess one initiation site or every SS-PGEA-Br chain contains about 14 initiation sites. No gelation during the subsequent ATRP was observed using such SS-PGEA-Br as the multifunctional initiators. In addition, the local concentration of initiation sites of SS-PGEA-Br can be tuned by adjusting the EA/cystamine feed ratio.

Well-defined comb-shaped SS-PGEA-*g*-PDMEAMA copolymers (SS-PGEADMs) were subsequently synthesized via ATRP of DMAEMA from SS-PGEA-Br (Figure 1). The SS-PGEADMs with different lengths of PDMEAMA side chains can be synthesized by varying the ATRP time. Table 1

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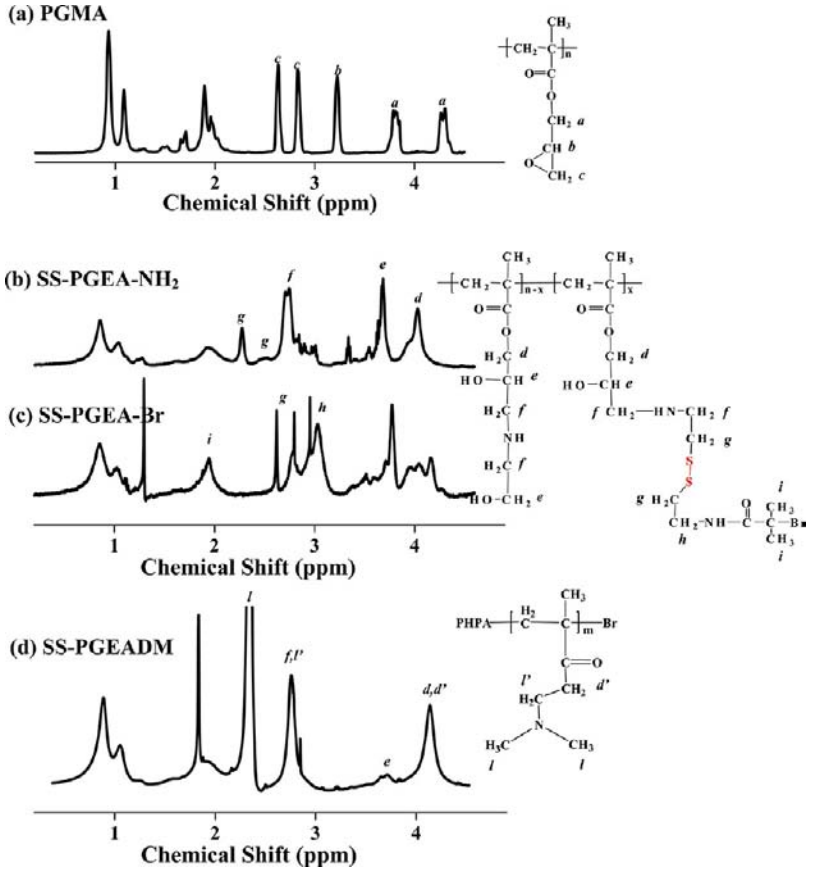

Figure 3. 300 MHz ^{1}H NMR spectra of (a) PGMA, (b) SS-PGEA-NH_2, (c) SS-PGEA-Br, and (d) PGEADM.

Table 1. Characterization of the Biocleavable PGEADM Polymers

sample	reaction time (min)	M_n[b] (g/mol)	PDI[b]	DMAEMA repeat units per side chain
SS-PGEA-Br[a]		1.73×10^4	1.82	
SS-PGEA-*g*-P(DMAEMA)1 or SS-PGEADM1[c]	5	4.82×10^4	1.78	14[d] 19[e]
SS-PGEA-*g*-P(DMAEMA)2 or SS-PGEADM2[c]	10	9.25×10^4	1.86	34[d] 33[e]
SS-PGEA-*g*-P(DMAEMA)3 or SS-PGEADM3[c]	20	1.21×10^5	1.90	47[d] 43[e]
SS-PGEA-*g*-P(DMAEMA)4 or SS-PGEADM4[c]	40	1.43×10^5	1.87	57[d] 51[e]

[a]About 6 functionalized GMA units of SS-PGEA-Br possess one initiation site or every SS-PGEA-Br chain contains about 14 initiation sites. The corresponding SS-PGEA-NH_2 were prepared using PGMA with about 75 repeat units (M_n = 1.06×10^4 g/mol, PDI = 1.32). [b]Determined from GPC results. PDI = weight average molecular weight/number average molecular weight, or M_w/M_n. [c]Synthesized using a molar feed ratio [DMAEMA (1 mL)]:[CuBr]:[Bpy] of 100:1:2.5 at room temperature in 5 mL of methanol/water (2/3, v/v) containing 0.10 g of SS-PGEA-Br. [d]Determined from M_n and the molecular weights of SS-PGEA-Br (1.73×10^4 g/mol), DMAEMA (157 g/mol), and 14 initiation sites of SS-PGEA-Br. [e]Determined from ^{1}H NMR data.

summarizes the GPC results of SS-PGEADM1 (from 5 min of ATRP), SS-PGEADM2 (from 10 min of ATRP), SS-PGEADM3 (from 20 min of ATRP), and SS-PGEADM4 (from 40 min of ATRP). With the increase in reaction time from 5 to 40 min, the M_n of SS-PGEADM from GPC increases from 4.82×10^4 to 1.45×10^5 g/mol, and the total number of DMEAMA repeat units per side chain increases accordingly from 14 to 57, based on the assumption of one initiation site out of every 6 functionalized GMA units of SS-PGEA-Br (Table 1). In addition, the PDIs of SS-PGEADMs are comparable to that of SS-PGEA-Br, indicating that the ATRP of DMEAMA is well-controlled. The typical C 1s core-level spectrum of SS-PGEADM as shown in Figure 2d can be curve-fitted into four peak components with BE's at about 284.6, 285.5, 286.2, and 288.4 eV, attributable to the C—H/C—C, C—N, C—O, and O═C—O species, respectively. In comparison with the C 1s core-level spectrum (Figure 2c) of SS-PGEA-Br, the increase in the [C—N]:[O═C—O] area ratios of SS-PGEADM is consistent with the presence of PDMEAMA. Figure 2d shows the typical ^{1}H NMR spectrum of SS-PGEADM. The typical chemical shifts at about 2.42 and 2.69 ppm are mainly attributable to the (*l*) N—CH_3 methyl and (*l*′) N—CH_2 methylene protons of the PDMAEMA side chains, respectively. The signal (*e*) associated with the SS-PGEA backbone had decreased substantially. From the ^{1}H NMR spectra of SS-PGEADMs, the average number of DMAEMA repeat units per side chain was also estimated and summarized in Table 1.

The disulfide bridge linkages between PDMAEMA side chains and PGEA-derivative backbones can make SS-PGEADM reductively breakable under reductive conditions. To demonstrate the responsiveness, SS-PGEADM was treated with 10 mM of DL-dithiothreitol (DTT), analogous to the intracellular redox potential.[9,15] Such DTT-induced degradation of the

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disulfide-linked SS-PGEADM was demonstrated using GPC analysis (Figure 4). After incubation with DTT for 2 h, the

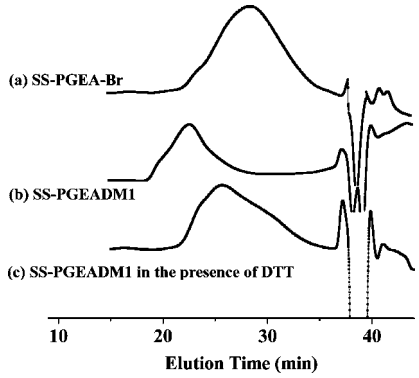

Figure 4. Aqueous GPC traces obtained for (a) SS-PGEA-Br, (b) SS-PGEADM1, and (c) SS-PGEADM1 in the presence of 10 mM DTT, where the incubation time with DTT was 2 h.

molecular weight of SS-PGEADM1 decreased substantially. The significant differences in the aqueous GPC traces of SS-PGEADM1 before and after the treatment with DTT clearly show that the disulfide-linked SS-PGEADM was responsive to the reductive agent DTT.

Biophysical Characterization of Cationic Polymer/pDNA Complexes. A successful gene delivery system requires that DNA must be condensed by polycation into nanoparticles small enough to facilitate cellular uptake. The ability to condense DNA is a prerequisite for polymeric gene vectors. In this work, the ability of the comb-shaped cationic copolymers to condense plasmid DNA (pDNA) into particulate structures was confirmed by agarose gel electrophoresis, particle size, and zeta potential measurements, as well as TEM imaging. Positively charged SS-PGEADM vectors were mixed with negatively charged plasmid pRL-CMV to form the polyplexes at various nitrogen (N)/phosphate (P) (or N/P) ratios via electrostatic interaction. The formation of the copolymer/pDNA complexes was first analyzed by their electrophoretic mobility on an agarose gel at various N/P ratios. Figure 5 shows the gel retardation results of the cationic polymer (SS-PGEA-NH_2 and SS-PGEADMs)/pDNA complexes with increasing N/P ratios, in comparison with that of the branched PEI (25 kDa)/pDNA complexes. SS-PGEA-NH_2 compacts pDNA completely at N/P ratios of 3.0 and above. SS-PGEADM1, SS-PGEADM2, SS-PGEADM3, and SS-PGEADM4 show increasing capability to condense pDNA, indicating that the increased cationic charges from the tertiary amine groups of PDMEAMA side chains could enhance the condensation capability. SS-PGEADMs with longer side chains exhibit similar condensation capability to that of the control PEI, which could inhibit the migration of pDNA at N/P ratios of 2.0 and above. In comparison with SS-PGEA-NH_2, the increased density of intramolecular amino groups as well as the flexible PDMAEMA side chains may render the SS-PGEADMs better complexation capability and interaction with pDNA.

The particle size and surface charge of complexes are important factors in modulating their cellular uptake. Formation of nanoparticles facilitates polyplex diffusion, extravasations through vascular fenestration, and cellular uptake.[16] The (a) particle sizes and (b) zeta potentials of the cationic polymer (SS-PGEA-NH_2 and SS-PGEADMs)/pDNA complexes at various N/P ratios were shown in Figure 6. All the

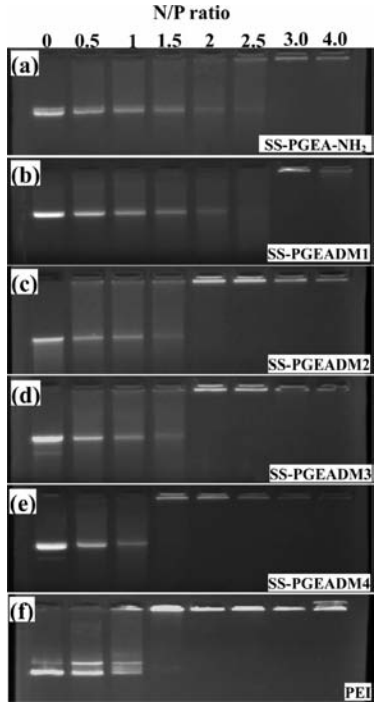

Figure 5. Electrophoretic mobility of plasmid DNA (pDNA) in the complexes of the cationic polymers ((a) SS-PGEA-NH_2, (b) SS-PGEADM1, (c) SS-PGEADM2, (d) SS-PGEADM3, (e) SS-PGEADM4, and (f) PEI) at various N/P ratios.

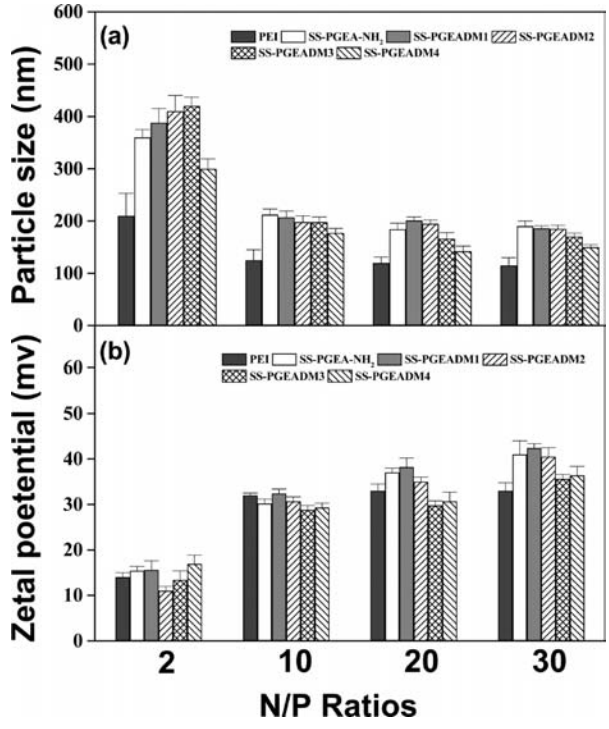

Figure 6. Particle size (a) and zeta potential (b) of the complexes between the cationic polymers (SS-PGEA-NH_2, SS-PGEADM1, SS-PGEADM2, SS-PGEADM3, SS-PGEADM4, and PEI) and pDNA at various N/P ratios.

cationic copolymers can efficiently compact pDNA into small particles. Generally, the hydrodynamic sizes of the complexes decrease with increasing N/P ratios. All vectors can efficiently compact pDNA into nanoparticles and showed decreased

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particle size with increasing N/P ratios. At the N/P ratio of 2.0, loose large aggregates were formed, due to the lower amount of cationic polymers. At higher N/P ratios, all vectors condense pDNA into nanoparticles in the diameter range of 100 to 200 nm. These complexes within this size range can readily undergo endocytosis.[17] In comparison with those of SS-PGEA-NH_2/pDNA, the particle sizes of SS-PGEADM4/pDNA were smaller at all various N/P ratios, which was consistent with the enhanced condensation ability of SS-PGEADMs. Figure 7

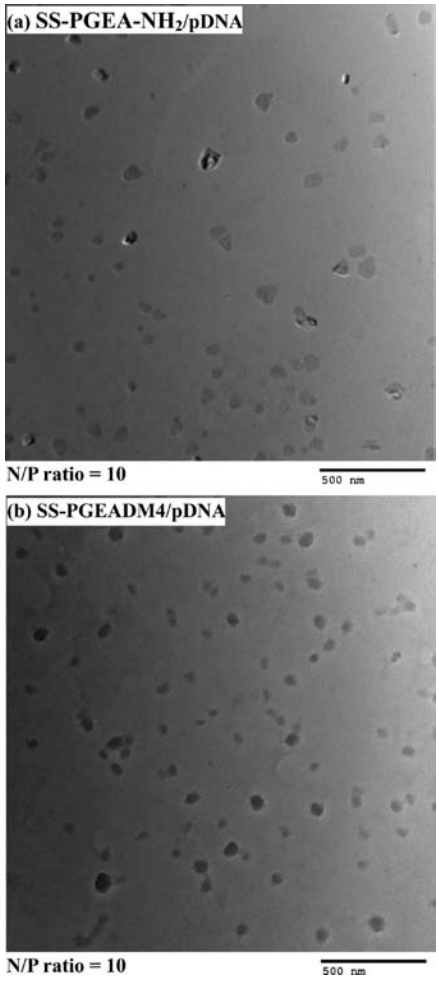

Figure 7. TEM images of the (a) SS-PGEA-NH_2/pDNA and (b) SS-PGEADM4/pDNA complexes at a ratio of 10.

shows the representative TEM images of the (a) SS-PGEA-NH_2/pDNA and (b) SS-PGEADM4/pDNA complexes at a ratio of 10. The images clearly reveal that the compacted complexes display a characteristic globular morphology and exist in the form of spherical nanoparticles. Zeta potential is an indicator of surface charges on the polymer/pDNA naoparticles. A positively charged surface allows electrostatic interaction with negatively charged cell surfaces, which can enhance cellular uptake by nonspecific endocytosis.[18] As indicated in Figure 6b, the zeta potentials of all polymer/pDNA complexes range from slightly positive to strongly positive with increasing N/P ratios, indicating the complex surface charge becomes positive upon the complete self-assembly of polycation and pDNA. The excess cationic polymers probably do not exert any significant effect on the particle size and zeta potential of the complexes (Figure 6).

As mentioned earlier, the disulfide bridge linkages between PDMAEMA side chains and PGEA-derivative backbones were responsive to the reductive agent, which can make SS-PGEADM breakable under reductive conditions (Figure 4). Such responsiveness may also have significant effect on pDNA release from the complexes under intracellular reductive conditions. The release of pDNA from the SS-PGEA-NH_2/pDNA and SS-PGEADM1/pDNA complexes at a ratio of 10 was studied with or without heparin under the presence or absence of DTT. After 12 h incubation at 37 °C (as shown in Figure 8) without heparin, the SS-PGEA-NH_2 and SS-

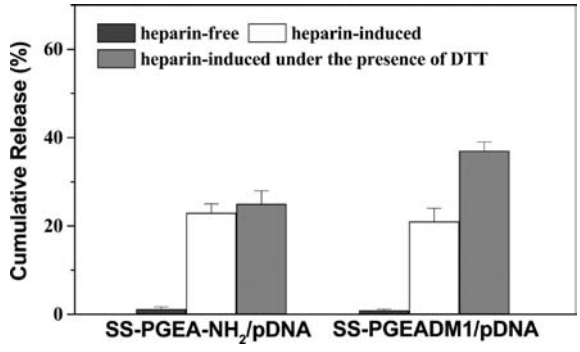

Figure 8. Cumulative release of pDNA from SS-PGEA-NH_2/pDNA and SS-PGEADM1/pDNA complexes at a ratio of 10 after 12 h incubation at 37 °C under different conditions (mean ± SD, n = 3).

PGEADM1 formulations could barely release DNA from their complexes. On the other hand, heparin-induced release was observed in the complexes, indicating that heparin, a competitor anionic sulfated sugar, could cause decomplexation of pDNA from the complexes.[14] As shown in Figure 8, the presence of DTT in the complex solution could further enhance pDNA release from SS-PGEADM1 formulation. However, no obvious effect on the SS-PGEA-NH_2/pDNA complex was observed under the same reductive condition. This phenomenon indicated that the cleavage of the short PDMAEMA side chains from the PGEA-derivative backbone under the reductive condition could lead to the unstable complexes. Such unstable complexes were readily decondensed via interexchange with polyanions to induce more DNA release. In cells, varieties of negatively charged macromolecules or cellular components (such as mRNA, sulfated sugars, and nuclear chromatin) exist, which as competitors similar to heparin can induce DNA release from the complex.[14] In addition, without addition of heparin, no more DNA release was observed in the presence of DTT, indicating that under the absence of polyanion competitors, the cleavable PDMAEMA side chains and PGEA-derivative backbones in the unstable complexes still could interact with DNA. The above results implied that the biocleavable nature of SS-PGEADMs may greatly facilitate pDNA release, and in turn, could modulate the gene expression in vitro or in vivo.

Cell Viability Assay. Cytotoxicity is one of the most important factors to be considered in selecting polymeric gene carriers. In general, high transfection efficiency is associated with a devastating toxicity. A successful delivery system should have high transfection efficiency and compromised toxicity. Figure 9 shows the *in vitro* MTT assay results of cytotoxicity of SS-PGEA-NH_2, SS-PGEADMs, and PEI in (a) HEK293, (b) COS7, and (c) L929 cells. All of the cationic polymers exhibit a dose-dependent cytotoxicity effect. As expected, SS-PGEA-NH_2

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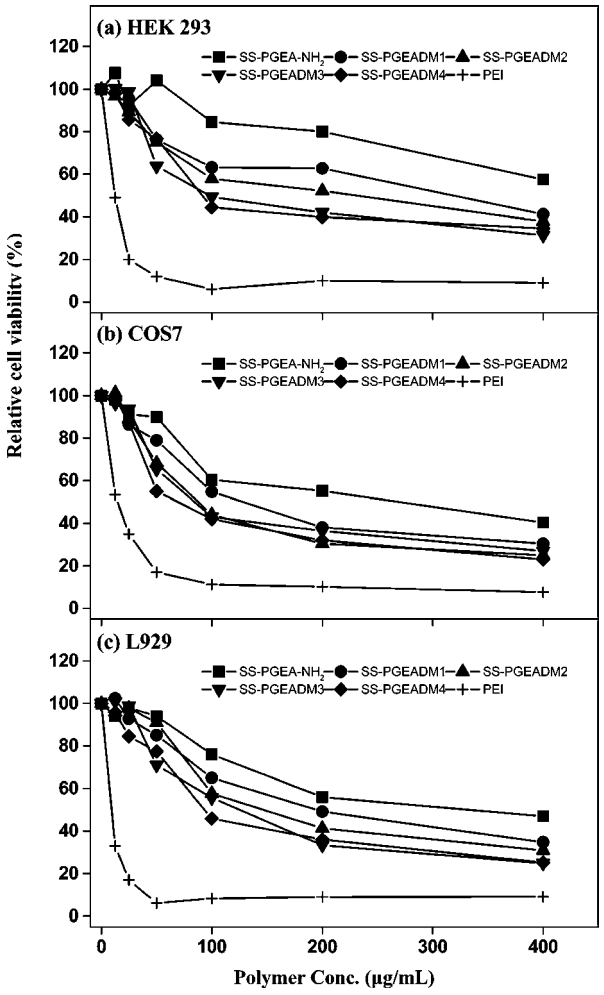

Figure 9. Cell viability assay in (a) HEK293, (b) COS7, and (c) L929 cells with various concentrations of SS-PGEA-NH_2, SS-PGEADM1, SS-PGEADM2, SS-PGEADM3, SS-PGEADM4, and PEI. Cell viability was determined by the MTT assay and expressed as a percentage of the control cell culture.

and SS-PGEAPDMs exhibit increasing toxicity. A high concentration of amino groups is always considered an important factor leading to high cytotoxicity. The introduction of the PDMEAMA side chains results in an increased concentration of amino groups and the subsequent cytotoxicity increase of SS-PGEAPDMs. Nevertheless, the slopes of the dose-dependent cytotoxicity curves for the control PEI are much steeper than those of all SS-PGEAPDMs.

The cell viability of the polymer/pDNA complexes as a function of N/P ratio was also evaluated in the HEK293 cell lines by using MTT assay (Figure 10). The N/P ratio has a profound impact on the cytotoxicity of complexes. The cell viability of all polymer/pDNA complexes was observed to decrease with increasing N/P ratios. At the same N/P ratio, the cell viability seemed to be highly dependent on the PDMAEMA side chain length. SS-PGEAPDM4 with the longest PDMAEMA side chains seems to be the most toxic, especially at high N/P ratios. It was well-known that the cytotoxicity of polycations increases with the molecular weight.[17] Due to the increased length in the PDMAEMA side chains of SS-PGEAPDM1 to SS-PGEAPDM4, their cytotoxicity shows the general upward trend as expected. The cytotoxicity

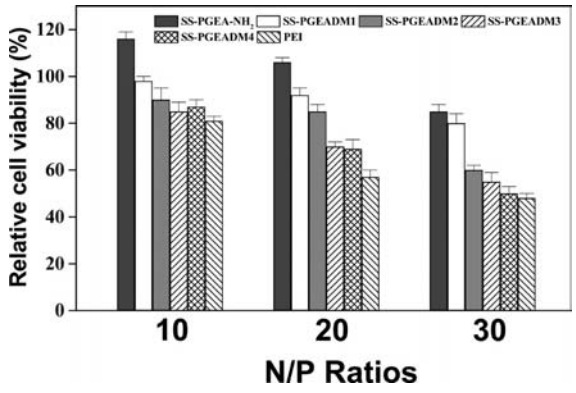

Figure 10. Cell viability of polymer/pDNA complexes at different N/P ratios in HEK293 cell line.

of SS-PGEAPDMs could be controlled by adjusting the length of the PDMEAMA side chains.

In Vitro Gene Transfection Assay. The in vitro gene transfection efficiency of the cationic polymers/pDNA complexes was assessed using luciferase as a gene reporter in HEK293, COS7, and L929 cell lines in the complete serum media. Figure 11 shows the gene transfection efficiency of SS-PGEA-NH_2 and SS-PGEADMs at various N/P ratios in comparison with those of PEI at a N/P ratio of 10, at which branched PEI (25 kDa) usually exhibits the highest transfection efficiency.[13,14] The transfection efficiency generally first increases at lower N/P ratios and then decreases slightly with the increase in N/P ratios. At lower N/P ratios, pDNA cannot be condensed efficiently by the polymers, and the resultant loose polymer/pDNA complex cannot enter the cell easily. At higher N/P ratios, the transfection formulation contains also free polymer, besides the compact and positively charged polymer/pDNA complexes. Due to the presence of an increasing amount of free cationic polymers with the increase in N/P ratios, the increasing cytotoxicity may result in a reduction in the transfection efficiency. Similar to PEGA reported earlier,[13] at the optimal N/P ratio, SS-PGEA-NH_2 exhibited transfection efficiency higher than or comparable to that of 'gold standard' PEI (25 kDa) in the different cell lines. With the increase in the side chain length of SS-PGEADMs, their optimal transfection efficiency generally increases in the three cell lines. The increase in the transfection capability generally follows the order SS-PGEA-NH_2 < SS-PGEADM1 < SS-PGEADM2 < SS-PGEADM3/SS-PGEADM4, with no further obvious enhancements in SS-PGEADM4. This observation also indicated that the transfection efficiency mediated by SS-PGEA-NH_2 could be further increased by introducing the moderate tertiary amine groups. The SS-PGEADMs were composed of PGEA-derivative backbones and disulfide-linked low-molecular-weight P(DMAEMA) side chains. As mentioned above, the disulfide bridge linkages were responsive to the reductive agent, making SS-PGEADM breakable (Figure 4 and Figure 8). Under intracellular reducible conditions, such responsiveness could lead the unstable complexes, which were readily decondensed to greatly facilitate pDNA release from the complexes and benefit the resultant gene expression.

■ CONCLUSIONS

The novel comb-shaped polymeric vectors (SS-PGEADMs) composed of PGEA-derivative backbones and disulfide-linked

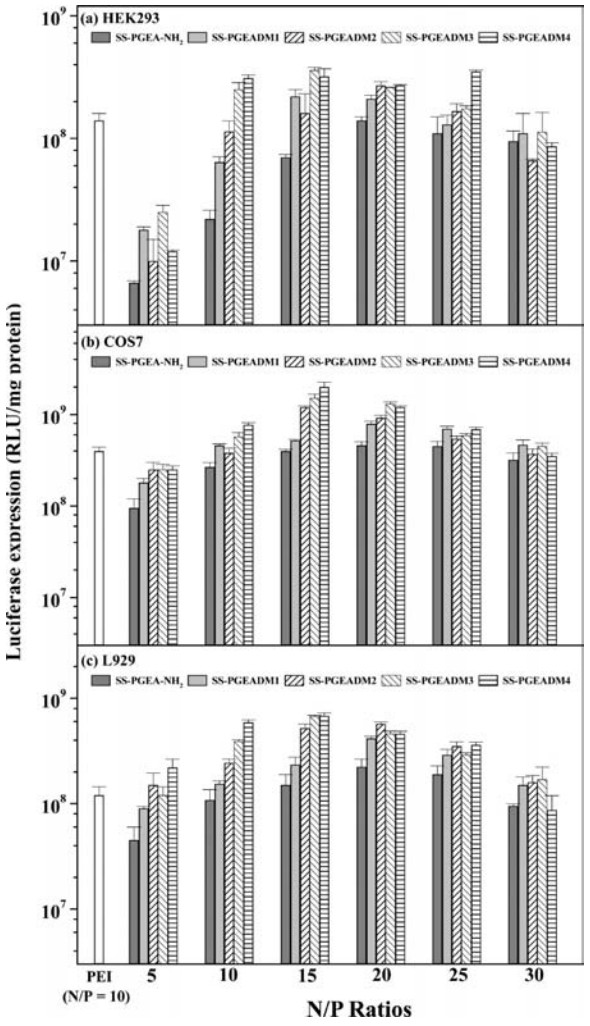

Figure 11. In vitro gene transfection efficiency of the cationic polymers (SS-PGEA-NH_2, SS-PGEADM1, SS-PGEADM2, SS-PGEADM3, and SS-PGEADM4)/pDNA complexes at various N/P ratios in comparison with that of PEI (25 kDa) in (a) HEK293, (b) COS7, and (c) L929 cells. The optimal transfection efficiency of PEI was obtained at the N/P ratio of 10.

PDMEAMA side chains were prepared by a combination of the ring-opening reaction and ATRP. The SS-PGEA-NH_2 backbones from the ring-opening reaction of PGMA with the amine moieties of ethanolamine/cystamine possess plentiful flanking secondary amine and hydroxyl groups and some flanking disulfide bond-containing cystamine derivatives. The disulfide-linked PDMEAMA side chains from ATRP possess pendant tertiary amine groups and could be biocleavable from the backbones. Such SS-PGEADMs with flanking multifunctional groups can effectively interact with pDNA. The resultant complexes were responsive to the reductive agent, facilitated pDNA release, and exhibited the enhanced gene transfection efficiencies in different cell lines. The disulfide-linked SS-PGEADM carriers having pendant multifunctional groups are potentially useful as efficient nonviral gene vectors for future gene therapy.

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Notes

The authors declare no competing financial interest.

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