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Novel Biodegradable Poly(disulfide amine)s for Gene Delivery with High Efficiency and Low Cytotoxicity

Mei Ou[†], **Xu-Li Wang**[‡], **Rongzuo Xu**[‡], **Chien-Wen Chang**[†], **David A. Bull**[§], and **Sung Wan Kim**[*,†]

Center for Controlled Chemical Delivery (CCCD), Department of Pharmaceutics and Pharmaceutical Chemistry, 421 Wakara Way, Suite 318, University of Utah, Salt Lake City, Utah 84112, and Division of Cardiothoracic Surgery, Room 3C127, School of Medicine, 30N 1900E, University of Utah, Salt Lake City, Utah 84132

Abstract

Novel biodegradable poly(disulfide amine)s with defined structure, high transfection efficiency, and low cytotoxicity were designed and synthesized as nonviral gene delivery carriers. Michael addition between *N*,*N*′-cystaminebisacrylamide (CBA) and three *N*-Boc protected diamines (*N*-Boc-1,2-diaminoethane, *N*-Boc-1,4-diaminobutane, and *N*-Boc-1,6-diaminohexane) followed by *N*-Boc deprotection under acidic condition resulted in final cationic polymers with disulfide bonds, tertiary amine groups in main chains, and pendant primary amine groups in side chains. Polymer structures were confirmed by ^{1}H NMR, and their molecular weights were in the range 3.3–4.7 kDa with narrow polydispersity (1.12–1.17) as determined by size exclusion chromatography (SEC). Acid–base titration assay showed that the poly(disulfide amine)s possessed superior buffering capacity to branched PEI 25 kDa in the pH range 7.4–5.1, which may facilitate the escape of DNA from the endosomal compartment. Gel retardation assay demonstrated that significant polyplex dissociation was observed in the presence of 5.0 mM DTT within 1 h, suggesting rapid DNA release in the reduction condition such as cytoplasm due to the cleavage of disulfide bonds. Genetic transfections mediated by these poly(disulfide amine)s were side-chain spacer length dependent. The poly (disulfide amine) with a hexaethylene spacer, poly(CBA-DAH), had comparable transfection efficiency to bPEI 25 kDa in the tested cell lines, i.e., 293T cells, Hela cells, and NIH3T3 cells. This same poly(disulfide amine) mediated 7-fold higher luciferase expression than bPEI 25 kDa in C2C12 cells (mouse myoblast cell line), a cell line difficult to transfect with many cationic polymers. Furthermore, MTT assay indicated that all three poly(disulfide amine)s/pDNA polyplexes were significantly less toxic than bPEI/pDNA complexes.

INTRODUCTION

Gene therapy has broad potential in the treatment of human genetic and acquired diseases through the delivery and application of therapeutic gene-based drugs. The use of safe, efficient, and controllable gene carriers is a requirement for the success of clinical gene therapy (1,2). Although viral vectors are very efficient in gene delivery, their potential safety and

*Correspondence to Dr. Sung Wan Kim, Center for Controlled Chemical Delivery (CCCD), Department of Pharmaceutics and Pharmaceutical Chemistry, University of Utah, Salt Lake City, Utah 84112, Phone: 801 581-6654, Fax: 801 581-7848, rburns@pharm.utah.edu.
[†]CCCD.
[‡]Department of Pharmaceutics and Pharmaceutical Chemistry.
[§]School of Medicine.

Supporting Information Available: FPLC data, ^{1}H NMR spectra, and experimental procedures. This information is available free of charge via the Internet at http://pubs.acs.org.

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immunogenicity concerns raise their risk in clinical applications (3). As an alternative to viral vectors, cationic polymers such as poly(L-lysine) (PLL), poly(ethylenimine) (PEI), poly (amidoamine)s dendrimers, and cationic liposomes have been synthesized as gene delivery carriers. The advantages of these cationic polymer carriers include safety, stability, large DNA and RNA loading capacity, and easy and large-scale production (4–6). The cationic polymers can condense negatively charged DNA into nanosized particles through electrostatic interactions, and the polymer/pDNA polyplexes can enter cells via endocytosis (7–9). As a result, the polymers can protect pDNA from nuclease degradation and facilitate cellular uptake to induce high gene transfection (10,11).

The currently available cationic polymers, however, have significant cytotoxicity concerns, mostly due to their poor biocompatibility and nondegradability under physical conditions. Consequently, the development of hydrolyzable cationic polymers was proposed, such as poly (β-amino ester)s (12–14), poly(amino ester)s (15,16), and poly(amido amine)s (17,18). For example, the library of poly(β-amino ester)s, containing ester bonds in structures, have been reported to improve biodegradability and transfection efficiency and decrease cytotoxicity (19,20). However, their degradation via hydrolysis or enzymatic cleavage is relatively slow in both extracellular oxidizing and intracellular reducing environments. At this point, disulfide bonds are preferred in the design and synthesis of biodegradable polymers. First, disulfide bonds are more hydrolytically stable than ester bonds in the extracellular environment, so the polycations with disulfide bonds can be used to prepare stable complexes with plasmid DNA. Second, disulfide bonds can be cleaved rapidly by glutathione and thioredoxin reductases in cytoplasm. As a result, DNA can be rapidly released from polyplexes to mediate efficient gene expression. Also, cytotoxicity will be decreased by avoiding high charge density and long-term polymer accumulation (21). Lin and co-workers have recently synthesized various linear poly (amido amine)s containing repetitive disulfide linkages in structures, which were proven to have significantly increased transfection efficiency and decreased cytotoxicity in COS-7 cells (22). However, poly(amido amine)s incorporated only tertiary amine groups, hydroxyl groups, and/or imidazole groups. The tertiary amine groups have limited DNA binding affinity due to steric hindrance, while hydroxyl and imidazole groups contribute little to binding of DNA. Consequently, transfection efficiencies would be decreased by their reduced ability to complex DNA. Therefore, we proposed to incorporate primary or secondary amine groups in such biodegradable polymers to increase nucleic acid binding affinity and following transfection efficiency. Our laboratory has previously synthesized a new form of poly(amido ethylenimine) s containing disulfide bonds and secondary amine groups in their structures (23). Those polymers have been proven to have significant transfection efficiency and low cytotoxicity for both DNA and siRNA delivery (23,24). During the synthesis, however, these polymers will form branches randomly, resulting in significant structural cross-linking and therefore poor solubility in water and some organic solvents.

In this paper, we propose to synthesize and evaluate poly-(disulfide amine)s, a novel series of linear cationic polymers with many of the characteristics of ideal polymeric gene delivery carriers that can mediate high gene transfection with low cytotoxicity. The advantages of these polymers are as follows: (1) Defined and improved polymer structures. Prepared by Michael addition and *N*-Boc deprotection under acidic condition, these polymers contain disulfide bonds, tertiary amine groups, and pendant primary amine groups in structures, and they do not form uncontrollable branches and cross-linking in synthesis. These structures aim to meet the fundamental design criteria of good gene carriers: reasonable biodegradability, strong DNA condensation ability, efficient gene transfection and low cytotoxicity. (2) Biodegradability. Poly(disulfide amine)s contain disulfide bonds in main chain, and are relatively stable in the extracellular oxidizing environment while being rapidly degraded in the intracellular reducing environment. Therefore, genetic materials in polyplexes will be released efficiently in the cytoplasm to allow for efficient gene expression. Meanwhile, cytotoxicity will decrease due

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to polymer degradation. (3) High nucleic acid binding affinity. Introducing unique primary amine side groups into poly(disulfide amine)s improves water solubility and enhances positive charge density. This allows plasmid DNA and other genetic materials, such as antisense oligonucleotides, peptide nucleic acids, and siRNA, to be stably condensed into nanosized particles under physiological pH, which will contribute to endocytosis and consequently efficient gene transfection. (4) High buffering capacity. The combination of tertiary and primary amine groups in poly(disulfide amine)s can promote endosomal–lysosomal escape based on the "proton sponge hypothesis". This characteristic gives poly(disulfide amine)s great potential in gene delivery. In this study, we synthesized poly(disulfide amine)s via Michael addition and *N*-Boc deprotection. Polymers were characterized by ^{1}H NMR, SEC, and acid–base titration. The properties of polymer/pDNA complexes were studied by dynamic light scattering and gel electrophoresis. *In vitro* transfection as well as *in vitro* cytotoxicty of polymer/pDNA complexes were evaluated by luciferase assay, BCA protein assay, and MTT assay using 293T cells (human renal epithelial cell line), Hela cells (human cervical cancer cell line), NIH3T3 (mouse embryonic fibroblasts), and C2C12 cells (mouse myoblast cell line).

MATERIALS AND METHODS

1. Materials

tert-Butyl-*N*-(2-aminoethyl)carbamate (*N*-Boc-1,2-diaminoethane, *N*-Boc-DAE), *tert*-butyl-*N*-(4-aminobutyl) carbamate (*N*-Boc-1,4-diaminobutane, *N*-Boc-DAB), *tert*-butyl-*N*-(6-aminohexyl)carbamate (*N*-Boc-1,6-diaminohexane, *N*-Boc-DAH), hyperbranched polyethylenimine (bPEI, M_w = 25 kDa), trifluoroacetic acid (TFA), triisobutylsilane (TIS), dithiothreitol (DTT), ethidium bromide (EtBr), and 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) were purchased from Sigma-Aldrich (St. Louis, MO). *N*,*N′*-Cystaminebisacrylamide (CBA) was purchased from PolySciences, Inc. (Warrington, PA). The plasmid pCMV-Luc, containing a firefly luciferase reporter gene which was inserted into a pCI plasmid vector driven by the CMV promoter (Promega, Madison, WI), was amplified in *E. coli* DH5α and isolated by standard Maxiprep kit (Invitrogen, Carlsbad, CA) according to manufacture's instruction. Dulbecco's Modified Eagle's Medium (DMEM), penicillin–streptomycin, fetal bovine serum (FBS), trypsin-like enzyme (TrypLE Express), and Dulbecco's phosphate buffered saline (PBS) were purchased from Invitrogen-Gibco (Carlsbad, CA). Luciferase assay system with reporter lysis buffer was purchased from Promega (Madison, WI). All materials and solvents were used as received without further purification.

2. Polymer Synthesis

The synthetic route of poly(disulfide amine)s was illustrated in Scheme 1. Here, the synthesis of poly(CBA-DAE) is described as the representative procedure. Briefly, *N*-Boc-DAE (0.160 g, 1 mmol) and CBA (0.260 g, 1 mmol) were added into a flask and dissolved in 1 mL MeOH/H_2O mixture (9/1 v/v). Polymerization was conducted in an oil bath at 60 °C in the dark under nitrogen atmosphere for 4 days. Then, 10% mol excess *N*-Boc-DAE was added into the reaction solution to consume any unreacted acrylamide functional groups, and the reaction was performed at 60 °C for at least additional 2 h. After that, the product was precipitated with 40 mL anhydrous diethyl ether and dried. Subsequently, the acid-liable *N*-Boc protection group was removed with a TFA/TIS/H_2O mixture (95/2.5/2.5 v/v) for 30 min. The crude was precipitated in 40 mL anhydrous diethyl ether and dried under vacuum. It was further purified by dialysis (MWCO = 1000) against MilliQ deionized water overnight, followed by lyophilization to obtain poly(CBA-DAE) as a solid powder. Poly(CBA-DAB) and poly(CBA-DAH) were synthesized similarly, except the polymerization took 3 days for them. The poly (disulfide amine)s were analyzed by ^{1}H NMR (400 MHz, D_2O) and the data are listed below.

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Poly(CBA-DAE): 2.91 ($NCH_2CH_2NH_2$, 2H), 2.64 (NCH_2 CH_2NH_2, 2H), 2.63 (NCH_2CH_2CO, 4H), 2.22 (NCH_2CH_2CO, 4H), 3.34 ($CONHCH_2CH_2SS$, 4H), 2.62 (CH_2SSCH_2, 4H).

Poly(CBA-DAB): 3.08 ($NCH_2CH_2CH_2CH_2NH_2$, 2H), 1.58 ($NCH_2CH_2CH_2CH_2NH_2$, 2H), 1.58 ($NCH_2CH_2CH_2CH_2NH_2$, 2H), 2.91 ($NCH_2CH_2CH_2CH_2NH_2$, 2H), 2.82 (NCH_2CH_2CO, 4H), 2.48 (NCH_2CH_2CO, 4H), 3.38 ($CONHCH_2CH_2SS$, 4H), 2.63 (CH_2SSCH_2, 4H).

Poly(CBA-DAH): 3.15 ($NCH_2CH_2CH_2CH_2CH_2CH_2NH_2$, 2H), 1.48 ($NCH_2CH_2CH_2CH_2CH_2CH_2NH_2$, 2H), 1.19 ($NCH_2CH_2$ $CH_2CH_2CH_2CH_2NH_2$, 2H), 1.19 ($NCH_2CH_2CH_2CH_2CH_2CH_2NH_2$, 2H), 1.48 ($NCH_2CH_2CH_2CH_2CH_2CH_2NH_2$, 2H), 2.85 ($NCH_2CH_2CH_2CH_2CH_2CH_2NH_2$, 2H), 2.81 ($NCH_2CH_2CO$, 4H), 2.52 ($NCH_2CH_2CO$, 4H), 3.35 ($CONHCH_2CH_2SS$, 4H), 2.65 ($CH_2SSCH_2$, 4H). The ^1H NMR spectra of poly(disulfide amine)s are given in the Supporting Information.

3. Polymer Characterization

The molecular weights of the polymers were determined by size exclusion chromatography (SEC) on an AKTA FPLC system (Amersham Biosciences, Piscataway, NJ) equipped with a Superose 12 column, and UV and refractive index detectors, eluted with Tris buffer (20 mM, pH 7.4) at a rate of 0.5 mL/min. Molecular weights were calibrated with standard poly[*N*-(2-hydroxypropyl)methacrylamide] (pHPMA). The FPLC data of polymers are given in the Supporting Information.

4. Buffering Capacity

The buffering capacities of the poly(disulfide amine)s were determined by acid–base titration. Briefly, 10 mL polymer solution was adjusted initially to pH 11.0 by 0.1 M NaOH. Then, the basic polymer solutions were titrated to pH 3.0 with aliquots of 0.01 M HCl. pH values of the solutions were measured after each addition. The buffering capacity is defined as the percentage of amine groups becoming protonated from pH 7.4 to 5.1 and can be calculated from the following equation (22):

$$\text{Buffering capacity }(\%)=\Delta V_{\text{HCl}}\times 0.01M/N\text{ mol}\times 100\%$$

Here, ΔV_{HCl} is the volume of 0.01 M HCl solution which brought the pH value of the polymer solution from 7.4 to 5.1, and *N* mol is the total moles of amine groups in the known amount of poly(disulfide amine)s.

5. Particle Size Measurement

Polyplexes were prepared by vortexing 1 μg pDNA (25 μL, 40 μg/mL) solution with an equal volume of polymer solution at predetermined nitrogen/phosphate (N/P) ratios, followed by 30 min incubation. The polyplexes were then diluted in 2 mL of dustfree diH_2O and the average particle sizes of polyplexes were measured using a BI-200SM dynamic light scattering instrument (DLS, Brookhaven Instrument Corporation, Holtsville, NY) at a 633 nm incident beam. Measurements were made at 25 °C at an angle of 90°. Measurements for each sample were repeated three times and reported as mean values ± standard deviations.

6. Gel Retardation Assay

Agarose gel (1%, w/v) containing 0.5 μg/mL ethidium bromide (EtBr) was prepared in TAE (Tris–acetate–EDTA) buffer. Poly(disulfide amine)/DNA complexes at predetermined N/P ratios were prepared in HEPES buffer as described above. The samples were mixed with 6 × loading dye and the mixtures were loaded onto an agarose gel. The gel was run at 100 V for

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30 min and the location of DNA bands was visualized with a UV illuminator using a Gel Documentation Systems (Bio-Rad, Hercules, CA). The DNA release from poly(disulfide amine)s/DNA polyplexes was evaluated by incubating polyplexes with 5 mM DTT at 37 °C for 1 h. The samples were then analyzed by gel electrophoresis as described above.

7. *In Vitro* Transfection

Synthetic poly(disulfide amine)s mediated transfection was evaluated on 293T cells (human renal epithelial cell line, ATCC), HeLa cells (human cervical cancer cell line, ATCC), NIH3T3 (mouse embryonic fibroblasts, ATCC), and C2C12 cells (mouse myoblast cell line, ATCC) by using the plasmid pCMV-Luc as reporter gene. Cells were maintained in DMEM containing 10% FBS, streptomycin (100 μg/mL), and penicillin (100 units/mL) at 37 °C in a humidified atmosphere with 5% CO_2. Cells were seeded 24 h prior to transfection in 24-well plates at initial density of 8.0×10^4, 4.0×10^4, 4.0×10^4, and 3.5×10^4 cells/well for 293T, HeLa, NIH3T3, and C2C12, respectively. DNA was complexed with the poly(CBA-DAE), poly (CBA-DAB), poly(CBA-DAH), and bPEI at predetermined N/P ratios in HEPES buffer and incubated for 30 min before use. At the time of transfection, the medium in each well was replaced with fresh serum-free medium. Polyplexes (0.5 μg DNA/well) were incubated with the cells for 4 h at 37 °C. The medium was then replaced with 500 μL of fresh complete medium, and cells were incubated for additional 44 h. The cells were then washed with prewarmed PBS, treated with 200 μL cell lysis buffer, and subjected to a freezing–thawing cycle. Cellular debris was removed by centrifugation at 14 000 *g* for 5 min. The luciferase activity in the cell lysate (25 μL) was measured using a luciferase assay kit (100 μL luciferase assay buffer) on a luminometer (Dynex Technologies Inc., Chantilly, VA). The relative luminescent unit (RLU) of luciferase expression was normalized against protein concentration in the cell extracts, measured by a BCA protein assay kit (Pierce, Rockford, IL). All transfection assays were carried out in triplicate.

8. Cytotoxicity

NIH3T3 cells were seeded in a 24-well plate at a density of 4.0×10^4 cells/well and incubated for 24 h. DNA was complexed with the poly(CBA-DAE), poly(CBA-DAB), poly(CBA-DAH), and bPEI at predetermined N/P ratios in HEPES buffer and incubated for 30 min before use. Polyplexes (0.5 μg DNA/well) were incubated with the cells for 4 h in serum-free medium followed by 20 h in complete medium. MTT solution (50 μL, 2 mg/mL) was then added, and cells were further incubated for 2 h. The medium was removed, and 300 μL DMSO was then added to each well. The absorption was measured at 570 nm using a microplate reader (model 680, Bio-Rad Laboratory, Hercules, CA). The percentage relative cell viability was determined relative to control (untreated) cells, which were not exposed to transfection system and taken as 100% cell viability. All cytotoxicity experiments were performed in triplicate.

RESULTS AND DISCUSSION

1. Synthesis and Characterization of Biodegradable Poly-(disulfide amine)s

Three novel biodegradable poly(disulfide amine)s were synthesized by Michael addition between *N*,*N*′-cystaminebisacrylamide (CBA) and three different *N*-Boc protected diamine monomers, *N*-Boc-DAE, *N*-Boc-DAB, and *N*-Boc-DAH. After removing *N*-Boc protection groups, three linear comblike polymers, poly(CBA-DAE), poly(CBA-DAB), and poly(CBA-DAH), were synthesized with one disulfide bond, one tertiary amine group in the main chain, and one pendant primary amine group in a side chain in each repeating unit (Scheme 1). All three poly(disulfide amine)s were purified by dialysis and lyophilized as a solid powder and were readily soluble in water, PBS buffer, HEPES buffer, Tris buffer, dimethyl sulfoxide (DMSO), and methanol, but not in chloroform, diethyl ether, and tetrahydrofuran. The final structures of poly(disulfide amine)s were confirmed by ^{1}H NMR (400 MHz, D_2O). The

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disappearance of signal peaks between δ 5 and 7 ppm indicated that the acrylamide end groups no longer existed in the final polymer products. Additionally, the two-step synthetic procedure avoided the possibility of forming branches or cross-links. These ^{1}H NMR results confirmed that our polymers have defined structures as expected and no branches were observed.

The molecular weight of polymers were measured by fast protein liquid chromatography (FPLC) and calibrated by pHPMA standards (Table 1). The range of the weight average molecular weight (M_w) of these polymers was from 3.34 to 4.72 kDa, while the range of the number average molecular weight (M_n) was from 2.85 to 4.23 kDa. The polydispersity index (PDI), ranging from 1.12 to 1.17, indicates that these poly(disulfide amine)s have a narrow molecular weight distribution.

Buffering capacity is an important factor for cationic gene carriers according to “proton sponge hypothesis” proposed by Behr et al. (10). It helps polymeric carriers to effectively compact and protect DNA after endocytosis and helps DNA escape from endosomes/lysosomes (25–27). Buffering capacities of poly(disulfide amine)s, measured by acid–base titration, were expressed as the percentage of amine groups being protonated from pH 7.4 to 5.1, mimicking the change from the high pH extracellular environment to the low pH endosomal environment (22,23,28). The results (Table 1 and Figure 1) show that all poly(disulfide amine)s have excellent buffering capacity, ranging from 52.61% to 61.20% protonation, which is much higher than the previously reported results of bPEI 25 kDa, 24% (22) and 13.5% (23). The high buffering capacities enable poly(disulfide amine)s to facilitate endosomal escape, contributing to an increase in gene transfection efficiency.

There are several attributes of linear poly(disulfide amine)s that make them particularly attractive for the development of new polymeric gene carriers: (1) the polymers contain disulfide bonds for fast degradation; (2) primary and tertiary amine groups can self-assemble with DNA, facilitating endosomal escape and release of DNA to the nucleus efficiently; (3) the presence of primary amine groups at each repeating units for high nucleic acid binding affinity and good water solubility; (4) the capacity to synthesize a variety of analogues directly from commercial available monomer materials; (5) amine concentration can be evaluated for more accurate and efficient gene transfection for the future library of poly(disulfide amine)s.

2. Characterization of Polymer/Plasmid DNA Complexes

To mediate endocytosis through cell membrane, cationic polymers need to condense DNA into nanosized particles via electrostatical interactions between the positively charged polymers and the negatively charged phosphates on DNA backbones (11,29). Dynamic light scattering (DLS) studies showed that three poly(disulfide amine)s can condense plasmid DNA into small particles with effective diameters of less than 300 nm at polymer/pDNA nitrogen/phosphate (N/P) ratios of 1:1 and above. In contrast, the diameters of bPEI/pDNA particles were larger at N/P ratios of 10:1 and 20:1 (336.5 and 484.5 nm) under the same conditions (Figure 2).

Gel retardation assay further verified that poly(disulfide amine)s can condense plasmid DNA at low N/P ratios. All poly(disulfide amine)s were dissolved in HEPES buffer solution (20 mM HEPES, pH 7.4, 5% glucose). One microgram samples of plasmid DNA (pCMV-Luc) with varying amounts of polymers were mixed and incubated at desired N/P ratios followed by performing agarose gel electrophoresis stained with EtBr (Figure 3). In the absence of DTT (Figure 3A), poly(CBA-DAE), poly(CBA-DAB), and poly(CBA-DAH) can completely retard plasmid DNA migration from N/P ratios of 5:1, 3:1, and 3:1, respectively. When the polyplexes were incubated with 5.0 mM DTT at 37 °C for 1 h, mimicking the intracellular reducing environment containing 0.1–10 mM glutathione, pDNA was released from all three poly (disulfide amine)s at all N/P ratios, with bands migrating toward the positive electrode in gel electrophoresis (Figure 3B). For the nondegradable control polymer bPEI 25 kDa, there was

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no pDNA released from bPEI/pDNA complexes in the presence of DTT. This gel retardation assay proved that all three poly(disulfide amine)s can release pDNA efficiently from polyplexes via disulfide bond cleavage, leading to increased DNA release and increased gene expression.

To facilitate efficient gene expression, cationic polymers should not only strongly condense plasmid DNA extracellularly, but also efficiently release DNA from polyplexes intracellulary. Previously, the hydrolyzable polymers, such as poly(β-amino amine)s and poly(amido amine)s, were synthesized by one-step Michael addition and only contained tertiary amines, hydroxyl, and/or imidazole groups (12,22). The tertiary amine groups have limited DNA binding affinity due to steric hindrance, while hydroxyl and imidazole groups contribute little in binding DNA. As a result, relatively high N/P ratios were required to completely condense DNA. For example, to retard DNA migration in gel electrophoresis, weight ratios equal to or higher than 40:1 were needed for poly(β-amino amine)s (20). Similarly, weight ratios of 24:1 or higher are required for poly(amido amine)s, such as pAPOL (22). For the poly(disulfide amine)s, on the contrary, the results of the gel retardation assay showed that they can form stable complexes with pDNA at N/P ratios as low as 3:1, suggesting that poly(disulfide amine)s with primary amines have stronger nucleic acid binding affinities than those hydrolyzable polycations as mentioned above. In addition, pendant primary amine groups are more nucleophilic than tertiary amine groups, which will facilitate more efficient gene transfection and expression (19). It is also well-known that disulfide bonds can be cleaved rapidly in the presence of a high intracellular concentration of glutathione and thioredoxin reductases. This rapid cleavage of disulfide bonds will ensure DNA release from complexes efficiently to facilitate nuclear import, gene transcription, and gene expression (22,23,30,31). In the presence of the reducing agent DTT, the cleavage of disulfide bonds of polymers was expected to dissociate polyplexes and release free DNA which were monitored by the agarose gel electrophoresis. Our poly(disulfide amine)s also showed the ability of rapid cleavage in the presence of DTT, mimicking the intracellular reducing environment. In summary, poly(disulfide amine)s demonstrated strong DNA condensing abilities by forming nanosized particles at low N/P ratios. They also showed rapid DNA releasing abilities by rapid disulfide bond cleavage in a reducing environment, which will facilitate the following gene expression (32–35).

3. *In Vitro* Transfection and Cytotoxicity of Poly(disulfide amine)s/Plasmid DNA Complexes

To evaluate *in vitro* transfection efficiency of biodegradable poly(disulfide amine)s, their complexes with reporter gene pCMV-Luc (0.5 μg/well) expressing luciferase were produced on four different cell lines, 293T, HeLa, NIH3T3, and C2C12, at five N/P ratios ranging from 5:1 to 80:1 in the absence of serum. bPEI (25 kDa)/pDNA complexes at an N/P ratio of 20:1 was used as a positive control. At this N/P ratio, bPEI showed the highest gene transfection efficiency while maintaining at least 70% cell viability. The transfection efficiency was quantitatively measured as the luciferase enzyme activity and normalized as the total cell protein concentration by BCA protein assay (Figure 4). Among these poly(disulfide amine)s, poly(CBA-DAH) showed the highest level of gene expression in all four cell lines. In 293T, HeLa and NIH3T3 cell lines, poly(CBA-DAH) had a comparable luciferase gene expression level to bPEI 25 kDa, at varying N/P ratios from 5:1 to 80:1. Interestingly, poly(CBA-DAH) expressed up to 7-fold higher gene transfection efficiency than bPEI in the C2C12 cell line at all N/P ratios, which was statistically different. The other two polymers showed overall lower luciferase expression than poly(CBA-DAH), while poly-(CBA-DAB) was N/P ratio dependent for gene expression in these four cell lines, suggesting that optimal N/P ratios for gene transfection are different for these three polymers. Until now, many biodegradable polymers containing disulfide bonds have been reported to reduce cytotoxicity; however, most of them have less or only comparable transfection efficiency compared to bPEI 25 kDa (22,23,34,36, 37). These results of high transfection efficiency of poly(disulfide amine)s, especially poly

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(CBA-DAH), support our future studies, since the primary mouse myoblast cell line (C2C12) is generally a cell line which is difficult to transfect with cationic polymers.

It is well-established that polymers containing disulfide bonds have good stability in an oxidative extracellular environment while they are efficiently degraded in reducing intracellular environment. Previous studies indicated that the better dissociation of plasmid DNA from polymers with disulfide bonds will facilitate gene expression (30,38,39). This characteristic gives our poly(disulfide amine)s a good ability to unpack DNA in the cells and improve transfection efficiency. Additionally, the primary amines are introduced into side chains of poly(disulfide amine)s, which can not only improve water solubility but is also expected to enhance stable complexation of plasmid DNA to mediate highly efficient gene delivery (31,40).

For the three poly(disulfide amine)s, the transfection efficiency sequence is poly(CBA-DAH) > poly(CBA-DAB) > poly(CBA-DAE). The main difference among the three polymers is their side chain lengths. Poly(CBA-DAH) has the longest alkyl side chain and may be more flexible and more hydrophobic than the other two polymers. It was observed previously that the polymers containing more hydrophobic side chains had higher transfection efficiency (22), which suggested that the side chains would influence gene delivery (20,22,31). While the exact mechanisms to explain and the precise methods to measure how side chain structures of polymers affect transfection are unknown, the more flexible and hydrophobic characteristics may assist pDNA complexation or some other unknown transfection steps (20). Some hypothesis indicated that the polymers with longer alkyl chains are more flexible and hydrophobic than stiffer polymers, which may contribute to more stable binding of nucleic acids (31,41). It is suggested that these more stable polyplexes will be distracted less by specific negatively charged cell surface proteins (proteoglycans), and therefore these polyplexes will remain intact during cellular endocytosis and consequently induce higher transfection efficiency (40,42).

In vitro cytotoxicity of poly(disulfide amine)s was evaluated by a standard MTT assay on NIH3T3 cells (Figure 5). The experiments were performed in the same manner as the transfection experiments described above, except that the MTT assay was performed at 24 h instead of 48 h post-transfection. As expected, poly(disulfide amine)s showed much lower cytotoxicity than bPEI 25 kDa. The overall profile in Figure 5 showed that bPEI 25 kDa has increasing cytotoxicity with increasing N/P ratios, while cell viability decreased to 7.7% at an N/P ratio of 80:1. In contrast, poly(disulfide amine)s showed no significant toxicity for cells even at an N/P ratio of 80:1, retaining 90% or higher cell viability relative to control cells (nontreated NIH3T3 cells). The results are consistent with the other three cell lines: 293T, HeLa, and C2C12. Reduced cytotoxicity is apparently a significant benefit of poly(disulfide amine)s, though only poly(CBA-DAH) showed superior transfection efficiency compared to bPEI 25 kDa. It suggested that the reduced toxicity is related to polymer degradation. bPEI 25 kDa would accumulate intracellularly, leading to undesirable toxicity. Introducing disulfide bonds into polymers would alleviate these concerns, because they will be degraded to nontoxic, small molecules through cleavage of disulfide bonds. Therefore, DNA would be released to mediate efficient gene transfection, and those low molecular weight fragments of polymers will be cleared easily from the body (30). Our transfection and cytotoxicity results of poly (disulfide amine)s accomplish the previous studies.

In summary, these novel poly(disulfide amine)s, especially poly(CBA-DAH), have high gene transfection efficiency and low cytotoxicity and great potential for gene delivery *in vitro*. From the above data, poly(CBA-DAH) has a significantly high gene transfection in primary mouse myoblast cell line (C2C12). This novel poly(disulfide amine) is a promising gene carrier in many other primary cells and stem cells. We have shown that poly(CBA-DAH) has high gene

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transfection efficiency on SVR cells (mouse pancreatic islet endothelial cells). Besides delivery plasmid DNA, poly(disulfide amine)s can be used as gene carriers to deliver other types of genetic material into human cells, such as antisense oligonucleotides, therapeutic genes, and small interfering RNA (siRNA). Furthermore, poly(disulfide amine)s can be modified with targeting moieties to specifically delivery genetic materials into certain cell types.

CONCLUSION

In this work, we have synthesized and investigated three novel linear and structurally defined poly(disulfide amine)s: poly-(CBA-DAE), poly(CBA-DAB), and poly(CBA-DAH). Those poly(disulfide amine)s are readily biodegraded in a reducing environment, releasing DNA rapidly due to the cleavage of disulfide bonds. They also have superior buffering capacity and nucleic acid binding ability based on acid–base titration and gel electrophoresis assay. In addition, all poly(disulfide amine)s showed much lower cytotoxicity than bPEI 25 kDa in NIH3T3 cells. The gene transfection efficiency of these polymers is side-chain spacer length dependent. Among the three polymeric carriers, poly(CBA-DAH), which contains a hexaethylene spacer, has the highest transfection efficiency in 293T cells, HeLa cells, NIH3T3 cells, and C2C12 cells. Specifically, it has a higher transfection efficiency than bPEI 25 kDa in C2C12 cells, a mouse primary myoblast cell line that is otherwise difficult to transfect. These poly(disulfide amine)s, especially poly(CBA-DAH), are promising for delivery of other genetic materials, such as therapeutic genes and siRNA. They also have potential in stem cell gene delivery. We are going to establish *in vivo* models using these poly(disulfide amine)s to further study their potential as gene carriers for gene delivery.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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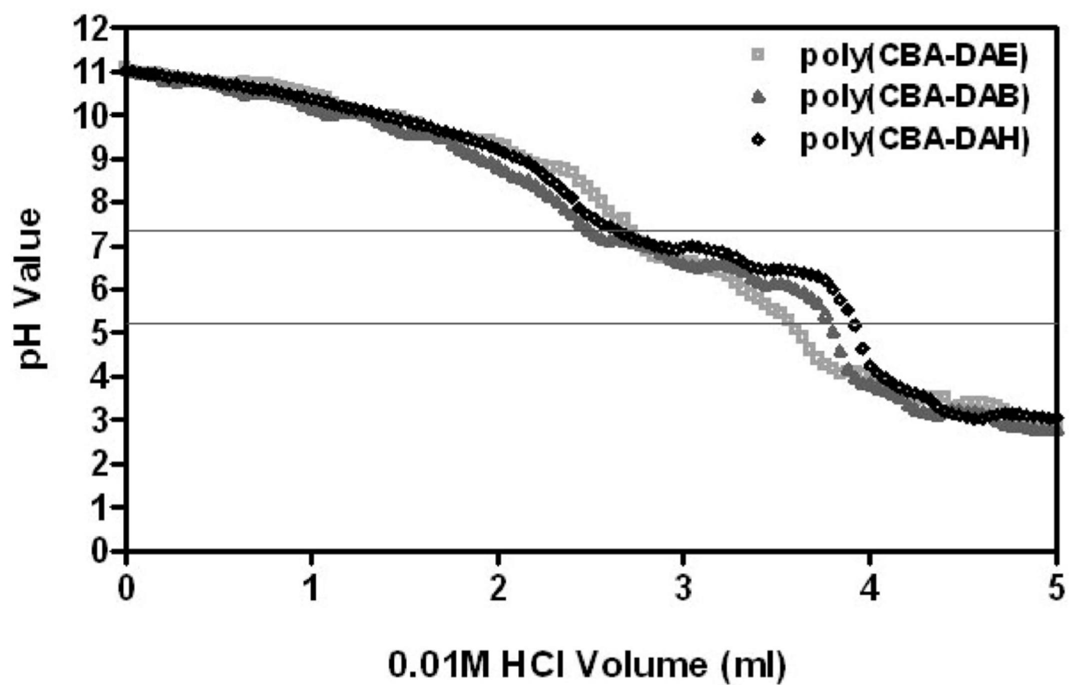

Figure 1.
Titration curves were obtained by titrating poly(disulfide amine)s aqueous solutions in 10 mL 0.1 M NaCl from pH 11.0 (initially adjusted by 0.1 M NaOH) to pH 3.0 using 0.01 M HCl. pH of the solutions was measured after each addition.

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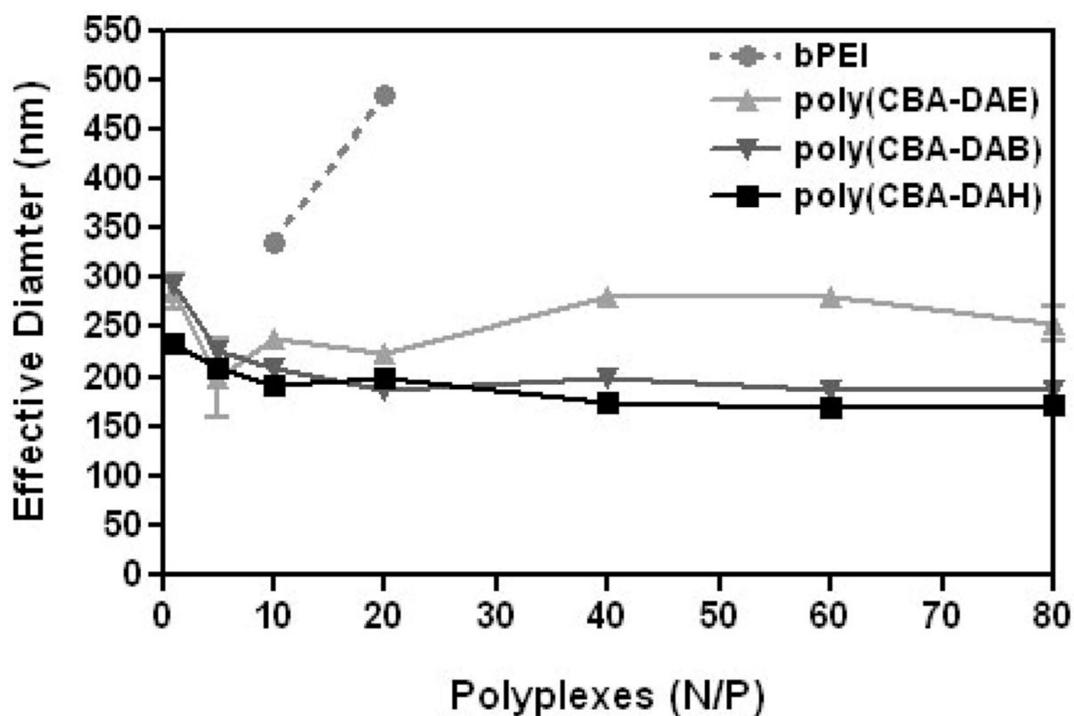

Figure 2.

Average particle sizes of poly(disulfide amine)s/pDNA complexes were measured at varying nitrogen/phosphate (N/P) ratios from 1:1 to 80:1, while bPEI (25 kDa)/pDNA complexes were measured at N/P ratios of 10:1 and 20:1. (●) bPEI; (▲) poly(CBA-DAE); (▼;) poly(CBA-DAB); (■) poly(CBA-DAH).

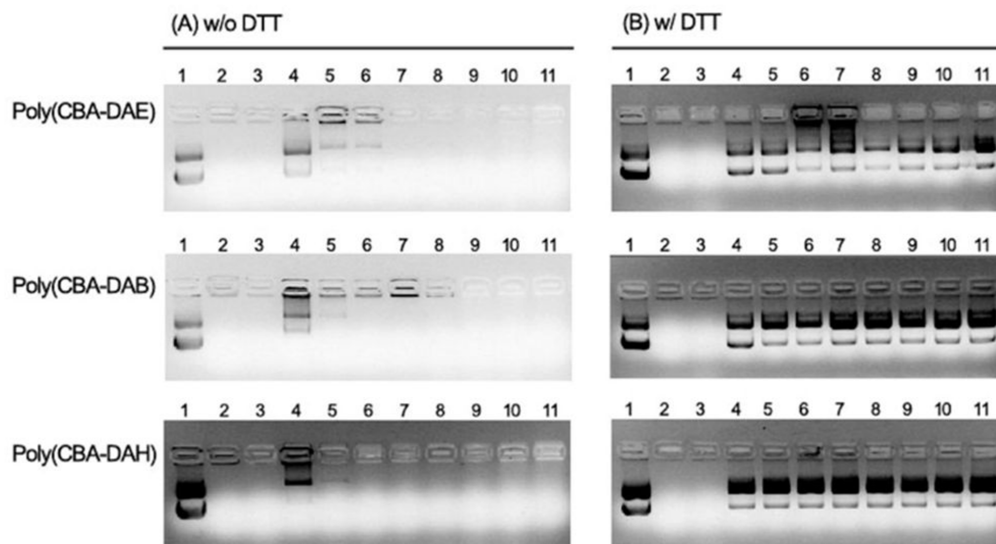

Figure 3.

Agarose gel electrophoresis of poly(CBA-DAE), poly(CBA-DAB), and poly(CBA-DAH) with plasmid DNA polyplexes at different nitrogen/phosphate (N/P) ratios at the conditions of (A) without DTT and (B) with 5.0 mM DTT incubation (37 °C, 1 h). Lane 1, naked pDNA; lanes 2 and 3, bPEI/pDNA at N/P ratios of 10:1 and 20:1; lanes 4–11, poly(disulfide amine)s/pDNA at N/P ratios of 1:1, 2:1, 3:1, 5:1, 10:1, 15:1, 20:1, and 40:1.

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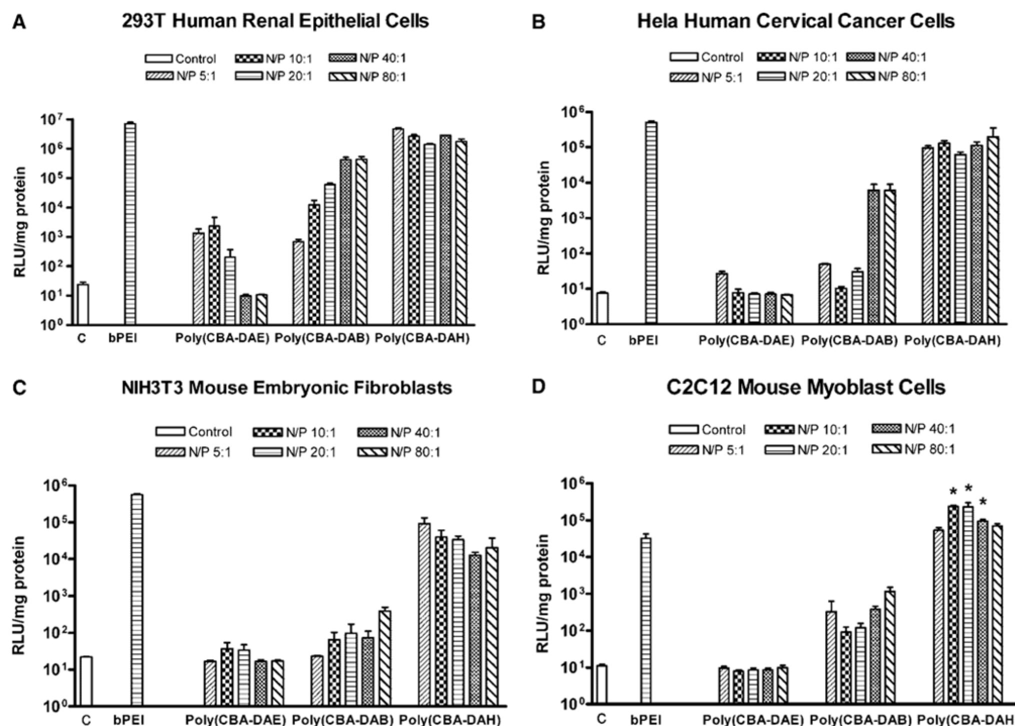

Figure 4.

Transfection efficiency of poly(disulfide amine)/pDNA polyplexes in four different cell lines at varying nitrogen/phosphate (N/P) ratios (0.5 μg pDNA/well). Negative control (C): nontreated cells group. Positive control: a bPEI 25 kDa group at N/P ratio of 20:1. The N/P ratios of poly(disulfide amine)/pDNA complexes were 5:1, 10:1, 20:1, 40:1, and 80:1, respectively. Transfection results are expressed in triplicate as the relative luminescent unit (RLU) of luciferase reporter gene expression normalized by the total cell protein content in each well as mean values ± standard deviations. (A) 293T cells: human renal epithelial cell line. (B) HeLa cells: human cervical cancer cell line. (C) NIH3T3: mouse embryonic fibroblasts cell line. (D) C2C12 cells: mouse myoblast cell line.

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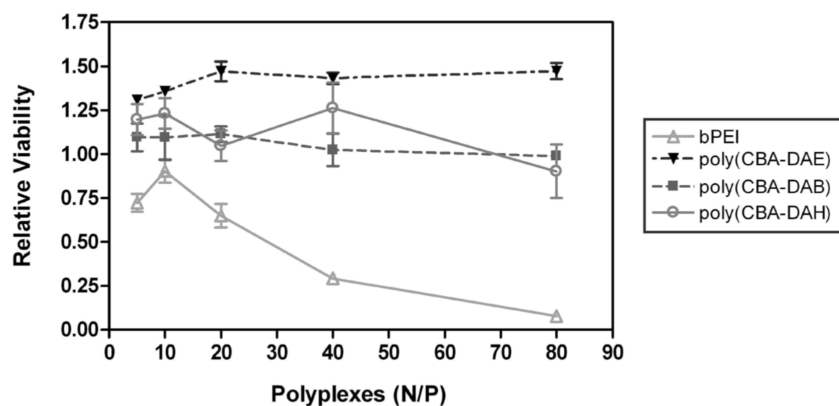

Figure 5.

Relative cell viabilities of poly(disulfide amine)s/pDNA polyplexes in NIH3T3 cells at varying nitrogen/phosphate (N/P) ratios compared to a nontreated control group and a bPEI 25 kDa treated group (0.5 μg pDNA/well): (△) bPEI, (▼;) poly(CBA-DAE), (■) poly(CBA-DAB), and (○) poly(CBA-DAH). Cytotoxicity was determined by MTT assay and represented triplicate as mean values ± standard deviations.

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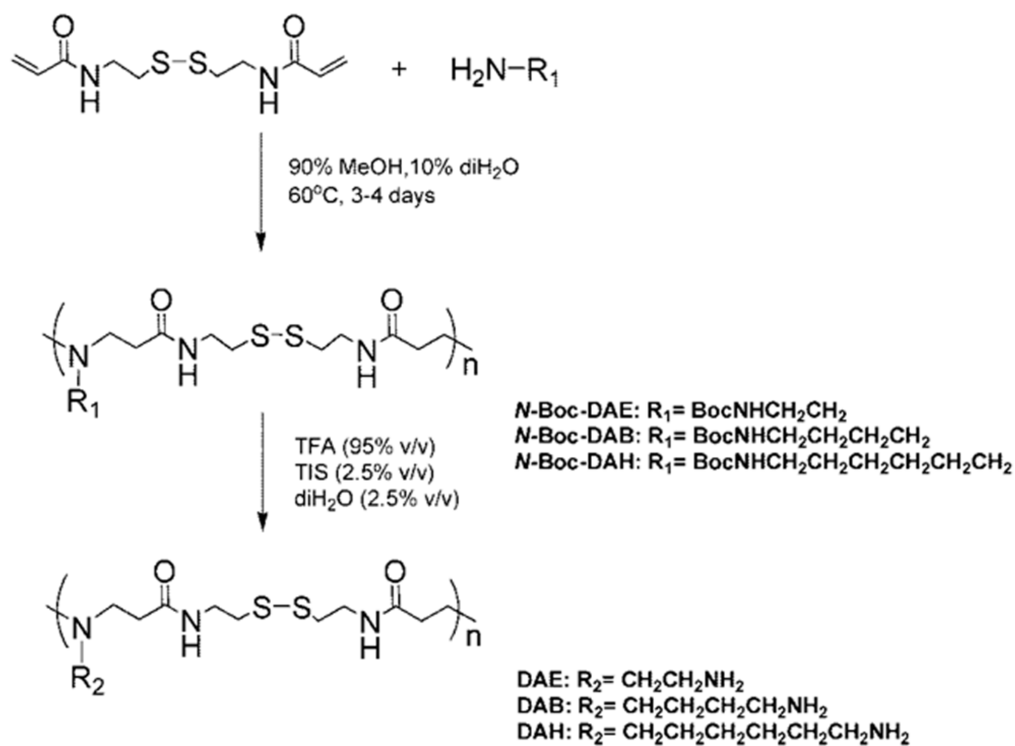

Scheme 1.
Synthesis of Biodegradable Poly(disulfide amine)s

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Table 1

Characteristics of Biodegradable Poly(disulfide amine)s[a]

polymers	M_n (kDa)	M_w (kDa)	PDI	buffering capacity in pH range 7.4–5.1 (%)
poly(CBA-DAE)	2.85	3.34	1.17	52.61
poly(CBA-DAB)	4.23	4.72	1.12	61.20
poly(CBA-DAH)	3.12	3.52	1.13	55.65

[a]FPLC conditions: poly(disulfide amine)s are dissolved in 0.5 mL Tris buffer (pH 7.4) as a concentration of 25 mg/mL. Superose 12 column is used to measure the molecular weight and polydispersity. Flow rate is 0.5 mL/min. Standard calibration is calculated using poly[*N*-(2-hydroxypropyl) methacrylamide] (pHPMA). Acid–base titration conditions: 10 mL of polymer solutions in 0.1 M NaCl was titrated with 0.01 M HCl from pH 11.0 to 3.0.