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Branched Polyethylenimine Derivatives with Reductively Cleavable Periphery for Safe and Efficient In Vitro Gene Transfer

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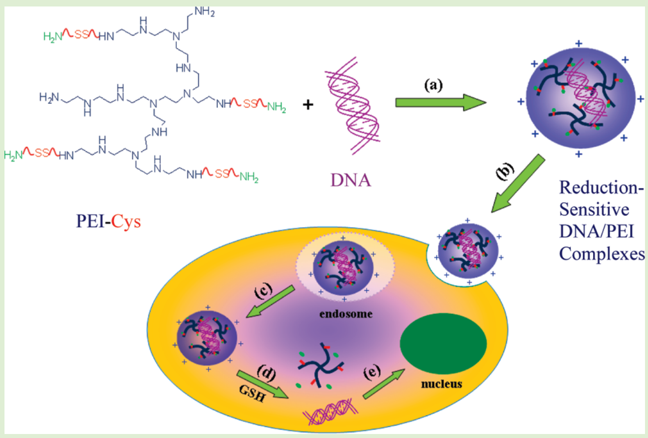
S *Supporting Information*



ABSTRACT: Twenty-five kDa polyethylenimine (PEI) is one of the most efficient nonviral gene transfer agents currently applied as a golden standard for in vitro transfection. In this study, novel 25 kDa PEI derivatives with reductively cleavable cystamine periphery (PEI-Cys) were designed to reduce carrier-associated cytotoxicity and to enhance further the transfection activity. The Michael-type conjugate addition of 25 kDa PEI with *N-tert*-butoxycarbonyl-*N*′-acryloyl-cystamine (Ac-Cys-^{t}Boc) and N-*tert*-butoxycarbonyl-*N*′-methacryloyl-cystamine (MAc-Cys-^{t}Boc) followed by deprotection readily afforded PEI-Cys derivatives, denoted as PEI-(Cys)x(Ac) and PEI-(Cys)x(MAc), with degree of substitution (DS) ranging from 14 to 34 and 13 to 38, respectively. All PEI-Cys derivatives had higher buffer capacity than the parent 25 kDa PEI (21.2 to 23.1% versus 15.1%). Gel retardation and ethidium bromide exclusion assays showed that cystamine modification resulted in largely enhanced interactions with DNA. PEI-(Cys)x(Ac) could condense DNA into small-sized particles of 80−90 nm at and above an N/P ratio of 5/1, which were smaller than polyplexes of 25 kDa PEI (100−130 nm). In comparison, PEI-(Cys)x(MAc) condensed DNA into somewhat larger particles (100−180 nm at N/P ratios from 30/1 to 5/1). Gel retardation and dynamic light scattering (DLS) measurements showed that PEI-Cys polyplexes were quickly unpacked to release DNA in response to 10 mM dithiothreitol (DTT). These PEI-Cys derivatives revealed markedly decreased cytotoxicity as compared with 25 kDa PEI with IC_{50} values of >100 mg/L and 50−75 mg/L for HeLa and 293T cells, respectively (corresponding IC_{50} data of 25 kDa PEI are ca. 11 and 3 mg/L). The in vitro transfection experiments in HeLa and 293T cells using pGL3 as a reporter gene showed that gene transfection activity of PEI-Cys derivatives decreased with increasing DS and PEI-(Cys)x(MAc) exhibited higher transfection activity than PEI-(Cys)x(Ac) at similar DS. Notably, polyplexes of PEI-(Cys)14(Ac) and PEI-(Cys)13(MAc) showed significantly enhanced gene transfection efficiency (up to 4.1-fold) as compared with 25 kDa PEI formulation at an N/P ratio of 10/1 in both serum-free and 10% serum-containing conditions. The modification of PEI with reductively cleavable periphery appears to be a potential approach to develop safer and more efficient nonviral gene vectors.

INTRODUCTION

In the past decade, cationic polymers as safer alternatives to viral vectors have received enormous attention for gene delivery.[1,2] The representing polymer is polyethylenimine (PEI), which has shown good transfection activity in different types of cells because of its unique combination of high charge density and endosomal pH buffer capacity.[3−5] The transfection performance of PEI depends on its macromolecular structures and molecular weights, in which 25 kDa branched PEI (in the following denoted as 25 kDa PEI) and 22 kDa linear PEI have turned out to be the best and are currently applied as golden standards for nonviral gene transfection.[6] These PEI reagents are, nevertheless, associated with varying levels of cytotoxicity. Moreover, as compared with viral vectors, the transfection activity of PEI remains moderate.

In recent years, different approaches have been actively explored to create better PEI-based gene carriers.[7] For example, on the basis of the fact that low-molecular-weight (LMW) PEIs have low cytotoxicity, various types of hydrolytically or reductively degradable PEI polymers and networks have been designed and investigated for the in vitro gene transfection.[8] In particular, reductively degradable polycations have appeared to be highly

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promising for the development of safe and efficient gene carriers.[9,10] Unlike hydrolytically degradable cationic polymers that are prone to degradation upon exposing to the moistures due to catalysis of amino groups, reductively degradable polycations are usually stable under physiological conditions (37 °C, pH 7.4) with degradation taking place rapidly and selectively under an intracellular reducing environment.[9] In general, there are three strategies to prepare reduction-sensitive cationic polymers for gene delivery, that is, incorporation of disulfide bonds in the cross-linker,[11,12] in the main chain,[13−15] or at the side chain.[16] For example, several groups reported that cross-linking of LMW branched PEI (e.g., 800 and 1800 Da) with different disulfide-containing cross-linking agents (e.g., dithiobis(succinimidylpropionate) (DSP), dimethyl-3,30-dithiobispropionimidate-2HCl (DTBP), and cystamine bisacrylamide (CBA)) led to significantly enhanced in vitro transfection activity as compared with the parent LMW PEI, with transfection efficiency approaching or in a few cases exceeding that of 25 kDa PEI control.[11,17−21] Kissel and coworkers reported that reversibly stabilized 25 kDa bPEI/DNA polyplexes, with or without PEGylation, obtained by cross-linking with DSP demonstrated improved stability in vitro and in vivo.[22−24] Seymour and coworkers reported that 25 kDa PEI/DNA complexes coated with poly[*N*-(2-hydroxypropyl) methacrylamide] (PHPMA) through disulfide bonds had 40- to 100-fold higher in vitro transfection efficiency than their irreversibly coated counterparts.[25] Goepferich and coworkers reported that reducible PEIs prepared from cross-linking of LMW linear PEI (2.6−4.6 kDa) using dithiodipropionic acid or cysteine linkages had superior transfection efficacies and substantially lower toxicities as compared with commonly used nonviral commercial transfection reagents including SuperFect, Lipofectamine, and JetPEI.[26] It should be noted, however, that the multiple reactive sites (i.e., primary and/or secondary amine groups) of PEIs often give rise to complex coupling reactions and to difficulties in synthesizing polymers with controlled characteristics such as molecular weight and degree of branching, which are known to have an immediate impact on the transfection activity. Park and coworkers designed and prepared structurally well-defined bioreducible linear PEIs (l-PEIS) by oxidative polycondensation of bismercapto ethylene imine oligomers (i.e., disulfide in the main chain), in which the transfection efficiency of l-PEIS increased with increasing amine density, approaching that of 25 kDa PEI formulation.[27] In contrast with ExGen 500, l-PEIS displayed low cytotoxicity and was completely degraded inside cells within 3 h.

The direct modification of PEIs, in particular with hydrophobic entities, represents another potential approach to generate structurally well-defined PEIs for nonviral gene transfection.[28] The hydrophobic modification of LMW PEIs was reported to yield enhanced transfection activity as compared with the parent unmodified PEIs, likely due to a delicate balance between DNA protection and release as well as improved interactions with the cellular membranes.[29−34] The extensive work on hydrophobic modification of 25 kDa PEI showed, nevertheless, mostly decreased transfection activity.[35] It should further be noted that hydrophobic modification is likely associated with the drawback of decreased water solubility. The modification of 25 kDa PEI with hydrophilic entities such as PEG was shown to give compromised results, which, on one hand, increased water solubility and decreased in vitro and in vivo toxicity of polyplexes but, on the other hand, significantly decreased its transfection activity.[36,37]

In this Article, we report on synthesis and gene transfection performances of novel 25 kDa PEI derivatives with reductively cleavable cystamine periphery (PEI-Cys) (Scheme 1). To the best of our knowledge, this represents a first design of bioreducible PEIs that contain cleavable disulfide bonds at the side chain. These new PEI-based vectors have the following features: (i) Unlike most other PEI derivatives, PEI-Cys conjugates have a defined structure with the degree of substitution (DS) controlled by feeding ratio of Boc-protected cystamine (meth)acrylate and PEI. (ii) The modification reaction transforms one primary amine into one primary and one secondary amine group or transforms one secondary amine into one primary and tertiary amine group. Hence, the key features of 25 kDa PEI as gene vector including water solubility, DNA condensation, and buffer capacity are not much altered by modification. (iii) The disulfide bonds in PEI-Cys conjugates while sufficiently stable in extracellular fluids (e.g., blood) are to be readily cleaved under an intracellular-mimicking reductive condition,[9] resulting in largely reduced charge density, which on one hand may lead to more efficient intracellular release of DNA than 25 kDa PEI giving enhanced gene transfection and may significantly decrease carrier-associated cytotoxicity. In this study, the synthesis, DNA condensation and reduction-triggered DNA release, in vitro gene transfection in HeLa and 293T cells, as well as cytotoxicity of PEI-Cys conjugates were investigated.

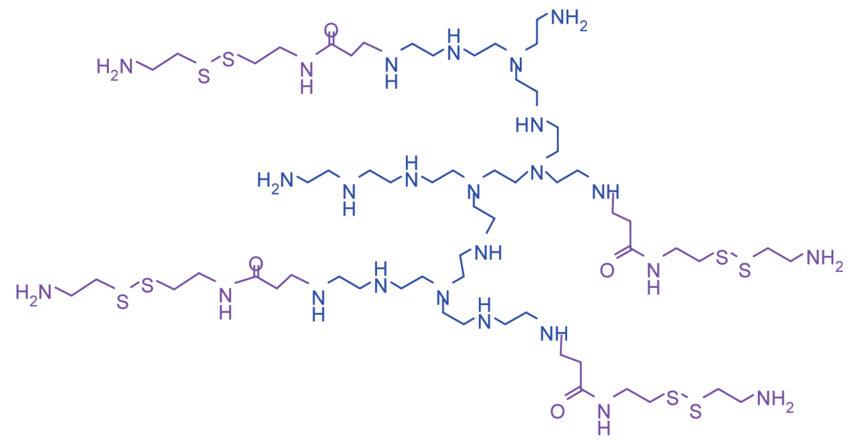
■ EXPERIMENTAL SECTION

Materials. We used 25 kDa branched polyethylenimine (25 kDa PEI, Aldrich), dithiothreitol (DTT, 99%, Merck), triethylamine (Et_3N, 99%, Alfa Aesar), cystamine dihydrochloride (Cytamine·2HCl, >98%, Alfa Aesar), acryloyl chloride (Alfa Aesar), di-*tert*-butyl dicarbonate (J&K Chemical), trifluoroacetic acid (TFA, Sinopharm Chemical Reagent), and methacryloyl chloride (Wuxi Chemical) without further purification. Dialysis membrane (MWCO 3500) was purchased from Beijing Biodee Biotechnology. Luciferase assay system and reporter lysis buffer were purchased from R-protagen (Suzhou, China). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), Dulbecco's phosphate buffered saline (PBS) and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenylterazolium bromide (MTT) were purchased from Invitrogen. The Micro BCA protein assay kit was purchased from Pierce.

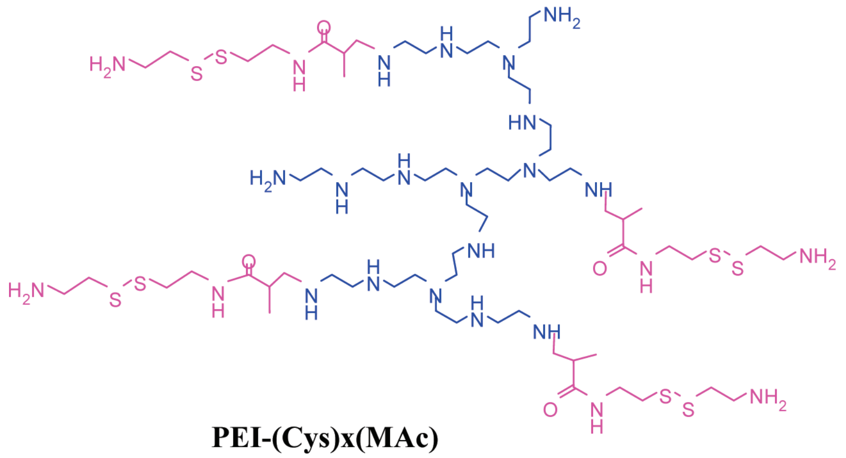
Synthesis of *N-tert*-Butoxycarbonyl-*N*′-scryloyl-cystamine (Ac-Cys-^{t}Boc) and *N-tert*-butoxycarbonyl-*N*′-methacryloyl-cystamine (MAc-Cys-^{t}Boc). Ac-Cys-^{t}Boc and MAc-Cys-^{t}Boc were synthesized in two steps (Supporting Information, Scheme S1). First, to a stirred solution of cystamine bis-hydrochloride (10.00 g, 44.40 mmol) in dry methanol (200 mL) at r.t. was added triethylamine (13.48 g, 133.25 mmol) and di-*tert*-butyldicarbonate (9.71 g, 44.45 mmol). The reaction was monitored by TLC (eluent: chloroform/isopropyl alcohol 1:1, $R_{f,\mathrm{product}}$ = 0.25). Following 2.5 h reaction, the solvent was evaporated, and 1 M KH_2PO_4 aqueous solution (300 mL, pH 4.2) was added. The aqueous phase was extracted with diethyl ether (2 × 50 mL) to remove *N,N*′-di-*tert*-butyloxycarbonyl cystamine, brought to pH 9 with 1 M NaOH, and extracted with ethyl acetate (6 × 30 mL). The combined organic phases were dried over $MgSO_4$ and filtered. The solvent was removed under reduced pressure to yield Cys-^{t}Boc (2.77 g, 25%) as a slightly yellowish oil. ^{1}H NMR (400 MHz, $CDCl_3$, Supporting Information, Figure S1A): δ 1.41 (s, 9H, Boc CH_3), 2.76 (t, 2H, CH_2−S), 2.83 (t, 2H, CH_2−S), 3.01 (t, 2H, NH_2−CH_2), 3.44 (t, 2H, CONH−CH_2).

In the next, to a stirred solution of acryloyl chloride (1.80 mL, 22.15 mmol) in dried chloroform (50 mL) under a nitrogen atmosphere was dropwise added a chloroform solution (50 mL) of triethylamine

Scheme 1. Schematic Representation of Reductively Cleavable PEI-Cys Conjugates



PEI-(Cys)x(Ac)



PEI-(Cys)x(MAc)

(3.21 mL, 23.12 mmol) and Cys-^{t}Boc (2.77 g, 10.97 mmol) over 1.5 h. The reaction was then allowed to equilibrate to room temperature and stirred for 24 h. The solvent was removed under reduced pressure, and the residue was washed with water (120 mL) and extracted with chloroform (3 × 50 mL). The combined organic fractions were dried to give Ac-Cys-^{t}Boc (3.30 g, 99%). Anal Calcd. for $C_{12}H_{22}N_2O_3S_2$: C, 47.03; H, 7.24; N, 9.14; S, 20.93%. Found: C, 46.88; H, 7.16; N, 8.90; S, 21.60%. ^{1}H NMR (400 MHz, $CDCl_3$, Supporting Information, Figure S1B): δ 1.44 (s, 9H, Boc CH_3), 2.77 (t, 2H, CH_2−S), 2.89 (t, 2H, CH_2−S), 3.44 (t, 2H, −NH−CH_2), 3.66 (t, 2H, −CH_2−NH−), 4.95 (1H, amide proton); 5.65 (m, 1H, vinyl proton); 6.18−6.35 (m, 2H, vinyl proton); 6.67 (1H, amide proton). Purity (HPLC): 98.39%. MAc-Cys-^{t}Boc was synthesized in a similar way from reaction of Cys-^{t}Boc and acryloyl chloride (^{1}H NMR, Supporting Information, Figure S1C).

Synthesis of PEI Derivatives with Reductively Cleavable Cystamine Periphery (PEI-Cys). In a typical experiment, to a brown reaction flask containing a solution of 25 kDa PEI in methanol (5 wt %, 3.6 mL) under nitrogen atmosphere was added Ac-Cys-^{t}Boc (267 mg, 0.87 mmol, theoretical DS = 25). The reaction mixture was stirred at r.t. for 6 days and then concentrated by rotary evaporation to yield a viscous liquid. Without further workup, deprotection of Boc group was performed in a mixture of CH_2Cl_2/TFA/1 M HCl (6 mL, v/v/v 1/1/1) for 4−6 h. The solution was diluted with water, its pH was adjusted to 4 with 4 M NaOH, and the product was purified by ultrafiltration (MWCO 3500). The PEI-Cys conjugate in HCl-salt form was collected as solid after freeze-drying (249 mg, yield: 60%). DS (^{1}H NMR) = 14.

Acid−Base Titration. PEI-Cys conjugates (0.1 mmol N) were dissolved in 5 mL of 150 mM NaCl aqueous solution. The pH of the polymer solution was brought to 2.0, and the solution was titrated with 0.1 M NaOH using a pH meter (DELTA 320). For comparison, 25 kDa PEI was also titrated in the same way. The buffering capacity, defined as the percentage of amine groups becoming deprotonated from pH 5.1 to 7.4, was calculated from equation

$$\text{buffer capacity}(\%) = 100(\Delta V_{\mathrm{NaOH}} \times 0.1\ \mathrm{M})/N\ \mathrm{mol}$$

wherein ΔV_{NaOH} is the volume of NaOH solution (0.1 M) required to bring the pH value of the polymer solution from 5.1 to 7.4 and N mol (0.1 mmol) is the total moles of protonable amine groups in the polymer.

Particle Size and Zeta-Potential Measurements. The polyplexes were prepared at varying N/P ratios of 5, 10, 15, 20, and 30 by adding a HEPES buffer solution (20 mM, pH 7.4) of polymer (600 μL, varying concentrations) to a HEPES buffer solution (20 mM, pH 7.4) of plasmid DNA (150 μL, 37.5 μg/mL), followed by vortexing for 5 s and incubating at r.t. for 30 min. The surface charge and the size of polyplexes were measured at 25 °C with 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 Electrophoresis Assay. The polyplexes were prepared as above at varying N/P ratios from 0.5/1 to 2.5/1. Low N/P ratios were employed to compare the DNA binding abilities of different PEG-Cys conjugates and 25 kDa PEI. Electrophoresis was carried out on 0.8%

agarose gel with a current of 100 V for 30 min in TAE buffer solution (40 mM Tris−HCl, 1% (v/v) acetic acid, and 1 mM EDTA). The retardation of the complexes was visualized by staining with ethidium bromide (EB).

Ethidium Bromide Exclusion Assay. The fluorescence of EB upon intercalation with DNA and subsequent decrease upon EB exclusion due to binding with 25 kDa PEI or PEI-Cys conjugates was measured. The experiments were carried out in the dark. In brief, to a plasmid DNA solution (5 μg/mL, 20 mM HEPES buffer, pH 7.4) was added an equimolar amount of EB solution (20 mM HEPES buffer, pH 7.4). The mixture was thoroughly mixed and incubated at r.t. for 1 h. To thus-prepared DNA/EB solution was added 25 kDa PEI or PEI-Cys solution in HEPES buffer resulting in different N/P ratios ranging from 0.5/1 to 5/1. The mixed solution was incubated at r.t. for 30 min. Fluorescence measurements were acquired using a Thermo multiskan flash with λ_{ex} = 510 nm, λ_{em} = 590 nm, and a 5 nm slit width. The pure EB solution and DNA/EB solution without cationic polymer were used as negative and positive controls, respectively. The percent relative fluorescence (%F) was determined using the equation $\%F = (F - F_{EB})/(F_0 - F_{EB})$, wherein F_{EB} and F_0 denote the fluorescence intensities of pure EB solution and DNA/EB solution, respectively.

Reduction-Triggered Dissociation of PEI-Cys/DNA Complexes Monitored by DLS and Gel Retardation Assays. Under a nitrogen atmosphere, a predetermined amount of DTT was introduced to a cuvette containing 750 μL of polymer/DNA complexes prepared, as described above (pH 7.4, 20 mM HEPES buffer), to yield a final DTT concentration of 10 mM. The cuvette was sealed with a septum, and sizes of polyplexes were monitored in time by DLS. For agarose gel retardation assays, 5 μL of dextran sodium sulfate (DSS, M_w = 500 000) solution in HEPES was added to suspensions of polyplexes to give DSS/DNA charge ratios of 1, 2, 4, and 6, respectively, and incubated for 30 min. Then, to 45 μL of polyplexes suspension in HEPES was added 5 μL of DTT solution in HEPES (20 mM, pH 7.4) to reach a final DTT concentration of 10 mM. After 1 h of incubation, polyplexes were electrophoresed through a 0.8% agarose gel containing EB at 100 V in TAE buffer solution (40 mM Tris−HCl, 1 vol % acetic acid, and 1 mM EDTA).

MTT Assays. The cytotoxicity of PEI-Cys conjugates was evaluated in HeLa and 293T cells by MTT assay. In brief, HeLa and 293T cells were seeded in a 96-well tissue culture plate at 1 × 10^4 cells/well in 100 μL of DMEM medium containing 10% FBS and grown to reach 70−80% confluence. PEI-Cys solutions were added to give varying polymer concentrations, and the cells were cultured for an additional 48 h. The medium was replaced with 200 μL of fresh medium. We added 25 μL of stock solution of MTT (4 mg/mL in PBS) to each well, and cells were further incubated for 4 h at 37 °C. The medium was aspirated, the MTT−formazan generated by live cells was dissolved in 150 μL of DMSO, and the absorbance at a wavelength of 490 nm of each well was measured using a microplate reader. The relative cell viability (%) was determined by comparing the absorbance at 490 nm with control wells containing only cell culture medium. Data are presented as average ± SD (n = 3).

In Vitro Gene Transfection. Transfection experiments were performed in HeLa and 293T cells using the plasmid pGL3 as a reporter gene. Transfections were conducted using polyplexes formed at N/P ratios of 5/1, 7.5/1, 10/1, and 15/1. The cells were plated in 24-well plates (cell density 7 × 10^4 cells/well) and maintained in DMEM supplemented with 10% FBS at 37 °C in a humidified atmosphere containing 5% CO_2 until 70% confluency. In a standard transfection experiment, the cells were rinsed with PBS and incubated with 100 μL of polyplex dispersion (1 μg of plasmid DNA per well) and 400 μL of culture medium with or without 10% serum for 4 h at 37 °C. Next, the polyplexes were removed, 500 μL of fresh culture medium containing 10% serum was added, and the cells were cultured for 2 days. Luciferase quantification was done using a commercial luciferase assay kit (R-Protagen) and TD-20/20 Luminometer (Promega). Transfection efficiency was expressed as relative light unit (RLU) per milligram of protein. Twenty-five kDa PEI/DNA formulation prepared at an optimal N/P ratio of 10/1 was used as a reference. All experiments were carried out in triplicate.

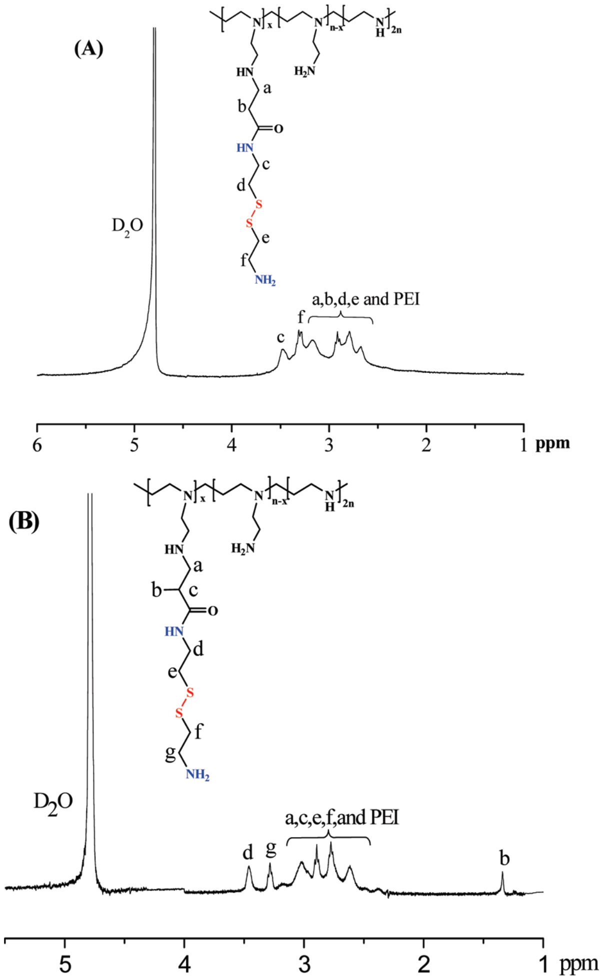


Figure 1. ^{1}H NMR spectra (400 MHz, D_2O) of (A) PEI-(Cys)14(Ac) (Table 1, entry 1) and (B) PEI-(Cys)19(MAc) (Table 1, entry 5).

RESULTS AND DISCUSSION

Synthesis of PEI-Cys Conjugates. A series of PEI-Cys derivatives were synthesized by Michael-type conjugate addition of 25 kDa PEI with Ac-Cys-^{t}Boc and MAc-Cys-^{t}Boc in methanol, followed by deprotection (Supporting Information, Scheme S2). The unconjugated cystamine were readily removed by ultrafiltration. Ac-Cys-^{t}Boc and MAc-Cys-^{t}Boc were prepared from cystamine in two straight steps (Supporting Information, Scheme S1). The mole percentages of Ac-Cys-^{t}Boc relative to the amino groups of PEI in the feed were set at 25, 50, and 70%. ^{1}H NMR in D_2O showed besides resonances of PEI (δ 2.25−3.24) the signals of cystamine moieties at δ 3.50 (methylene protons next to the amide) and 3.32 (methylene protons close to the terminal amine) (Figure 1A). The degrees of substitution (DS, defined as mole percentage of cystamine moieties in the final conjugates relative to the amino groups of PEI) of resulting conjugates, denoted as PEI-(Cys)x(Ac), were determined to be 14, 27, and 34, which

Table 1. Synthesis of PEI-Cys Derivatives

entry	PEI-Cys	cys in feed (mol %)[a]	DS[b]	N^1:N^2:N^3 mole ratio[c]	buffer capacity (%) [d]
1	PEI-(Cys)14(Ac)	25	14	22:56:22	21.2
2	PEI-(Cys)27(Ac)	50	27	21:58:21	21.7
3	PEI-(Cys)34(Ac)	70	34	22:56:22	21.9
4	PEI-(Cys)13(MAc)	30	13	22:56:22	22.5
5	PEI-(Cys)19(MAc)	50	19	21:58:21	23.0
6	PEI-(Cys)38(MAc)	70	38	28:44:28	23.1
7	25 KDa PEI			25:50:25	15.1

[a] Mole percentages of (M)Ac-Cys-^{t}Boc monomer relative to the amino groups of PEI. [b] DS (degree of substitution, defined as mole percentage of cystamine moieties in the final PEI-Cys conjugates relative to the amino groups of PEI) determined by ^{1}H NMR. [c] Mole ratios of primary/secondary/tertiary amines calculated assuming that primary amine is more reactive than secondary amine. [d] Buffer capacity (defined as the percentage of amine groups becoming deprotonated from pH 5.1 to 7.4) determined by acid−base titration.

increased with increasing mole percentages of Ac-Cys-^{t}Boc in the feed (Table 1, entries 1−3). Similarly, PEI-(Cys)x(MAc) conjugates with DS of 13, 19, and 38 were synthesized (Table 1, entries 4−6). ^{1}H NMR in D_2O displayed besides resonances of PEI three additional peaks at δ 1.32 (methyl protons of MAc moiety) and δ 3.48 and 3.30 (methylene protons of cystamine moieties) (Figure 1B).

The transfection activity of 25 kDa PEI intimately relies on its unique combination of primary, secondary, and tertiary amine groups, in which the primary amines help to condense DNA into positively charged small-sized nanoparticles, whereas secondary and tertiary amines with high buffer capacity at the low pH range are assumed to facilitate endosomal escape via the "proton sponge effect". It is important, therefore, to maintain the compositions of different amine groups in PEI. To assume that primary amine is more reactive than secondary amine in Michael-type conjugate addition reactions as previously reported by us and others,[12,38] mole ratios of primary-to-secondary-to-tertiary amines of PEI-Cys derivatives could be calculated. It turned out that PEI-Cys derivatives had very similar amine compositions to the parent PEI (Table 1). Notably, acid−base titration measurements (Supporting Information, Figure S2) showed that all PEI-Cys derivatives had somewhat higher buffer capacity in the endosomal pH range (pH 5.1 to 7.4) than the parent 25 kDa PEI (21.2 to 23.1 versus 15.1%) (Table 1).

Biophysical Characterization of DNA Polyplexes. The plasmid DNA condensation abilities of PEI-Cys derivatives were studied using gel electrophoresis, EB exclusion assay, DLS, and zeta potential measurements. Interestingly, gel retardation assays revealed that cystamine modification of PEI led to enhanced interaction with DNA (Supporting Information, Figure S3). For example, PEI-(Cys)14(Ac) and PEI-(Cys)27(Ac) were able to inhibit completely DNA migration at an N/P ratio of 2/1 and 1/1, respectively, whereas a higher N/P ratio of 2.5/1 was needed for 25 kDa PEI. In a similar way, PEI-(Cys)x(MAc) with DS of 13, 19, and 38 resulted in complete inhibition of DNA migration at an N/P ratio of 2/1, 1.5/1, and 1/1, respectively. The results of EB exclusion assays showed clearly that all PEI-Cys conjugates interacted more strongly with DNA than the parent 25 kDa PEI (Figure 2). Furthermore, PEI-(Cys)x(MAc) displayed in general higher DNA binding affinities as compared with PEI-(Cys)x(Ac),

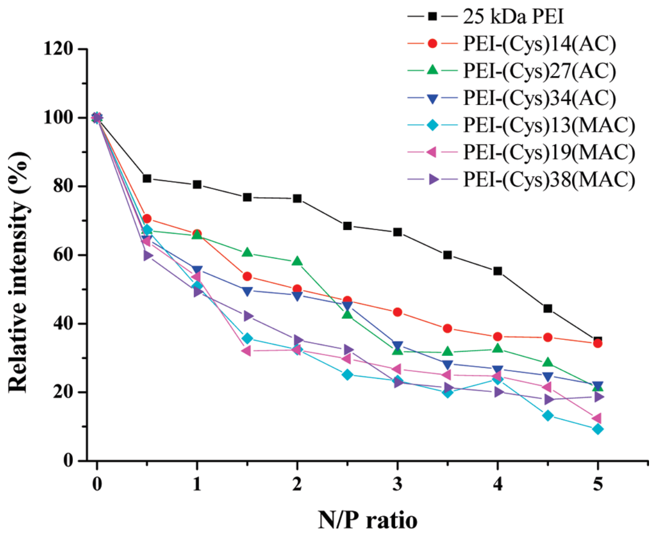


Figure 2. DNA binding of PEI-Cys conjugates measured by the ethidium bromide (EB) exclusion assay (20 mM HEPES buffer, pH 7.4). 25 kDa PEI was used as a control.

indicating that hydrophobic interactions play a role in DNA condensation. The N/P ratios required for PEI-(Cys)x(MAc), PEI-(Cys)x(Ac), and 25 kDa PEI to inhibit 50% of the relative EB-DNA fluorescence were determined to be 1.0 to 1.1, 1.5 to 2.2, and 4.2, respectively. This is in contrast with acetylation of 25 kDa PEI that was reported to weaken PEI−DNA interactions.[30]

DLS results showed that all PEI-Cys derivatives, along with 25 kDa PEI, were able to condense DNA into nanosized particles (<180 nm) at and above an N/P ratio of 5/1 (Figure 3A). Notably, PEI-(Cys)x(Ac) conjugates afforded, in general, smaller particles than 25 kDa PEI at N/P ratios ranging from 5/1 to 30/1 (with average diameters of 80−90 nm versus 100−130 nm). In contrast, PEI-(Cys)x(MAc) gave somewhat larger particles (120−180 nm) than 25 kDa PEI at N/P ratios ranging from 5/1 to 15/1. The sizes of PEI-(Cys)x(MAc) complexes decreased with increasing N/P ratios from 5/1 to 30/1. When N/P ratios were higher than 20/1, PEI-(Cys)x(MAc) condensed DNA into particles of similar sizes to 25 kDa PEI. The extent of cystamine substitution (DS), however, had little influence on the sizes of polyplexes (Figure 3A). Zeta potential measurements revealed that polyplexes of PEI-(Cys)x(Ac) derivatives had marginally lower surface charges as compared with 25 kDa PEI at and above an N/P ratio of 10/1, in which positive charges of 17−28 mV were observed depending on N/P ratios (Figure 3B). In comparison, polyplexes of PEI-(Cys)x(MAc) derivatives showed, in general, lower surface charges (+13 ∼ +17 mV) than 25 kDa PEI.

Reduction-Triggered Dissociation of Polyplexes. The aim of this study was to develop novel 25 kDa branched PEI-based gene vectors that contain reductively cleavable cystamine periphery. Under intracellular reducing conditions, the cleavage of the periphery would take place, resulting in largely decreased charge density, which would in turn decrease carrier-associated cytotoxicity as well as facilitate intracellular unpacking of polyplexes leading to an enhanced transfection activity.

To confirm the reduction-sensitivity of PEI-Cys derivatives, we monitored the change of polyplex sizes in response to 10 mM dithiothreitol (DTT) at pH 7.4 using DLS. The results showed

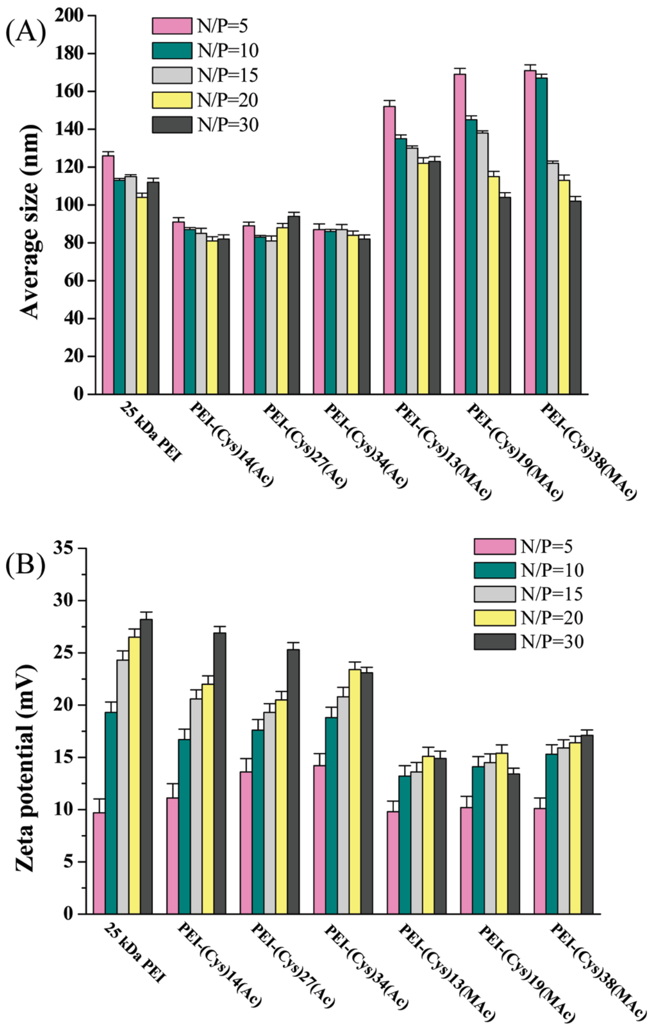


Figure 3. (A) Average particle size and (B) zeta potential of PEI-Cys/DNA polyplexes prepared at different N/P ratios ranging from 5/1 to 30/1 in 20 mM HEPES buffer at pH 7.4.

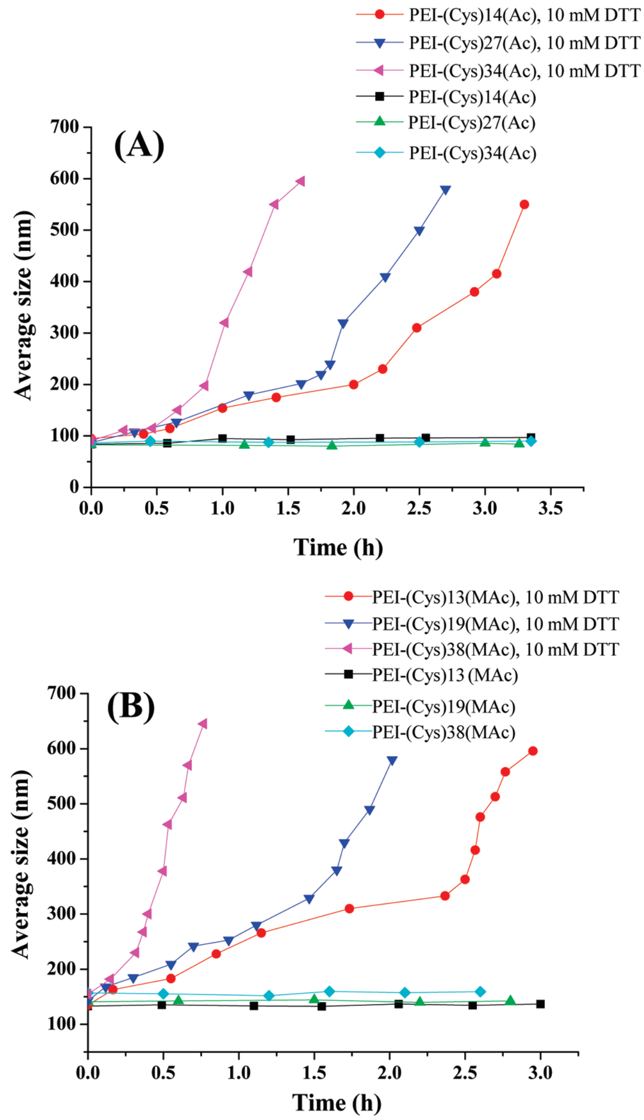


Figure 4. Influence of 10 mM DTT on the sizes of polyplexes prepared from (A) PEI-(Cys)x(Ac) and (B) PEI-(Cys)x(MAc) at an N/P ratio of 10/1.

that under nonreductive conditions, the polyplex sizes of PEI-Cys derivatives prepared at an N/P ratio of 10/1 did not change over a period of 3 h. However, in the presence of 10 mM DTT under otherwise the same conditions, rapid increase in particle sizes to an average diameter of >600 nm was observed (Figure 4). This is likely because reductive cleavage of cystamine moieties markedly decreases the charge density and hence DNA condensation ability of PEI-Cys derivatives. For both PEI-(Cys)x(Ac) and PEI-(Cys)x(MAc) series, the higher the DS, the faster the change of polyplex sizes. For instance, increase in polyplex sizes to ~600 nm was observed at 3, 2, and 0.75 h for PEI-(Cys)x(MAc) derivatives with DS of 13, 19 and 38, respectively. This is in line with our expectation that higher substitution would result in more significant reduction of charge density upon cleavage of the disulfide bonds under a reductive condition. Furthermore, polyplexes of PEI-(Cys)x(MAc) revealed slightly faster change of particle sizes in response to 10 mM DTT as compared with those of PEI-(Cys)x(Ac) at a similar DS.

Reduction-triggered DNA release from polyplexes of PEI-Cys derivatives was further studied by gel retardation assays. The polyplexes prepared from PEI-(Cys)14(Ac) and PEI-(Cys)13(MAc) at an N/P ratio of 5/1 were stable against exchange with six-fold excess of negatively charged DSS relative to DNA phosphate groups, confirming high stability of PEI-Cys polyplexes. However, in the presence of 10 mM DTT, release of DNA was observed upon the addition of two- and one-fold excess of DSS for PEI-(Cys)14(Ac) and PEI-(Cys)13(MAc), respectively (Figure 5). The polyplexes of PEI-(Cys)13(MAc) appeared to be more readily unpacked to release DNA in response to 10 mM DTT than PEI-(Cys)14(Ac).

In Vitro Cytotoxicity and Transfection. The cytotoxicity of PEI-Cys derivatives was investigated in HeLa and 293T cells by MTT assays. Notably, all PEI-Cys derivatives exhibited markedly reduced cytotoxicity as compared with 25 kDa PEI (Figure 6). There was practically no difference in toxicity between PEI-(Cys)x(Ac) and PEI-(Cys)x(MAc). Remarkably, 25 kDa PEI had an IC_{50} value of ~11 mg/L in HeLa cells, whereas over 65% cells were viable at the highest tested concentration (100 mg/L) of PEI-Cys derivatives (i.e., IC_{50} > 100 mg/L) (Figure 6A). These PEI-Cys conjugates along with 25 kDa PEI showed higher cytotoxicities to 293T cells, in which IC_{50} data of approximately

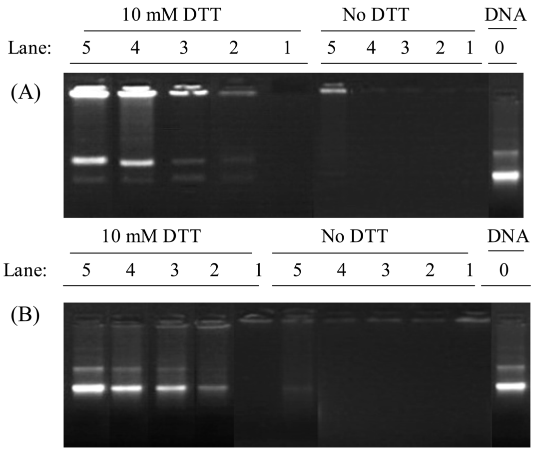


Figure 5. Agarose gel electrophoresis of (A) PEI-(Cys)14(Ac)/DNA and (B) PEI-(Cys)13(MAc)/DNA complexes prepared at an N/P ratio of 5/1 with or without 10 mM DTT. Lane 0 is free DNA; lane 1 is polyplexes formed at an N/P ratio of 5/1; and lanes 2−5 correspond to polyplexes formed at an N/P ratio of 5/1 with addition of DSS at varying sodium sulfate/DNA phosphate ratios of 1, 2, 4, and 6, respectively.

50−75 and 3 mg/L were determined for PEI-Cys conjugates and 25 kDa PEI, respectively (Figure 6B). This largely decreased cytotoxicity of PEI-Cys derivatives as compared with the parent 25 kDa PEI is most likely due to reductive degradation of disulfide bonds inside the cells leading to significantly reduced charge density. Branched PEI has ∼25% primary, 50% secondary, and 25% tertiary amine groups. The primary amines have relatively high pK_a, whereas both secondary and tertiary amines are known to have low pK_a. Hence, even with the lowest DS of 13 or 14, reductive degradation results in the loss of over half of their primary amines that play a major role in the charge density and cytotoxicity of PEI. The detailed toxicity investigation on different polycations by Kissel and coworkers revealed that cationic charge density is one of the key parameters for cell damage.[39]

The in vitro transfection activity of PEI-Cys derivatives was evaluated in HeLa and 293T cells using pGL3 as a reporter gene at four different N/P ratios (i.e., 5/1, 7.5/1, 10/1, and 15/1). The results showed that in the absence of serum, the transfection activity of PEI-Cys polyplexes in HeLa cells first increased and then decreased with increasing N/P ratios, and like 25 kDa PEI, the highest transfection efficiency was observed at an N/P ratio of 10/1 (Figure 7). It should be noted that for both PEI-(Cys)x-(Ac) and PEI-(Cys)x(MAc), the transfection efficiency decreased with increasing DS of cystamine. In addition, PEI-(Cys)x(MAc) exhibited, in general, somewhat higher transfection activity than PEI-(Cys)x(Ac) at comparable DS. Remarkably, PEI-(Cys)14(Ac) and PEI-(Cys)13(MAc) displayed 2.2- and 4.1-fold enhancement of gene transfection efficiency, respectively, as compared with 25 kDa PEI at an N/P ratio of 10/1 (Figure 7).

The transfection performance of PEI-(Cys)14(Ac) and PEI-(Cys)13(MAc) in 10% serum media followed similar trends to transfection without serum, resulting in comparable to or higher transfection efficiencies (up to 1.8-fold enhancement) relative to 25 kDa PEI at an N/P ratio of 10/1 in both HeLa and 293T cells (Figure 8). It should be noted that polyplexes of 25 kDa PEI that formed at an N/P ratio of 15/1 were very toxic to both HeLa and 293T cells (cell viabilities <60%). The better transfection activity of PEI-(Cys)x(MAc) as compared with PEI-(Cys)x(Ac) could be due to its slightly more hydrophobic nature, which, as

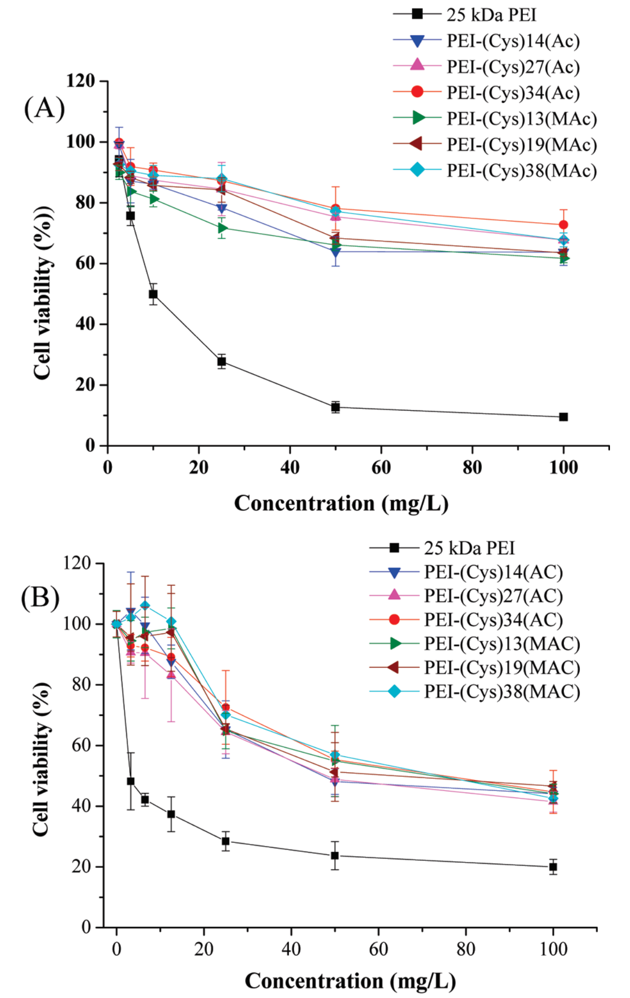


Figure 6. Cytotoxicity of PEI-Cys derivatives determined by MTT assays ($n = 3$). 25 kDa PEI was used as a control. (A) HeLa cells and (B) 293T cells.

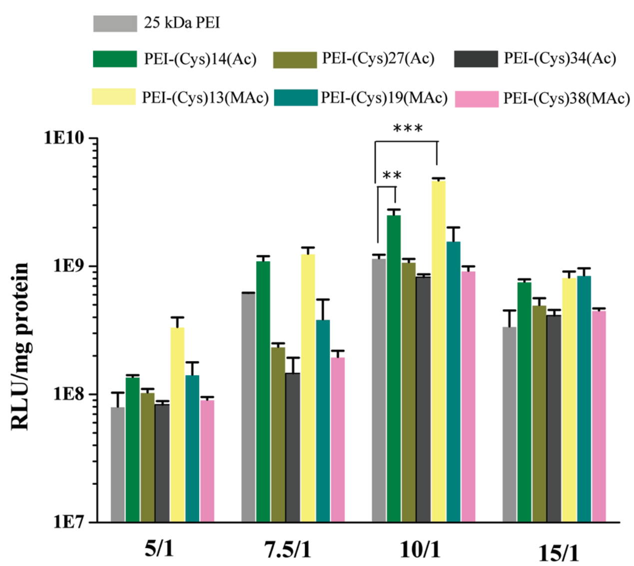
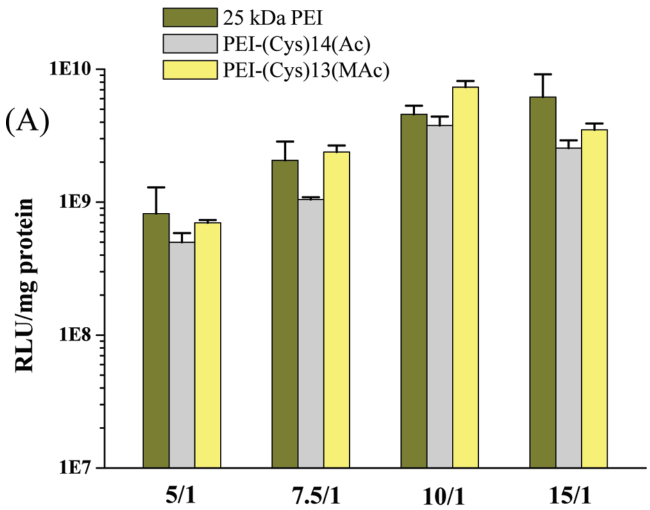


Figure 7. Transfection efficiencies of PEI-Cys polyplexes in HeLa cells at N/P ratios of 5/1, 7.5/1, 10/1, and 15/1 in serum-free media. 25 kDa PEI formulation was used as a control (Student's t test, $^{**}p < 0.01$, $^{***}p < 0.001$).



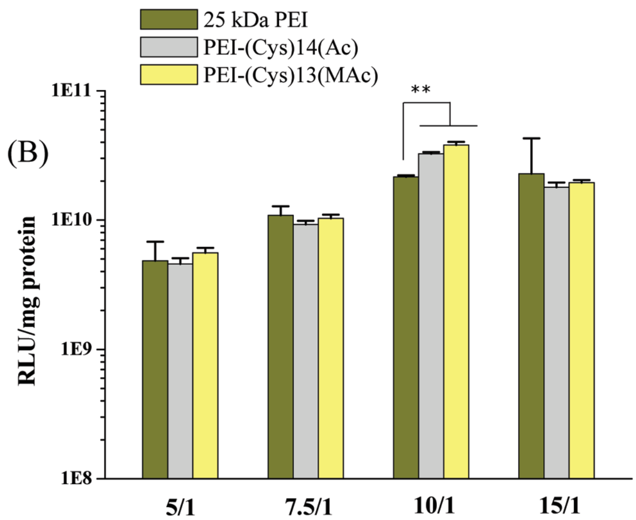


Figure 8. Transfection efficiencies of PEI-(Cys)14(Ac) and PEI-(Cys)13(MAc) polyplexes in (A) HeLa cells and (B) 293T cells at N/P ratios of 5/1, 7.5/1, 10/1, and 15/1 in 10% serum media. 25 kDa PEI formulation was used as a control (Student's *t* test, $^{**}p < 0.01$).

previously reported, would possibly induce enhanced interactions with cellular membranes.[29−34] The other possible reason might be that polyplexes of PEI-(Cys)x(MAc) could release DNA more easily than those of PEI-(Cys)x(Ac) under the intracellular reducing environment, as shown by DLS and gel retardation measurements (Figures 4 and 5).

The results of this study showed that modification of 25 kDa PEI with reductively cleavable cystamine periphery to a certain extent could indeed significantly improve its transfection and toxicity profiles, highlighting the importance of manipulating charge density of PEI vectors in cells. However, modification of PEI to a further extent results in opposite effects, although our conjugation approach does not much alter amine compositions of PEI. The possible explanation could be that significant modification of PEI with cystamine has led to largely reduced charge-to-mass ratios.

CONCLUSIONS

We have demonstrated for the first time that modification of 25 kDa branched PEI with reductively cleavable cystamine moieties results in not only significantly reduced cytotoxicity but also markedly enhanced in vitro transfection under both serum-containing and serum-free conditions at an N/P ratio of 10/1. These novel PEI derivatives can be readily prepared with defined structures and controlled degree of substitution. The very unique feature of this work is that several key properties of 25 kDa branched PEI pertinent to its gene transfection performance are not much altered by the modification. This new way of PEI modification is highly appealing for the development of safer and more efficient nonviral gene vectors.

ASSOCIATED CONTENT

Supporting Information. Synthesis schemes for monomers and PEI-Cys derivatives, ^{1}H NMR spectra, acid−base titration curves, and agarose gel electrophoresis results. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

§Y.W. and M.Z. made equal contributions to this work.

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