Revisiting Complexation between DNA and Polyethylenimine: Does the Disulfide Linkage Play a Critical Role in Promoting Gene Delivery?[a]

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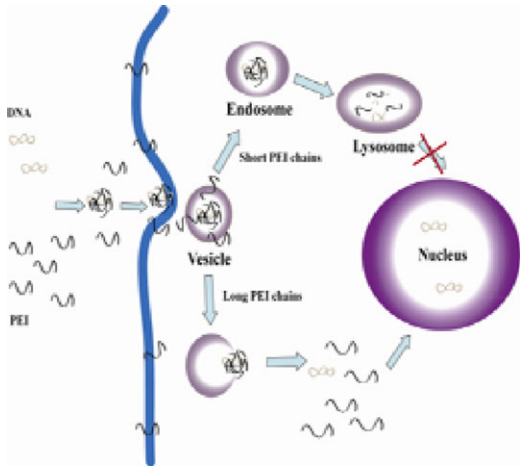
Despite its cytotoxicity, polyethylenimine (PEI) is still used as a golden reference in gene transfection. Long PEI chains are more effective but also more cytotoxic. To solve this problem, an alternative strategy is to link short PEI chains into a longer PEI with disulfide bonds because they are degradable in the cell. However, how PEI promotes gene transfection is still unclear. Also, the chain length of PEI is also increased as disulfide bonds are formed. Therefore, it is important to investigate whether the increase in transfection efficiency is attributable to the disulfide linkage, chain size, or both. To distinguish between such factors, a novel method is developed here to make longer linear PEI with disulfide bonds (lPEI$_{s\text{-}s}$) by linking the mercapto groups of short linear PEI (lPEI$_i$). By comparing the physiochemical properties and the transfection efficiencies of short lPEI$_i$, long lPEI$_{s\text{-}s}$, and un-degradable long PEI, it is found that introducing disulfide bonds instead of directly using longer PEI chains has less effect on gene transfection, and it is the chain length that plays a key role in promoting gene transfection.



1. Introduction

Gene vectors have received much attention because of their applications in the field of gene therapy.[1,2] In the last two decades, they have been developed in different forms, including both viral[3–5] and non-viral vectors (cationic lipids[6] and polymers[7–11]). In comparison with other polymers, cationic polymer chains are attractive because of their low immunogenicity, large packing size, and construction flexibility.[12,13] Among thousands of cationic polymers synthesized, polyethylenimine (PEI) remains one of the most efficient and simplest non-viral vectors.[14–16] It is known that large PEI chains (branched and linear PEI 25k) are more effective than their small counterparts, but large PEI chains exhibit high cytotoxicity, which limits the application of PEI.[17–21] To solve this problem, linking short PEI chains via disulfide bonds into a longer chain was suggested and applied for the following reasons. It is much better for longer PEI chains to combine, stabilize,

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and protect DNA in the extracellular space. They can be quickly degraded in the intracellular redox environment wherein the glutathione concentration is 50–1000 times higher than that in the extracellular space.[22–24] Long PEI chains are degraded into small ones that are less cytotoxic and bind less to DNA after their disulfide bonds are cleaved inside the cell. Such a strategy has been tried by various groups. For example, Liu et al.[25] crosslinked branch PEI chains using click chemistry, Peng et al.[26] prepared disulfide crosslinked PEI clusters via thiolation of small branched ones, Breunig et al.[27] used dithiobis(succinimidyl propionate) (DSP) as a crosslinking agent, and Lee et al.[28] synthesized longer degradable linear PEI chains by several coupling-hydrolysis cycles. Most of these efforts, as expected, resulted in a lower cytotoxicity and higher gene transfection efficiency. However, the exact role of the disulfide bonds during the gene transfection remains unclear, though the assumptions made to date seem reasonable.

A few years ago, our group developed a novel laser light-scattering method to in situ monitor the disulfide linking reaction of short branch chains ($\overline{M}_w \sim 2\,kg\,mol^{-1}$, denoted as *b*PEI-2k).[29] It was found that linking 3-4 *b*PEI-2k to form the larger PEI could significantly enhance the gene transfection by a factor of 10^5–10^7 times, depending on the PEI/DNA ratio. It was also found that most of the PEI chains did not combine with DNA in the solution mixture of polymer and DNA, especially when the N/P ratio was higher than six,[30] and it is those free PEI chains with a size larger than ≈ 15 nm (contour length)[31] that effectively promote the gene transfection.[32,33] Recently, we also found that DNA was continuously released from the polyplexes prior to the disulfide bond's cleavage.[34] Therefore, the disulfide bond's effect during gene transfection should be reconsidered. It was important to investigate whether the increase in transfection efficiency (10^5–10^7 times) is due to the disulfide bond cleavage[35] or to the effect of chain length.

To summarize the reported studies, most of them focused on linking small branched PEI to obtain an effective vector. Compared to its small precursor, the disulfide-linked branched PEI showed a higher transfection efficiency. However, comparative studies of disulfide-linked linear PEIs are few in number. In this study, we designed and developed a novel method to prepare longer, structure-defined linear PEI chains with degradable disulfide linkages (*l*PEI$_{s\text{-}s}$). In comparison to branched PEI, the chain length and structure of linear PEI were much easier to define. We purposely chose our initial linear chain (*l*PEI$_i$) with a longer chain length (≈ 15 nm) to test whether the extension of the chain length with degradable disulfide linkages could indeed promote gene transfection. We also synthesized a non-degradable linear PEI (*l*PEI-4.2k, ≈ 44 nm) with a weight-average molar mass similar to *l*PEI$_{s\text{-}s}$ (≈ 45 nm) so that we could comparatively investigate their transfection efficiency and cytotoxicity under identical conditions to probe the effects of disulfide bonds.

2. Experimental Section

2.1. Materials and Cell Lines

Linear polyethylenimine (*l*PEI-25k, $\overline{M}_w = 25\,kg\,mol^{-1}$) was purchased from Polysciences. Hydrobromic acid solution (33 wt% in acetic acid, HBr/AcOH), branched polyethylenimine (*b*PEI-25k, $\overline{M}_w = 25\,kg\,mol^{-1}$), p-toluenesulfonyl chloride (p-TsCl), ethylene glycol, and potassium thioacetate from Sigma-Aldrich were used without further purification. Methyl p-toluenesulfonate purchased from Sigma-Aldrich was distilled and then stored under nitrogen. 2-Ethyl-2-oxazoline from Sigma-Aldrich was purified by vacuum distillation over CaH_2. Acetonitrile from RCI Labscan was dried over CaH_2 and distilled under dry nitrogen. All other chemicals and solvents were used as received without further purification.

Initial Plasmid DNA (pGL3) with an SV40 promoter and an enhancer sequence encoding firefly luciferase was purchased from Promega (Madison, USA). A large amount of this plasmid DNA was prepared with a Qiagen Plasmid Maxi Kit (Qiagen, Germany). Fetal bovine serum (FBS), penicillin-streptomycin, and Dulbecco's modified Eagle's medium (DMEM) were obtained from GIBCO (NY, USA). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Sigma-Aldrich (Germany). 293T cells and HepG2 cells were grown at 37 °C/5% CO_2 in DMEM supplemented with 10% FBS, penicillin at 100 units mL^{-1}, and streptomycin at 100 $\mu g\,mL^{-1}$, respectively.

2.2. Characterization

NMR spectra were recorded on a Bruker Avance III 400 spectrometer. The chemical shifts (δ) were reported in ppm with the solvent resonance as the internal standard relative to chloroform (δ 7.26) or D_2O (δ 4.79) for ^1H. All the ^1H NMR measurements were carried out at room temperature. Mass spectra (ESI-MS) were obtained with a HP 5989B spectrometer and determined at an ionizing voltage of 70 eV; relevant data were tabulated as *m/z*. Matrix-assisted laser desorption time of flight mass spectrometry (MALDI-TOF, Voyager-DE STR, AB SCIEX) was used to characterize the molar mass of all the PEI samples. Trans-4-hydroxy-3-methoxycinnamic acid was used as the matrix. The solvent was acetonitrile and 3% TFA in deionized water.

2.3. Synthesis of 1,2-Bis(p-tolylsufonyloxy)ethane

To a stirred solution of ethylene glycol (0.3 g, 4.8×10^{-3} mol) and TEA (2 mL) in dry methylene chloride (20 mL), a solution of p-TsCl (3.6 g, 19.2×10^{-3} mol) in dry methylene chloride (5 mL) was added dropwise at 0 °C. After overnight stirring, the solution was filtered and re-crystallized in $CHCl_3$/methanol solution. The product was dried in vacuo (yield 95%, 1.9 g).[36] ^1H NMR (400 MHz, $CDCl_3$, δ): 7.74–7.72 (d, 4H, $-C_6\underline{H}_4CH_3$), 7.34–7.32(d, 4H, $-C_6\underline{H}_4CH_3$), 4.18 (s, 4H,

–O–C$\underline{H}_2$C$\underline{H}_2$–O–), 2.45 (s, 3H, –$C_6H_4C\underline{H}_3$) (Figure S1 in the Supporting Information). HRMS (ESI) m/z: $[M+Na]^+$ calcd for 1,2-bis(p-tolylsufonyloxy)ethane, 393.4405; found,: 393.4408.

2.4. Synthesis of Poly(2-ethyl-2-oxazoline)-bis(thioacetate) (AcS-PETOZ-SAc)

A solution of 1,2-bis(p-tolylsufonyloxy)ethane (1.08 g, 2.7×10^{-3} mol) and 2-ethyl-2-oxazoline (10 mL, 100×10^{-3} mol) in dry acetonitrile (30 mL) was heated to 90 °C and stirred for 48 h under vacuum in a Schlenk flask. The solution was then cooled down and stored at 4 °C to synthesize polymer chains with p-toluenesulfonate end groups. 24 h later, the polymer solution was precipitated in diethyl ether. The precipitate was washed three more times with diethyl ether and vacuum dried to obtain a yellow powder of poly(2-ethyl-2-oxaline)-bis(tosylate) (Ts-PETOZ-Ts) with a yield of 90% (8.9 g).[37,38] ^{1}H NMR (400 MHz, $CDCl_3$, δ): 7.71–7.69 (d, –$C_6\underline{H}_4CH_3$), 7.16–7.14 (d, –$C_6\underline{H}_4CH_3$), 3.46 (s, –C$\underline{H}_2$C$\underline{H}_2$NH–), 2.45–2.30 (m, –COC$\underline{H}_2CH_3$ and –$C_6H_4C\underline{H}_3$), 1.21–1.11 (m, –$COCH_2C\underline{H}_3$) (Figure S2 in the Supporting Information).

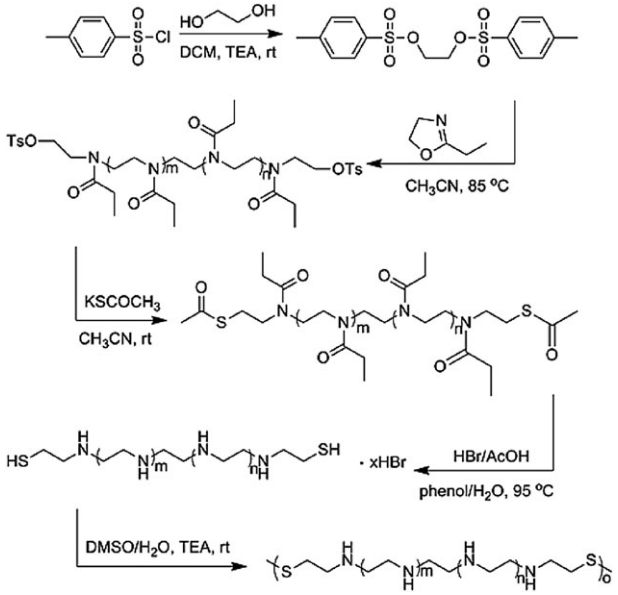
A solution of Ts-PETOZ-Ts (5 g, 1.3×10^{-3} mol) and potassium thioacetate (0.74 g, 6.5×10^{-3} mol) in acetonitrile (35 mL) was stirred in a round bottom flask at 25 °C for 12 h before it was diluted with CH_2Cl_2 (65 mL) and further stirred for 1 h. The solution was then washed with saturated NaCl solution (3 × 100 mL) and deionized water (100 mL). The resultant solution was dried with anhydrous magnesium sulfate. Finally, AcS-PETOZ-SAc was harvested by precipitation in cold diethyl ether (yield 90%, 4.4 g).[38] ^{1}H NMR (400 MHz, $CDCl_3$, δ): 3.46 (s, –C$\underline{H}_2$C$\underline{H}_2$NH–), 3.0–2.9 (t, –SCO–C$\underline{H}_3$), 2.45–2.30 (m, –COC$\underline{H}_2CH_3$), 1.21–1.11 (m, –$COCH_2C\underline{H}_3$) (Figure S3 in the Supporting Information). $\overline{M}_n = 3.74$, PDI = 1.37.

2.5. Synthesis of Linear Thiolated Poly(ethylenimine) Precursor (*l*PEI$_i$)

AcS-PETOZ-SAc (4 g, 1×10^{-3} mol) and phenol (0.045 g, 0.5×10^{-3} mol) were dissolved in 33% HBr/AcOH (20 mL) and further diluted with deionized water (20 mL). After refluxing for 24 h, a pale brown precipitate was obtained. The precipitate was washed with ethanol several times and refluxed in ethanol for 2 h. The mixture was re-precipitated in diethyl ether and filtered to obtain linear PEI with two functional ends.[28] ^{1}H NMR (400 MHz, D_2O, δ): 3.65–3.54 (m, –C$\underline{H}_2$C$\underline{H}_2$NH–), 2.62–2.55 (m, –C$\underline{H}_2$SH) (Figure S4 in the Supporting Information). $\overline{M}_n = 1.66$, PDI = 1.12.

2.6. Synthesis of Linear Poly(ethylenimine) with Degradable Disulfide Linkages (*l*PEI$_{s\text{-}s}$)

*l*PEI$_i$ (0.5 g, 0.3×10^{-3} mol) and triethylamine (0.1 mL) were dissolved in a mixture of DMSO and deionized water (1:1 v/v, 2 mL). The solution was stirred at 25 °C for 7 d to ensure a sufficient reaction. After adjusting to pH 10 with 5 M NaOH, a white precipitate was obtained. The crude product was re-dissolved in water (pH = 4) and dialyzed against a large amount of water (for 2 d) using a dialysis bag with a cut-off molar mass ≈1 kg mol^{-1} (MWCO). The final product was freeze-dried. ^{1}H NMR (400 MHz, D_2O, δ): 3.65–3.53 (m, –C$\underline{H}_2$C$\underline{H}_2$NH–), 2.54–2.48 (m, –C$\underline{H}_2$S–) (Figure S5 in the Supporting Information). $\overline{M}_n = 3.96$, PDI = 1.43. Scheme 1 shows a schematic of the synthesis.



Scheme 1. Synthesis of degradable linear polyethylenimine (*l*PEI$_{s\text{-}s}$) (TsO: tosylate).

2.7. Synthesis of Linear Polyethylenimine (*l*PEI-4.2k) without a Degradable Disulfide Bond

A solution of methyl p-toluenesulfonate (0.186 g, 1×10^{-3} mol) and 2-ethyl-2-oxazoline (12 mL, 0.12 mol) in dry acetonitrile (30 mL) was heated to 90 °C and stirred for 48 h under vacuum in a Schlenk flask before it was cooled down and precipitated in cold diethyl ether. The precipitate was washed three times with diethyl ether and then vacuum dried, which resulted in a yellow powder of poly(2-ethyl-2-oxaline) (PETOZ) with a yield of 80% (9.5 g). ^{1}H NMR (400 MHz, $CDCl_3$, δ): 7.67–7.65 (d, –$C_6\underline{H}_4CH_3$), 7.16–7.14 (d, –$C_6\underline{H}_4CH_3$), 3.44 (s, –C$\underline{H}_2$C$\underline{H}_2$NH-), 2.39–2.29 (m, –COC$\underline{H}_2CH_3$ and –$C_6H_4C\underline{H}_3$), 1.11 (m, –$COCH_2C\underline{H}_3$). GPC (THF as eluent): $\overline{M}_n = 9.8$ kg mol^{-1}, PDI = 1.31.

Then, PETOZ (5 g, 0.5×10^{-3} mol) was dissolved into 15% HCl (30 mL) solution. After refluxing for 24 h, a white precipitate was filtered and re-dissolved in 30 mL of water, whose pH was adjusted to ~9 by the addition of 5 M sodium hydroxide (NaOH). The precipitate was washed with water until pH ≈ 7 was reached. The final product was obtained by freeze-drying (yield 90%, 1.9 g). ^{1}H NMR (400 MHz, D_2O, δ): 3.52–3.46 (m, –CH_2CH_2NH–). $\overline{M}_n = 4.2$ kg mol^{-1} (as calculated from the GPC result for PETOZ).

2.8. Laser Light Scattering

A commercial LLS instrument (ALV5000) with a vertically polarized 22 mV He-Ne laser (632.8 nm, Uniphase) was used to detect the degradation of *l*PEI$_{s\text{-}s}$ in real time and characterize the hydrodynamic radius of polyplexes mediated by different PEIs. In

static LLS, the weight-average molar mass ($\overline{M}_w$) and the z-average root-mean square radius of gyration ($<R_g>_z$) of scattering objects can be obtained in a sufficiently dilute solution/dispersion from the angular and concentration dependence of the excess absolute scattering intensity (Rayleigh ratio $R_{vv}(q)$) as $KC/R_{vv}(q) \approx (1 + q^2 R_g^2/3)/\overline{M}_w$ where $K = 4\pi^2 n^2 (dn/dc)^2/(N_A \lambda_0^4)$ and $q = 4\pi n/\lambda_0 \sin \theta/2$, with dn/dc, N_A, λ_0, n and θ being the specific refractive index increment, the Avogadro constant, the incident wavelength in a vacuum, the refractive index of the solvent, and the scattering angle, respectively. Note that we have neglected the concentration correction here.

In dynamic LLS, the Laplace inversion of each measured intensity-intensity time correlation function ($G^{[2]}(q,t)$) in the self-beating mode can be related to a line-width distribution $G(\Gamma)$. For a diffusion relaxation, Γ is further related to the translational diffusion coefficient D by $(\Gamma/q^2)_{c\to 0, q\to 0} = D$. Therefore $G(\Gamma)$ can be converted to into a translational diffusion coefficient distribution G(D) or a hydrodynamic radius distribution f(Rh) using the Stokes-Einstein equation, $R_h = k_B T/6\pi\eta D$, where k_B, T, and η are the Boltzmann constant, the absolute temperature, and the solvent viscosity, respectively. To measure the hydrodynamic radius of the PEI/DNA polyplexes, the solution of DNA in TE was diluted to a concentration of 20 μg μL^{-1} with 0.03 M NaCl solution and then clarified using a 0.45 μm filter. The small volume of dust-free PEI solution was gently added stepwise to the DNA solution to make PEI/DNA polyplex dispersions with different desired N/P ratios. The average hydrodynamic radius of the PEI/DNA polyplexes was detected by dynamic LLS at a low scattering angle of 20°.

2.9. Ellman's Assay Test

To check for the presence of mercapto groups, the Ellman reagent was prepared by dissolving 5,5'-dithiobis(2-nitrobenzoic acid) in 0.1 M phosphate buffer (pH = 8.0) with a concentration of 0.4 mg mL^{-1} (1.0×10^{-3} mol mL^{-1}). 4 mg of lPEI$_i$ were dissolved in 2 mL of phosphate buffer. 1 mL of Ellman's solution was then added. The reaction mixture was shaken for 15 min in the dark at room temperature. 1 mL of the reaction mixture was diluted with deionized water (2 mL) and analyzed using UV-vis spectrometry.

2.10. Cytotoxicity of PEI Vectors

The MTT assay was used to test the cytotoxicity of each polymer used. PEI solutions with different concentrations were prepared in DMEM. 293T cells were seeded in 96-well culture plate at 10^4 cells/well in 100 μL DMEM medium containing 10% FBS and antibiotics (penicillin at 100 units mL^{-1} and streptomycin at 100 μg mL^{-1}). 24 h later the PEI solution being studied was added to each well. The treated cells were incubated in a humidified environment with 5% CO_2 at 37 °C for 48 h. The MTT reagent (in 20 μL PBS, 5 mg mL^{-1}) was added to each well and further incubated at 37 °C for 4 h. The medium was then removed and replaced with 100 μL of DMSO. The plate was gently agitated for 30 min before the absorbance (A) at 490 nm was recorded with a microplate reader (Bio-rad, USA). The cell viability was calculated from Equation (1):

$$\text{Viability} = (A_{\text{treated}} - A_{\text{blank}})/(A_{\text{control}} - A_{\text{blank}}) \times 100\% \quad (1)$$

where A_{treated}, A_{blank}, and A_{control} are the absorbance of the cells treated with PEI, fresh culture medium, and without any treatment, respectively. Each experiment condition was repeated in triplicate. The data is shown as the mean value ± a standard deviation (± SD).

2.11. Degradation Test of Long lPEI$_{s\text{-}s}$

lPEI$_{s\text{-}s}$ (40 mg) and 1,4-dithio-D,L-threitol (DTT, 1 mg) were dissolved in PBS (2 mL). The mixture was clarified with a 0.45 μm filter to remove dust particles. The final concentration of DTT was 3×10^{-3} mol L^{-1}. LLS was used to in situ monitor the average molar mass of lPEI$_{s\text{-}s}$, i.e., its degradation, in solution by measuring the decrease in the scattering intensity. The weight average molar mass of lPEI$_{s\text{-}s}$ can be calculated using Equation (2):

$$\overline{M_w} = \overline{M_{lPEI_{s-s}}} \left(C_{lPEI_{s-s}} / _{\langle I_0 \rangle lPEI_{s-s}} \right) / \left(C / _{\langle I_0 \rangle} \right), \quad (2)$$

where $C_{\text{lPEIs-s}}$ and C are the concentrations of the initial lPEI$_{s\text{-}s}$ and the degraded lPEI$_{s\text{-}s}$, respectively, and $<I_0>_{\text{lPEIs-s}}$ and $<I_0>$ are the time average scattering intensities of the initial lPEI$_{s\text{-}s}$ and the degraded lPEI$_{s\text{-}s}$ at the zero scattering angle, respectively.

2.12. Gel Retardation Assay

PEI/DNA polyplexes with different desired N/P ratios were prepared by adding an appropriate amount of PEI (5 μL) to 0.4 μg of DNA (5 μL) in PBS. The resultant polyplex dispersions were incubated at room temperature for 5 min and loaded on a 0.8% (w/v) agarose gel containing ethidium bromide in TAE (tris-acetate) buffer. The gel electrophoresis was carried out at 100 V. After 1 h, the DNA bands in the gel were visualized with a UV (254 nm) illuminator and photographed with a Vilber Lourmat imaging system.

2.13. Zeta-Potential Measurements of PEI/DNA Polyplexes

Polyplexes with different N/P ratios were prepared by adding an appropriate amount of PEI (20 μL) to 1.6 μg of DNA (20 μL) in PBS. The resultant polyplex dispersions were further diluted with 1 mL of PBS. The average mobility (μ_E) of the polyplexes under an electric field in an aqueous solution was determined from the frequency shift in a laser Doppler spectrum using a commercial zeta-potential spectrometer (ZetaPlus, Brookhaven) with two platinum-coated electrodes and one He-Ne laser as the light source. Each data point presented in the mobility measurement was averaged over 5 times at 25 °C. The zeta-potential ($\xi_{\text{potential}}$) can be calculated from $\mu_E = 2\varepsilon\xi_{\text{potential}} f(\kappa R_b) / _{(3\eta)}$, where ε is the permittivity of water and $1/\kappa$ is the Debye screening length.

2.14. In Vitro Gene Transfection

The gene transfection experiments were performed in 293T and HepG2 cells using the plasmid pGL3 as an exogenous reporter gene. The gene transfection was conducted by using the PEI/DNA polyplexes formed with different N/P ratios (10/1, 20/1, 30/1, 40/1, and 50/1). The cells were seeded in a 48-well plate at an initial

density of 5×10^4 per well (293T cells) or 3×10^4 per well (HepG2 cells) for 24 h before the gene transfection experiments were conducted. Each PEI/DNA polyplex dispersion with a desired N/P ratio was diluted in serum-free DMEM medium (200 μL) or serum-containing DMEM medium (200 μL) and then administered to the cells at a final concentration of 0.4 μg DNA per well. After 4 h, the transfection medium was removed and the complete DMEM medium (containing FBS and antibiotics, 500 μL per well) was added. The cells were further cultured for 48 h before the transgene expression level was evaluated. A GloMax 96 microplate luminometer (Promega, USA) and the Bio-Rad protein assay reagent were used to determine the transfection efficiency of the PEI/DNA polyplexes, which is expressed as a relative luminescence unit (RLU) per cellular protein (mean ± SD of triplicates).

3. Results and Discussion

Previously, coupling small branch PEI chains via disulfide linkage sometimes resulted in large PEI clusters with a network-like structure and a lower transfection efficiency. Recently, Lee et al.[28] reported a method to prepare degradable linear PEI chains without network formation, but they only checked the transfection efficiency of some large PEIs ($\overline{M}_n \approx 10$ kDa). The small precursor of these PEIs could not be tested. In our study, we purposely linked short linear PEI chains (*l*PEI$_i$) into a long one (*l*PEI$_{s\text{-}s}$) so that we were able to compare their gene transfection efficiencies and find out whether it is the disulfide bonds or the chain length that dominantly affects gene transfection. As discussed before, the initiator was synthesized by the sulfonylation of ethylene glycol and was then used to initiate cationic ring-opening polymerization from both ends, resulting in a linear (Ts-PETOZ-Ts) chain with two terminal tosyl ester ends that were further substituted by thioesters. The acid hydrolysis of such a chain led to the initial linear PEI chain (*l*PEI$_i$) with two mercapto-ends, as shown in Figure 1, where adding the Ellman reagent led to an absorbance at ~412 nm in comparison with the untreated *l*PEI$_i$ solution. After obtaining the thiol terminated *l*PEI$_i$ chains, we coupled them together to form longer linear *l*PEI$_{s\text{-}s}$ chains.

The number- and weight-average molar masses of AcS-PETOZ-SAc, the resultant *l*PEI$_i$, and the coupled *l*PEI$_{s\text{-}s}$ were obtained using GPC and MALDI-TOF-MS (Table S1 in the Supporting Information). The number average molar mass of *l*PEI$_i$ estimated from the GPC results for AcS-PETOZ-SAc was ≈ 1.59 kg mol^{-1}, fairly close to that measured with MALDI-TOF-MS. The resultant long *l*PEI$_{s\text{-}s}$ has a three times higher molar mass than the initial short *l*PEI$_i$, indicating that, on average, each *l*PEI$_{s\text{-}s}$ chain contains three shorter linear *l*PEI$_i$ chains. Their contour lengths (if stretched) are ≈ 15 and ≈ 45 nm, respectively.

Figure 2 shows the kinetics of the reduction of the disulfide bonds inside *l*PEI$_{s\text{-}s}$ after the addition of DTT in terms of the decrease in weight average molar mass ($\overline{M}_w$) measured by LLS. Here we emphasize that the molar mass is not the absolute value because of the polyelectrolyte's nature. It clearly shows that the cleavage of the disulfide bonds inside *l*PEI$_{s\text{-}s}$ takes about 48 h in PBS, although DTT is less effective at pH ≤ 7. The decrease of $\overline{M}_w$ from 5.6 kg mol^{-1} to 2 kg mol^{-1} indicates that longer linear *l*PEI$_{s\text{-}s}$ chains can be nearly degraded into initial shorter *l*PEI$_i$ chains. Due to the redox gradient across the cell membrane, it is expected that longer *l*PEI$_{s\text{-}s}$ chains will be degraded after the polyplexes enter the intracellular space.

Figure 3 shows that, as expected, the cytotoxicity of *l*PEI$_i$, *l*PEI-4.2k, and *l*PEI$_{s\text{-}s}$ increases with the polymer concentration, where large *b*PEI-25k and long *l*PEI-25k are used as controls. Note that in the working concentration range of gene transfection (10^{-3}–10^{-2} mg mL^{-1}), large *b*PEI-25k and *l*PEI-25k are much more cytotoxic than small *l*PEI$_i$, *l*PEI$_{s\text{-}s}$ and *l*PEI-4.2k. The cytotoxicity of large cationic polymer chains has been studied and explained before, and is presumably due to their disruption of anionic cell and organelle

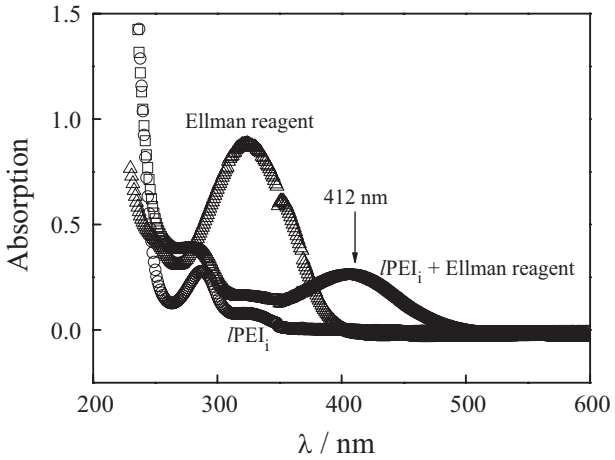


Figure 1. UV-vis spectra of Ellman reagent and *l*PEI$_i$ solutions before and after addition of Ellman reagent.

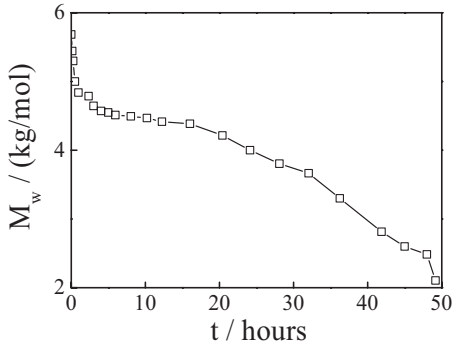


Figure 2. Time dependence of weight average molar mass of *l*PEI$_{s\text{-}s}$ (20 mg mL^{-1}) after the addition of D,L-dithiothreitol (DTT, 3×10^{-3} mol L^{-1}).

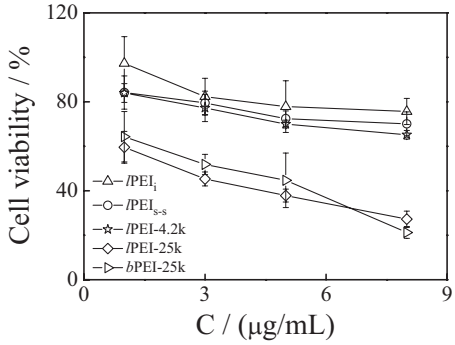


Figure 3. Relative cell viabilities of lPEI$_i$, lPEI$_{s\text{-}s}$, lPEI-4.2k, lPEI-25k, and bPEI-25k in 293T cells at different working concentrations.

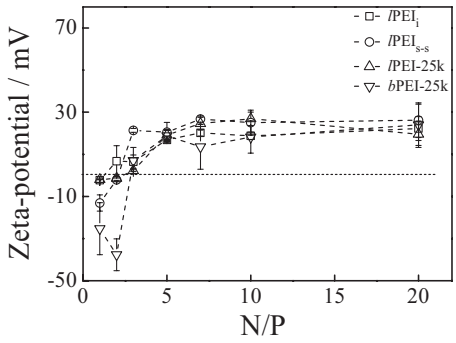


Figure 5. N/P ratio dependence of zeta-potential of PEI/DNA complexes in PBS. (N/P = 1, 2, 3, 5, 7, 10, 20.)

membranes so the cell undergoes necrosis.[17] It should also be noted that linking a few short linear lPEI$_i$ chains together to form a longer lPEI$_{s\text{-}s}$ chain slightly increases its cytotoxicity. However, lPEI$_{s\text{-}s}$ has a similar cytotoxicity with non-degradable lPEI-4.2k, indicating that it is the chain length rather than the degradable –S–S– linkage that mainly determines the cytotoxicity of cationic polymer chains.

Figure 4 shows that short linear lPEI$_i$ can completely condense DNA at N/P ≈ 5, reflected in the disappearance of free DNA strips, while longer linear lPEI$_{s\text{-}s}$ starts to retard DNA at N/P ≈ 3 and also completely condenses DNA at N/P ≈ 5. This indicates that using the disulfide bonds to link short lPEI$_i$ chains together to form a longer lPEI$_{s\text{-}s}$ chain only slightly helps the formation of PEI/DNA polyplexes. It is worth noting that lPEI$_{s\text{-}s}$ has similar condensation ability to large bPEI-25k and lPEI-25k (results not shown) in condensing anionic DNA to form stable polyplexes, suggesting that the condensation ability remains once the chain reaches a certain length.

Figure 5 shows that the zeta potential of the resultant PEI/DNA complexes changes from –30 mV to 20 mV when more cationic PEI chains are added into the solution mixture of PEI and DNA, i.e., as the N/P ratio increases. In accordance with the gel retardation results, the addition of cationic PEI chains inverses the zeta-potential from negative to positive at N/P ≈ 3; furthermore, the zeta potential reaches a plateau at N/P ≈ 5. This clearly indicates that the complexes becomes positively charged at N/P ≈ 3 and are nearly covered by cationic PEI chains at N/P ≈ 5. At higher N/P ratios, most of the PEI chains added must be free in the solution mixture.[32,33] Our previous results have shown that it is these free chains that promote gene transfection.

Figure 6 shows that most of the PEI/DNA polyplexes prepared at different N/P ratios are smaller than 400 nm and the average hydrodynamic radius ($<R_h>$) reaches its maximum at N/P ≈ 5. Further addition of PEI apparently leads to a smaller $<R_h>$. It is worth noting that in the past such a decrease of $<R_h>$ has often been attributed to further condensation of the polyplexes. However, those free PEI chains present in the complex solution have a smaller size, which leads to a decrease in the average hydrodynamic size, especially at the higher N/P ratio. In our cases here, most of the added PEI chains are free in the solution mixture after N/P > 5. Therefore, a decrease in $<R_h>$ is expected at higher N/P ratios.

Figure 7 and Figure 8 reveal that, in the absence or presence of serum, all of the PEI vectors (short lPEI$_i$, long lPEI$_{s\text{-}s}$, lPEI-4.2k, and large but more cytotoxic bPEI-25k and

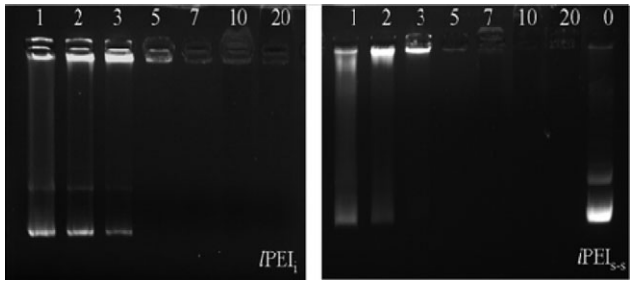


Figure 4. Gel retardation assay of lPEI$_i$/DNA and lPEI$_{s\text{-}s}$/DNA complexes (N/P = 1, 2, 3, 5, 7, 10, 20), where naked DNA (N/P = 0) is used for comparison (as a control).

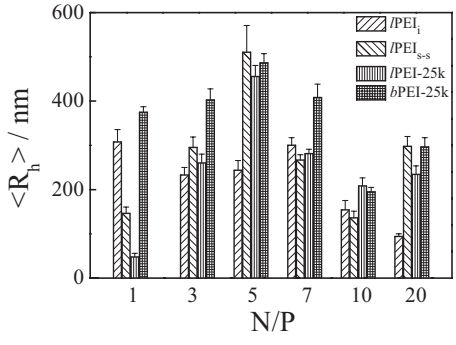


Figure 6. Average hydrodynamic radius of PEI/DNA complexes prepared with different N/P ratios (N/P = 1, 3, 5, 7, 10, 20) in 0.03 M NaCl, where $<R_h>$ was measured by dynamic laser light scattering at 20°.

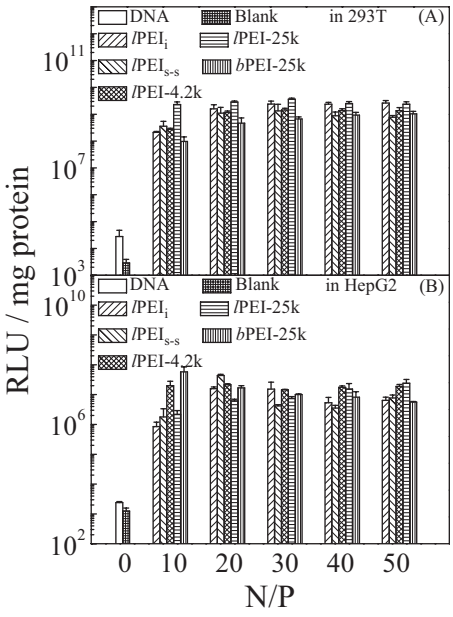


Figure 7. In vitro gene transfection efficiency of PEI/DNA complexes prepared with different PEIs at various N/P ratios (N/P = 0, 10, 20, 30, 40, 50) in serum-free medium.

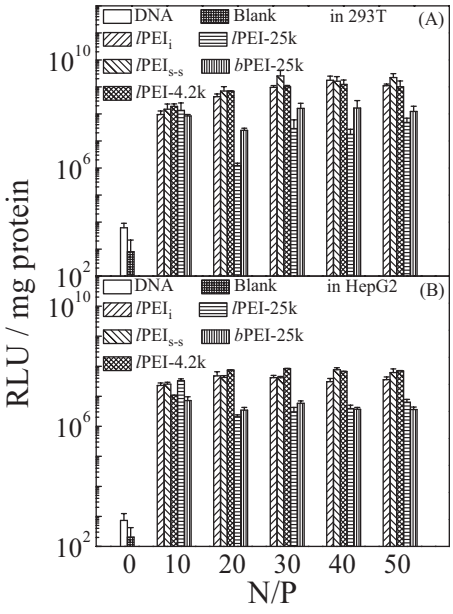
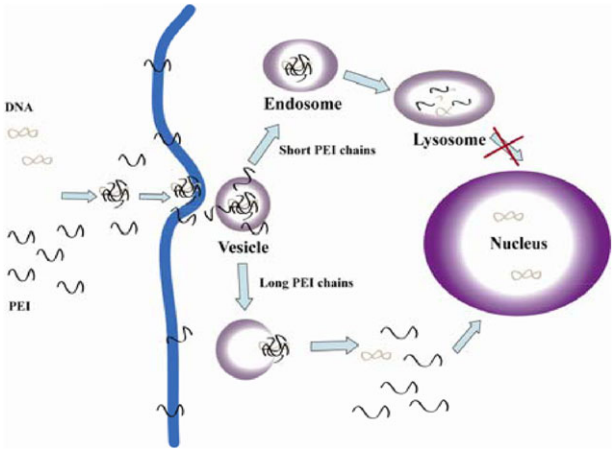


Figure 8. In vitro gene transfection efficiency of PEI/DNA complexes prepared with different PEIs at various N/P ratios (N/P = 0, 10, 20, 30, 40, 50) in serum-containing medium.

*l*PEI-25k chains) have similar gene transfection efficiency. Unexpectedly, coupling a few short *l*PEI$_i$ chains into a longer *l*PEI$_{s\text{-}s}$ does not make it better than short *l*PEI$_i$ and non-degradable *l*PEI-4.2k chains with a similar length. The maximum efficiency occurs at N/P ≈ 20 in serum-free medium and at N/P ≈ 30 in serum-containing medium. We also tested the transfection efficiencies in CHO and COS-7 cells and obtained similar results (Figure S6 and Figure S7 in the Supporting Information). It should also be noted that *l*PEI$_{s\text{-}s}$ showed similar cytotoxicity to non-degradable *l*PEI-4.2k. This indicates that disulfide bonds might not play an important role in increasing the transfection efficiency and reducing the cytotoxicity. The real role of disulfide bonds should be re-considered.

As shown in Figure 7 and Figure 8, the transfection efficiencies of *l*PEI$_i$, *l*PEI$_{s\text{-}s}$, and *l*PEI-4.2k are comparable, which is very different to our previous study, in which *b*PEI-7k with 2–3 disulfide linkages was 10^5–10^7 times more effective than its precursor, small branched *b*PEI-2k.[29] The difference between these two studies is that the chain length of branched *b*PEI-2k is much shorter than that of linear *l*PEI$_i$ though they have a similar weight average molar mass. The question then is why did the chain coupled via the degradable disulfide bonds have a huge effect in a previous study but nearly no effect here? Our answer is that it is the chain length of PEI rather than the disulfide bonds that plays an important role in gene delivery.

Firstly, according to our previous studies,[32,33] there are two kinds of PEI chains in the solution mixture – those bound to DNA and those free in the solution mixture. The bound chains condense DNA to form polyplexes, providing charge neutrality. The free chains can significantly increase the uptake rate constant of the polyplexes, depending on the chain length. In our previous experiments,[29] the initial branched PEI-2k chains were too short to increase the uptake rate and protect DNA from degradation while disulfide linked *b*PEI-2k, i.e., *b*PEI-7k, was much longer so could work well (probably helping DNA to escape from the endosome-lysosome pathway). This explains why simple chain extension results in a huge increase in the gene transfection efficiency. In the current study, the initial linear *l*PEI$_i$ chains are already long enough because of their linear structure. Further chain extension with disulfide bonds has little effect on gene transfection efficiency, but leads to a higher cytotoxicity. Secondly, our recent research indicated that DNA continuously releases from the polyplexes after intracellular uptake.[34] Some polyplexes can escape from the endosome-lysosome pathway; others, as well as the disulfide bonds, will be destroyed in the lysosome. There is no strong correlation between the cleavage of the disulfide bonds and the release of DNA from the polyplexes. Meanwhile, our current study proved that degradable *l*PEI$_{s\text{-}s}$ does not show a higher transfection efficiency than non-degradable *l*PEI-4.2k. A combination of all these studies clearly shows that it is the chain length rather than the disulfide bond that plays a dominant role in promoting gene transfection. Finally, it should be also noted that long PEI chains also induce higher cytotoxicity. It is therefore important to control the chain



Scheme 2. Schematic of the effect of chain length on intracellular trafficking. Long PEI chains can effectively help DNA to escape from the endosome-lysosome pathway, but short PEI cannot.

length to balance the transfection efficiency and cytotoxicity. Scheme 2 summarizes the effect of chain length on intracellular trafficking.

4. Conclusion

By purposely synthesizing an initial linear polyethylenimine (*l*PEI$_i$, $\overline{M}_w \approx 2\,kg\,mol^{-1}$) that is long enough and coupling a few of them together to form a longer *l*PEI$_{s\text{-}s}$ chain via degradable disulfide bonds, we conducted a comparative study of their cytotoxicity and in vitro gene transfection efficiency in different cell lines. Our results revealed that the extension of the chain length makes longer *l*PEI$_{s\text{-}s}$ chains slightly more cytotoxic but has nearly no effect on gene transfection, which is different from our previous study where the gene transfection efficiency increased by ≈5–7 orders of magnitude when three to four small branched PEI chains ($\overline{M}_w \sim 2\,kg\,mol^{-1}$) were coupled together to form a larger one (>15 nm). Such a drastic difference enables us to hypothesize that free cationic PEI chains with a sufficient length may increase the uptake rate and protect DNA from degradation. In the current study, initial linear *l*PEI$_i$ chains were already long enough, so further chain extension via disulfide bonds had nearly no effect on gene transfection, while in our previous study, the initial branched *b*PEI-2k chains with a similar molar mass of *l*PEI$_i$ were much smaller, so chain extension greatly affected gene transfection. A combination of our previous and current results clearly shows that it is the chain length that plays the dominant role in promoting gene transfection rather than the degradable disulfide linkage. Our results also revealed that in the development of non-viral vectors it is important to control the chain length to balance the gene transfection efficiency and cytotoxicity.

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