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A Comparative Evaluation of Disulfide-Linked and Hydrophobically-Modified PEI for Plasmid Delivery

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Abstract

Non-viral gene therapy has become an important approach for treatment of hereditary and acquired diseases as a result of better understanding of molecular mechanisms involved in disease development. To design more effective gene carriers, plasmid DNA (pDNA) delivery to 293T cells was investigated by using two types of polymeric carriers; polymer constructed with disulfide (–S–S–) linkages and polymers modified with hydrophobic moieties. The base polymer used for this study was 2-kDa poly(ethylene imine) (PEI2), a relatively cell-compatible but ineffective gene carrier. The –S–S– linking was achieved *via* Michael addition reaction using cystamine bisacrylamide (CBA), whereas hydrophobic modification by *N*-acylation of PEI2 amines with palmitoyl chloride (PA). The cytotoxicity of the polymers was found to be lower than that of the 25-kDa branched PEI, but both types of modifications increased the toxicity of PEI2 to some extent. The polymers were able to form polyplexes with pDNA with variable hydrodynamic sizes (130–600 nm) and ζ-potential (3.6–20.9 mV). Based on the expression of the reporter gene Enhanced Green Fluorescent Protein (EGFP), disulfide linking significantly increased the efficiency of native PEI2, which was not effective on its own. The PA-modified PEI2 was also effective for gene delivery, but disulfide linkage of this polymer did not increase its efficiency any further. Our results showed that hydrophobic modification of 2-kDa PEI significantly improved its transfection efficiency but improvements in transfection efficiency as a result of disulfide linking was dependent on the nature of the polymeric building blocks.

Keywords

Gene therapy, non-viral vector, poly(ethylene imine), disulfide-linkage, hydrophobic modification

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1. Introduction

Non-viral gene carriers are actively explored for delivery of therapeutic genes due to their flexible chemistry, facile manufacturing and acceptable safety properties [1]. The efficiency of these gene carriers depends on their ability to deliver plasmid DNA (pDNA) into the cell nucleus and to sustain subsequent transgene expression. Cationic polymers, such as poly(L-lysine) (PLL), poly(ethylene imine) (PEI) and poly(amido amine) (PAMAM) dendrimers and cationic lipids have been investigated since they can condense long, string-like pDNA molecules into nano- to micrometer-sized spherical complexes *via* electrostatic interactions [2]. Condensation of pDNA into polyelectrolyte complexes provides an easy route for cellular uptake by endocytosis while sterically protecting the pDNA from nucleolytic enzymes during delivery [3, 4]. However, wide-spread application of non-viral carriers is hindered by their low efficiency and general cytotoxicity [5]. To improve their performance, different functionalities were incorporated into the carriers by chemical modifications [6]. The overall goal is to utilize the beneficial effect of the functional groups on major bottlenecks of gene delivery, such as stronger binding to cell surface, escape from endosomal–lysosomal network and vector unpacking [1, 2, 5].

Low molecular weight (MW) cationic polymers have been cross-linked with stimuli responsive linkages so as to create a delivery mechanism that can respond to environmental conditions [7–10]. Disulfide (–S–S–)-linked polymers is one class of polymers recently explored for gene delivery, since they can exploit the redox-potential gradient between the extra- and intracellular space; while stable in extracellular milieu, the complexes are destabilized intracellularly once exposed to the highly reductive medium [8, 11]. The intracellular destabilization of the complexes consequently facilitates the release pDNA and increases the gene expression [12]. Low-MW PEIs have been typically employed for designing redox-sensitive carriers because of their acceptable biocompatibility and structural composition analogous to 25-kDa branched PEI (PEI25), an effective non-viral carrier. *In vitro* transfection efficiency of 800-Da PEI was shown to increase significantly as a result of –S–S– linking of the polymer with DMSO-mediated oxidation of thiol end-capped groups [13]. Other cross-linkers, such as dithiobis(succinimidylpropionate) (DSP), dimethyl 3,3′-dithiobispropionimidate (DTBP), bis(2-methacryl-oyloxyethyl) disulfide and cystamine bisacrylamide (CBA), were employed to create (–S–S–)-linked PEIs to enhance the transgene expression [9, 11, 14]. Cross-linking of 800-Da PEI with CBA made its transfection efficiency comparable to PEI25, with marginal toxicity [15]. The DSP-linked 800-Da PEI was superior to the DTBP-linked polymers in transfecting CHO cells, indicating the direct impact of the linker moiety [9]. In a different study, Lin *et al.* reported a series of (–S–S–)-linked polymers and observed a general correlation between the transfection efficiency and the hydrophobicity of the side-groups in polymers [14]. The effect of hydrophobicity on transfection efficiency of CBA-linked polymers was also reported by an independent group; the

hydrophobicity of the side-chain was found to be more predominant than the hydrophobicity of the polymer backbone [16]. However, increased hydrophobicity was also reported to have an adverse effect on transfection efficiency for certain types of parent molecules; increasing alkyl spacer from ethylene to propylene between the amino units in the polymeric side-chains resulted in lower transfection [17], suggesting that molecular details are as critical as the polymer hydrophobicity for transfection efficiency.

To further elucidate the role of hydrophobicity on the effectiveness of disulfide-linked polymers, this study prepared and evaluated disulfide-linked polymers from a hydrophobically-modified low-MW (2000) branched PEI (PEI2). We previously explored hydrophobic modification of polycations (PLL and PEI) with aliphatic lipids and investigated a correlation between the extent of lipid substitution and transfection efficiency [18, 19]. This approach efficiently and conveniently converted a non-effective polymer to an effective one for *in vitro* gene delivery. Unlike other studies that employed small amine building blocks that were not effective gene carriers on their own, this study employed a hydrophobically-modified building block that is expected to be effective on its own, allowing us to evaluate if disulfide linking improves the effectiveness of an already effective polymer. The (–S–S–)-linked carriers were designed using native PEI2 and a PEI2 substituted with palmitic acid (PEI2–PA). PA is an endogenous fatty acid that plays a major role on protein membrane interactions and protein trafficking [21]. The (–S–S–)-linked polymers were obtained by Michael addition of CBA to amines of polymers [20]. In this way, the effectiveness of the disulfide-linked *vs* hydrophobically-modified PEIs was also directly compared to explore the relative merit of each approach in gene delivery.

2. Materials and Methods

2.1. Materials

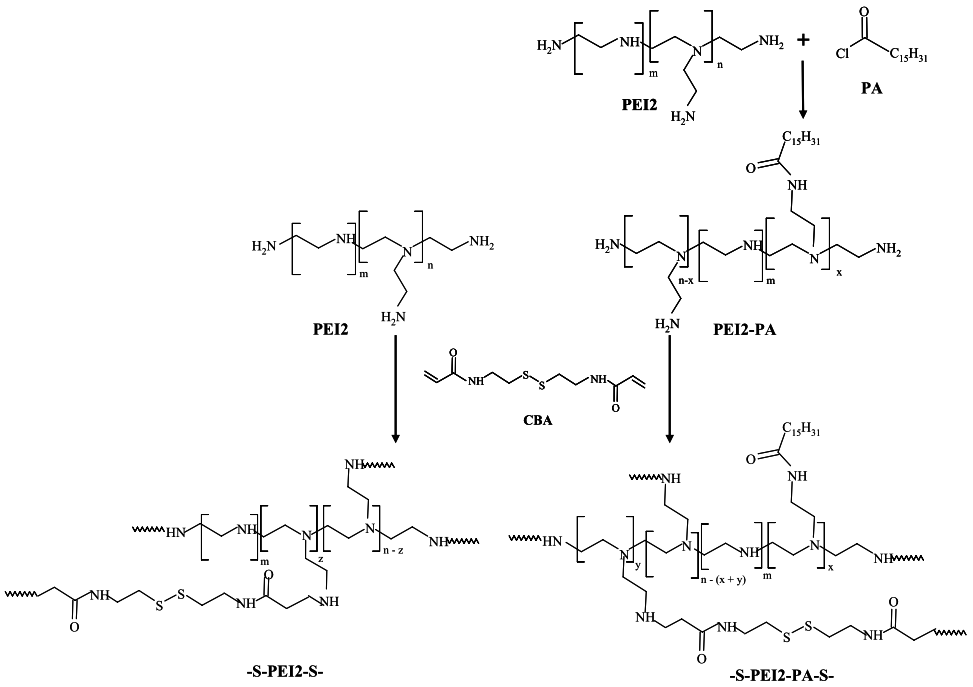
Cystamine · 2HCl, acryloyl chloride, branched PEI25, branched PEI2, palmitoyl chloride (PA) and trypsin/EDTA were obtained from Sigma-Aldrich. The PEI2 (50% aqueous solution) was dried by lyophilization before any reaction. CBA was synthesized in-house according to a previous report [22] and its identity was confirmed by ^{1}H-NMR. Dulbecco's Modified Eagle Medium (DMEM; high glucose with L-glutamine), penicillin (1×10^4 U/ml) and streptomycin (10 mg/ml) were from Invitrogen. Fetal bovine serum (FBS) was from VWR. The plasmids gWIZ (blank plasmid with CMV promoter) and gWIZ–GFP (AcGFP expressing plasmid with CMV promoter) used in transfection studies were purchased from Aldevron. SYBR green II was purchased from Cambrex BioScience.

2.2. Polymer Synthesis and Characterization

The synthesis of (–S–S–)-linked PEI2 (–S–PEI2–S–) was by Michael addition reaction with CBA according a protocol described elsewhere [20]. Briefly, 20 mg

PEI2 was dissolved in 5 ml MeOH/H_2O solution (9:1, v/v) and stirred in a three-necked flask equipped with a condenser at 45°C under N_2. A different amount of CBA solution in methanol was dropped into polymer solutions and mixture was stirred in the dark at 45°C under N_2 atmosphere (Scheme 1). After 24 h of stirring, 10% (w/w) excess PEI2 (1 mg/ml) was added to consume any un-reacted CBA and stirred for another 6 h. The reaction mixture was then diluted to 10 ml with distilled water and acidified to pH 4 with 10 M HCl. The un-reacted polymers were removed by dialysis (MWCO = 3500) against distilled water for 48 h. The final product was collected as a white powder after lyophilizing for 24 h.

The PEI2–PA was synthesized by *N*-acylation of PEI2 with PA as described previously [23]. Briefly, PEI2 (100 mg) was dissolved in $CHCl_3$ (100 ml) and stirred in a three-necked flask under N_2. After 30 min of stirrng, triethylamine (80 µl) was added to the solution, allowed to stir for 30 min, the reaction mixture was cooled to approx. 4°C, and different amounts of PA solution (10, 25 and 50 µl of 1 mg/ml in $CHCl_3$) was added drop-wise under stirring. The reaction mixture was stirred at room temperature for 12 h and the final product was collected by precipitation in excess ether for lyophilization. The (–S–S–)-linked PEI–PA (–S–PEI2–PA–S–) was synthesized under identical conditions to that of the synthesis of –S–PEI2–S– (Scheme 1).



Scheme 1. Schematic representation of the synthesis of (–S–S–)-linked polymers. Native (PEI2) and or PA-substituted PEI2 (PEI2–PA) were reacted with CBA at 45°C in methanol/water (9:1, v/v) under N_2 environment for the preparation of disulfide-linked co-polymers.

The structural composition of the polymers was analyzed by ^{1}H-NMR (Bruker 300 MHz) in D_2O. The integrated values of the characteristic chemical shifts corresponding to PA (δ approx. 0.8 ppm; –C$\underline{H}_3$), PEI2 (δ approx. 2.5–3.0 ppm; –NH–C$_2\underline{H}_4$–) and CBA (δ approx. 3.5 ppm; –S–C$\underline{H}_2$–) were used to obtain the extent of conjugation and cross-linking [22, 23]. The MWs and polydispersities of the polymers were determined by size-exclusion chromatography (SEC). Polymer solution (20 µl, 1 mg/ml in water) was injected into a 30 cm × 4.6 mm column (60 Å, ES Industries) which was attached to a Waters 600E pump. The mobile phase was 150 mM NaCl (pH 2.0) with a flow rate of 0.3 ml/min and the elution pattern was detected by a refractive index (Waters Model 410) and a light scattering detector (PD2020; Precision Detectors). The MW of the polymers was estimated using Precision MW Analysis software according to the following formula: $\mathrm{MW} = (K_2 \times \mathrm{ILS})/(K_1 \times I_{\mathrm{RI}} \times (\mathrm{d}n/\mathrm{d}c))$, where ILS is light scattering signal intensity, I_{RI} refractometer signal intensity, $\mathrm{d}n/\mathrm{d}c$ refractive index increment, and K_2 and K_1 are calibration constants [24]. The calibration constants were obtained by using a PEI2 solution (1 mg/ml in water) as a standard.

2.3. Acid–Base Titration

Buffering capacity of the polymers was determined by acid–base titration as described elsewhere [14]. Briefly, polymer solution (0.2 mg/ml) was prepared in 150 mM NaCl and the pH was set to 10 with aqueous NaOH (0.1 M). Then, the solution was titrated from pH 10 to 2 with 0.1 M HCl and pH values were measured continuously. For comparison, 0.2 mg/ml PEI25 in 150 mM NaCl and a 150 mM NaCl solution without any polymer were titrated under identical conditions.

2.4. Dye Exclusion Assay

The pDNA complexation of the polymers was measured by a dye exclusion assay. The stock polymer solution (5 µg/ml) was diluted with NaCl (150 mM) in polypropylene tubes to give final concentrations of 0.1 to 4 µg/ml. Subsequently, 4 µl pDNA (25 µg/ml) was added to each tube to produce complexes of mass ratios 0.025 to 1.0. After 30 min incubation, 1.0 ml SYBR green II (1×) was added to each tube and 250 µl of each sample was read on a 96-well plate (Fluoroskan Ascent; Thermo Labsystems) at λ_{EX} of 485 nm and λ_{EM} of 527 nm to quantify the amount of free pDNA. The fluorescence values so obtained (in triplicate) was normalized with the fluorescence of free pDNA solution (i.e., in the absence of polymers) and plotted as a function of polymer/pDNA ratio.

2.5. Particle Size and ζ-Potential Measurements

Size and ζ-potential of the complexes were determined using dynamic light scattering (DLS) and electrophoretic light scattering (ELS) using a Zetasizer 3000 HS (Malvern Instrument) equipped with a He–Ne laser and operated at 10 mW. Polymer solutions (20 µg/ml) were diluted with 150 mM NaCl to give final concentrations of 8.3 and 16.6 µg/ml in a volume of 300 µl. Subsequently, 40 µl pDNA

(25 μg/ml) was added to each sample to give complexes with mass ratios of 2.5 and 5.0. The complexes were incubated for 30 min at room temperature and the measurements were performed after diluting each sample to 3 ml of 150 mM NaCl.

2.6. Atomic Force Microscopy (AFM) Measurement

Size and surface morphology of the complexes prepared in 150 mM NaCl (mass ratio 5.0) was observed by MFP-3D AFM (Asylum Research) with inverted optical microscope in contact mode using Si cantilever with a spring constant of 3.5 N/m and a resonance frequency of 75 kHz. AFM samples were prepared by dropping 10 μl of the complexes on mica (PELCO® Mica Discs) and drying overnight before assessing the AFM images.

2.7. Cytotoxicity Evaluation by MTT Assay

The cytotoxicity of the polymers was tested in 293T cells. Cells were seeded in 48-well flat-bottomed multiwell plates and allowed to grow for 24 h in 500 μl DMEM with 10% serum, 100 U/ml penicillin and 100 μg/ml streptomycin in a 37°C incubator with 95/5% air/CO_2. The cell confluence was typically 80–90% at the time of testing. Different amounts of polymers (1 mg/ml in H_2O) were directly added to each well (in triplicate) to give final polymer concentrations of 1.25 to 20 μg/ml and the cells were incubated for 24 h at 37°C. MTT solution (100 μl, 5 mg/ml in HBSS) was added to each well and further incubated for approx. 2 h. The medium was then replaced with 500 μl DMSO to dissolve the MTT crystals formed. The optical density was measured at 570 nm using a microplate reader. Cell viability (%) was calculated by normalizing the absorbance of the polymer-treated samples with the absorbance of untreated cells, which was used as a reference control (i.e., 100% cell viability).

2.8. Transfection Studies

The complexes were prepared in 150 mM NaCl at three different mass ratios (1.0, 2.5 and 5.0) and used for *in vitro* transfection of 293T cells. Typically, 7.5 μl pDNA (0.4 μg/μl) was added to polymer solutions at desired mass ratios (total volume 60 μl), and incubated for 30 min at room temperature before addition to the cells. The 293T cells were seeded in 24-well plates (500 μl/well) and allowed to grow for 24 h prior to transfection. The medium was replaced just before transfection with the fresh medium containing 500 μl DMEM supplied with 10% serum, penicillin (100 U/ml) and streptomycin (100 μg/ml). The complexes (20 μl) were directly added to each well in triplicate and incubated for 24 h. Cell culture media was then replaced with fresh media. After 2 and 7 days, the cells were trypsinized and fixed using 3.5% formaldehyde in HBSS without phenol red (300 μl). The GFP gene expression was quantified using a Beckman Coulter QUANTA™ SC Flow Cytometer using the FL1 channel (3000–5000 events/sample). Instrument settings were calibrated for each run so as to obtain a background level of GFP gene expression of 1–2% for control samples (i.e., untreated cells). The mean fluorescence/cell for the GFP positive cells was also determined.

2.9. Cell Uptake Studies

Plasmid uptake experiments were assessed in 293T cells using FITC-labeled gWIZ and flow cytometry under experimental procedures that were similar to the *in vitro* transfection study. For plasmid labeling, 20 µl gWIZ (5 µg/µl) was incubated with 30 µl FITC (100 mM in DMSO) in 300 µl aqueous DMSO (1:1, v/v) for 3 h at 37°C. The labelled plasmid was precipitated with 825 µl ethanol (95%) in the presence of 30 µl sodium acetate (3 M). The final mixture solution was cooled to −20°C for 15 min and centrifuged for 10 min at 13.8×10^3 rpm, and washed with 95% ethanol (×2). The precipitated plasmid was dissolved in ddH_2O to prepare 0.4 mg/ml gWIZ–FITC solution. The complexes (mass ratio of 2.5 and 5.0) were prepared by mixing 1 mg/ml polymer solutions (see the figure legends for exact polymers used) with 0.4 mg/ml gWIZ–FITC in 150 mM NaCl. The complexes (10 µl) were directly added to each well (in triplicate) containing 293T cells grown in 24-well plates. After 24 h of incubation, cells were washed once and treated with 100 IU/ml heparin for 30 min at 37°C in the presence of 75 mM sodium azide to remove any extracellularly-bound complexes. Further processing and flow cytometry were performed as in the transfection studies.

3. Results

3.1. Polymer Synthesis and Characterization

The (–S–S–)-linked PEI2 (–S–PEI2–S–) was synthesized by Michael addition of CBA to the primary amines of PEI2 at three different mole ratios (CBA/PEI = 0.9, 1.8 and 3.6; Table 1). The complete disappearance of the resonance peaks corresponding to –C<u>H</u>=C<u>H</u>$_2$ at δ approx. 5–7 ppm and appearance of resonance peak corresponding to –S–C<u>H</u>$_2$– at δ approx. 3.5 ppm of CBA along with the characteristic resonance peaks of PEI2 in ^{1}H-NMR spectra confirmed the expected identity of the final products. The disulfide content (CBA/PEI2) of the final polymers, ob-

Table 1.
Preparation conditions for the polymers synthesized in this study and their properties

Sample ID	Polymer (mg)	CBA (mg)	CBA/PEI2		$M_w \times 10^{-3}$	$M_n \times 10^{-3}$	PDI
			Feed ratio	Calculated from ^{1}H-NMR spectrum			
–S–PEI2–S-I	20	9.76	3.7	2.67	5.1	3.7	1.4
–S–PEI2–S-II	20	4.88	1.8	1.75	8.2	4.3	1.9
–S–PEI2–S-III	20	2.44	0.9	0.82	9.3	5.0	1.9
PEI2–PA	–	–	–	–	15.0	4.3	3.5
–S–PEI2–PA–S-I	20	9.76	3.7	2.5	22.0	7.14	3.0
–S–PEI2–PA–S-II	20	4.88	1.8	1.7	30.2	6.7	4.5
–S–PEI2–PA–S-III	20	2.44	0.9	0.72	30.0	3.82	7.85

tained from the ^{1}H-NMR spectra (δ approx. 3.5 ppm for –S–C$\underline{H}_2$– and δ approx. 2.5–3.0 ppm for –NH–C$_2\underline{H}_4$–), was 2.67, 1.75 and 0.87, and corresponded to the feed ratios of 3.6, 1.8 and 0.9, respectively. The MW of the polymer was inversely proportional to the CBA content in reaction mixture and ranged between 5100 and 9300 (Table 1). Incubating the polymers with 10 mM mercaptoethanol for 2 h resulted in reduction of the MW to approx. 2000 (SEC), indicating the cleavable nature of the prepared polymers (not shown).

Hydrophobic modification of PEI2 was carried out at 4°C using low PEI2 concentration due to rapid reaction kinetics. The selected feed ratios for the modifications were 0.72, 1.8 and 3.6 (lipid/PEI2) since the products at higher mol ratios (>3.6) were insoluble in water, making them not useful for gene delivery. ^{1}H-NMR spectra were in line with the expected structural composition of the polymers. The characteristic resonance peaks corresponding to the palmitoyl Hs, –C$\underline{H}_3$ (δ approx. 0.8 ppm), γ-C$\underline{H}_2$ (δ approx. 1.26 ppm), β-C$\underline{H}_2$ (δ approx. 1.6 ppm) and α-C$\underline{H}_2$ (δ approx. 2.16 ppm) were clearly resolved in the ^{1}H-NMR spectra. The extent of conjugations (lipid/PEI) calculated using the ratio of peaks corresponding to –C$\underline{H}_3$ (δ approx. 0.8 ppm for PA) and –NH–C$_2\underline{H}_4$– (δ approx. 2.5 to 3.0 ppm for PEI2) were 0.2, 0.6 and 2.9 for the three mol ratios used. Among the three different PEI2–PAs obtained, the polymer with the highest lipid content (2.9 lipid/PEI) was selected for synthesis of the (–S–S–)-linked polymers. As with –S–PEI2–S, ^{1}H-NMR spectra of –S–PEI2–PA–S– exhibited the characteristic peaks corresponding to CBA, PEI and PA. The disulfide content of the final polymers was 2.5, 1.7 and 0.72 CBA/PEI2–PA for the feed ratios of 3.6, 1.8 and 0.9, respectively. The assessment of the MW of PEI2–PA-based polymers was attempted under various chromatography conditions. However, the PEI2–PA yielded a MW that was higher than the expected MW (approx. 15×10^3 *vs* the theoretical MW of approx. 2000), possibly due to lipid-mediated aggregation of polymers in aqueous media and/or lower solubility of the polymers (i.e., incomplete dispersion) under aqueous elution conditions. Although the MWs of –S–PEI2–PA–S– polymers were found to be higher than the MW of the PEI2–PA under the same conditions (as expected), the obtained values were not considered to be accurate. No organic solvent could be identified either that provided a clear elution profile for this amphiphilic polymer, so that our MW analysis of the hydrophobically-modified polymers were considered inconclusive.

3.2. Titration, Complex Formation and Size/ζ-Potential of Polyplexes

The endosomal escape of PEI-based polyplexes is facilitated by the buffering capacity of the polymers under endosomal pH conditions. All tested polymers showed a significant buffering capacity as they were titrated from pH 10 to 2 (Fig. 1). There were no differences in the buffering capacity of PEI25, PEI2 and PEI2–PA (from synthesis III), indicating that the buffer capacity did not depend on the MW of the polymer. Since only approx. 18% of primary amines of PEI2–PAs were modified, this did not seem to make a significant difference in the buffering

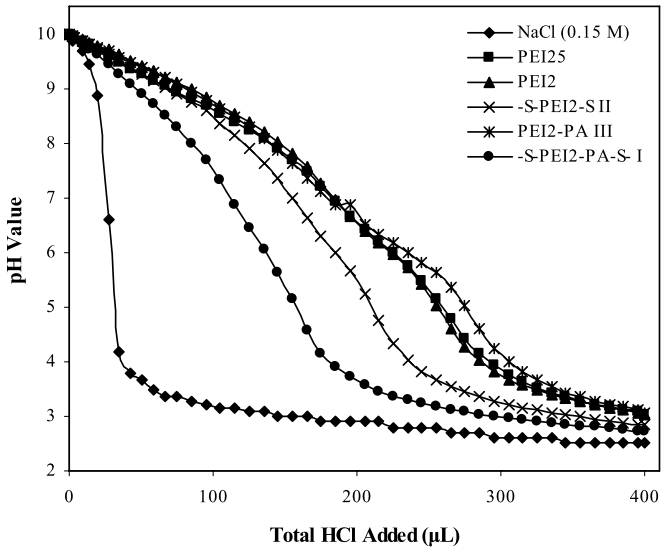
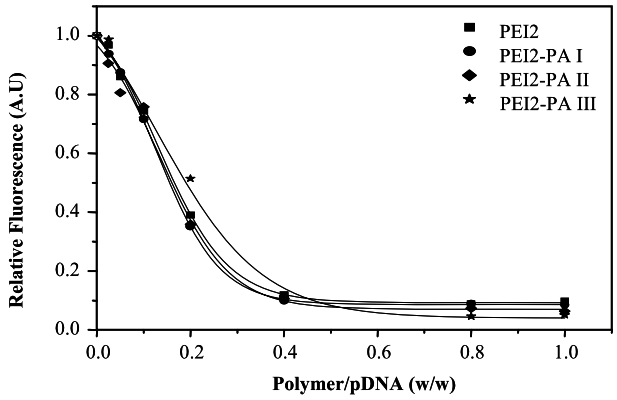
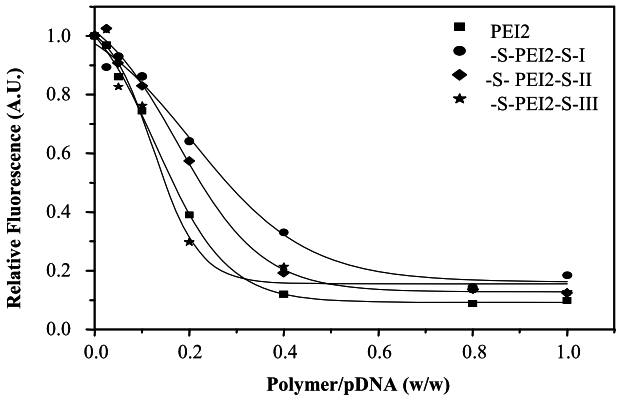


Figure 1. Acid titration profiles of 150 mM NaCl, PEI25, PEI2, –S–PEI2–S–, PEI2–PA (from synthesis III) and –S–PEI2–PA–S– solutions. Polymer solutions at pH 10 were titrated with 0.1 M HCl up to pH approx. 2 at room temperature, while recording the changes in pH value.

capacity. The buffering capacities of –S–PEI2–S– and –S–PEI2–PA–S– were significantly decreased, in line with the reduced density of protonable amine groups. The pDNA binding by the polymer was evaluated by measuring the amount of free pDNA remaining after complex formation. The fluorescence intensity of the dye–pDNA intercalated complex was decreased with the polymer concentration in solution, indicating an efficient polyplex formation (Fig. 2). The pDNA binding by PEI2 was not affected after PA grafting onto the polymer (Fig. 2A). The –S–PEI2–S– and –S–PEI2–PA–S– polymers generally displayed a lower affinity to the pDNA (indicated by a right-ward shift in Fig. 2B and 2C). Whereas increased CBA/PEI2 ratios in –S–PEI2–S– polymers resulted in less affinity towards the pDNA, no clear relationship was evident with the CBA/PEI2–PA ratios in the case of –S–PEI2–PA–S– polymers. The influence of polymer type and its content on complex size was determined through dynamic light scattering and AFM analysis. At higher mass ratio (polymer/pDNA = 5.0) the mean hydrodynamic diameter of PEI2/pDNA and –S–PEI2–S–/pDNA complexes ranged from 500 to 600 nm, with no obvious effect of disulfide linking on the size. However, PEI2–PA/pDNA and –SPEI2–PA–S–/pDNA were more tightly condensed, indicated by the lower particle sizes of 130–200 nm (Fig. 3A). AFM micrographs of the complexes (polymer/DNA = 5.0) indicated discrete, smooth, and homogenous particles with variable diameters. The PEI2/pDNA and –S–PEI2–S–/pDNA complexes were slightly larger in AFM (>100 nm), but PEI2–PA/pDNA and –S–PEI2–PAS–/pDNA complexes were more condensed (<100 nm) (Fig. 4). Based on the ζ-potential measurement, all polyplexes had positive charges ranging from +3.6 to +20.9 mV



(A)



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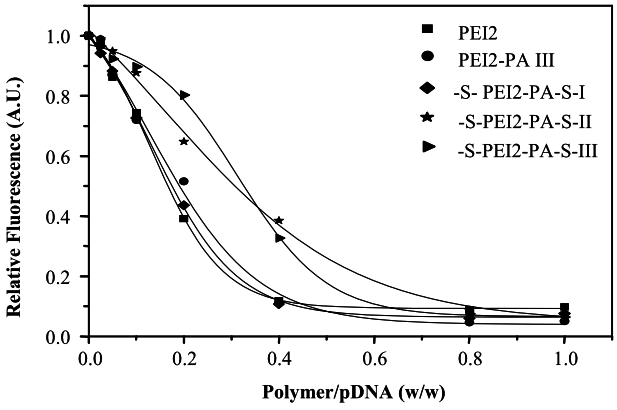


(C)

Figure 2. pDNA complexation with PEI2–PA (A), –S–PEI2–S– (B) and –S–PEI2–PA–S– (C) polymers at polymer/pDNA mass ratios of 0.0 to 1.0 (w/w). Different concentrations of polymers were incubated with pDNA in 150 mM NaCl for 30 min and subsequently treated with SYBER GREEN II for the fluorescence measurement. The obtained fluorescence values were normalized with the fluorescence of free pDNA (i.e., no polymer addition).

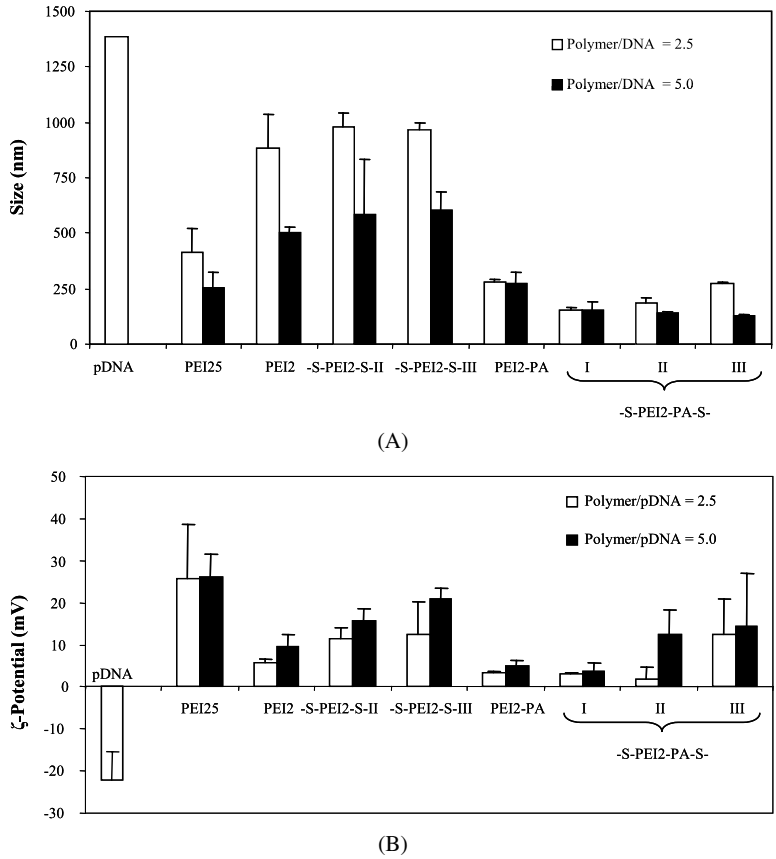


Figure 3. Hydrodynamic size (A) and ζ-potential (B) of the complexes prepared with polymer/pDNA mass ratios of 2.5 and 5.0 (w/w). The complexes were prepared by incubating the polymers with pDNA in 150 mM NaCl. The indicated values represent the size and ζ-potential as means $\pm$ SD of triplicate measurement.

(Fig. 3B). The ζ-potential of PEI2/pDNA (+9.4 mV) was increased to +15.8 (–S–PEI–S– from synthesis II) upon disulfide linking, while it was slightly decreased to +5.0 mV (PEI2–PA from synthesis III) upon hydrophobic modification. The ζ-potential of the complexes with –S–PEI2–PA–S– (from synthesis III), however, was slightly increased to +14.3 mV, similar to the properties of –S–PEI2–S–.

3.3. Biological Assessments

The cytotoxicity of the polymers on 293T cells is summarized in Fig. 5. The PEI2 was observed to exhibit negligible toxicity on the cells with cell viability >90% at concentrations of 1.25 to 20 μg/ml. This was unlike the PEI25, which displayed a significant cytotoxicity starting at 5 μg/ml. The cell viability was slightly decreased (65–85%) with –S–PEI2–S– polymers, whereas the PA substitution resulted in a more dramatic decrease in cell viability (20–90%) with the selected polymer

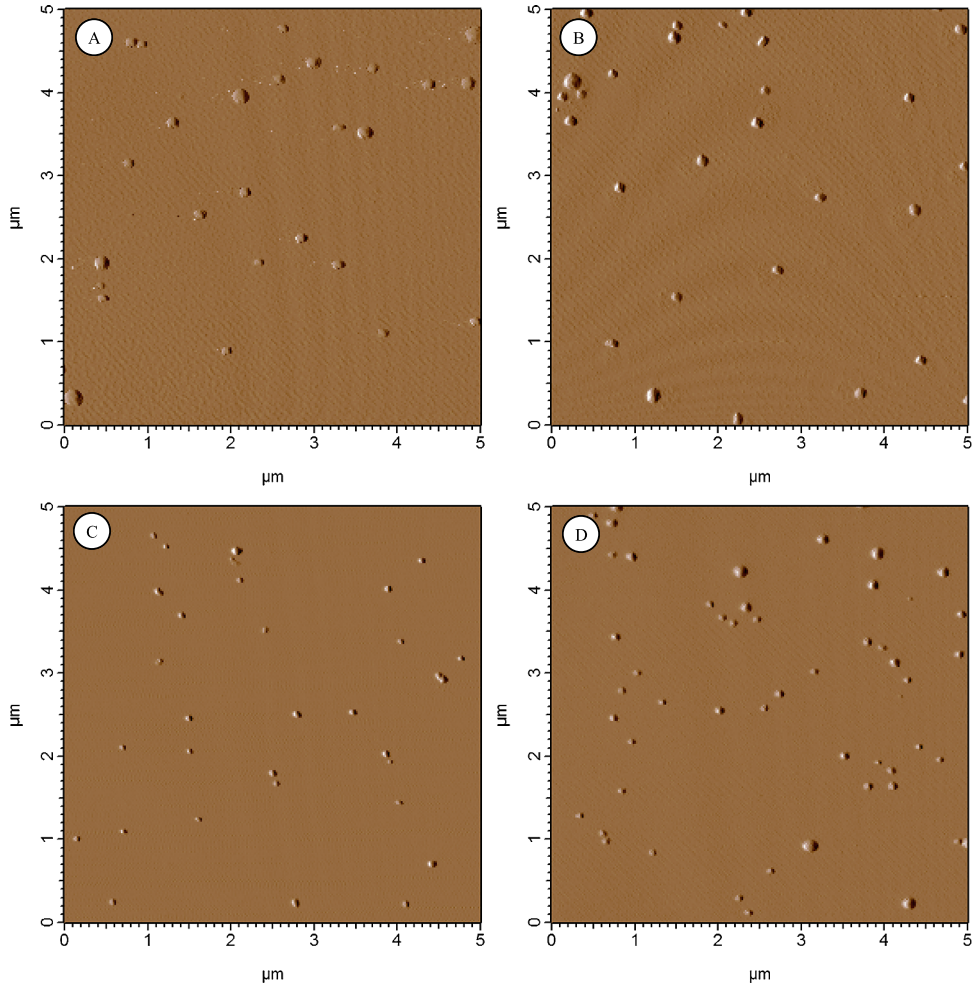


Figure 4. AFM micrographs of PEI2/pDNA (A), –S–PEI2–S–/pDNA (B; polymer from synthesis II), PEI2–PA/pDNA (C; polymer from synthesis III) and –S–PEI2–PA–S–/pDNA (D; polymer from synthesis I) complexes. The complexes were prepared in 150 mM NaCl by incubating the polymers with pDNA at polymer/pDNA mass ratio of 5.0. This figure is published in colour in the online edition of this journal, that can be accessed *via* http://www.brill.nl/jbs

concentrations. Although the PEI2–PA with the highest disulfide content (from synthesis III) was clearly cytotoxic, the –S–S– linking of this PEI2–PA resulted in less cytotoxic polymers (cell viability 30–95%, depending on the polymer concentration). Transfection experiments were performed with a GFP plasmid after forming complexes at three different mass ratios (1.0, 2.5 and 5.0). The complexes with the PEI25 was prepared at optimized mass ratios of 1.0 and 2.5, and cells without any complexes were used a negative control. A blank plasmid (gWIZ) with no reporter gene was initially used as a control treatment. Our previous studies using human

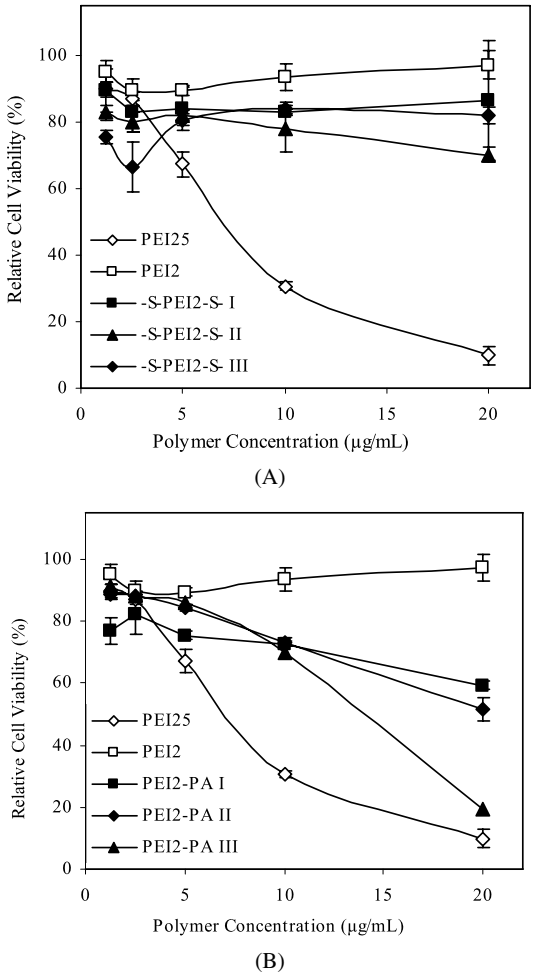
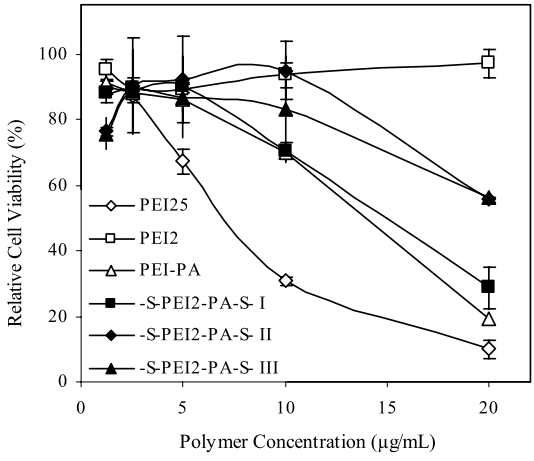


Figure 5. The relative viability of 293T cells in (–S–S–)-linked PEI2 (A), PA substituted PEI2 (B) and (–S–S–)-linked PEI2–PA polymers at different polymer concentrations (20, 10, 5, 2.5 and 1.25 µg/ml), exposed for 24 h. The relative cell viabilities shown (mean ± SD of triplicate wells) were obtained from the MTT assay and they were expressed relative to the cells without polymers (=100%).

fibroblasts indicated a significant auto-fluorescence with simple exposure of the cells to the complexes, and treatment of cells with gWIZ complexes was intended to account for this background in the current study [19]. The results showed negligible auto-fluorescence in the 293T cells under current experimental conditions (not shown), indicating relatively insensitivity of these cells to the polyplex treatment. The PEI2 was not effective for GFP expression but the –S–PEI2–S– polymers (in particular polymer II) gave a significant GFP expression both on days 2 and 7 (Fig. 6A and 6B, respectively). A gradual increase in transgene expression was observed with increasing polymer/pDNA mass ratio. The transfection efficiency of –S–PEI2–S-II (polymer/pDNA = 5.0, w/w) was 35-fold higher than PEI2 on day



(C)

Figure 5. (Continued.)

2 and 55-fold higher on day 7 based on the GFP-positive cells obtained. Similar conclusions were reached based on the mean fluorescence in the cell population. The PEI25 was more effective than the –S–PEI2–S– polymers in this system on both days. The PA substitution on PEI2 was beneficial in transgene expression in 293T cells and transfection efficiency was proportional to the PA content of polymers described in Table 1. The PEI2–PA from synthesis III with the highest lipid content (lipid/PEI = 2.9) was selected for further study since it yielded the best transfection efficiency among the PA-substituted polymers. Transfection efficiency of this PEI2–PA (based on percent GFP-positive cells) was approx. 55- and approx. 65-fold higher than the efficiency of PEI2 on day 2 and day 7, respectively (Fig. 7). The mean fluorescence intensity of cells exposed with the complexes of PEI2–PA was also significantly higher than the unmodified PEI2. However, the disulfide linking of PEI2–PA did not lead to improved transfection efficiency. Although –S–PEI2–PA–S– from synthesis I displayed slightly higher transfection than the parent polymer at the polymer/pDNA mass ratio of 1.0, PEI2–PA gave the highest GFP expression as compared to the other disulfide-linked PEI2–PAs at mass ratios of 2.5 and 5.0. The mean fluorescence intensity of the cells treated with the complexes of disulfide-linked PEI2–PA also gave a similar result. Based on the uptake of FITC–gWIZ, the plasmid uptake by PEI2 was relatively low and it did not vary with the polymer/pDNA mass ratio in the complexes (Fig. 8). This was unlike PEI25 and PEI2–PA that displayed a higher plasmid uptake as a function of polymer/pDNA mass ratio in the complexes. The plasmid uptake with the –S–PEI2–S– polymers was similar to the PEI2 and comparatively lower than PEI25 complexes at polymer/pDNA mass ratios of 2.5 and 5.0 (Fig. 9A). All disulfide-linked PEI2–PA polymers showed a similar level of uptake as the PEI2–PA (Fig. 9B), whose plasmid delivery was equivalent to that of the PEI25.

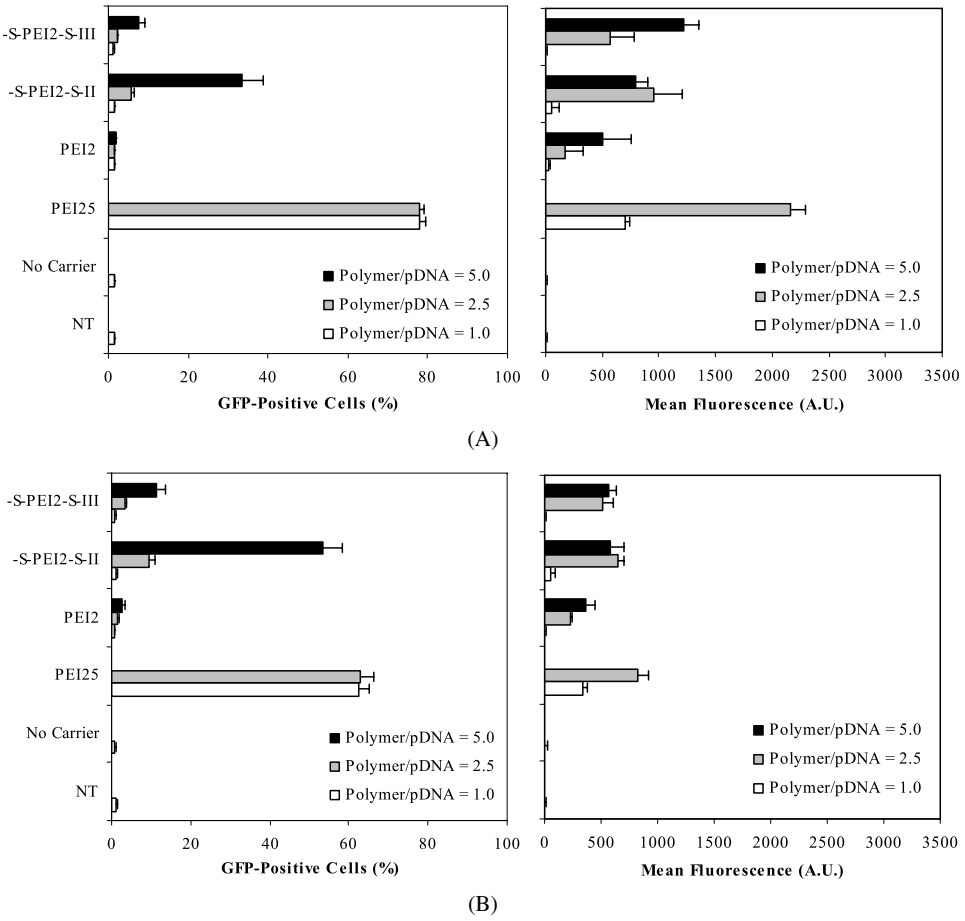


Figure 6. Transfection efficiency in 293T cells treated with the complexes of PEI25, PEI2 and –S–PEI2–S– polymers (II and III) on day 2 (A) and day 7 (B). The polymer/pDNA mass ratios were 1.0, 2.5 and 5.0 (w/w) for PEI2 and –S–PEI2–S– complexes, and 1.0 and 2.5 for PEI25 complexes. No treatment (NT) and plasmid without any carrier served as negative controls, while PEI25 served as a positive control. The results are summarized as either percentage of GFP-positive cells or mean fluorescence of cell population (mean ± SD of triplicate wells).

4. Discussion

The basic requirements for clinical application of non-viral gene carriers are efficiency and safety. The efficiency of a non-viral carrier is directly affected by several factors, in particular cell binding affinity of the complexes and rate of intracellular pDNA release [1, 2, 5]. To address these issues, chemical modifications of low-MW cationic polymers have been employed since such polymers are relatively cell-compatible. The low gene-delivery efficiency of these carriers has been addressed by hydrophobic-modification or –S–S– linking [11, 18, 19]. The hydrophobic modification is expected to enhance the cell binding affinity and plasma membrane

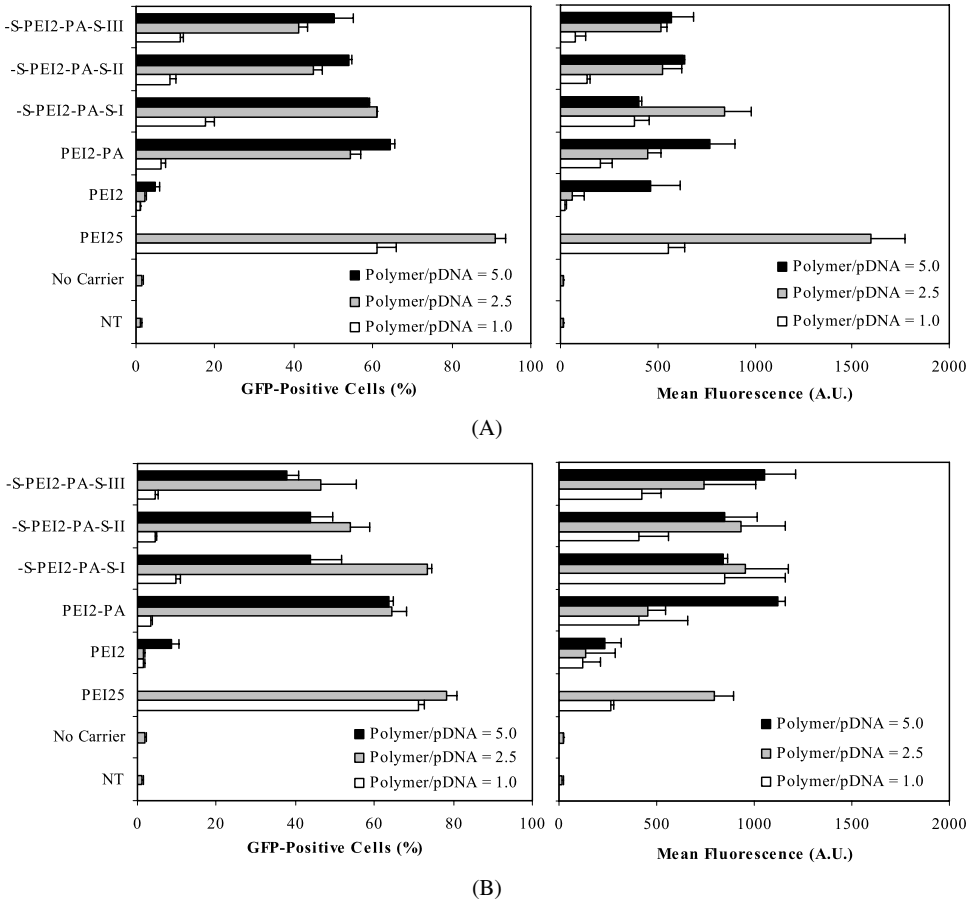


Figure 7. Transfection efficiency in 293T cells treated with the complexes of PEI25, PEI2, PEI2–PA and –S–PEI2–PA–S– polymers (I, II and III) on day 2 (A) and day 7 (B). The polymer/pDNA mass ratios were 1.0, 2.5 and 5.0 (w/w) for PEI2, PEI2–PA and –S–PEI2–PA–S– complexes, and 1.0 and 2.5 for PEI25 complexes. No treatment (NT) and plasmid without any carrier served as negative controls, while PEI25 served as a positive control. The results are summarized as either percentage of GFP-positive cells or mean fluorescence of cell population (mean ± SD of triplicate wells).

permeability of the complexes, whereas disulfide linking is expected to facilitate intracellular reductive degradation and subsequent release of DNA [11, 18]. Since PEI25 is considered to be effective but too cytotoxic for clinical use, we considered the 2-kDa PEI as a good starting point for the desirable chemical modifications. We observed that the –S–S– linking of PEI2 decreased the pDNA binding affinity to some extent, likely due to lower density of amino groups. This was supported by the decreased buffering capacity of the polymer, which is essentially a measure of the available amine groups for H^+ binding [14].

Transfection efficiency of PEI2 was significantly increased after –S–S– linking, clearly indicating the beneficial effect of the modification in pDNA delivery, as

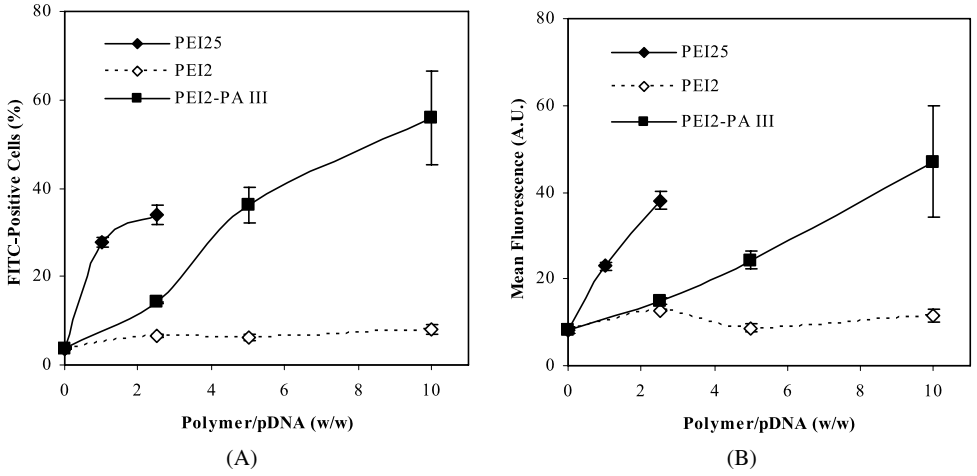


Figure 8. Plasmid uptake in 293T cells treated with the complexes of PEI25, PEI2 and PEI2–PA III with polymer/gWIZ–FITC mass ratios 1.25 to 10.0. The complexes were prepared with FITC labelled pDNA and incubated with the cells for 24 h, after which the cell levels of the plasmid was determined by flow cytometry. The results (mean ± SD of triplicate wells) were summarized as either FITC-positive cells (A) or mean fluorescence of the cell population (B). Untreated cells (NT; not shown) and cells exposed to the pDNA without any carrier gave background levels of uptake (approx. 3.5% FITC-positive cells; mean fluorescence approx. 8).

observed by others [9, 11, 15]. The enhanced transfection efficiency of disulfide-linked PEI2 might be due to increased plasmid delivery due to higher MW, and/or reductive cleavage of disulfide linkages in the cytoplasm to release the pDNA [11, 25]. However, the latter was reported to be the dominant mechanism by others [9, 11]. We note that the parent polymer (PEI2), which is expected to be liberated after intracellular cleavage, should still be capable of pDNA binding on its own. In fact, our data (not shown) did not indicate released pDNA upon treating the complexes with disulfide-cleaving mercaptoethanol. We believe the enhanced transfection efficiency of disulfide-linked PEI2 might be due to increased MW, as well as disassembly of complexes [25]. Since –S–S– linking did decrease the pDNA binding efficiency, the polymer might display increased propensity to disassemble on its own once internalized.

The PA substitution on PEI2 showed a small undesirable effect on pDNA complexation ability of the polymer. The decreased amine content and/or increased steric hindrance might have been the reason for this effect. However, the significant decrease in the size of the complexes formed with the PEI2–PA showed a dramatic effect of PA substitution. This was most evident when the complex sizes were assessed in hydrated state (i.e., from dynamic light scattering). The particle sizes measured with the dynamic light scattering were larger than the sizes assessed by AFM, but this could be attributed to the need to dry the complexes for AFM analysis, which would artificially shrink the complexes. The hydrophobic interactions among the substituted PAs were presumably the major driving force for the

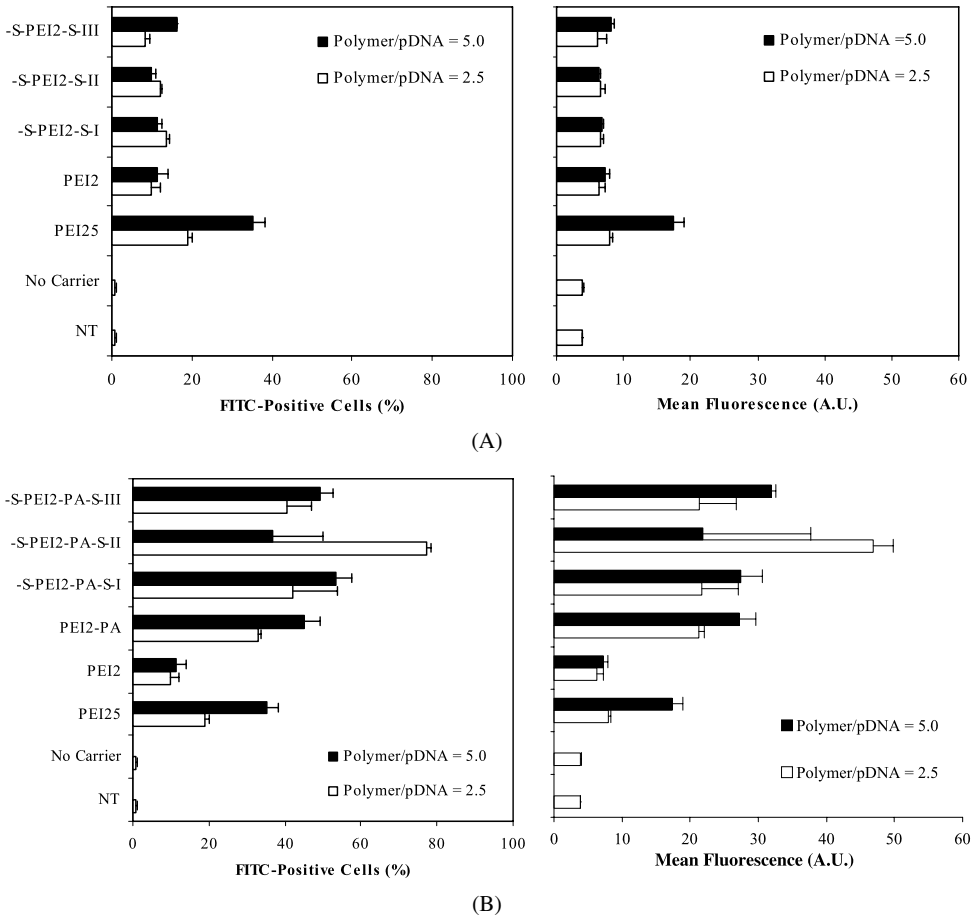


Figure 9. Plasmid uptake in 293T cells treated with the complexes of different polymers (PEI25, PEI2, PA-substituted PEI2 (PEI2–PA) and corresponding (–S–S–)-linked polymers) with gWIZ–FITC (mass ratios: 2.5 and 5.0). The results (mean ± SD of triplicate wells) were summarized as FITC-positive cells and mean fluorescence of the cell population. No treatment (NT) and plasmid without carrier gave similar results (approx. 1%). PEI25, PEI2–PA III and (–S–S–)-linked PEI2–PA display significantly higher cell uptake (A) as compared to the PEI2 and its (–S–S–)-linked derivatives (B).

reduced particle sizes. The disulfide linking of PEI2–PA led to complex formation with slight changes in size and ζ-potential, but this effect was not as significant as the initial PA substitution on PEI2.

The beneficial effect of lipid substitution was clearly observed by the increased transfection efficiency in 293T cells (i.e., approx. 65-fold higher percent GFP-positive cells as compared to native PEI2). The linear increase in the transfection efficiency of PEI2 with the PA content is consistent with our previous result, which implied that the lipid content in cationic polymers was the main reason for betterment of the carrier efficiency [18, 19]. The mechanism behind the beneficial effect of lipid substitution was expected to be enhanced cell uptake of complexes, and our

results were coherent with this hypothesis. The effects of lipid substitution on internalization route of the complexes as well as their fusogenic properties need further exploration. The –S–S– linking of PEI2–PA was not beneficial when such polymers were used to transfect 293T cells, unlike the –S–S– linking of PEI2. The lack of a beneficial effect in the case of –S–S– linking of PEI2–PA might be due to steric interference of the relatively long PA side-chain in pDNA binding, ultimately reducing the plasmid delivery to cells. Having lipids with shorter chain lengths (e.g., myristic acid with C14) on the polymer backbone might make the disulfides more accessible for cleavage. Although the transfection efficiency of a disulfide cross-linking polymers was inversely correlated with the length of alkyl side-chain, the reason behind this effect was not previously explored [17]. Limited endosomal escape might be another reason for the low efficiency of disulfide-linked PEI–PA as a direct consequence of decreased buffering capacity after disulfide linking, since the other physicochemical characteristics, such as particle size, surface charge and stability, were not so different from the parent PEI–PA.

We additionally explored the efficiency the polymers in another cell type, human breast cancer cell line MDA435 cells, but found both types of polymers to be ineffective, indicating the cell-type dependence of the transfection by the polymeric carriers [11]. The polymers obtained by disulfide cross-linking and PA substitution of PEI2 displayed an equivalent gene expression with PEI25 on 293T cells for a transfection period up to one week. However, based on head-to-head comparisons, the effect of PA substitution seems to result in better performance of PEI2 as compared to the –S–S– linking of the polymer.

5. Conclusions

Disulfide-linked polymers were synthesized by Michael addition of CBA to the primary amines of 2-kDa native PEI2 and a PA-substituted PEI2. Buffering capacity of the PEI2 was decreased upon disulfide linking while hydrophobic modification did not seem make a difference in this aspect. All polymers formed complexes with pDNA, which was condensed into discrete cationic particles of variable sizes (130–600 nm) and ζ-potentials (3.5–20 mV). While disulfide linking of PEI2 did not make any difference in the mean hydrodynamic diameter of the complexes, the PA substitution on PEI2 significantly decreased the particle size to $<$200 nm. Transfection efficiency of PEI2 in 293T cells was increased upon both disulfide linking and PA substitution of PEI; however, disulfide linking of PEI2–PA did not increase the efficiency of the PEI2–PA any further. We propose that hydrophobic modification might be a simpler alternative to disulfide linking when one attempts to improve the efficiency of gene carriers.

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References

1. D. W. Pack, A. S. Hoffman, S. Pune and P. S. Stayton, *Nature Rev. Drug Discov.* **4**, 581 (2005).
2. M. A. Mintzer and E. E. Simanek, *Chem. Rev.* **109**, 259 (2009).
3. I. A. Khalil, K. Kogure, H. Akita and H. Harashima, *Pharmacol. Rev.* **58**, 32 (2006).
4. H. G. Abdelhady, S. Allen, M. C. Davies, C. J. Roberts, S. J. B. Tendler and P. M. Williams, *Nucleic Acids Res.* **31**, 4001 (2003).
5. M. Morille, C. Passirani, A. Vonarbourg, A. Clavreul and J. P. Benoit, *Biomaterials* **29**, 3477 (2008).
6. R. Kircheis, L. Wightman and E. Wagner, *Adv. Drug Deliv. Rev.* **53**, 341 (2001).
7. A. Napoli, M. Valentini, N. Titelli, M. Muller and J. A. Hubbell, *Nature Mater.* **3**, 183 (2004).
8. S. Bauhuber, C. Hozsa, M. Breunig and A. Gopferich, *Adv. Mater.* **21**, 3286 (2009).
9. M. A. Gosselin, W. Guo and R. J. Lee, *Bioconj. Chem.* **12**, 989 (2001).
10. D. M. Lynn and R. Langer, *J. Am. Chem. Soc.* **122**, 10761 (2000).
11. M. Breunig, U. Lungwitz, R. Liebl and A. Goepferich, *Proc. Natl. Acad. Sci. USA* **104**, 14454 (2007).
12. M. L. Read, K. H. Bremner, D. Oupicky, N. K. Green, P. F. Searle and L. W. Seymour, *J. Gene Med.* **5**, 232 (2003).
13. Q. Peng, Z. Zhong and R. Zhuo, *Bioconj. Chem.* **19**, 499 (2008).
14. C. Lin, Z. Zhong, M. C. Lok, X. Jiang, W. E. Hennink, J. Feijen and J. F. J. Engbersen, *Bioconj. Chem.* **18**, 138 (2007).
15. Y. X. Sun, X. Zeng, Q. F. Meng, X. Z. Zhang, S. X. Cheng and R. X. Zhuo, *Biomaterials* **29**, 4356 (2008).
16. M. Ou, R. Xu, S. H. Kim, D. A. Bull and S. W. Kim, *Biomaterials* **30**, 5804 (2009).
17. C. Lin, C. J. Blaauboer, M. M. Timoneda, M. C. Lok, M. van Steenbergen, W. E. Hennink, Z. Zhong, J. Feijen and J. F. J. Engbersen, *J. Control. Rel.* **126**, 166 (2008).
18. A. Neamnark, O. Suwantong, R. Bahadur K. C., C. Y. M. Hsu, P. Supaphol and H. Uludag, *Mol. Pharm.* **6**, 1798 (2009).
19. M. Abbasi, H. Uludag, V. Incani, C. Olson, X. Lin, B. A. Clements, D. Rutkowski, A. Ghahary and M. Weinfeld, *Biomacromolecules* **8**, 1059 (2007).
20. L. V. Christensen, C. W. Chang, W. J. Kim and S. W. Kim, *Bioconj. Chem.* **17**, 1233 (2006).
21. M. E. Linder and R. J. Deschenes, *Biochemistry* **42**, 4311 (2003).
22. E. Emilitri, E. Ranucci and P. Ferruti, *J. Polym. Sci. (A1)* **43**, 1404 (2005).
23. M. D. Brown, A. Schatzlein, A. Brownlie, V. Jack, W. Wang, L. Tetley, A. I. Gray and I. F. Uchegbu, *Bioconj. Chem.* **11**, 880 (2000).
24. H. Uludag, B. Norrie, N. Kousinioris and T. Gao, *Biotechnol. Bioeng.* **73**, 510 (2001).
25. M. Breunig, U. Lungwitz, R. Liebl, C. Fontanari, J. Klar, A. Kurtz, T. Blunk and A. Goepferich, *J. Gene Med.* **7**, 1287 (2005).