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PAPER

Biodegradable cross-linked poly(amino alcohol esters) based on LMW PEI for gene delivery†

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Polyethylenimine (PEI, especially with M_w of 25 000) has been known as an efficient gene carrier and a gold standard of gene transfection due to its high transfection efficiency (TE). However, high concomitant cytotoxicity limited the application of PEI. In this report, several cationic polymers derived from low molecular weight (LMW) PEI (M_w 600) linked with diglycidyl adipate (DA–PEI) or its analogs (diglycidyl succinate, DS–PEI and diglycidyl oxalate, DO–PEI; D–PEIs for all 3 polymers) were prepared and characterized. GPC gave M_ws of DA–PEI, DS–PEI and DO–PEI as 6861, 16 015 and 35 281, respectively. Moreover, degradation of the ester-containing DS–PEI was also confirmed by GPC. In addition, hydroxyls in these polymers could improve their water solubility. These polymers exhibited good ability to condense plasmid DNA into nanoparticles with the size of 120–250 nm. ζ-potentials of the polyplexes were found to be around +10–20 mV under weight ratios (polymer/DNA) from 0.5 to 32. Agarose gel retardation showed that DNA could be released from the polyplexes after being pre-incubated for 30 h. *In vitro* experiments were carried out and it was found that DS–PEI showed about 5 times of TE compared to that of the PEI/DNA polyplex under a weight ratio of 1 in A549 cells. Meanwhile, the cytotoxicity of D–PEIs assayed by MTT is lower than that of 25 kDa PEI in HEK293 cells. These results suggested that this series of PEI derivatives would be promising non-viral biodegradable vectors for gene delivery.

Introduction

The success of gene therapy largely depends on the availability of delivery vehicles. Although viral vectors display rather good transfection properties both *in vitro* and *in vivo*, there are a large number of problems associated with the use of these vectors.[1] Frequently studied cationic gene delivery polymers[2] include polyethylenimine (PEI), poly(2-dimethylaminoethyl methacrylate) (pDMAEMA), poly-L-lysine (pLL), *etc*. Our group also prepared some linear[3] and reticular[4] cyclen-based polymers which have *in vitro* transfection efficiencies (TE) close to that of PEI (25 kDa). Among these polymers, PEI is the most studied material for DNA delivery because of its strong buffering capability in the pH region of 7.4–5.1 together with high binding capability towards DNA and a relatively high TE. Therefore, 25 kDa PEI has been considered as the gold standard of gene transfection. However, the high cytotoxicity partly caused by its high molecular weight and charge density seriously hampered its therapeutic use. On the other hand, low molecular weight (LMW) PEI (M_w < 2000), by contrast, has been demonstrated to have a relatively low cytotoxicity. But it also cannot be used as gene vectors due to rather unsatisfying TE. To overcome this inconsistency, the use of biodegradable polymers, which can break down into low molecular weight (LMW) segments, is a logical choice. Several functional groups, such as polycaprolactone diacrylate linkage,[5] cyclodextrin (CD),[6] dextran,[7] and dithiobis (succinimidylpropionate),[8] were introduced to modify LMW PEI. And some non-PEI-based biodegradable gene carriers such as linear poly(β-amino ester) (PBAE),[9] poly (4-hydroxy-L-proline ester),[10] hyperbranched poly(amino ester),[11] poly[α-(4-aminobutyl)-L-glycolic acid] (PAGA)[12] and poly(2-aminoethyl propylene phosphate) (PPE-EA)[13] have also been prepared and applied to gene delivery. Although the TEs of these materials were comparable to that of 25 kDa PEI and the cytotoxicity was reduced to some degree, it remains a challenge to obtain efficient gene vectors combining the advantages of high TE and low cytotoxicity by careful molecular design and simple preparation.

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Recently, the poly(amino alcohol esters) were found to be ideal gene delivery vectors. With the biodegradable polyester backbone and the hydrophilic hydroxyl groups, this type of cationic polymers showed relatively lower cytotoxicity and good water solubility.[14] He and co-workers reported novel PEI-grafted polycarbonates, and high TE was obtained by using these polymers as gene delivery vectors.[15] Dong and Wei also synthesized cross-linked poly(amino alcohol esters) of glycidyl acrylates and PEI 600, however, the TE of these polymers in *in vitro* transfection was low.[16] In this report, we would like to introduce a series of cross-linked PEI 600 by using diglycidyl esters with different chain lengths as linkage. Their interaction with plasmid DNA was investigated, and the *in vitro* TEs in different cell-lines were studied. The relative quantitative structure–activity relationship (QSAR) was discussed.

Experimental section

Materials and methods

All chemicals and reagents were obtained commercially and were used as received. Anhydrous ethanol and dichloromethane were dried and purified under nitrogen by using standard methods and were distilled immediately before use. LMW PEI (branched, average molecular weight 600 Da, 99%) was purchased from Aladdin (Shanghai, China). 25 kDa PEI (branched, average molecular weight 25 kDa) and MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) were purchased from Sigma-Aldrich (St. Louis, MO, USA). The plasmids used in the study were pGL-3 (Promega, Madison, WI, USA, coding for luciferase DNA) and pEGFP-N1 (Clontech, Palo Alto, CA, USA, coding for EGFP DNA). The Dulbecco's Modified Eagle's Medium (DMEM), 1640 Medium and fetal bovine serum were purchased from Invitrogen Corp. The MicroBCA protein assay kit was obtained from Pierce (Rockford, IL, USA). The luciferase assay kit was purchased from Promega (Madison, WI, USA). The endotoxin free plasmid purification kit was purchased from TIANGEN (Beijing, China).

MS-ESI spectral data were recorded on a Finnigan LCQ^{DECA}. IR spectra were measured with a Shimadzu FTIR-4200 spectrometer. ^{1}H-NMR spectra were obtained on a Bruker AV400 spectrometer. $CDCl_3$ or D_2O was used as the solvent and TMS as the internal reference. The molecular weight of polyamine was determined by gel permeation chromatography (GPC) (Waters 515 pump, Waters 2410 Refractive Index Detector (25 °C, incorporating Shodex columns OHPAK KB-803). A filtered mixture of 0.2 mol L^{-1} HAc/NaAc buffer which contained 20% CH_3CN (volume ratio) was used as the mobile phase with a flow rate of 0.5 mL min^{-1}. Molecular weights were calculated against poly(ethylene glycol) standards of number average molecular weights ranging from 200 to 80 000.

Preparation of diglycidyl esters 2

These compounds were prepared following ref. 17. In a typical procedure, allyl alcohol (0.22 mol) and adipic acid (0.100 mol) were mixed and refluxed at 105 °C in the presence of 0.1% (v/v) sulfuric acid for 24 h. Excess sulfuric acid was neutralized with sodium carbonate. Unreacted allyl alcohol was evaporated off at 70 °C for 1 h under vacuum. The product, diallyl adipate, was extracted with ethyl acetate and dried with anhydrous Na_2SO_4. After filtration, the crude product was collected by evaporation under reduced pressure and was used in the next step without further purification.

Diallyl adipate (11.34 g, 0.05 mol) was oxidized by *meta*-chloroperoxybenzoic acid (*m*-CPBA, 19.02 g, 0.11 mol, 99% purity) in 100 mL of methylene chloride. The mixture was refluxed at 55 °C for 8 h. After the reaction, the byproduct *meta*-chlorobenzoic acid was crystallized at −20 °C overnight and filtered from the solution. The remaining byproducts were removed by filtration followed by column chromatography with ethyl acetate and petroleum ether (v/v 1 : 2) as eluent.

Diglycidyl adipate (DA, **2a**). Yield 46.8%. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 1.684–1.691 (d, J = 2.8 Hz, 4H), 2.386–2.393 (d, J = 2.8 Hz, 4H), 2.635–2.659 (m, 2H), 2.841–2.868 (m, 2H), 3.203–3.213 (t, J = 1.6 Hz, 2H), 3.883–3.941 (m, 2H), 4.399–4.442 (m, 2H). ^{13}C NMR (400 MHz, $CDCl_3$): δ = 23.90, 33.25, 44.28, 49.00, 64.60, 172.59. MS (ESI): m/z = 281.18 $[M + Na]^+$. IR (KBr, cm^{-1}): 3062.09, 3004.15, 2948.90, 2874.25, 1731.00, 1419.08, 1135.94, 1081.34, 909.12, 798.01, 762.12.

Diglycidyl succinate (DS, **2b**). Yield 26.0%. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 2.649–2.668 (m, 2H), 2.705 (s, 4H), 2.852 (t, J = 4.4, 2H), 3.198–3.238 (m, 2H), 3.957 (dd, J = 12.4, 2.4 Hz, 2H), 4.445 (dd, J = 12.4, 2.8 Hz, 2H). ^{13}C NMR (400 MHz, $CDCl_3$): δ = 28.62, 44.39, 49.05, 65.03, 171.72. MS (ESI): m/z = 253.17 $[M + Na]^+$. IR (KBr, cm^{-1}): 3013.30, 2934.80, 1733.95, 1425.43, 1352.74, 1310.09, 1148.86, 996.73, 908.58, 861.75.

Diglycidyl oxalate (DO, **2c**). Yield 45.0%. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 2.724 (dd, J = 4.8, 2.4 Hz, 2H), 2.900 (t, J = 4.8 Hz, 2H), 3.294–3.334 (m, 2H), 4.175 (dd, J = 12.4, 6.4 Hz, 2H), 4.595 (dd, J = 12.4, 3.2 Hz, 2H). ^{13}C NMR (400 MHz, $CDCl_3$): δ = 44.63, 48.54, 67.35, 156.89. MS (ESI): m/z = 225.02 $[M + Na]^+$. IR (KBr, cm^{-1}): 3006.82, 2961.11, 1743.86, 1447.60, 1189.20, 901.42, 749.16, 545.72.

Preparation of D–PEIs (title polymers)

In a typical procedure, PEI 600 (1.50 mmol), diglycidyl adipate or its analogs (1.33 mmol) and 3 mL of anhydrous ethanol were mixed in a flask with magnetic stirring and refluxed for 24 h (besides, 6 h, and 12 h for DS–PEI-6 and DS–PEI-12, respectively) in an oil bath. After the reaction, the mixture was diluted with 3 mL of anhydrous methanol, and the crude product was precipitated by the addition of anhydrous dichloromethane/cyclohexane (v/v 2 : 1). The precipitation was collected and dried in vacuum to get the product as colorless oil. The molecular weights of D–PEIs were measured by GPC.

DA–PEI (**3a**). Yield 46.5%. ^{1}H-NMR (400 MHz, D_2O): δ = 1.477 (br, 4H), 2.135–2.358 (br, 4H), 2.358–2.609 (m, 58H), 3.174–3.212 (m, 4H), 3.346–3.465 (m, 4H), 3.678–3.801 (m, 2H). M_w = 6861 (PDI = 1.82). IR (KBr, cm^{-1}): 3346.15, 2934.37, 2843.64, 1618.86, 1470.31, 1311.66, 1110.20, 1045.87, 816.39.

DS–PEI (**3b**). Yield 24.9%. ^{1}H-NMR (400 MHz, D_2O): δ = 2.410–2.644 (m, 58H), 3.190–3.232 (m, 4H), 3.371–3.475 (m, 4H) 3.704–3.854 (m, 2H). M_w = 16 015 (PDI = 3.35). IR (KBr, cm^{-1}): 3284.25, 2931.77, 2837.02, 1628.08, 1562.40, 1472.60, 1311.25, 1110.07, 1046.09, 815.96.

DO–PEI (**3c**). Yield 13.3%. ^{1}H-NMR (400 MHz, D_2O): δ = 2.556–2.672 (m, 54H), 3.243–3.314 (m, 4H), 3.393–3.517 (m, 4H), 3.716 (br, 2H). M_w = 35 281 (PDI = 3.78). IR (KBr, cm^{-1}): 3292.71, 2933.38, 2825.86, 1665.42, 1510.41, 1462.91, 1358.94, 1301.32, 1048.27, 810.89.

Acid–base titration

In this assay, briefly, D–PEIs (0.25 mmol of amino groups) was dissolved in 5 mL of 150 mM NaCl aqueous solution, and 1 N HCl was added to adjust the pH to 2.0. Aliquots (50 μL for each) of 0.1 M NaOH were added, and the solution pH was measured with a pH meter (pHS-25) after each addition. For comparison, PEI (25 kDa) was used under same experimental conditions.

Cell culture

HEK (human embryonic kidney) 293 cells and human nonsmall-cell lung carcinoma A549 cells were incubated, respectively, in Dulbecco's Modified Eagle's Medium (DMEM) and 1640 Medium containing 10% fetal bovine serum (FBS) and 1% antibiotics (penicillin–streptomycin, 10 000 U mL^{-1}) at 37 °C in a humidified atmosphere containing 5% CO_2.

Amplification and purification of plasmid DNA

pGL-3 and pEGFP plasmids were used. The former one as the luciferase reporter gene was transformed in *E. coli* JM109 and the latter one as the green fluorescent protein gene was transformed in *E. coli* DH5α. Both plasmids were amplified in terrific broth media at 37 °C overnight. The plasmids were purified by an EndoFree TiangenTM Plasmid Kit. Then the purified plasmids were dissolved in TE buffer solution and stored at −20 °C. The integrity of plasmids was confirmed by agarose gel electrophoresis. The purity and concentration of plasmids were determined by ultraviolet (UV) absorbance at 260 and 280 nm.

Agarose gel electrophoresis

D–PEI/DNA complexes at different weight ratios ranging from 0.06 to 0.2 were prepared by adding an appropriate volume of D–PEIs (in 150 mM NaCl solution) to 0.8 μL of pEGFP–N1 DNA (120 ng μL^{-1} in 40 mM Tris–HCl buffer solution). The complexes were diluted by 150 mM NaCl solution to a total volume of 6 μL, and then the complexes were incubated at 37 °C for 30 min. After that the complexes were electrophoresed on the 0.7% (W/V) agarose gel containing EB and with Tris–acetate (TAE) running buffer at 110 V for 30 min. DNA was visualized with a UV lamp using a Bio-Rad Universal Hood II.

The property of DNA release associated with the degradation of D–PEIs was further investigated by agarose gel electrophoresis. D–PEI/DNA complexes at different weight ratios ranging from 0.1 to 0.5 were prepared by adding an appropriate volume of D–PEIs (in 150 mM NaCl solution) to 0.8 μL of pEGFP–N1 DNA (120 ng μL^{-1} in 40 mM Tris–HCl buffer solution). Posterior procedures were the same as above except that the incubation times were 0.5 and 30 h.

Polymer degradation study

For the polymer degradation study, DS–PEI (M_w = 7860, smaller than that used for transfection) was dissolved in a single-strength (1×) phosphate-buffered saline (PBS) solution and was constantly shaken in a 37 °C incubator at 100 rpm. The DS–PEI solution was withdrawn at different time points and then lyophilized. The relative molecular mass of degraded products was determined by GPC using a Waters 515 pump and a Waters 2410 refractive index detector (25 °C).

Particle size and ζ potential measurements

Particle size and ζ potential measurements of polyplexes were carried out using a Nano-ZS 3600 (Malvern Instruments, USA) with a He–Ne Laser beam (633 nm, fixed scattering angle of 90°) at 25 °C. D–PEI/DNA polyplexes at weight ratios ranging from 0.5 to 32 were prepared by the same method with above-mentioned agarose gel electrophoresis. After 30 min incubation in 100 μL ultrapure water, polyplex solutions were diluted to a final volume of 1 mL before measurements.

Cell viability assay

Toxicity of D–PEIs toward 293 cells and A549 cells was determined by using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) reduction assay following literature procedures. The 293 cells (6000 cells per well) and A549 cells (14 000 cells per well) were seeded into 96-well plates. The cells were then incubated in a culture medium containing D–PEIs with a particular concentration for 24 h. After that, the medium was replaced with 200 μL of fresh medium, and 20 μL of sterile filtered MTT (5 mg mL^{-1}) stock solution in PBS was added to each well. After 4 h, the unreacted dye was removed by aspiration. The formazan crystals were dissolved in 150 μL DMSO per well and measured spectrophotometrically in an ELISA plate reader (model 550, Bio-Rad) at a wavelength of 570 nm. The cell survival was expressed as follows: cell viability = $(OD_{treated}/OD_{control}) \times 100\%$.

In vitro transfection

Luciferase assay. The 25 kDa PEI was used as the positive control due to its high TE *in vitro* and *in vivo*. The plasmid pGL-3 was used as a reporter gene. Transfections of pGL-3 plasmid mediated by D–PEIs in 293 cells and A549 cells were studied as compared with 25 kDa PEI. 293 cells or A549 cells were seeded at a density of 6×10^4 cells per well in the 24-well plate with 0.5 mL of medium containing 10% FBS and incubated at 37 °C for 24 h. Then the complexes were prepared at different weight ratios by adding 1.5 μg of plasmid DNA to an appropriate volume of the D–PEI solution. Before transfection, the cells were washed by a serum-free medium, and then the D–PEI/DNA complexes were added with the serum-free medium for 4 h at 37 °C. Then the serum-free medium was replaced by the flash medium containing 10%

FBS, and the cells were further incubated for 24 h. After that, the medium was removed. The luciferase assay was performed according to manufacture's protocols (Promega). Relative light units (RLUs) were measured with a chemiluminometer (FLOROSKAN ASCENT FL). The total protein was measured according to a BCA protein assay kit (Pierce). Luciferase activity was expressed as RLU per mg protein. Data are shown as mean ± standard deviation (SD) based on 3 independent measurements. The statistical significance between two sets of data was calculated using Student's *t*-test. An AP value < 0.05 was considered statistically significant.

Green fluorescent protein assay. Transfections of the pEGFP-N1 plasmid mediated by D–PEIs in 293 cells and A549 cells were also evaluated. The best weight ratios in 293 cells and A549 cells determined from the luciferase assay were used. 293 cells and A549 cells were inoculated at a density of 2.4×10^5 and 3×10^5 cells per well in 24-well plates, respectively, 24 h prior to transfection. D–PEI/DNA complexes were prepared by adding an appropriate volume of D–PEIs solution (in 150 mM NaCl solution) to 50 μL pEGFP-N1 DNA solution (30 μg mL^{-1} in 40 mM Tris–HCl buffer solution) with a final volume of 100 μL. Then the complexes were incubated at room temperature for 30 min. The plates were washed with PBS twice, and 100 μL D–PEI/DNA complexes were then added to a well with an additional 150 μL of medium without FBS. The final concentration of DNA in the complexes was calculated to be 6 μg mL^{-1}. After 4 h of incubation, the D–PEI/DNA complex containing medium was replaced with 0.5 mL of fresh medium containing 10% FBS, and the cells were further incubated for 24 h at 37 °C. The cells were directly observed by an inverted microscope (Olympus IX 71). The microscopy images were obtained at a magnification of 100× and recorded using Viewfinder Lite (1.0) software.

Results and discussion

Synthesis and characterization of title polymer D–PEIs

The preparation route of D–PEIs is shown in Scheme 1. In a typical procedure, adipic acid reacted with allyl alcohol in the presence of sulfuric acid to give the diester compound **1a**. Subsequent oxidation of the double bonds by *meta*-chloroperbenzoic acid (*m*-CPBA) led to diglycidyl esters **2a**. We also tried the direct route to prepare **2a** by using sodium salt of succinic acid and epichlorohydrin. But this method failed as the succinic acid anions would lead to the ring-opening of epichlorohydrin. The polymerization was simply processed by mixing **2a** and PEI 600 in ethanol under reflux temperature. The crude product was recrystallized for 3 times to ensure their polydispersity. In the polymerization reaction, the amount of PEI 600 should be slightly more than that of the diesters, otherwise the reaction mixture would change to gel-like materials. This could be attributed to the uncontrollable crosslinking between the diglycidyl esters and LMW PEI when excessive diesters were used. To optimize the M_w of D–PEIs **3**, which might strongly influence their transfection efficiency and cytotoxicity, we prepared DS–PEI (**3a**) through different reaction times of 6 h (DS–PEI-6), 12 h (DS–PEI-12), 24 h (DS–PEI), and 48 h (DS–PEI-48). GPC gave the corresponding M_ws of 6065, 8689, 16 015, 61 600, for DS–PEI-6, DS–PEI-12, DS-PEI and DS–PEI-48, respectively, indicating that the molecular weights of these polymers increased associated with reaction time. According to the data of luciferase assay (Fig. 6), we chose 24 h as the reaction time in the preparation of DA–PEI (**3a**) and DO–PEI (**3c**). The obtained polymers showed significant water solubility, which was required for the subsequent studies. GPC gave M_ws of DA–PEI, DS–PEI and DO–PEI as 6861 (PDI = 1.82), 16 015 (PDI = 3.35) and 35 281 (PDI = 3.78), respectively. The increasing M_ws from DA–PEI to DO–PEI suggested that the polymerization would be more difficult for the longer bridge (**2a**). Additionally, the degradation of the ester-containing polymer was also studied by using DS–PEI (M_w 7860 Da) as an example *via* GPC. As shown in Fig. 1, the M_w of DS–PEI decreased from 7860 to

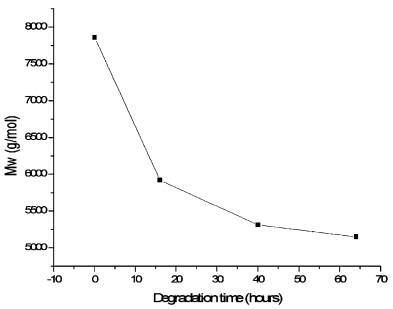
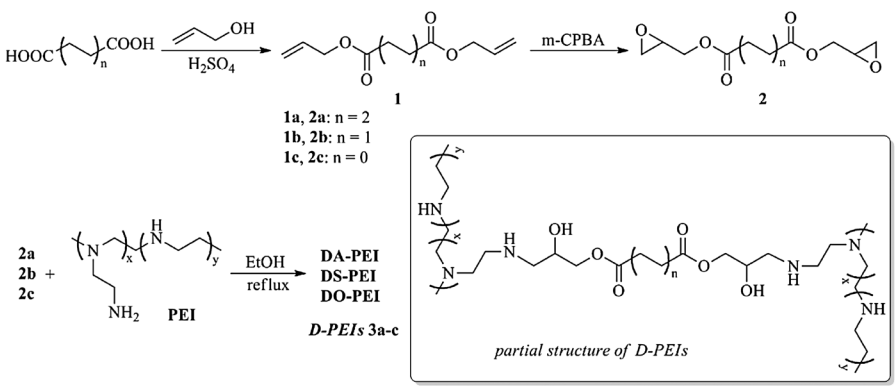


Fig. 1 Change of molecular weight of DS–PEI (M_w = 7860 Da) with degradation time in PBS (pH = 7.4).



Scheme 1 Preparation route of D–PEIs.

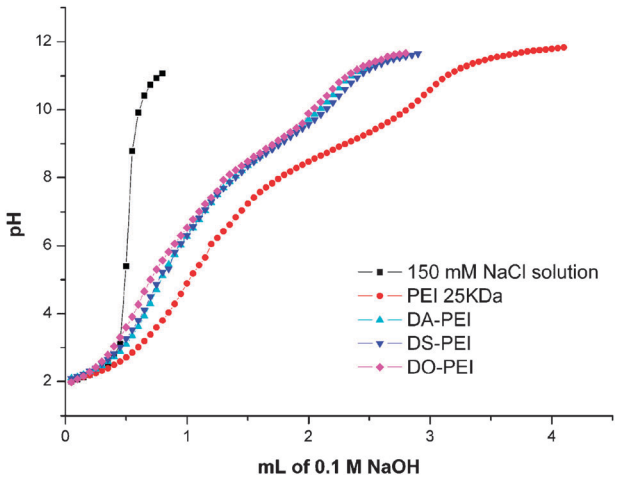


Fig. 2 Acid–base titration profiles of PEI 25 KDa and D–PEIs. D–PEIs or PEI 25 KDa (0.25 mmol of amino groups) was first treated with 1 N HCl to adjust pH to 2.0, and then the solution pH was measured after each addition of 50 μL of 0.1 N NaOH. The relatively flat curve of PEI indicates its higher buffer capability than those of D–PEIs.

5900 Da within 12 h. Further extension of time resulted in a relatively slight decrease of M_w, and the M_w of 5312 Da was detected after 36 h.

Buffer capability

Acid–base titration was performed to evaluate the proton buffering effect of D–PEIs. PEI 25 kDa is well known for its strong proton-sponge effect, leading to the disruption of endosome in the transfection process.[18] In this study, the buffer capabilities of D–PEIs and 25 kDa PEI were evaluated by acid–base titration. As shown in Fig. 2, the buffer capability of D–PEIs is slightly lower than that of PEI. It was reported that the buffer capabilities of these polycations mainly depend on the presence of primary, secondary and tertiary amines groups. Since D–PEIs have a lower molecular weight and less density of amino groups in the structure relative to 25 kDa PEI, the lower buffer capacities of D–PEIs are reasonable. The titration profiles between **3a–c** were almost identical, indicating that the length of bridged diacid chains has little effect on the buffer capacity.

Formation of D–PEI/DNA complexes

DNA condensation capability is essential for polymeric gene vectors. Gel retardation analysis was performed to confirm the affinity between D–PEIs and plasmid DNA. As shown in Fig. 3, the electrophoretic mobility of DNA was retarded by the introduction of D–PEIs, and total DNA retardation was detected at and above weight ratios of 0.1. The results suggested that D–PEIs can bind to DNA through electrostatic interactions between the DNA backbone and the cationic nitrogen atoms in D–PEIs.

The potential advantage of biodegradable gene vectors as compared to their non-degradable counterparts is their reduced cytotoxicity and the avoidance of accumulation of the polymer in the cells after repeated administration.[19] The degradability of the polymers can be helpful as a tool to release the plasmid DNA into the cytosol. Hence, the release of pDNA from the polyplexes was also studied by gel retardation analysis. Comparative experiments about D–PEIs pre-incubated in 150 mM NaCl solvent at 37 °C for different periods of time were performed. Fig. 4A shows that the polymers and pEGFP-N1 plasmid could form stable complexes even under a weight ratio of 0.1. After incubation alone for 30 h, these polymers could not efficiently retard DNA, indicating that no distinct condensation was processed (Fig. 4B). This might be attributed to the cleavage of ester bonds in the polymers, which resulted in the degradation to small segments that could not efficiently bind to DNA. For DA–PEI, the degradation rate might be slower than those of other two materials, and as a result, it could still condense and retard DNA under the weight ratio of 0.5 (Fig. 4B, lane 6).

The appropriate size of polymer/DNA nanoparticles is of critical importance for polyamines as gene vectors into the target cells. The desirable *in vivo* vectors should efficiently

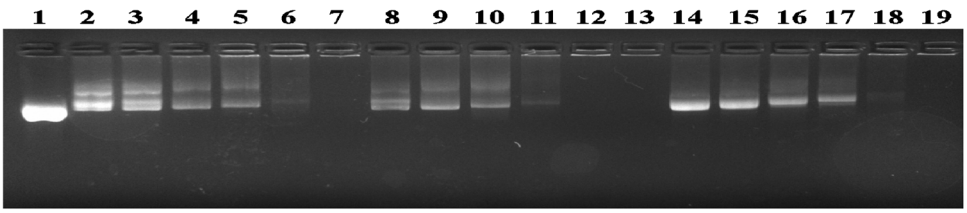


Fig. 3 Electrophoretic mobility of pEGFP-N1 in the presence of DA–PEI (lanes 2–7), DS–PEI (lanes 8–13) and DO–PEI (lanes 14–19) (ethidium bromide staining). Lane 1: DNA control. The weight ratios of D–PEI/DNA are 0.06, 0.07, 0.08, 0.09, 0.1, 0.2 for lanes 2–7, 8–13 and 14–19.

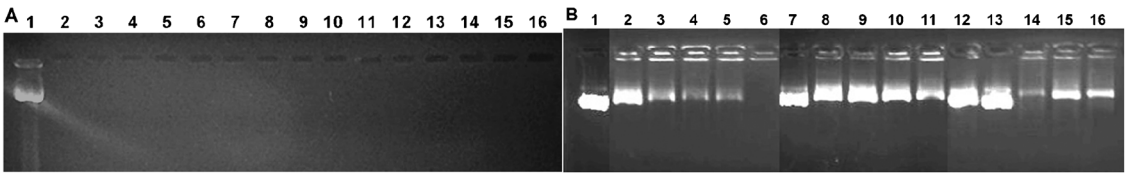


Fig. 4 Electrophoretic mobility of pEGFP-N1 plasmid after pre-incubation for 0 h (A) and 30 h (B) of D–PEIs alone (ethidium bromide staining). Lanes 2–6: DA-PEI/pDNA with a weight ratio of 0.1, 0.2, 0.3, 0.4, 0.5, respectively; lanes 7–11: DS-PEI/pDNA with a weight ratio of 0.1, 0.2, 0.3, 0.4, 0.5, respectively; lanes 12–16: DO-PEI/pDNA with a weight ratio of 0.1, 0.2, 0.3, 0.4, 0.5, respectively; lane 1: pDNA control.

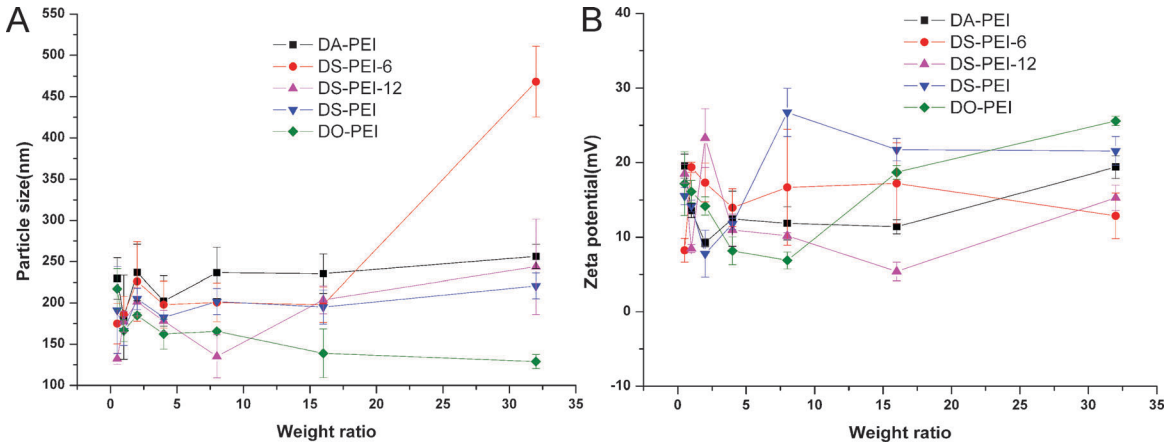


Fig. 5 Average particle size (A) and ζ-potential (B) of D–PEI/DNA at weight ratios of 0.5, 1, 2, 4, 8, 16 and 32 (mean ± SD, $n = 3$).

compact pDNA into small (below 200 nm) nanoparticles, which was very useful for particle stabilization, efficient endocytosis and gene transfer.[20] The particle size of D–PEIs/pDNA complexes was measured at various weight ratios, and the results are shown in Fig. 5A. D–PEIs could efficiently compact pDNA into small nanoparticles with the sizes of around 120–250 nm in diameter at the weight ratio from 0.5 to 16. Distinctly smaller particles of D–PEIs were observed at the weight ratios of 1 compared to those obtained at adjacent weight ratios (0.5 and 2). Further increase of weight ratio might lead to the accretion of nanoparticle sizes, especially for DS–PEI-6 which has a relatively low molecular weight caused by shorter preparation time, and it might be more liable to aggregate under high concentration. Of the three polymers, DO–PEI could form the polyplex with around 140 nm in diameter, which was much smaller than those formed by DNA and other D–PEIs. This could be explained by the larger molecular weight and ion density of DO–PEI induced by its shorter diester bridge. In a word, D–PEIs could compact pDNA to form nanoparticles with proper sizes that are prone to endocytosis.[3]

ζ-potential is an indicator of surface charges on the polymer/pDNA nanoparticles. A positively charged surface allows electrostatic interaction with anionic cell surfaces and facilitates cellular uptake.[21] The ζ-potential values of D–PEI/DNA complexes did not display clear trends associated with the weight ratio (0.5–32, Fig. 5B), and the range of +5–20 mV is slightly lower than the most reported PEI/DNA complexes (~ +25–30 mV) and is higher than the results of PEG–PEI copolymers which also act as effective gene carriers.[22] This relatively low ζ-potential might be attributed to the repeated hydroxyl and ester groups which can screen the positive charge of amino groups in the structure of D–PEIs.

Cytotoxicity

The cytotoxicity of cationic polymers is thought to be caused by the damage due to the interaction with plasma membrane or other cellular compartments, and researches have found a rough correlation between toxicity and TE.[23] The *in vitro* cytotoxicity of D–PEIs was evaluated in A549 and 293 cells by MTT assay, and the 25 kDa PEI was used as the control. As shown in Fig. 6, 25 kDa PEI displayed serious cytotoxicity in two cell-lines and the relative cell viability of PEI were less than 20% when its concentration was over 20 μg mL^{-1}. To our surprise, D–PEIs showed high cytotoxicity in A549 cells, especially under higher concentration (Fig. 6A). However, under the concentrations in subsequent transfection studies (up to 6.0 μg mL^{-1}), the cytotoxicities were low. On the other hand, D–PEIs indicated relatively high cell viabilities in 293

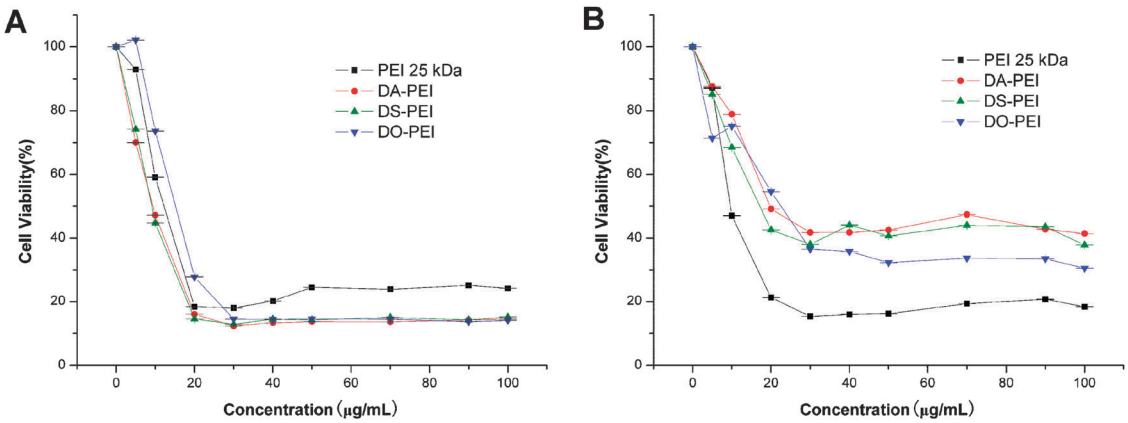


Fig. 6 Relative cell viabilities of D–PEIs and 25 kDa PEI in A549 (A) and 293 (B) cells.

cells, and about 40% cell viability was found even at 100 μg mL^{-1} (Fig. 6B). DO–PEI displayed higher cytotoxicity than DA–PEI and DS–PEI, and this might be attributed to the higher positive charge, which was caused by the shorter diester bridge and therefore higher density of amino groups on the surface of the complexes. The higher M_w of DO–PEI might also contribute to its higher cytotoxicity. It is evident that cell-dependent cytotoxicities were found for D–PEIs, and A549 cells showed a relatively severe weakness against the cytotoxicity of both PEI and D–PEIs.

In vitro transfection

The gene transfection efficiency of D–PEI/DNA complexes was assessed by *in vitro* delivery experiments of luciferase reporter gene (plasmid pGL-3) into A549 and 293 cells. PEI 25 kDa was used for comparison because of its high TE and easy availability. PEI/DNA polyplexes were prepared at an N/P ratio of 10 (weight ratio of 1.39, which is the accepted optimal PEI usage for transfection), and D–PEI/DNA complexes were prepared at various weight ratios including 0.5, 1 and 2 (D–PEI concentrations of 1.5, 3.0, 6.0 μg mL^{-1}, respectively). Firstly, the effect of molecular weight of DS–PEI on the TE was investigated, and the results are shown in Fig. 7.

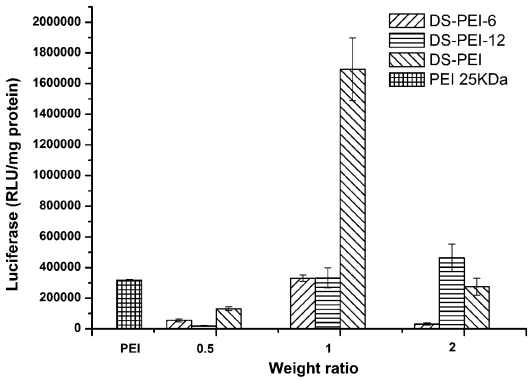


Fig. 7 Effect of molecular weight of DS–PEI and weight ratio on the TE (luciferase expression) in A549 cells (mean ± SD, $n = 3$). For comparison with Fig. 6, the concentrations of DS–PEIs in the transfection experiments were 1.5, 3, 6 μg mL^{-1} for the weight ratio of 0.5, 1 and 2, respectively.

To our delight, D–PEIs displayed comparable TEs with PEI in A549 cell-line. Generally, the increase of the molecular weight led to higher TE. However, for the experiments with a weight ratio of 2, TE was decreased for DS–PEI, which was obtained after 24 h reaction. This might be attributed to the increased cytotoxicity under higher concentration and higher molecular weight. The best result was achieved when using DS–PEI under a weight ratio of 1, in which more than 5 times of TE was obtained compared to that of the PEI/DNA polyplex. Meanwhile, although DS–PEI could bind and retard DNA under a weight ratio of 0.5 in gel retardation analysis, low TEs were observed at this weight ratio.

The TEs of three prepared D–PEIs in different cell-lines were then studied, and the results are shown in Fig. 8. D–PEIs can act as effective non-viral gene vectors with high TEs. By using PEI as standard, better results were obtained in A549 cells than those achieved in 293 cells. As shown in Fig. 8A, all three polymers showed 3–5 times of TE compared to that of the PEI/DNA complex, and the best result belonged to DS–PEI under a weight ratio of 1. For DA–PEI, the best TE was obtained under a weight ratio of 0.5. In 293 cell-line, the weight ratio of 1 was also the optimized usage, and under this condition, DO–PEI displayed the best result, which was about 60% TE compared to that of the PEI/DNA complex (Fig. 8B). We speculated that the relatively small particles formed at the weight ratio of 1 (<200 nm, Fig. 5A) might facilitate the endocytosis and subsequent gene expression. Additionally, compared to PEI, the D–PEIs showed better cell selectivity towards the cancer cell A549.

To directly visualize the infected cells expressing pEGFP-Nl, enhanced green fluorescent protein expression in A549 and 293 cells was observed by an inverted fluorescent microscope. According to the results of the luciferase assay, D–PEI/DNA and PEI 25 kDa per DNA (as control) complexes were used at the optimal weight ratio of 1 (0.5 for DA–PEI in A549 cells) and the N/P ratio of 10, respectively (Fig. 9). In A549 cell-line, the images showed that the density of transfected cells by DS–PEI/DNA and DO–PEI/DNA were similar to that caused by 25 kDa PEI/DNA complexes (Fig. 9A–D), while DA–PEI showed weaker GFP expression. On the other hand, the images in Fig. 9E–H showed that the density of transfected

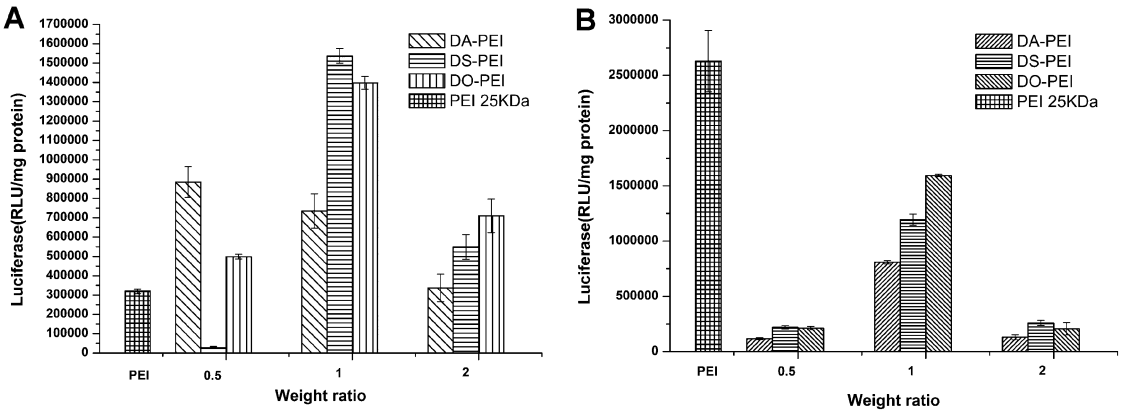


Fig. 8 Luciferase expression in A549 (A) and 293 (B) cells transfected by 25 kDa PEI/DNA (N/P = 10) and D–PEI/DNA complexes at different weight ratios (mean ± SD, $n = 3$).

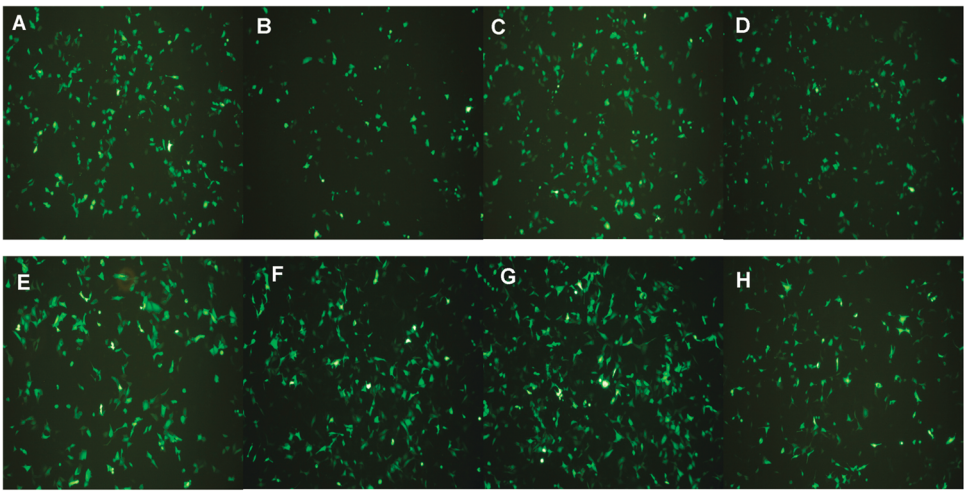


Fig. 9 Fluorescent microscope images of pEGFP-transfected in A549 (A–D) and 293 (E–H) cells. N/P of PEI/DNA was 10 (weight ratio of 1.39), D–PEI/DNA weight ratios were 1 : 1 except B (0.5): (A, E) PEI 25 kDa, (B, F) DA–PEI, (C, G) DS–PEI, (D, H) DO–PEI. These 4 cm-wide images were obtained after 68-fold reduction from original pictures, which were recorded at the magnification of 100×.

293 cells by DS-PEI/DNA (Fig. 9G) was somewhat enhanced compared to that caused by 25 kDa PEI/DNA complexes. In a sense, the types of both the cells and guest genes would affect the TEs of D–PEI. Further increase or decrease of the weight ratio led to much lower TE, fewer cells that expressed GFP were observed by microscopy (data not shown). Similar results were obtained in luciferase expression experiments. This low TE was attributed to the liability to degradation under a low weight ratio and the difficulty to release DNA and higher cytotoxicity under a high weight ratio. The transfection results exhibited that the bridge length may have large influence on the TE, and distinct difference in TE in both cell-lines could be observed between the three D–PEI/DNA polyplexes.

Conclusions

Several cationic polymers derived from low molecular weight (LMW) PEI (M_w 600) linked with diglycidyl diacid esters (D–PEIs) were prepared. D–PEIs reserve the good binding ability and buffer capacity of PEI, and at the same time, reduce the cytotoxicity and facilitate DNA release according to its biodegradability. These new polymeric materials showed enough ability to condense DNA into nanoparticles with proper sizes and ζ-potentials. *In vitro* transfections of reporter genes of luciferase and enhanced green fluorescent protein against A549 and 293 cell-lines suggested that the TEs of D–PEI/DNA complexes were higher than that of 25 kDa PEI/DNA complexes. In A549 cell-line, all three polymers showed 3–5 times of TEs compared to that of the PEI/DNA polyplex. The best result belonged to DS–PEI under a weight ratio of 1. These results indicate that the new type of polyamine could be a promising non-viral polycationic reagent for gene delivery.

Acknowledgements

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