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Comprehensive comparison of two new biodegradable gene carriers

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ABSTRACT

Safety and high transfection efficiency are the prerequisites for an ideal gene vector. Polyethylenimine (PEI), especially PEI 25k (25 kDa), is a well-known cationic gene carrier with high transfection efficiency. However, the high toxicity, depended on its molecular weight, has limited its use as a potential gene carrier. In our research, for the purpose of reducing the toxicity and increasing the transfection efficiency and further to inspect where the degradation of these biodegradable polymers take place would be more beneficial, in cytoplasm or in endocytic vesicles, two kinds of degradable polymers were synthesized. One is an acid-liable PEI derivate (PEI-GA) which was cross-linked by PEI 2k with glutadialdehyde (GA) through imine linkages and the other one (PEI-TEG) was cross-linked PEI 2k with modified triethyleneglycol (TEG) through biscarbamate linkages and can be degraded at neutral environment. By the use of a series of assay methods both *in vitro* and *in vivo*, the results showed that PEI-TEG was found to be biodegradable at neutral environment and exhibit high transfection ability with low toxicity, which indicated its potential as a candidate carrier for gene therapy.

1. Introduction

In the past decades, non-viral gene delivery systems, such as cationic liposomes, peptides, and polymers have attracted great attention due to their superiorities over viral systems, for incidence, the lack of specific immune response, no restrictions in the size of DNA, and the ease of large scale production (Mintzer and Simanek, 2009; Thomas and Klibanov, 2003; Yu and Wagner, 2009). Among cationic polymers, polyethylenimine (PEI), a commercially available cationic polyamine first introduced by Boussif et al. (1995), is one of the most promising and widely studied gene carriers for its highly efficient delivery of DNA (Godbey et al., 1999a), which were ascribed to the buffering capacity of PEI, thus protecting DNA from nuclease degradation and facilitating endosomal escape of PEI/DNA complexes ("proton sponge hypothesis") (Boussif et al., 1995). In addition, due to its high transfection efficiency, branched PEI 25k has been used as a standard reference to compared with other newly designed polymers (Kunath et al., 2003). However, significant *in vitro* as well as *in vivo* toxicity is frequently associated with such nucleic acid carriers. The transfection efficiency and toxicity of PEI are depended on its molecular weight and it is generally accepted that PEI with a higher molecular weight (HMW) (i.e. 25 kDa) shows higher transfection efficiency and higher toxicity compared with a low molecular weight PEI (LMW) (i.e. 2 kDa) (Fischer et al., 1999; Godbey et al., 1999b).

To enhance the transfection efficiency and/or decrease the toxicity of PEI, two major vector modification strategies have been reported. The first one was based on the modification of HMW-PEI by coating with human serum albumin (Chen et al., 1994) and dextran (Tseng and Jong, 2003; Tseng et al., 2004), PEGylation (Lutz et al., 2008; Nimesh et al., 2006; Remaut et al., 2007; Sung et al., 2003; Tang et al., 2003; Zhang et al., 2008) and acylation (Forrest et al., 2004) to improve the polymer's biocompatibility. However, most of this kind of modification showed inferior transfection efficiency than non-modified PEI. By contrast, the other strategy was the direct chemical modification of nontoxic LMW-PEI to increase its gene transfer efficiency (Arote et al., 2007; Forrest et al., 2003; Jiang et al., 2007; Kim et al., 2005; Lee et al., 2003; Park et al., 2005; Tang et al., 2006; Thomas et al., 2005; Thomas and Klibanov, 2002; Wen et al., 2009; Xu et al., 2008; Zhang and Vinogradov, 2010), especially cross-linking the LMW-PEI with biodegradable linkers (Thomas and Klibanov, 2002; Forrest et al., 2003; Kim et al., 2005; Park et al., 2005; Thomas et al., 2005; Xu et al., 2008). Lower toxicity of PEI has generally been achieved by this strategy, but the improvement in transfection efficiency varied from reports (Forrest et al., 2003; Kim et al., 2005; Park et al., 2005; Thomas et al., 2005; Xu et al., 2008).

On the other hand, for the design of biodegradable gene delivery polymers, many questions remained. One of the most important questions was where the degradation of these polymers take place would be more beneficial, in cytoplasm or in endocytic vesicles.

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Up to now, hundreds of newly designed polymers have been synthesized as carriers for the gene delivery. However, no research has really compared these two kinds of degradation pattern in parallel. To address this, we synthesized two kinds of polymers PEI-GA (polymer A) and PEI-TEG (polymer B). Among them, PEI-GA was cross-linked by PEI 2k with glutadialdehyde (GA) through imine linkages and could be degraded in endocytic vesicles (Kim et al., 2005). The other one PEI-TEG was cross-linked PEI 2k by biscarbamate linkages, which can be degraded at neutral environment. This polymer was newly designed and synthesized in our group. Currently, the most commonly used linkages for biodegradable PEI derivates were ester bonds (Thomas and Klibanov, 2002; Forrest et al., 2003; Park et al., 2005; Thomas et al., 2005). However, the ester bond could generate acids upon degradation, which was disfavored for buffering the endosomal environment. Thus, we synthesized the polymer PEI-TEG. Unlike the ester bond, the degradation of each biscarbamate linkage only generated alcohols, CO_2 and two amino groups (Xu et al., 2008).

In this work, polymer PEI-GA and polymer PEI-TEG were tested by acid–base titration, DNA-binding assay, *in vitro* cell transfection studies, cytotoxicity assays, lactate dehydrogenase (LDH) release measurement, hemolytic activity assay and *in vivo* transfection and toxicity assays, and compared with PEI 25k and lipofectamine 2000.

2. Materials and methods

2.1. Materials

Branched PEI 2k, branched PEI 25k, 4-nitrophenyl chloroformate, 3-(4,5-dimethyl-thiazole-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), agarose, HEPES, Tris were procured from Sigma Chemical Co., St. Louis, MO, USA. Gold-view was purchased from Solarbio (Beijing, China). Triethyleneglycol (TEG) was purchased from Kelong (Chengdu, China). The pORF-lacZ and pGL3 plasmid DNA were isolated and purified from DH5-α *Escherichia coli* using the Qiagen Giga Endo-free plasmid purification kit (CA, USA). DNA concentration and purity were quantified by UV absorbance at 260 and 280 nm on a Varian CARY 100 Conc UV Spectrophotometer. Cell culture medium DMEM was obtained from Gibco Co. (USA). β-Gal assay kit was from Invitrogen (USA). BCA assay kit was from KeyGEN (Nanjing, China). The 293T, HepG2 and L929 cell lines were obtained from Shanghai Cell Institute, China Academy of Sciences. All the other chemicals and reagents used were of the analytical grade obtained commercially.

2.2. Synthesis and characterization of polymers

2.2.1. Glutadialdehyde as the crosslinker

The synthesis method of the acid-liable PEI derivate was presented as Kim et al. (2005). Briefly, 2.0 g PEI 2k was introduced in 20 mL anhydrous ethylene dichloride (EDC) solution and stirred vigorously to dissolve. After PEI became clearly dissolved in the reaction solution, 0.1 g glutadialdehyde (GA) dissolved in 20 mL anhydrous EDC was dropwise added with vigorous stirring at room temperature. After stirring for another 4 h, the solution was evaporated to remove the solvent. The viscous residue was dissolved again in water and dialyzed through a cellulose membrane of molecular weight cut off of 3500 in deionized water for 3 days, and then, after 2 days of lyophilization, we got yellowish product as semisolid with high viscosity. The product was named as A or PEI-GA.

2.2.2. Triethyleneglycol as the crosslinker

The new PEI derivate cross-linked by triethyleneglycol through biscarbamate linkages. Briefly, 0.15 g anhydrous triethyleneglycol (TEG, 1.0 mmol) was dissolved in anhydrous ethylene dichloride (25 mL) and afterwards 604 mg 4-nitrophenyl chloroformate (NPC, 3.0 mmol) was added. The solution was stirred and 415 μL triethylamine (3.0 mmol) was added drop-wise through a syringe over a period of 5 min. The reaction mixture was allowed to stir at room temperature for 6 h. Subsequently, it was washed with saturated sodium chloride solution and the organic phase was collected and dried over anhydrous sodium sulfate. Then the solvent was dropwise added to the ethylene dichloride solution of PEI 2k (2.0 g). After stirring for another 16 h, the solution was evaporated to remove the solvent. The viscous residue was dissolved again in double-distilled water and dialyzed through a cellulose membrane of molecular weight cut off of 3500 for 3 days, and then, after 2 days of lyophilization, we got yellowish product or albicans product as solid or semisolid with high viscosity. The product was named as B or PEI-TEG.

2.3. Characterization and measurements of the new PEI derivates

To confirm the synthesis of the conjugations, NMR and FT-IR were used. ^{13}C NMR was recorded on liquid samples (D_2O, Sigma–Aldrich) in an AVANCE AV II-400 MHz NMR Spectrometer (Bruker, German). To avoid any influence of the Nuclear Overhauser effect (NOE), all ^{13}C spectra used for quantitative analysis were recorded using inverse gated decouplig pulse sequences. The ^{1}H NMR experiment of PEI-TEG was also collected on liquid sample (D_2O, Sigma–Aldrich) in a Varian UNITY INOVA400 NMR Spectrometer at 400 MHz. FTIR spectra were recorded on VECTOR 22 FT-IR (Bruker, German), with the following scan parameters: scan range 4400–400 cm^{-1}: number of scan 16: resolution 4.0 cm^{-1}: interval 1.0 cm^{-1}: units %T. Nitrogen content was assayed by elemental analyzer (Euro EA 3000, Euro Vector S.P.A.).

The molecular weight and polydispersity of the PEI derivates were determined by GPC relative to PEO standards (Polymer Labs) (Lin et al., 2007). In short, GPC measurements were performed using a Waters 515 isocratic HPLC pump and thermostated (40 °C) OHpak SB 803 gel-permeation chromatography column (Shodex, Japan). Data were collected using a differential refractometer (model 2410).

The buffering capacity of the new synthesized PEI derivates from pH 10 to 2.5 was determined by acid–base titration. Briefly, each polymer was dissolved in 10 mL of 0.15 M sodium chloride aqueous solution to give a final concentration of total amino groups in the polymer of 5 mM, the pH of the polymer solution was brought to 10 with NaOH, and the solution was subsequently titrated with 0.1 M HCl at 25 °C. The pH values were recorded with a pH Meter (Five Easy, Mettler Toledo, Switzerland). The buffering capacity was defined as the percentage of amine groups becoming protonated from pH 7.4 to 5.1 (mimic the pH change from the extracellular environment to the lower pH of the endosomes), and can be calculated from the following equation:

$$\text{Buffer capacity}\,(\%) = \frac{\Delta V \times 0.1M}{\text{N mol}} \times 100\%$$

wherein ΔV is the volume of HCl solution (0.1 M) required to bring the pH value of the polymer solution from 7.4 to 5.1, and N mol (0.05 mmol) is the total moles of protonable amine groups in the known amount of cationic polymers.

2.4. Polyplex preparation

All PEI derivates/DNA complexes were freshly prepared before use. Plasmid DNA was condensed with standard PEI 25k or the new PEI derivates at various mass ratios. PEI/DNA complexes were prepared at a final DNA concentration of 40 μg/mL as described by Kloeckner et al. (2006). Briefly, indicated amounts of plasmid DNA and PEI were each diluted in 5% glucose solution to the same vol-

ume and then the DNA solution was added into the solution of PEI derivates, and rapidly mixed with gentle vortex for 10 s. PEI/DNA complexes were allowed to stand for at least 20 min at room temperature before use.

2.5. Particle size and zeta potential analysis

PEI/plasmid DNA complexes at a polymer/DNA mass ratio of 0.25, 0.5 and 1 were prepared by adding a 5% glucose solution of plasmid DNA (125 μL, 80 μg/mL) to a 5% glucose solution of PEI (125 μL), followed by vortex for 10 s and incubating at room temperature for 30 min, and 5% glucose solution (750 μL) was added to a final volume of 1.0 mL. The mean particle size and zeta potential of the complexes were measured by photon correlation spectroscopy (PCS) (Zetasizer Nano ZS90, Malvern instruments Ltd., UK).

2.6. Gel retardation assay

To confirm DNA condensation ability of the PEI derivates, electrophoresis was performed. A known amount of plasmid DNA (0.3 μg) was condensed by these polymers. The mass ratio of PEI/DNA was designed as 0.25 and 0.5. The formed complexes thus were mixed with 6× loading buffer (Generay Biotech, Shanghai, China) and analyzed on a 1.0% agarose gel (Tris–acetic acid buffer, TAE, containing 0.06% gold-view) at 80 V for 45 min, and the bands corresponding to plasmid DNA were visualized under UV light.

2.7. DNase I protection assay

Protection and release of DNA in complexes were carried out by electrophoresis according to the method of Park et al. (2005). Briefly, 2 μL of DNase I (1 units) or PBS was added to 10 μL of naked plasmid DNA (0.5 μg) or complexes solutions (the mass ratio of PEI/DNA was 1/1), and incubated at 37 °C with shaking at 100 rpm for 1 h. For DNase inactivation, all samples were treated with 4 μL of EDTA (250 mM) for 10 min and mixed with 10 μL sodium dodecyl sulfate (SDS) dissolved in 0.1 M NaOH (pH 7.2) (1%). The final samples were incubated for 1 h at room temperature and electrophoresis was performed with 1.0% agarose gel in TAE running buffer for 45 min at 80 V.

2.8. Polymer degradation study

The hydrolytic cleavage of the new PEI derivates was performed as Kloeckner et al. (2006). Briefly, polymers were prepared with a concentration of 5 mg/mL, and adjusted to pH 5.1 and 7.4 by acetate buffer or PBS buffers, respectively, and incubated at 37 °C for up to 16 days. After hydrolysis of different time spaces, the pH value was adjusted to pH 7.0 and polymer/DNA complexes were formed at weight ratio of 0.5 in 5% glucose. After stand for 30 min at room temperature, samples were analyzed by agarose gel electrophoresis.

2.9. In vitro transfection assay

Transfection experiments were performed on 293T cells and HepG2 cells using the plasmid pORF-LacZ as the reporter gene. Two parallel transfection series were prepared, one for the determination of reporter gene expression (β-galactosidase) and the other for the evaluation of cell viability by MTT assay. 293T and HepG2 cells were cultured in DMEM with 10% bovine serum, streptomycin (100 mg/mL) and penicillin (100 U/mL). For transfection experiments, the cells were seeded at 1×10^5 cells/well onto 24-well Plates 24 h before transfection. The cells were about 70% confluence at the time of transfection. After the cells were washed twice by PBS, 0.2 mL medium with or without serum was added into each well. For each well in a transfection, 0.05 mL PEI/DNA complexes (different polymer/DNA mass ratios ranging from 1/1 to 12/1) containing 2 μg of pORF-1acZ were gently overlaid onto the cells. The cells were incubated with the PEI/DNA complexes at 37 °C in a 5% CO_2 incubator. After 4 h of incubation, the transfection medium was replaced with 1 mL fresh complete medium and cells were further incubated for 48 h under the same conditions. Expression of β-galactosidase genes was measured with β-gal assay kit according to the manufacturer's instruction. The transfected 293T cells and HepG2 cells were washed twice with PBS and lysed with lysis buffer (ripa, 400 μL/well). Cell debris was removed by centrifugation at 12,000 × *g* for 10 min and 10 μL of the supernatant added to 50 μL of cleavage buffer containing β-mercaptoethanol and 17 μL of ONPG solution. After incubation for 30 min at 37 °C, the absorbance at 415 nm was measured. The total protein concentrations in cell lysates were determined using BCA assay. The metabolic activity of transfected Cells was assayed by MTT method.

To investigate the endosomolytic property of the polymers, 293T cells and HepG2 cells were seeded in 24-well plate at a density of 1×10^5 cells/well, and cultured for 24 h. The endosome proton pump inhibitor, bafilomycin A1 (Sigma; final concentration was 400 nM) diluted in culture medium without serum was pipetted into wells. After 10 min incubation period, transfection and analysis were carried out as described above.

2.10. MTT assay

The MTT assay on the cytotoxicity of the plain polymers was carried out in varying concentrations from 2 to 80 μg/mL were incubated with L929 cells. After the incubation for 24 h, 20 μL of MTT (5 mg/mL) solution was added into each well and was allowed to react for 4 h at 37 °C. Then the medium of each well was replaced with 150 μL of DMSO and the plate was incubated for 10 min at room temperature. Absorbance at 570 nm was measured with a microplate reader (Bio-Rad Model 550, USA). The MTT value of untreated cells was taken as 100% cell viability. All transfection and toxicity assays were carried out in triplicate.

2.11. Lactate dehydrogenase (LDH) measurement

To assess the toxicity of the PEI derivates to the cell membrane, the released LDH was measured using a commercial kit (Jian Chen Bio, Nanjing). Briefly, 2×10^4 L929 cells were seeded in 96-well plates and allowed to attach overnight. Cells were washed twice by PBS, and then treated with PEI derivates of 5–20 μg/mL in serum free medium for 4 h at 37 °C and 5% CO_2. At the end of incubation, 60 μL of supernatants were transferred into another 96-well plate, treated with 60 μL of reconstituted substrate mix and incubated for 15 min at 37 °C in the darkness, and incubated with 50 μL phenylhydrazine at 37 °C for another 15 min. Absorbance was recorded at 450 nm following the addition of the stop solution. Maximal LDH release in control experiments performed with 1% triton X-100 was set as 100% cytotoxicity. The results are expressed as the mean % LDH release ± SD.

2.12. Hemolytic activity measurement

Human erythrocytes were isolated from fresh citrate-treated blood (West China Hospital, Sichuan University, PR China) and washed in HEPES-buffered saline (HBS) by four centrifugation cycles, each at 2200 rpm for 10 min at 4 °C. The erythrocyte pellet was diluted in HBS to a final concentration of 4% erythrocytes (5×10^8 erythrocytes/mL, with or without 10% serum). Suspension of the red blood cells was always freshly prepared and used within 24 h. 80 μL polymers of 1.0 mg/mL diluted in HBS were added in a V-bottom 96-well plate. For 100% lysis,

control wells contained buffer with 1% Triton X-100. Erythrocyte suspension (80 μL) was added to each well and the plates were incubated at 37 °C for 45 min under constant shaking. After centrifugation at 2200 rpm for 10 min, 80 μL supernatant was analyzed for hemoglobin release at 450 nm using a microplate reader (Bio-Rad Model 550, USA). To simulate the *in vivo* situation, the hemolytic activity of the samples which were used to assay the transfection efficiency *in vivo* were also measured simultaneously. Experiments were performed in triplicates. The study was approved by the Ethics Committee of West China Medical School, Sichuan University.

2.13. Erythrocyte aggregation assay

PEI derivates/DNA complexes were formed in 5% glucose. Then 20 μL 2% erythrocytes and 20 μL complexes were mixed and incubated for 10 min. Then the erythrocyte pellet was resuspended in 200 μL HBS and transferred to a 24-well plate containing 1 ml HBS per well. Erythrocyte aggregation was visualized under inverted microscope (Axiovert 40 CFL, Carl Zeiss, oberkochem, Germany) at 400× magnification.

2.14. In vivo transfection and toxicity assays

BALB/c mice (female; body weight, 18–22 g), purchased from West China Experimental Animal Center of Sichuan University (China), were maintained in a germ-free environment and allowed free access to food and water. All animal experiments were approved by the Institutional Animal Care and Use Committee of Sichuan University. The polymer complexes (different polymer/DNA mass ratios ranging from 1/1 to 12/1) containing 50 μg pGL3 at a concentration of 250 μg/mL DNA in 5% glucose were injected into the tail vein and PBS was injected as the control group. After 24 h of injection, animals were sacrificed and indicated tissues were resected and stored at −80 °C. Tissues of 0.2–0.3 g were suspended in 500 μl of lysis buffer prepared by mixing 4 mL of 5× Passive Lysis Buffer (Promega), 800 μL of 8.7 mg/mL PMSF (Sigma–Aldrich) in methanol, 400 μL of the protease inhibitor cocktail (Sigma–Aldrich), and 14.8 mL of water. The samples were homogenized, freeze-thawed and centrifuged. Then 10 μL supernatants were mixed with 50 μL of the Luciferase Assay Reagent (Promega), and the luminescence was measured using an LMax II^{384} luminometer (Molecular Devices, USA). Protein concentrations were determined using the BCA assay. The results were expressed as a mean ± SD, $n = 5$.

To investigate the toxicity of these complexes *in vivo*, the plasma was collected when the mice were sacrificed and the plasma level of ALT (liver enzyme alanine aminotransferase) were assayed by ALT assay kit (Jian Chen Bio, Nanjing). On the other hand, the toxicity to the lung and liver were further evaluated by histological examination. Samples were fixed in 10% buffered formalin and embedded in paraffin. Five-micrometer-thick sections were placed on glass slides and stained with hematoxylin and eosin (H&E). The stained slides were evaluated under inverted microscope (Axiovert 40 CFL, Carl Zeiss, Oberkochem, Germany) at 100× magnification.

2.15. Statistical analysis

In vitro transfection, MTT assay, LDH measurement and hemolytic activity assays were performed in triplicate unless specified. The data were illustrated as mean ± standard deviation (SD). Data were analyzed using the Student t test and one-way analysis of variance (ANOVA) and considered significant difference at the level of $p < 0.05$ and very significant difference at the level of $p < 0.01$.

Table 1
Characteristics of the PEI derivates: nitrogen content and the percentage of different kinds of nitrogen.

Samples	Primary amine (%)	Secondary amine (%)	Tertiary amine (%)	*N* (%)
PEI 2k	36.30	34.42	29.28	34.87
A	33.14	33.60	33.27	26.50
B	35.88	33.86	30.26	22.20
PEI 25k	31.94	32.09	35.97	34.08

3. Results and discussion

3.1. Synthesis and characterization of polymers

PEI-GA (polymer A) and PEI-TEG (polymer B) linked by two kinds of cross-linkers, imine bonds and biscarbamate linkages respectively, were synthesized successfully. Fig. 1 was the simple flow-sheet of the synthesis methods. Structures of the synthesized copolymers were analyzed by NMR and FT-IR. For polymer A, in the ^{1}H NMR spectra, the methylene groups with different amine substituents were not obvious to identify the new PEI derivate. Thus, ^{13}C NMR was used to testify the structure of PEI derivates (Kim et al., 2005). As shown in Fig. 2a, the chemical shift of the peak changed from 197.3 to 164.4 ppm due to the disappearance of aldehyde carbonyl peak (CH=O) at 197.3 ppm and the appearance of signals for the imine carbon (C=N) at 164.4 ppm. For polymer B, the ^{13}C NMR spectra in D_2O was composed of δ = 158.17 ppm (–O–CO–N–), 63.95–69.43 ppm (–CH_2–O–) and 36.31–53.63 ppm (–CH_2–N–); the ^{1}H NMR spectra at 400 MHz (Fig. 2b) included δ = 4.23 ppm (NHCOOCH_2CH_2OCH_2, ester linker), 3.77 ppm (NHCOOCH_2CH_2OCH_2CH_2, ether linker), 3.73 ppm (NHCOOCH_2CH_2OCH_2, ether linker), 3.48 ppm (OHCH_2CH_2OCH_2, hydrolyzed ester) and 3.33–2.5 ppm (–NHCH_2CH_2–, PEI ethylene), and the FTIR spectra (KBr) ν (cm^{-1}) consisted of 3419 (amino stretching), 1698 (carbonyl stretching), 1472, 1384, 1305 (ether twisting), 1124 (ether stretching).

To determine the nitrogen content, the polymers were assayed by elemental analyzer (von Harpe et al., 2000). The relative ratio of different amino functions was calculated from the signal intensities in ^{13}C NMR analysis. As the result shows (Table 1), compared with PEI 2k, the nitrogen content of polymer A and polymer B decreased due to the conjugation of the cross-linkers, which also led to the ratio of primary amine and secondary amine groups decreased and the ratio of tertiary amine groups increased (Table 1).

Gel permeation chromatography (GPC) measurements showed that these new PEI derivates had the weight-average molecular weights (Mw) of 3.12 kDa and 3.68 kDa respectively, which was much lower than that of the PEI 25k. On the other hand, the PDI values of these new PEI derivates were also much lower than that of the PEI 25k (Table 2 and Fig. 2d), which means they had much narrower molecular weights distribution.

It was assumed that the buffering effect of cationic polymers facilitates the endosomal escape of complexes (proton sponge hypothesis). Therefore, the buffering capacity of the PEI derivates was examined by acid–base titration (Fig. 2c). The results revealed that polymer A and polymer B showed 31.32% and 38.32% protona-

Table 2
Characteristics of the PEI derivates: weight-average molecular weight (Mw), Polydispersity index (PDI) and buffering capacity.

Polymers	Mw (kDa)	PDI	Buffering capacity (%)
A	3.12	1.36	31.32
B	3.68	1.64	38.32
PEI 25k	13.10	2.71	19.00

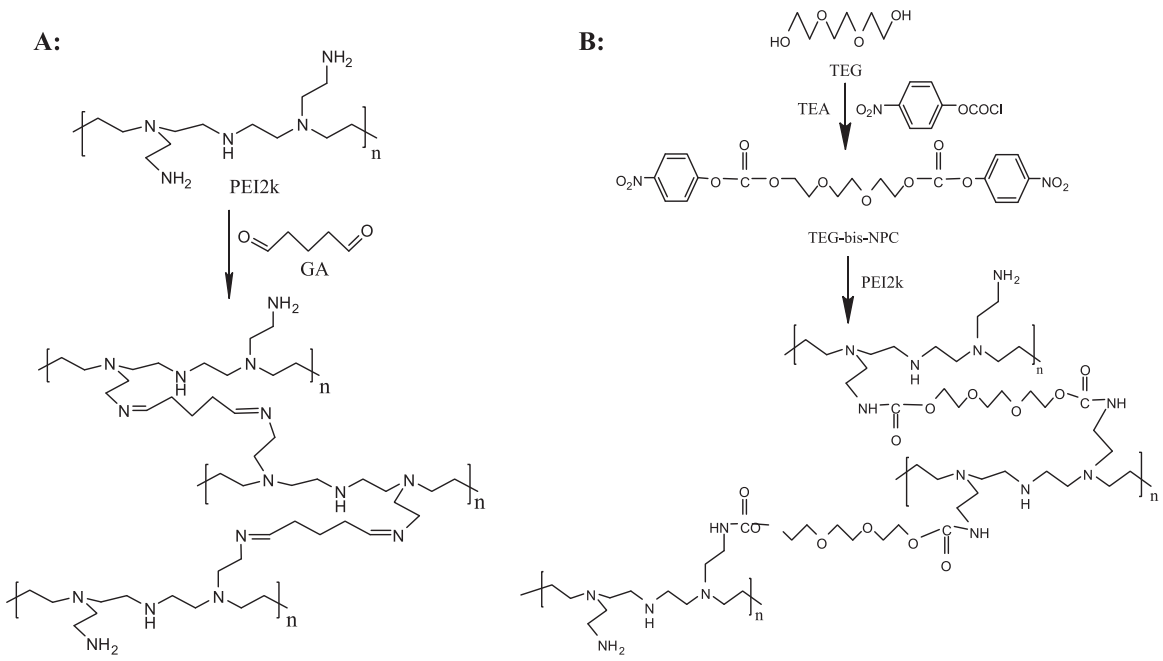

Fig. 1. Reaction scheme of the biodegradable PEI derivates. a: PEI-GA; b: PEI-TEG.

tion respectively compared with 19.00% of PEI 25k in the pH ranging from 5.1 to 7.4, suggesting their excellent buffering capacities.

The degradation of these PEI derivates was assayed by agarose gel electrophoresis. The hydrolytic conditions were chosen at 37 °C in phosphate buffer (pH 7.4) or acetate buffer (pH 5.1), mimicking the pH of the cytoplasm and endosomal vesicles, respectively. It could be concluded from Fig. 3a and b that at pH 5.1, polymer A degraded faster than that in buffer condition of pH 7.4, which was in accordance with the previous report (Kim et al., 2005). On the other hand, polymer B was more readily degraded at pH 7.4 than at pH 5.1. The result was similar with that by Park et al. (2005) and Zhong et al. (2005), who assumed that at pH 7.4 a higher fraction

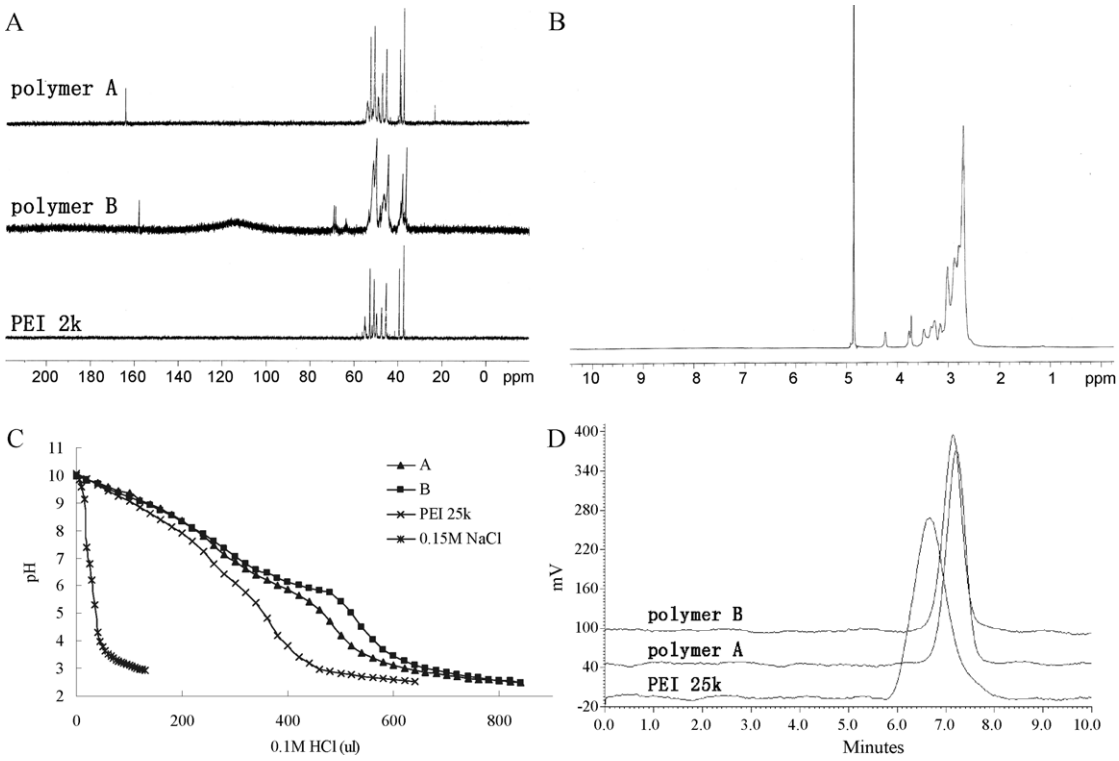

Fig. 2. a: ^{13}C NMR spectra of polymer A (PEI-GA) in D_2O: δ = 164.41 ppm (–C═N–), 37.42–54.15 ppm (–CH_2–N–); ^{13}C NMR spectra of polymer B (PEI-TEG) in D_2O: δ = 158.17 ppm (–O–CO–N–), 63.95–69.43 ppm (–CH_2–O–), 36.31–53.63 ppm (–CH_2–N–). B: ^{1}H NMR spectrum (400 MHz) of polymer B in D_2O: δ = 4.23 ppm (NHCOOC**H**$_2$$CH_2OCH_2$, ester linker), 3.77 ppm (NHCOOCH_2C**H**$_2$OCH_2, ether linker), 3.73 ppm (NHCOOCH_2CH_2OC**H**$_2$, ether linker), 3.48 ppm (HOC**H**$_2$$CH_2OCH_2$, hydrolyzed ester) and 3.33–2.5 ppm (–NHC**H**$_2$C**H**$_2$–, PEI ethylene). C: Titration curves obtained by titrating aqueous solutions of PEI derivates (~5 mM amino nitrogen atoms) in 150 mM aqueous NaCl with 0.1 M HCl. D: GPC spectra of the polymers.

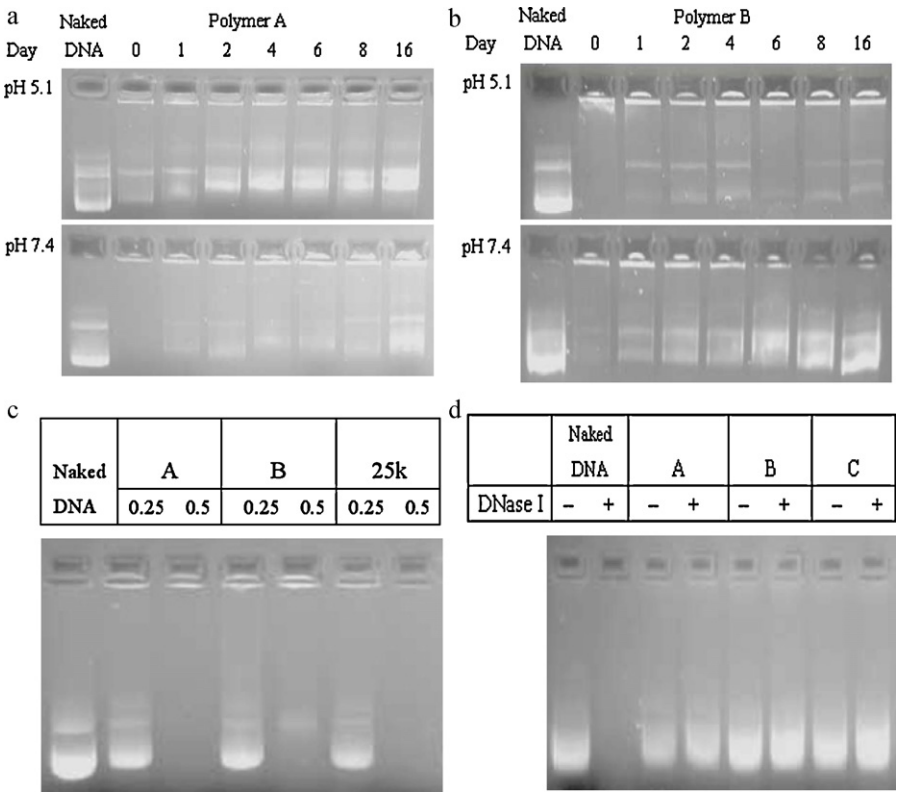

Fig. 3. a and b: pH-dependent hydrolysis of PEI derivates. The DNA-binding abilities of the hydrolysised PEI derivates were examined by agarose gel electrophoresis. c: DNA-binding assay. The DNA-binding abilities of all copolymers were examined by agarose gel electrophoresis. The polymers/plasmid mass ratios were 0.25 and 0.5 respectively. d: DNase I protection assay. PEI/DNA complexes were prepared at 1:1 (w/w) and tested for their ability to protect DNA against the DNase I degradation.

of unprotonated amino groups catalyze the hydrolysis of the ester groups in the polymer, contributing to the more rapid degradation at this pH.

3.2. Characterization of copolymer/DNA complexes

The interactions between the cross-linked PEI and plasmid DNA lead to the neutralization of the negative charge of DNA, which subsequently retards its mobility under the influence of electric field. Thus, agarose gel electrophoresis was performed and the retardation of DNA mobility was checked to confirm the formation of cross-linked PEI/DNA complexes. All the polymers were assayed at two different mass ratios (0.25 and 0.5) (Lynn et al., 2001). The retardation was analyzed on 1.0% agarose gel. As shown in Fig. 3c, when the mass ratio was 0.25, all polymers, including PEI 25k, can not retard DNA completely; however, when the mass ratio was increased to 0.5, polymer A and PEI 25k can retard DNA completely, while very little of the DNA was not retarded by polymer B under the influence of electric field. Therefore, it can be concluded that PEI 25k and polymer A have higher DNA binding activities than polymer B. On the other hand, for the efficient transfection, DNA should be protected against degradation by nucleases in the biological fluids. Agarose gel electrophoresis assay also demonstrated that polymer A, B and PEI 25k were able to protect DNA from enzymatic hydrolysis at a low PEI/DNA mass ratio of 1.0, even treated with DNase-I for 1 h (Fig. 3d).

The average diameter, PDI and zeta-potential of PEI/DNA complexes were measured at the mass ratios of 0.25, 0.5 and 1, respectively (Fig. 4a and b). The data showed that the average sizes of all the particles were smaller than 250 nm except PEI 25k/DNA complexes and polymer B/DNA complexes at the mass ratios of 0.25 and 0.5 respectively, which were over micrometers. Moreover, as the mass ratio increased, zeta-potential of each polymer/DNA complexes raised accordingly. When the mass ratio increased to 1.0, the particles of all the polymers/DNA complexes were nano-sized and with a narrow size distribution (PDI <0.2), which would be favorable for gene delivery.

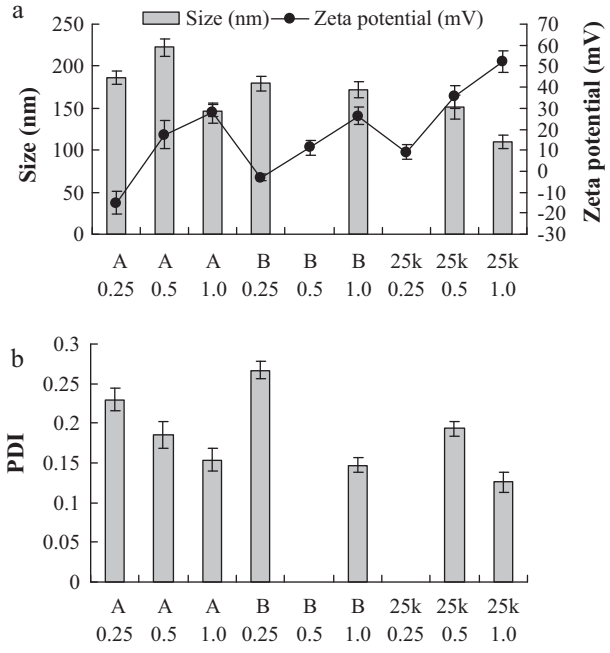

Fig. 4. Physicochemical properties of polymer/DNA complexes were measured at various mass ratios, (a) particle size and Z-potential, (b) PDI values. The sizes and PDI of polymer B/DNA and PEI 25k/DNA complexes at mass ratio of 0.5 and 0.25 respectively, were not present because they were over micrometers ($n = 3$).

3.3. In vitro transfection activity and cytotoxicity assay

To evaluate the transfection efficiency of the PEI derivates, the *in vitro* transfection efficiency was measured on 293T cells and HepG2 cells (Fig. 5a and c). PEI/pORF-lacZ complexes were formulated at various mass ratios ranging from 1/1 to 12/1 in 5% glucose and transfected on 293T cells and HepG2 cells, and the metabolic activities of transfected cells were measured by MTT assay (Fig. 5b and d). It was demonstrated in our experiments that the transfection efficiency was enhanced with an increased mass ratio of polymer/DNA complexes at the beginning. But when the mass ratio was increased to a certain value, the transfection efficiency of polymer/DNA complexes decreased reversely, which might be because of the corresponding increase of their cytotoxicity (data not present). The optimal mass ratios of polymer A/DNA complexes, polymer B/DNA complexes and PEI 25k/DNA complexes were 4/1, 8/1 and 2/1 respectively both in 293T cells and in HepG2 cells (with the size and zeta potential were 123.5 ± 13.1 nm, 115.8 ± 14.3 nm, 105.4 ± 16.2 nm and 58.74 ± 6.14 mV, 45.64 ± 5.62 mV, 67.53 ± 4.78 mV respectively, and the PDI values were all smaller than 0.15). The lipofectamin 2000 was transfected according to the product guidelines. Fig. 5b and d showed that, at the optimal mass ratio, the relative cell viabilities of the complexes prepared by the PEI derivates were all over 85%. In contrast, the complexes made by PEI 25k or lipofectamin 2000 exhibited the relative cell viability less than 60% and 50% in the absence of serum respectively. For transfection activity assay (Fig. 5a and c), the complexes made by polymer B exhibit a remarkably higher transfection efficiency in the serum free medium compared with the complexes made by the acid-liable PEI derivate, PEI 25k and lipofectamin both in 293T and in HepG2 cells, which was 12.7 fold, 56.3 fold, 81.3 fold higher in 293T cells and 11.0 fold, 13.7 fold, 63.8 fold higher in HepG2 cells, respectively ($p < 0.01$). Moreover, in the medium with serum, the transfection efficiency of polymer B/DNA complexes was also higher than that of other formulations in the medium with or without serum ($p < 0.05$).

The result of that polymer A/DNA complexes exhibited lower transfection efficiency than polymer B/DNA complexes, might suggest that polymer degrading faster at neutral conditions was more applicable than the acid-liable polymer. This can be explained by at least two reasons: first, since polymer B had alleviated cytotoxicity than acid-liable polymers, a higher mass ratio can be used to maximize its transfect potential (the optimal mass ratio of polymer A/DNA complexes and B/DNA complexes was 4/1 and 8/1 respectively), as it has been reported that free cationic polymers can promote transfection efficiency sharply (Boeckle et al., 2004). Secondly, although DNA condensed with acid-liable polymers may easily escape from endocytic vesicles and be unpacked, the unprotected DNA was not stable against the enzymes in endosomes or in the cytoplasm, which was one of the key barriers for successful gene delivery. On the other hand, the most important reason of the higher transfection efficiency mediated by polymer B and A than PEI 25k, might be that the intracellular positively charged carriers may interact with negatively charged nuclear molecules or translation-related proteins in the cytoplasm and thus inhibit transfection efficiency (Ruponen et al., 2009). The biodegradable polymers (polymer A or polymer B), which might have weaker binding capacity to negatively charged nuclear molecules (for example mRNA) than non-degradable polymers (i.e. PEI 25k) after intracellular degradation, would inhibit transcription in a less degree and thus possess higher transfection efficiency.

To determine the transfection mechanism mediated by the new PEI derivates, 293T cells and HepG2 cells were treated with bafilomycin A1 during the transfection. As a specific inhibitor of vacuolar type H^+ ATPase, bafilomycin A1 can inhibit the endo-/lysosomal proton pump and further decrease the transfection mediated by PEI (Kichler et al., 2001). As shown in Fig. 5e and), the transfection efficiencies of the complexes made by polymer A, polymer B and PEI 25k were drastically decreased after bafilomycin A1 treatment ($p < 0.01$). However, the transfection efficiency of lipofectamin 2000/DNA complexes only slightly decreased after the same treatment. The dramatical decline of the transfection efficiency of the new PEI derivates/DNA complexes in the presence of bafilomycin A1 suggested that their transfection mechanism was based on the buffering capacity to facilitate endosomal escape (Kichler et al., 2001).

An applicable gene carrier should be non-toxic or low-toxic. Therefore, MTT assay was used to measure the cytotoxicity of plain polymers. For all polymers, the cytotoxicity was increased as the concentration increased. As shown in Fig. 6a, the relative cell viability curve of polymer B was above that of polymer A, and much higher than that of PEI 25k. In other words, when the concentration was over 20 μg/mL, the magnitude of cytotoxicity was significantly different between these three polymers, which was PEI 25k > A > B ($p < 0.05$).

LDH is a stable cytosolic enzyme released upon cell lysis, and its release was studied to estimate the early-stage cytotoxicity associated with plasma membrane disruption. The increased levels of LDH are directly proportional to the extent of damage caused by toxic agent to the cell membrane. Therefore, LDH release induced by the polymers at the concentration of 5-20 μg/mL was measured after their incubation with L929 cells for 4 h. Consistent with MTT assay, Fig. 6b indicated that there is an ascending trend in the LDH release as the polymer concentration increased, and it was notable that polymer B showed lower LDH release levels compared with polymer A and PEI 25k at the same concentration ($p < 0.05$).

A potential gene delivery carrier should also be biocompatible and have minimal interactions with blood components. The positively charged compounds tend to induce membrane damage due to the electrostatic interaction with negatively charged membrane proteins (Petersen et al., 2002). Therefore, the release of hemoglobin was assayed to quantify hemolytic activity caused by polymers at neutral condition, and PEI 25k was used as control. As shown in Fig. 6c, the hemolytic activity of polymer B was about 2.5 fold less than that of polymer A and 4 fold less than that of PEI 25k in the HBS solution without serum ($p < 0.05$). However, for all polymers, the presence of serum inhibited the hemolytic activity possibly due to the binding of cationic polymers with proteins, which greatly reduced the incidence of their interactions with erythrocytes (Kloeckner et al., 2006). As demonstrated in Fig. 6c, in the presence of serum, the hemolytic activity of polymer B was also the lowest one ($p < 0.05$). In all it was in accordance with the results of MTT assay and LDH release measurements, the hemolytic activity was PEI 25k > A > B.

The erythrocyte aggregation assay was another important way to evaluate the biocompatibility of the new polymers, especially for the intravenous administration samples. The cationic PEI/DNA complexes can induce erythrocyte aggregation. After incubated with erythrocyte, PEI 25k/DNA complexes induced strong erythrocyte aggregation (Fig. 7b), whereas the complexes made by polymer A and polymer B had a drastically decreased interaction even at a higher mass ratio (Fig. 7c and d), which means the polymer A and B had much higher biocompatibility than PEI 25k.

3.4. In vivo transfection activity and toxicity assay

To evaluate the transfection efficiency of the new PEI derivates/DNA complexes *in vivo*, BALB/c mice were intravenously injected with 50 μg DNA complexed with polymer A, polymer B and PEI 25k. The optimal mass ratios of polymer A/DNA complexes, polymer B/DNA complexes and PEI 25k/DNA complexes were 4/1, 6/1 and 1.32/1 respectively. The organs were harvested 24 h later

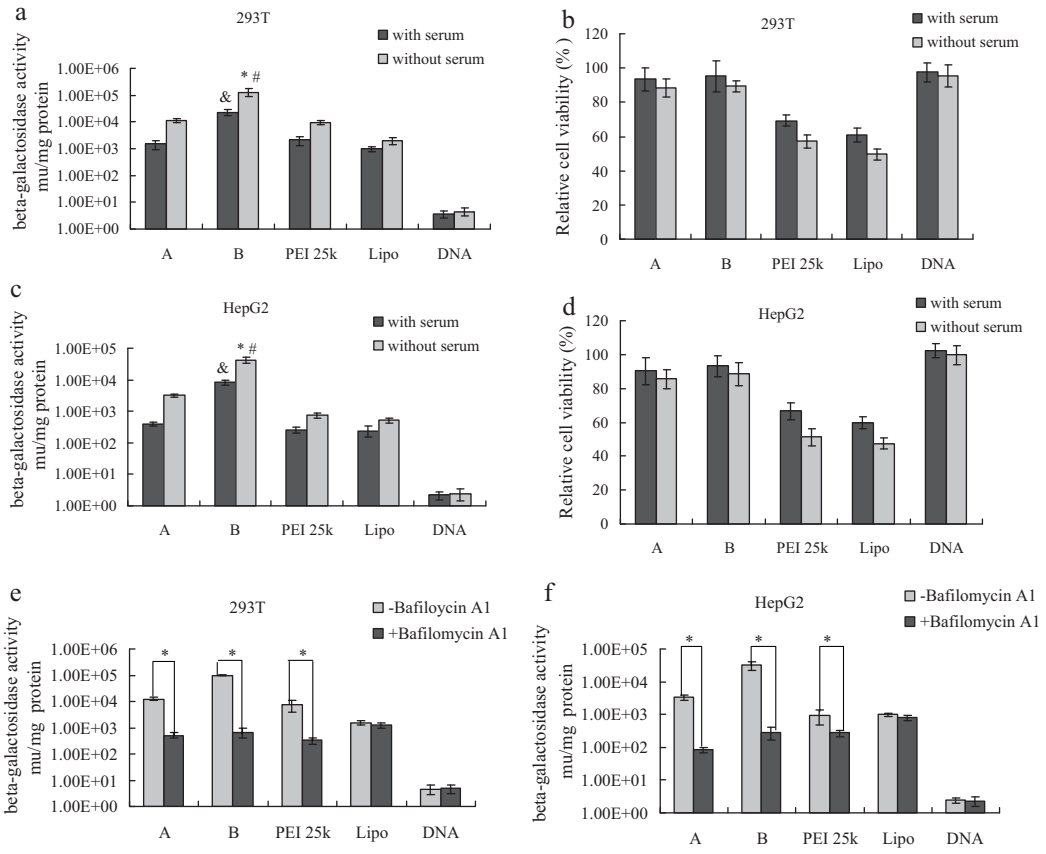

Fig. 5. (a–d) Comparison of the optimal transfection efficiency and the corresponding cell viability of the polymer/DNA complexes on 293T cells and HepG2 cells in the absence or presence of 10% serum. $^{*}p < 0.01$, *vs* the polymer B in the medium with serum; $^{\#}p < 0.01$, *vs* the group of other polymers in the medium with or without serum; $^{\&}p < 0.05$, *vs* the group of other polymers in the medium with or without serum. (e and f) Effect of bafilomycin A1 on PEI-mediated transfection efficiency. 293T cells (e) and HepG2 cells (f) were transfected in the absence or in the presence of the proton pump inhibitor, bafilomycin A1 (final concentration was 400 nM), $^{*}p < 0.01$. For all the figures (a–f), polymer/DNA mass ratios for polymer A, polymer B and PEI 25k were 4/1, 8/1 and 2/1 respectively. "Lipo" means lipofectamine 2000, which was prepared for transfection according to the product guidelines, and the naked DNA was used as the blank control. Data points represent the mean ± S.D of three experiments.

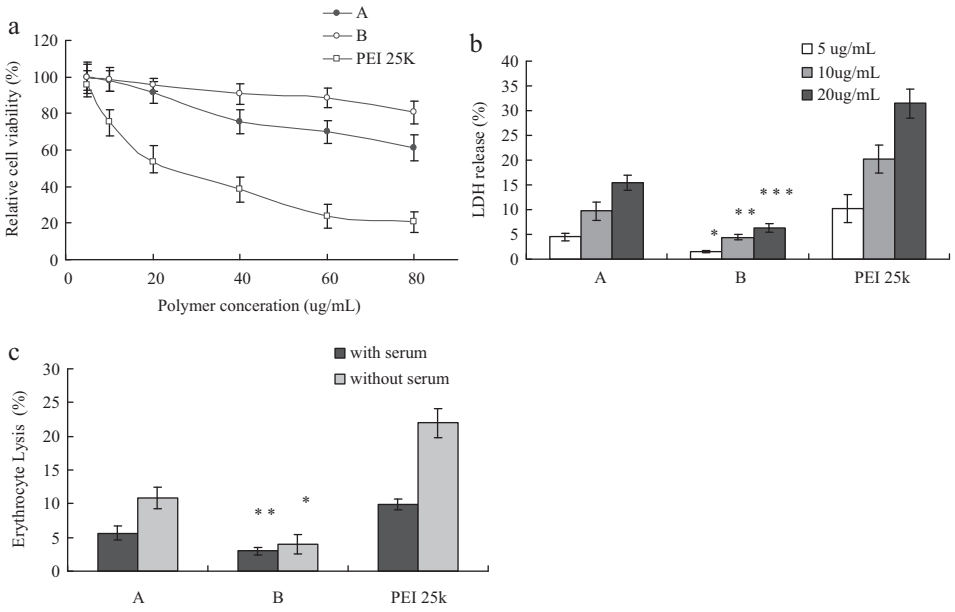

Fig. 6. (a) Relative cellular viability was determined using MTT assay where results were compared with an untreated control ($n = 3$). (b) LDH release assay of the new PEI derivates on L929 cells ($n = 3$). $^{*}p < 0.05$, *vs* the group of the other polymers at the concentration of 5 μg/mL; $^{**}p < 0.05$, *vs* the group of the other polymers at the concentration of 10 μg/mL; $^{***}p < 0.05$, *vs* the group of the other polymers at the concentration of 20 μg/mL. (c) Hemolytic activity assay. Human erythrocytes with or without serum were incubated with plain polymers (final polymer concentration was 0.5 mg/mL) for 45 min at 37 °C and hemoglobin release was quantified at 450 nm. Lysis (100%) refers to triton X-100 treatment of erythrocytes; % erythrocyte lysis is presented as mean values ± SD of triplicates. $^{*}p < 0.05$, *vs* the group of polymer A and PEI 25k in the HBS solution without serum; $^{**}p < 0.05$, *vs* the group of polymer A and PEI 25k in the HBS solution with serum.

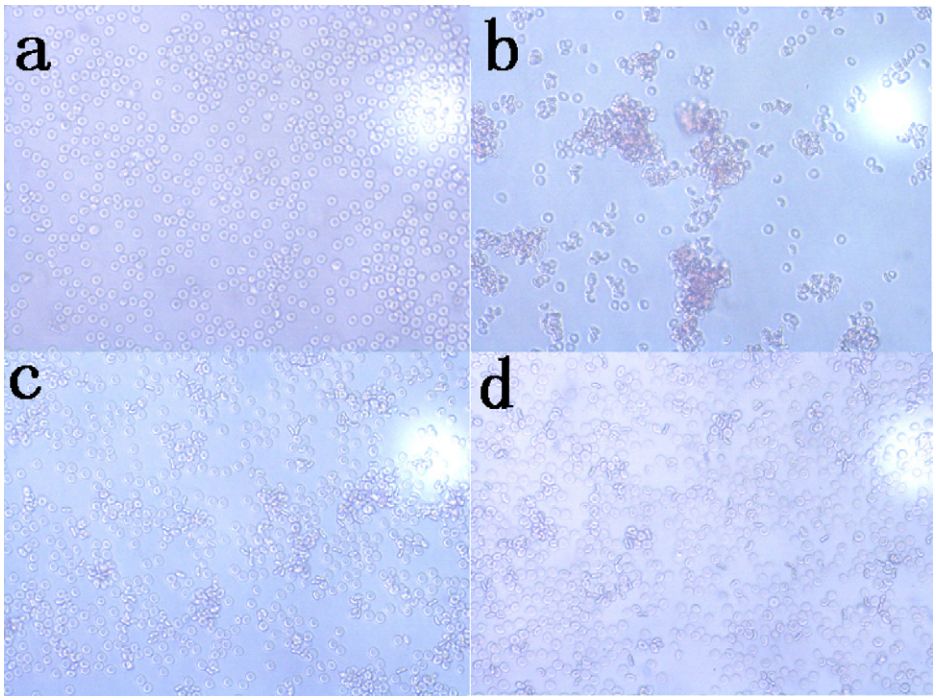

Fig. 7. Erythrocyte aggregation assay of the polymer/DNA complexes. a: PBS groups; b: the group of PEI 25k/DNA complexes (the mass ratio was 1.32/1); c: the group of polymer A/DNA complexes (the mass ratio was 4/1); d: the group of polymer B/DNA complexes (the mass ratio was 6/1).

and luciferase activity per milligram of total protein was determined. As shown in Fig. 8a, all the polymers/DNA complexes at their optimal mass ratio had the highest gene expression in lungs and the gene expression of the complexes made by polymer A and polymer B was higher than that of PEI 25k/DNA complexes in lungs ($p < 0.05$). And in other organs, they all had very low gene expression except in the liver and in spleen.

The *in vivo* toxicity of the polymer/DNA complexes was tested by ALT assay kit and histological examination. As shown in Fig. 8b, the serum ALT level of the PEI 25k group was much higher than that of other groups ($p < 0.01$). ALT was an indicator of hepatocyte toxicity. The high ALT level induced by PEI 25k/DNA complexes suggested their severe toxicity to hepatocyte. On the other hand, the serum ALT levels in the polymer A and B groups only slightly increased compared with the naked DNA group, which indicated their low liver toxicity. For the histological examination, the lung sections of the polymers/DNA complexes showed blood congestion, disintegration of alveolar structures, perivascular or interstitial edema, neutrophil intravascular adhesion and accumulation as well as neutrophil infiltration of edematous alveolar structures (Fig. 9d, f and h). Judging from these pictures, the damage of the PEI 25k groups was much heavier than that of the other groups, and for the group of polymer B, although there were some blood congestion in the lung sections (Fig. 9f), the alveolar structure was more integrity and with much less neutrophil infiltration than the other polymer groups, which suggested it had the lowest toxicity to the lungs of the three polymers/DNA complexes. Meanwhile, the liver sections also showed some blood congestion, interstitial edema, neutrophil infiltration and necrosis or apoptosis (Fig. 9c, e and g). Comparing these figures, the damage of the liver by PEI 25k/DNA complexes was also the strongest one, and the complexes made by polymer B had the lowest toxicity to the liver among the three polymers/DNA complexes. The result was in accordance with ALT assay. Taken the ALT assay and histological examination together, it could be concluded that the new PEI derivates/DNA complexes had much lower toxicity than that of PEI 25k/DNA complexes *in vivo*, and the complexes made by polymer B had the lowest toxicity among these polymers.

Compared with PEI 25k/DNA complexes, the new PEI derivates/DNA complexes could increase the gene expression in lungs after intravenous injection ($p < 0.01$). However, the enhancement was less dramatically than that *in vitro*. The huge different results between *in vitro* and *in vivo* might be because the complicated environment in body. The cationic nanoparticles could not reach the target cells effectively by intravenous injection due to the non-specific binding with the anionic components of the body and/or be uptaken by the phagocytic cells (Nishikawa and Huang, 2001). For the PEI polymers, there might be another reason for the

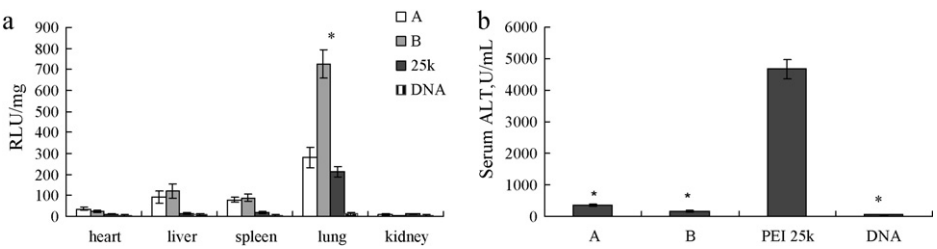

Fig. 8. (a) Gene expression after systemic gene delivery in BALB/c mice. Transfection complexes were injected into the tail vein (50 μg/mouse); transgene expression at 24 h after application was measured by luciferase assay. Luciferase activity represents as mean values ± SD of five mice. *$p < 0.01$, *vs* the group of A and PEI 25k. (b) Serum ALT levels of mice injected with PEI derivates/DNA complexes via tail vein (mean ± S.D.; $n = 5$). *$p < 0.01$, *vs* the group of PEI 25k, and the naked DNA was used as the blank control.

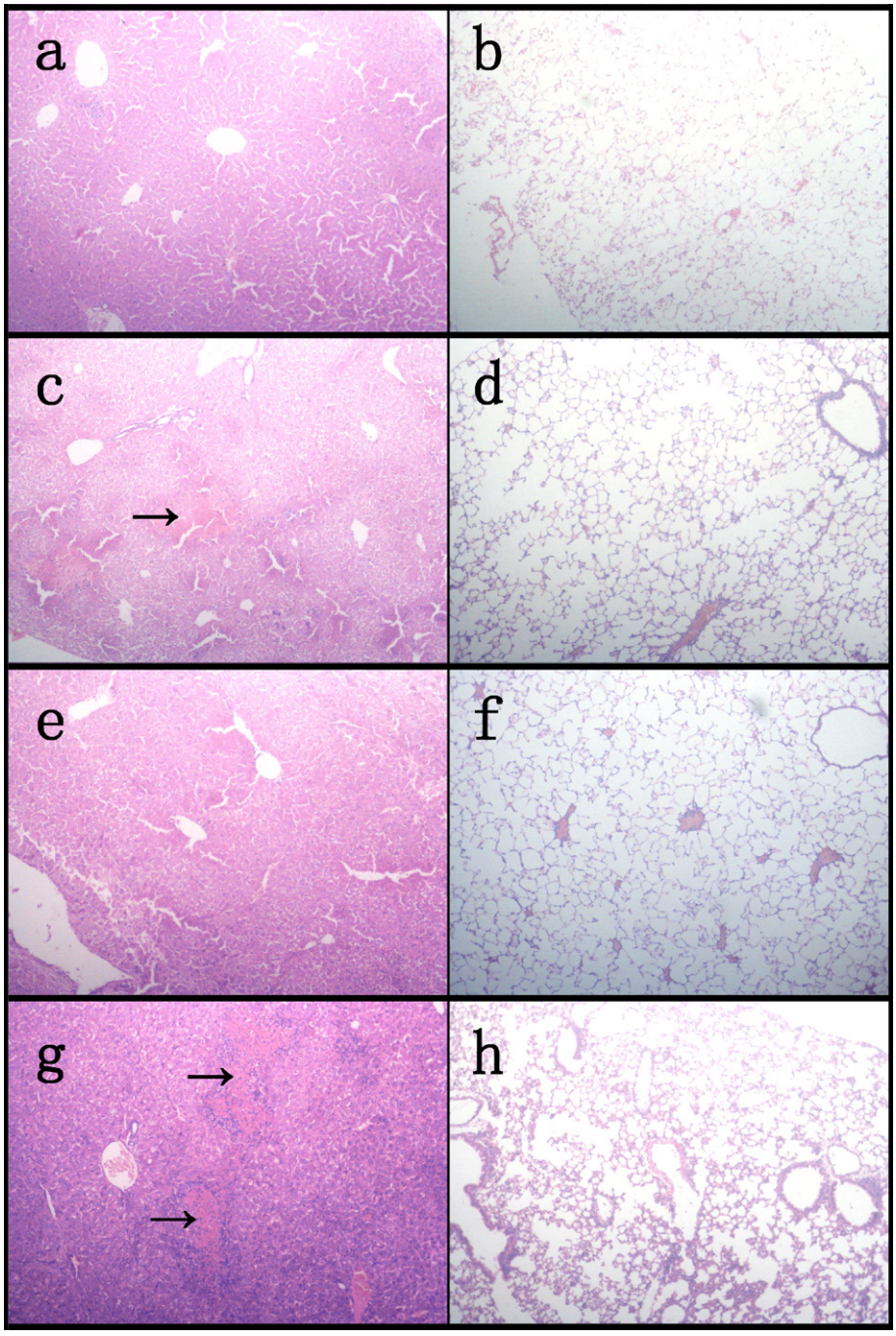

Fig. 9. Sections of livers and lungs of mice 24 h after injection of the polymers/DNA complexes. The left pictures were liver paraffin sections stained with H&E. The right pictures were lung paraffin sections stained with H&E. (a) and (b) were the animal groups injected with PBS; (c) and (d) were the animal groups injected with polymer A/DNA complexes; (e) and (f) were the animal groups injected with polymer B/DNA complexes; (g) and (h) were the animal groups injected with PEI 25k/DNA complexes. Magnification for all panels is 100×. Black arrows in panel (c and g) indicate the areas of damage. These are representative sections from five animals analyzed for each condition.

lower transfection ability *in vivo*. That was the effect of the free polymers in the formulation, which was reported could promote the transfection efficiency sharply *in vitro* (Boeckle et al., 2004). It was very difficult for the free cationic polymers to reach the targeted cells together with the complexes *in vivo*, so they could not improve the transfection efficiency as well as they did *in vitro*. To further improve the *in vivo* transfection ability of the new PEI derivates/DNA complexes, they may need to be conjugated with targeting ligands and modified to be anionic or non charged particles. Nevertheless, one of the promising results observed by the new PEI derivates/DNA complexes in this study was their much lower toxicity and higher tansfection efficiency than PEI 25k. Both the *in vitro* and the *in vivo* results suggested that the polymer B had the lowest toxicity and the highest transfection efficiency among the polymers under investigation, which indicated its potential as a candidate carrier for gene therapy.

4. Conclusions

In the present study, the new PEI derivates, PEI-GA and PEI-TEG, linked by imine linkages and biscarbamate linkages respectively, were successfully prepared and evaluated for their potential as

novel gene carriers. In general, polymer PEI-TEG, which exhibited the lowest cytotoxicity and the highest gene transfection efficiency both *in vitro* and *in vivo*, was demonstrated as the most applicable and promising gene delivery vector for its superiorities over PEI 25k and lipofectamin 2000. And as a gene carrier, the polymer degrading faster at neutral conditions might be more applicable than the acid-liable one.

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