Pharmaceutical Research, Vol. 26, No. 9, September 2009 (© 2009)
DOI: 10.1007/s11095-009-9928-9

Research Paper

Fabrication of a Novel Core-Shell Gene Delivery System Based on a Brush-Like Polycation of α, β–Poly (L-Aspartate-Graft-PEI)

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Received January 2, 2009; accepted June 15, 2009; published online June 26, 2009

Purpose. A novel core-shell gene delivery system was fabricated in order to improve its gene transfection efficiency, particularly in the presence of serum.
Materials and Methods. α, β–poly (L-aspartate-graft-PEI) (PAE) was simply synthesized by ring-opening reaction of poly (L-succinimide) with low molecular weight (LMW) linear polyethylenimine (PEI, Mn = 423). PAE/DNA nanoparticles were characterized. Condensation and protection ability of plasmid by PAE were confirmed by agarose gel electrophoresis assay. Cytotoxicity of the polymer and polymer/DNA nanoparticles were measured by MTS assay. Gene transfection efficiencies were evaluated both *in vitro* and *in vivo*.
Results. Core-shell nanoparticles assembled between DNA and PAE showed positive zeta potential, narrow size distribution, and spherical compact shapes with size below 250 nm when N/P ratio is above 10. Cytotoxicity of PAE was rather lower than that of PEI 25K, while the most efficient gene transfection and serum resistant ability of PAE/DNA complexes were higher than that of PEI 25K. Bafilomycin A1 treatment suggested "proton sponge" mechanism of PAE-mediated gene transfection. PAE/pEGFP-N2 nanoparticles also showed good gene expression *in vivo* and were dominantly distributed in kidney, liver, spleen and lung after intravenous administration.
Conclusions. The results demonstrated the potential use of PAE as an effective gene carrier.

KEY WORDS: core-shell nanoparticles; gene delivery; poly (L-succinimide); polyethylenimine; α, β–Poly (L-aspartate-graft-PEI).

INTRODUCTION

Gene therapy, the treatment or prevention of inherited or acquired human diseases at the genetic level through the application of nucleic acid-based drugs such as plasmid DNA or siRNA, has the potential to arouse a new revolution in medical fields (1–3). Although more than one thousand clinical trials have been done during the past two decades, no gene therapy protocol has yet been approved by the Food and Drug Administration in America (4). Since DNA is prone to be degraded by nuclear enzyme and is not easily internalized by cells due to its negative charge nature, it is still a major challenge to find how to efficiently and safely deliver the DNA to the target position (2). Viral carriers, used in most clinical trials due to their relatively high gene transfection efficiency, are always being oppugned by their clinical safety, such as their immunogenicity, oncogenicity, and random DNA insertion (5,6). Therefore, non-viral gene carriers for gene therapy have been increasingly proposed as safer alternatives to viral carriers owing to their improved safety, easier production and purification, flexibility of design, and relatively large gene-carrying capacity (7,8).

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ABBREVIATIONS: DCC, *N, N′*-dicyclohexylcarbodiimide; DMEM, Dulbecco's modified Eagle's medium; DMF, *N, N*-dimethylformamide; DMSO, dimethyl sulfoxide; EGFP, enhanced green fluorescent protein; FBS, fetal bovine serum; HGF, (hepatocyte growth factor); LMW, low molecular weight; MPS, mononuclear phagocytic system; PAE, α, β–poly (L-aspartate-graft-PEI); PEI, polyethylenimine; PSI, poly (L-succinimide); RLUs, Relative light units.

Of all the non-viral gene carriers, polyethylenimine (PEI) has been known as an effective and widely studied polymeric gene carrier (9, 10). Since PEI contains varying levels of primary, secondary, and tertiary amines that can be protonated under physiological pH, the DNA can be condensed into nanoparticles and protected from enzymatic degradation via complexes formed between negative DNA and positive PEI, thus facilitating the cell uptake and endolysosomal escape (11). Previous reports showed that high transfection efficiency of PEI, as well as its cytotoxicity, greatly depended on its molecular weight and geometries (12, 13). Branched PEI with a molecular weight higher than 25 KDa displays high transfection efficiency due to its good plasmid condensation ability and lysosomal buffering capacity, and has been used as a positive benchmark to which new gene carriers are often compared. On the other hand, high toxicity resulted from its non-degradability and high charge density, limiting its practical applications in clinical gene therapy.

To enhance the gene transfection efficiency and to maintain its low toxicity, many polycations based on low molecular weight PEI (LMW PEI) have been synthesized and used as gene carriers. These LMW PEI-based polycations showed negligible cytotoxicity and superior gene transfection efficiency in comparison with PEI 25K (14–18). However, their low transfection efficiency in the presence of serum is still an open problem. Herein, to improve the gene transfection efficiency in the presence of serum, a novel core-shell gene delivery system was fabricated from a brush-like polycation. Particularly, α, β–poly (L-aspartate-graft-PEI) (PAE) was simply synthesized by ring-opening reaction of poly (L-succinimide) (PSI) with LMW linear PEI (Mn = 423), and characterized by gel permeation chromatography with multi-angle laser scattering (GPC-MALS) and ^{1}H-nuclear magnetic resonance (NMR). DNA condensation and protection ability of the PAE and PAE/DNA core-shell nanoassembly were explored. Cytotoxicity of the polymer and polymer/DNA nanoparticles was measured and compared with that of PEI at the same concentration or N/P ratio. Gene transfection efficiency was investigated both *in vitro* and *in vivo*. It will be expected that a brush-like polycation resulting from the high grafting ratio of small PEI to α, β–poly (L-aspartate) will form the core-shell nanoparticles with hydrophilic shell assembled between PAE and DNA to enhance their serum-resistant ability.

MATERIALS AND METHODS

Materials

L-aspartic acid, phosphoric acid, linear polyethylenimine (PEI, Mn: 423), *N, N*-dimethylformamide (DMF), *N, N′*-dicyclohexylcarbodiimide (DCC), dimethyl sulfoxide (DMSO), agarose, ethidium bromide (EtBr), branched polyethylenimine (PEI, Mw: 25K), Williams's E medium, bafilomycin A1 and calf thymus DNA were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM) and fetal calf serum (FCS) were from GIBCOBRL-Life Technologies (Paris, France). Cell Titer 96 Aqueous One Solution Cell Proliferation Kit (MTS) for cell viability, Luciferase Reporter 1,000 Assay System for *in vitro* transfection assay and pGL3-control vector with SV-40 promoter and enhancer encoding firefly (*Photinus pyralis*) luciferase were obtained from Promega (Madison, WI, USA). Plasmid pEGFP-N2, which has the early promoter of CMV and enhanced green fluorescent protein (EGFP) gene, were obtained from Clontech (Palo Alto, CA, USA). Plasmids were amplified with a competent *Escherichia coli* bacterial strain DH5α, and their purification was performed using a QIAGEN (Chatsworth, CA, USA) kit. The concentration and purity of plasmid were determined by measuring UV absorbance at 260 and 280 nm, respectively. All samples showing an A_{260}/A_{280} ratio of 1.9–2.0 were stored at −20°C before use.

Cell Lines and Culture

HeLa (human cervix epithelial carcinoma cells), 293T (human kidney cells), and HepG2 (human hepatoblastoma cells) were thawed and cultured in Dulbecco's modified Eagle medium (DMEM, Gibco BRL, Paris, France) supplemented with 10% fetal bovine serum (FBS, HyClone, Logan, Utah), streptomycin at 100 μg/ml, and penicillin at 100 U/ml. All cells were incubated at 37°C in humidified 5% CO_2 atmosphere. Cells were split by using trypsin/EDTA solution when almost confluent.

Primary hepatocytes were isolated from the liver of a male ICR mouse by collagenase perfusion technique of Seglen (19). Briefly, the mouse was anesthetized, then the liver was perfused with prewarmed Hanks's balanced salt solution and collagenase solution. After that, the liver was dissected and filtered to remove debris and cell clumps. Dead cells were separated by a density gradient using percoll solution, and viable cells were cultured with Williams's E medium, containing antibiotics, HEPES, HGF (hepatocyte growth factor), and insulin.

Synthesis of PAE

Poly (L-succinimide) (PSI) was synthesized according to the similar methods described by Neri *et al.* (20). Briefly, finely ground L-aspartic acid (25 g) was mixed with 12.5 g of 85% phosphoric acid in a 1,000 ml flask. The flask was placed in a rotary evaporator and heated for 3 h under reduced pressure in an oil bath at 180°C. The obtained mass was dissolved in 150 ml of N, N-dimethylformamide. The solution was filtered and poured slowly into a beaker containing 1,000 ml of distilled water. The precipitate was collected by filtration, rinsed with distilled water several times, and dried in a vacuum at 50°C for 48 h (yield: 93%).

To increase the molecular weight of PSI prepolymer, the prepolymerized PSI was condensed further with DCC. Five hundred mg of DCC was added to the 100 ml of DMF solution containing 10 g of PSI prepolymer. The mixture was stirred at room temperature for 48 h. After removal of dicyclohexylurea by filtration, PSI was precipitated by distilled water, washed with distilled water and ethanol, and dried in a vacuum at 50°C for 48 h (yield: 98%).

PAE was synthesized by ring-opening polymerization of PSI with PEI 423. In a typical reaction procedure, PEI 423 and PSI were separately dissolved in DMF. The solutions of PEI and PSI were mixed in molar ratio of succinimide rings to PEI of 1:3 (Su/PEI) and maintained at room temperature with constant stirring for 22 h. After filtration, the solution was thoroughly

dialyzed using Spectra/Pro membrane (MWCO=3500) against deionized water at room temperature for 2 days. The lyophilized products were collected, and stored at −20°C.

Characterization of PAE

The structure of PAE was characterized by ^{1}H nuclear magnetic resonance (^{1}H-NMR) (Avance ^{TM} 500, Bruker, Germany). For NMR measurement, the sample concentration in D_2O was 10 mg/ml. The molecular weights of samples were measured by gel permeation chromatography with multiangle laser scattering (GPC-MALS) with 690 nm laser wavelength (Dawn Eos, Wyatt, USA). Sodex Ohpak SB-803 HQ (phenomenox, USA) column was used. The column temperature was maintained at 25°C. Mobile phase was 0.5 M ammonium acetate (pH 5.5), and the flow rate was 0.5 ml/min.

Gel Retardation Assay

Complex formation of the PAE and plasmid DNA were examined by the electrophoretic mobility of the complexes. Briefly, polymer/DNA complexes with various N/P ratios were prepared freshly before use by gently vortexing a mixture of pGL3-control and polymer solution. The complexes were incubated at room temperature for 20 min and then followed by addition of 6× agarose loading dye mixture (Biosesang, Seongnam, Korea). After further incubation for 10 min, the mixture solutions were loaded onto 0.8% agarose gels with EtBr (0.1 μg/ml) and run with Tris-acetate (TAE) buffer at 100 V for 40 min. The gel was analyzed on UV illuminator to show the location of the DNA.

Protection and Release Assay of DNA

A 2 μl of PAE/DNA complexes at N/P ratio 5 and naked plasmid DNA (pGL3-control) were separately incubated with 1 μl of PBS or DNase–I (1unit) in DNase/Mg^{2+} digestion buffer (50 mM Tris–Cl, pH 7.6 and 10 mM $MgCl_2$) at 37°C with shaking at 100 rpm for 30 min as the similar method previously described by Gebhart *et al.* (21). For DNase inactivation and DNA release, all samples were treated with 250 mM EDTA for 10 min and mixed with sodium dodecyl sulfate (SDS) dissolved in 0.1 M NaOH (pH 7.2). Finally, samples were incubated at room temperature for 2 h, followed by addition of 6× agarose loading dye mixture (Biosesang, Seongnam, Korea). After further incubation for 10 min, the mixture solutions were loaded onto 0.8% agarose gels with EtBr (0.1 μg/ml) and run with Tris-acetate (TAE) buffer at 50 V for 1 h. The gel was analyzed on UV illuminator to show the location of the DNA.

Particle Size and Zeta Potential Measurement

The particle sizes and zeta potential of polymer/DNA complexes were measured using an electrophoretic light scattering spectrophotometer (ELS8000, Otsuka Electronice, Osaka, Japan), with 90 and 20° scattering angles, respectively. Polymer/DNA complexes were prepared in water at N/P ratios of 1, 5, 10, 20, and 25, respectively, and allowed to incubate at room temperature for at least 25 min. The calf thymus DNA concentration for each sample was 40 μg/ml.

Morphology Observation with Energy-Filtering Transmission Electron Microscopy

For energy-filtering transmission electron microscopy (EF-TEM) measurement, the final concentration of DNA in complex solution was 20 μg/ml. A 10 μl of polymer/DNA complexes with N/P ratio 10 was carefully dropped onto clean copper grid and negatively stained with 1.5 wt−% phosphotungstic acid (pH 7.4) for 5 sec. The copper grid surface was dried at room temperature for 5 min before imaging on EF-TEM (LIBRA 120, Carl Zeiss, Germany).

Cell Viability Assays

In vitro cytotoxicity was investigated by Cell Titer 96 A_{queous} One Solution Cell Proliferation Kit (Promega). Cells were seeded in 96 well plate at an initial density of 1×10^4 (HeLa, 293T and Primary hepatocytes) or 2×10^4 (HepG2) cells/well in 200 μl growth medium and incubated for 18–20 h to reach 80% confluency at the time of treatment. Growth medium was replaced by 100 μL fresh, serum-free media, containing various amounts of polymers or various N/P ratios of polymer/DNA complexes. Cells were further incubated for 24 h and followed by the addition of 20 μl of Cell Titer 96 A_{queous} One Solution Reagent. After further incubation for 2–4 h, the absorbance was measured at 570 nm using an ELISA plate reader (GLR 1000, Genelabs Diagnostics, Singapore) to evaluate the metabolic activity of the cells. Cell viability (%) = (OD_{sample} / $OD_{control}$) × 100, where OD_{sample} represents an OD value from a well treated with polymer or polymer/DNA complexes and $OD_{control}$ from a well treated with medium only.

In Vitro Luciferase Activity and Transfection Mechanism Assay

Cells were seeded in 24-well plates at an initial density of 1×10^5 (HeLa and 293T) or 2×10^5 (HepG2) cells per well in 1 ml growth medium and incubated for 18–20 h to reach 70–80% confluency at the time of transfection. The medium was replaced with 500 μl serum-free or 10% serum-containing media with polymer/pGL3-control (1 μg) complexes at various N/P ratios (5, 10, 20, 30, and 40) and additionally incubated for 6 h. Then, the media were changed with fresh media containing serum and allowed to incubate for 24 h. The luciferase assay was performed according to manufacturer's protocols. Relative light units (RLUs) were measured with a chemiluminometer (Autolumat LB953, EG and G, Berthold, Germany). Protein quantification was determined by the BCA method, and RLUs were normalized to protein concentration in the cell extracts (22). Each transfection experiment was carried out in triplicate, and transfection activity was expressed as relative light units.

To explore the gene transfection mechanism of PAE, HeLa cells were incubated with 500 μL of 200 nM bafilomycin A1 (2 μg of bafilomycin A1 was dissolved in 1 ml DMSO, then 15 ml serum-free media was added and sterilized by filteration) for 10 min before transfection with polymer/pGL3-control complexes at N/P ratios of 10 and 20.

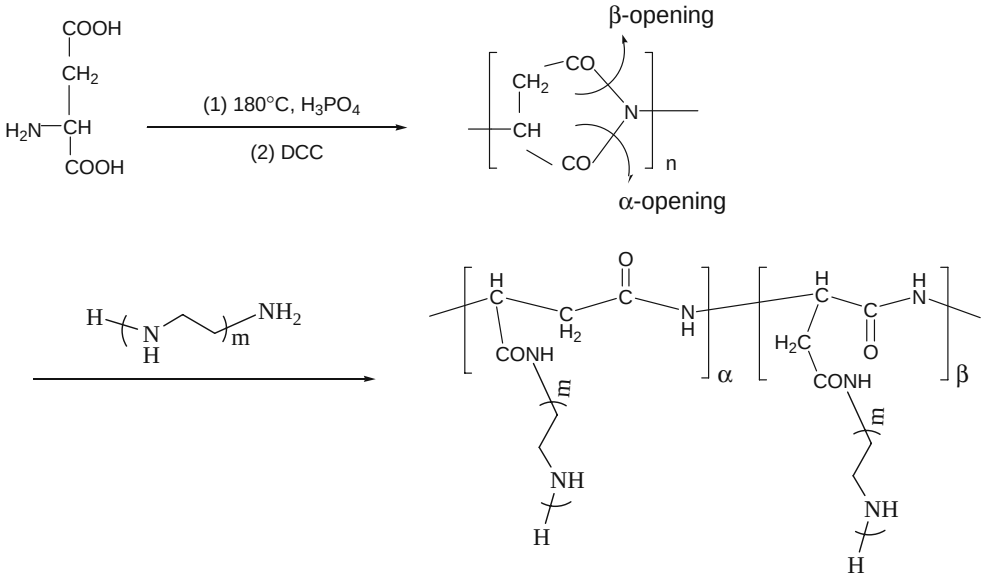

Fig. 1. The synthesis scheme of PAE.

Fluorescent Microscopy Observation

The HeLa cells were seeded in 24-well plates at 5×10^4 cells/well in 1 ml growth medium. The cells were incubated for 18–20 h to reach 80% confluency at the time of treatment. Growth medium was replaced by 500 μl fresh, serum-free media, containing polymer/pEGFP-N_2 (1 μg) complexes at N/P ratio 40 (PAE and PEI 423) or 10 (PEI 25K), and additionally incubated for 6 h. Then, the media were changed with fresh media containing serum and allowed to incubate for 24 h. Thereafter, the cells transfected with pEGFP-N2 were observed under bright field and UV using GFP filter at 200×magnification under an inverted fluorescent microscopy to observe the expression of green fluorescent protein.

Gene Expression *In Vivo*

Three groups of BALB/c mice (n=3) were treated with 20 μg pEGFP-N2 plasmid DNA in PBS solution, PAE/pEGFP-N2 complexes (N/P=40), or PEI 25K/pEGFP-N2 complexes (N/P=10), respectively. Mice injected with PBS were used as a control. All groups of mice were injected through intramuscle (i.m.) in the posterior tibialis muscle on one leg. The muscles from each mouse in the groups were harvested at day 4, and perfused in 4% phosphate-buffered formaldehyde, then 30% glucose. Furthermore, the muscles were embedded in Tissue-Tek OCT (Sakura, Torrance, CA) and frozen in −80°C freezer. Five micrometers of tissue cryosections were cut with micro-

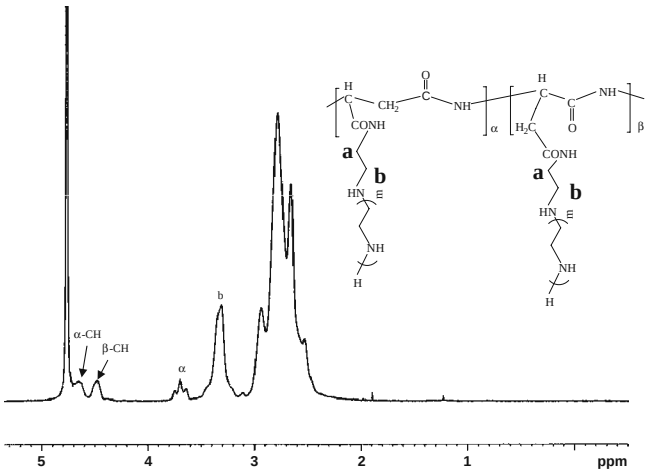

Fig. 2. ^{1}H-NMR spectrum of PAE in D_2O: 4.65-4.48 (-NHCHCH2CONH-); 3.75-3.64 (-CONHCH2CH2N-); 3.31 (-CONHCH2CH2N-) and 3.11-2.53(-NHCHCH2CONH-, -NHCH2CH2NH-).

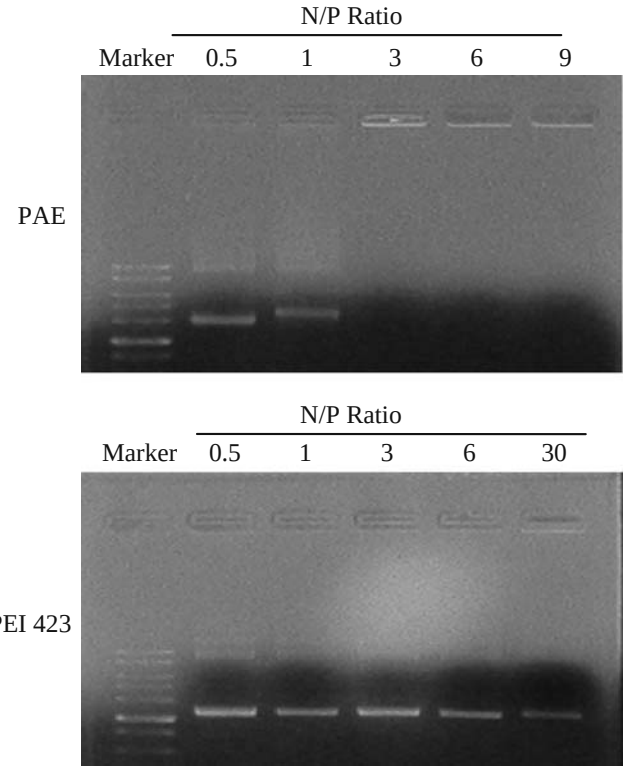

Fig. 3. Agarose gel electrophoresis of polymer/DNA (pGL3-control) complexes at various N/P ratios.

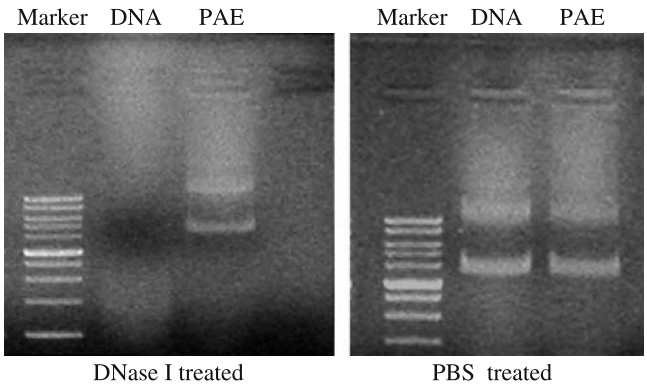

Fig. 4. Protection and release assay of PAE/DNA (pGL3-control) complexes. DNA was released by adding 1% SDS to PAE/DNA complexes at N/P ratio 5.

tome (Leica, Nussloch, Germany) and mounted on slides for further fluorescent microscope observation.

Biodistribution Assay

The biodistribution of PAE/DNA and PEI/DNA complexes after i.v. administration were also measured by labeling the polymer with ^{99m}Tc. The radio labeling method has been reported previously (23). Briefly, ^{99m}Tc pertechnetate (37~48 MBq) in normal saline was mixed with $SnCl_2$ (in 0.01 NHCl) and PAE (concentration = 5 mg/ml). The mixture was allowed to react for 15 min with shaking. The pH of the final mixture was approximately 7.4. $^{99m}TcO_4^-$ was analyzed by chromatography on ITLC-sg strips eluted with saline. After development, the chromatographic strips were scanned on an automatic TLC scanner (BIOSCAN, Washington D.C., USA).

Specific-pathogen-free female BALB/C mice (4 weeks old) were administered with ^{99m}Tc- PAE/DNA complexes and ^{99m}Tc- PEI/DNA complexes by i.v. (tail vein) injection. At predetermined time points, mice (n=4 at each time point) were sacrificed, and viscera and blood were collected. The amounts of radioactivity in the tissues were counted on a γ-counter (Packard Cobra Gamma Counter, Minnesota, USA) with injection standards, and were expressed as a percentage of injected dose per gram (% ID/g).

All animal experiments were approved by the Chonbuk National University School of Medicine Committee and were performed in accordance with their guidelines.

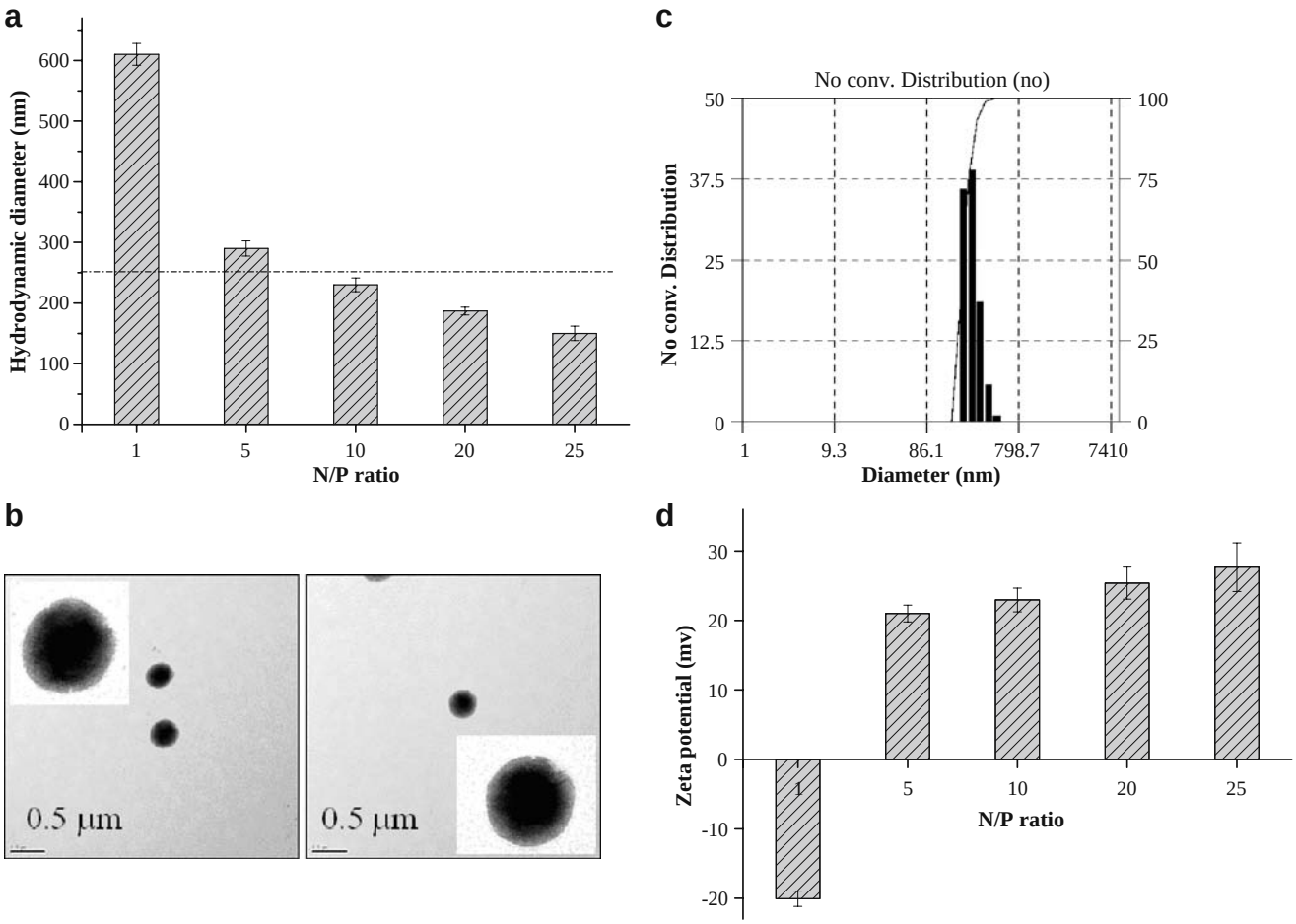

Fig. 5. (**a**) Particle sizes of polymer/DNA complexes in distilled water at various N/P ratios (mean ± SD n=3); (**b**) EF-TEM images of PAE/DNA complexes at N/P ratio 10 (about 230 nm): phosphotungstic acid was used as negative staining agent; (**c**) size distribution of PAE/DNA complexes in distilled water at N/P ratio 10 (about 230±11 nm); and (**d**) zeta potential of polymer/DNA complexes in distilled water at various N/P ratios (mean ± SD n=3).

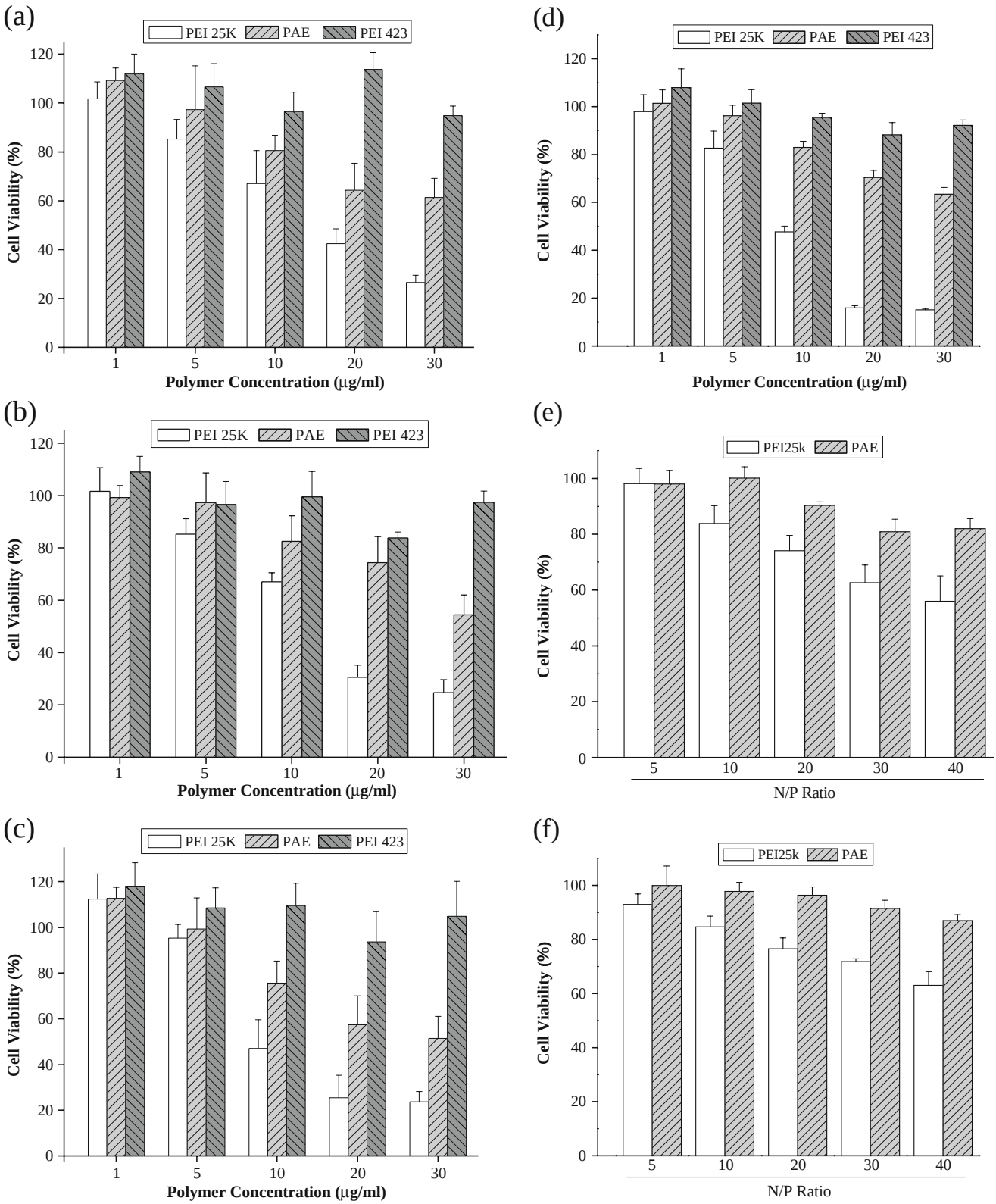

Fig. 6. Cell viability of copolymers at various concentrations and N/P ratios in different cell lines (mean ± SD n=3): (a) 293T; (b) HeLa ; (c) HepG2; (d) primary hepatocytes; (e) HeLa; and (f) primary hepatocytes.

Statistical Data Analysis

Statistical data analysis was performed using the Student's *t*-test with $p<0.05$ as the level of significance.

RESULTS AND DISCUSSION

Synthesis and Characterization of PAE

To fabricate core-shell gene delivery system, PAE with high grafting ratio was successfully synthesized through ring-opening of PSI with PEI as shown in Fig. 1. Since the feed molar ratio of succinimide rings to amino groups in PEI reached 1:6, the reaction mixture was homogeneous without gel formation during the whole reaction process, indicating that only one primary amino group per PEI molecule participated in the ring-opening reaction, and another intact primary amino group was able to interact with DNA. After removal of superfluous unreacted PEI by thorough dialysis, the lyophilized PAE was white powder with good water-soluble nature. The molecular weight of PAE measured by GPC-MALS was 3.5×10^4, which is appropriate as a gene carrier. The structure of PAE was also confirmed by ^{1}H-NMR, and the detail chemical shift data of PAE were shown in Fig. 2. The disappearance of the peak at 5.3 ppm assigned to methine protons of succinimide units clearly indicated that all the rings in PSI were opened by the amino groups in PEI (24). The two chemical shift values of the methine protons in PAE were caused by the α- and β-ring-opening manners. The ratio of α- and β-openings calculated from the integral area ratio of methine peak at 4.65 ppm (α-CH) to 4.48 ppm (β-CH) was 55/45, indicating that the α-openings predominantly occurred rather than the β-openings.

Characterization of PAE/DNA Complexes

As aforementioned, polycations are able to interact with the negatively charged phosphate groups of DNA, which results in the formation of neutral polyelectrolyte nanoparticles unable to migrate under the influence of electric field in agarose gel. As shown in Fig. 3, it was revealed that PEI 423 could not efficiently condense DNA, even at N/P ratio 30, due to its low molecular weight. Whereas the N/P ratio of PAE/DNA complexes was around 3, the migration of DNA was completely retarded, suggesting its good DNA condensation ability.

Effective condensation is a key issue for DNA stability against degradation by nucleases (25). Agarose gel electrophoresis assay showed that naked DNA was completely degraded by DNase-I within 30 min (Fig. 4). On the other hand, after the formation of complexes with PAE at N/P ratio 5, DNA was efficiently protected from the enzymatic hydrolysis, which is one of the crucial factors for efficient gene delivery *in vitro* as well as *in vivo*.

In polymeric gene delivery systems, the sizes of complexes assembled between polymers and DNA are known to dramatically affect gene transfection efficiency. As shown in Fig. 5a, PAE condensed DNA into nano-sized particles. At N/P ratio 1, where the nanoparticles could not form compactly, the particle sizes were very large. The sizes of the particles decreased sharply from 610 to 290 nm with increasing N/P

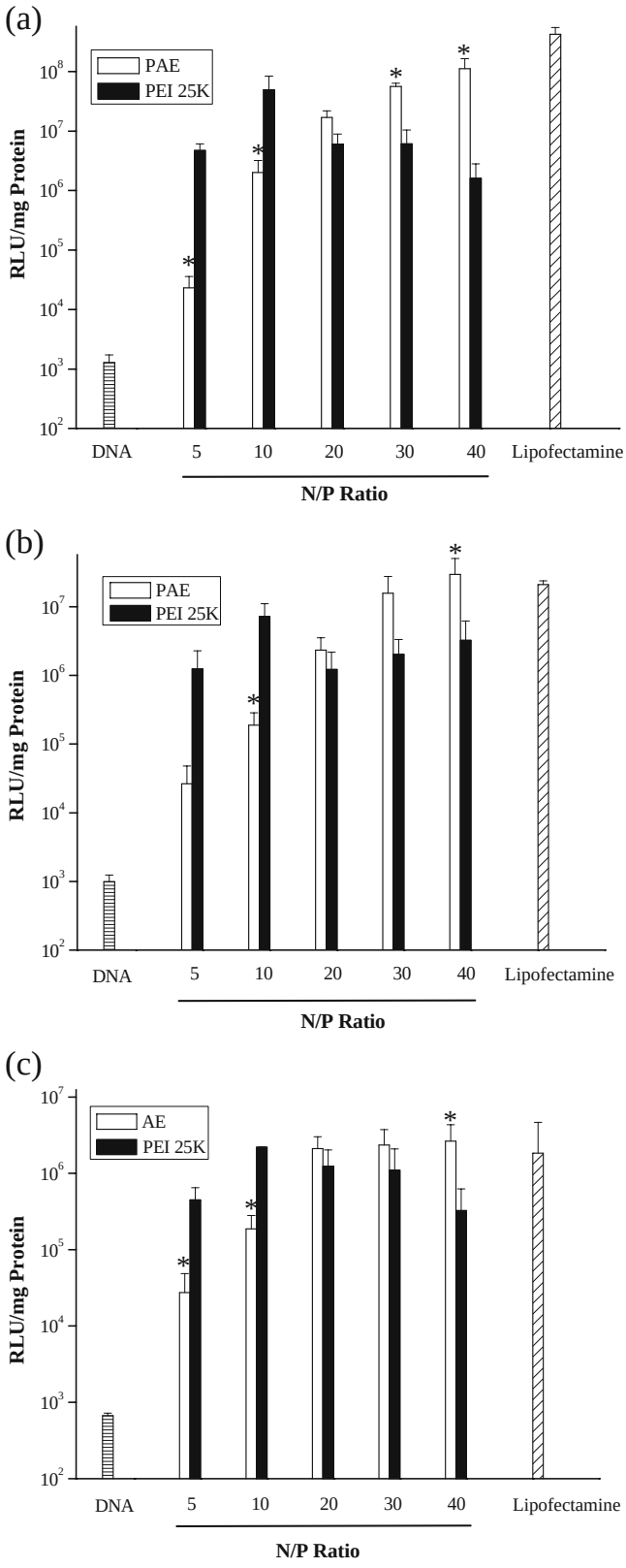

Fig. 7. Transfection efficiency of copolymer/pGL3-control at various N/P ratios and in different cell lines (mean ± SD $n=3$; *$p<0.05$): (a) 293T; (b) HeLa; and (c) HepG2.

ratios from 1 to 5. Then the sizes of particles decreased gradually from 290 to 150 nm with increasing N/P ratios from 5 to 25. When N/P ratio was abound 10, the sizes of the particles were less than 230 nm, suggesting their endocytosis potential (26).

The particle sizes of the PAE/DNA complexes in the absence and presence of serum at N/P ratio 40 were also measured. The particles sizes were around 142±4, 192±12, 123±7, and 115±6 nm for the serum concentration of 0, 10, 30, and 50%, respectively, indicating that the PAE/DNA complexes were relatively stable even in the presence of serum. The particles sizes of PAE itself were 223±13, 96.1±6, and 78.5±10 nm for the serum concentration of 10, 30, and 50%, respectively, indicating that the particle sizes of PAE decreased with an increase of serum, although the particle size of the PAE could not be measured in the absence of serum. It might be due to the interaction of cationic PAE with serum proteins such as albumin, etc..

The formation of PAE/DNA nanoparticles was also proved by the observation of the morphology. Representative EF-TEM images of the PAE/DNA nanoparticles at N/P ratio 10 were shown in Fig. 5b. Since cationic PEI and anionic DNA can form poor water-soluble nanoparticles (27), the self-assembling of PAE with DNA produced nanoparticles with core-shell structure is expected, where PEI/DNA was sequestered toward the particle core and α, β–poly (L-aspartic acid) derivative backbone formed the hydrophilic shell. The hypothesis of core-shell nanoparticles was confirmed by the enlarged figure inserted in Fig. 5b, where the dark region was wrapped by the light corona. The spherical shapes and good dispersity of nanoparticles were also shown in Fig 5b. Sizes observed from EF-TEM images were about 230 nm, very similar to those measured by dynamic light scattering.

The good dispersity of PAE/DNA nanoparticles was further proved by the unimodal size distribution shown in Fig. 5c. As aforementioned, the electrostatic interaction between polymers and DNA resulted in nanoparticles. When the absolute values of zeta potential are above 30 mV, the strong charge repulsive forces prevent aggregation among nanoparticles, resulting in narrow size distribution. However, the condition was not just the case for PAE/DNA nanoparticles. As discussed later, the zeta potential of PAE/DNA nanoparticles was less than 30 mV at all the tested N/P ratios (Fig. 5d). Therefore, besides the zeta potential repulsive forces, the hydrophilic shell of PAE/DNA nanoparticles might be an additional benefit to their enhanced stability and dispersity.

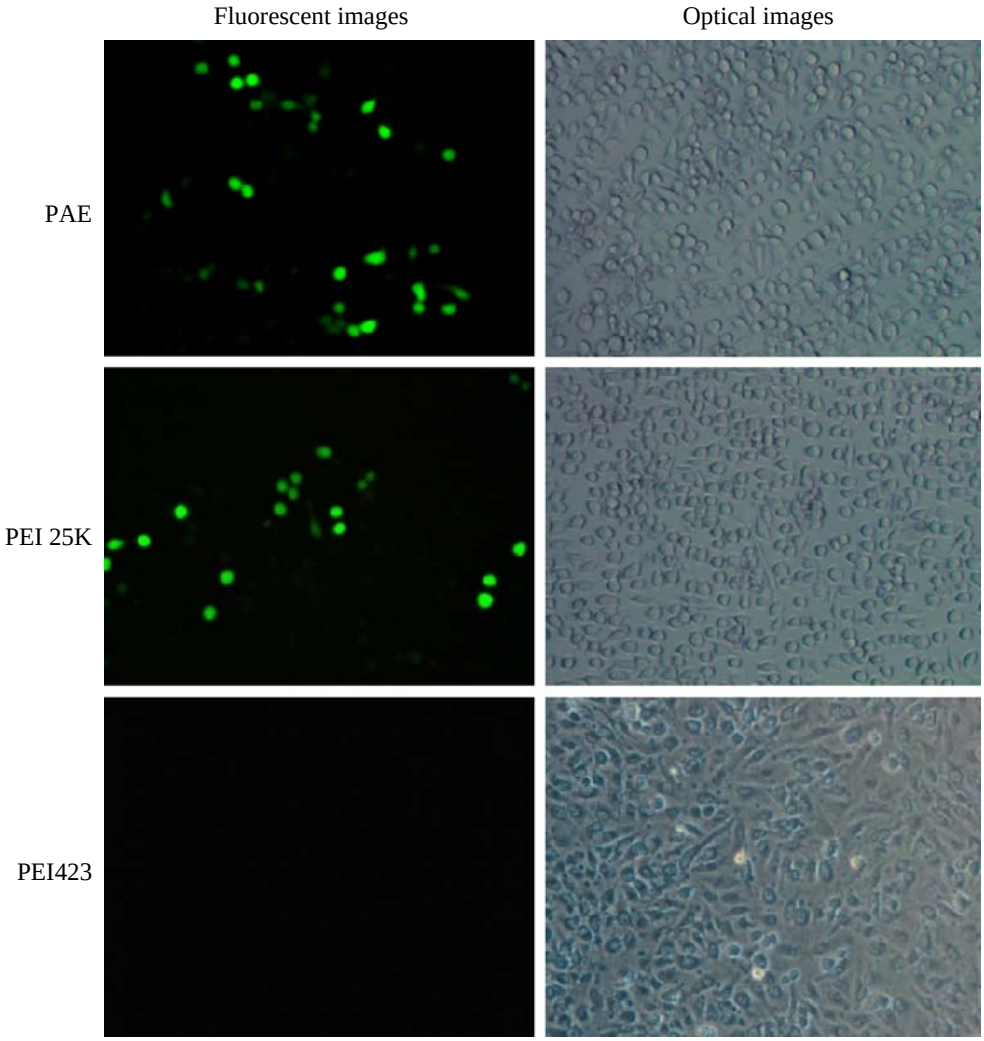

Fig. 8. EGFP expression in HeLa cell lines transfected with polymer/pEGFP-N2 complexes at N/P ratio 40 (PAE and PEI 423) and 10 (PEI 25K) (magnification× 200).

To know better the zeta potential of PAE/DNA nanoparticles depending on N/P ratios, the zeta potentials of the nanoparticles at various N/P ratios were measured. As shown in Fig. 5d, at N/P ratio 1, the zeta potential of PAE/DNA nanoparticles was negative, whereas zeta potentials rapidly increased from −20 mV to +21 mV with increasing N/P ratio from 1 to 5, then increased gradually from +21 mV to +27 mV with increasing N/P ratio from 5 to 25. A positive zeta potential of untargeted polyplexes is necessary for the attachment to anionic cell surfaces, which consequently facilitates uptake by the cell (28).

Cytotoxicity of PAE

Cytotoxicity of polymeric gene vectors is an important factor that affects the transfection efficiency. The cytotoxicity of polycation is probably caused by polymer aggregation on cell surface due to the strong electrostatic interaction with plasma membrane, which results in destabilization and ultimately impairing the cell membrane functions (29,30). Many attempts have been performed to reduce the cytotoxicity of gene carrier and hence enhance its gene transfection efficiency. Amongst them the most favorable one is to develop LMW PEI-based degradable polycation as a gene carrier. As shown in Fig. 6, PEI 423 showed almost non-toxic in 293T, HeLa, HepG2 cell lines and primary hepatocytes by the MTS assay at all the tested polycation concentration, while the dose-dependent cytotoxicity was observed with increasing concentration of PEI 25K or PAE. At the same concentration, PAE was found to be much less cytotoxic than PEI 25K, especially at higher dose. It is thought that the low charge density and degradable peptide backbone were the key factors to the low cytotoxicity of PAE. The cytotoxicity of the polymer/DNA complexes was reduced when the cationic polymers were complexed with DNA owing to masking of cationic charges of the polymers because the cytotoxicity arises from the charges of the cationic polymers. Also, because of the core shell structure, the positive charges of the PAE/DNA nanoparticles could be partially shielded by the shell, which resulted in their low surface charge along with low cytotoxicity. As shown in Fig. 6(e) and (f), the cell viabilities of the PAE/DNA nanoparticles were higher than 80% at all tested N/P.

In Vitro Transfection Efficiency Assay of PAE/DNA Complexes

In vitro transfection efficiencies of PAE in 293T, HeLa and HepG2 cells are shown in Fig. 7. It was found that the transfection efficiency of PAE in three cell lines showed a tendency to increase monotonously with an increase of N/P ratios from 5 to 40. The most efficient gene expression of PAE occurred at N/P ratio 40, even higher than those of PEI 25K (in 293T, HeLa and HepG2 cells) and Lipofectamine (in HeLa and HepG2 cells). However, the transfection efficiency of PEI 25K showed a changeful character with an increase of N/P ratios from 5 to 40. Its transfection efficiency increased with increasing the N/P ratios from 5 to 10, which contributed to the increased surface charge of PEI 25K/DNA complexes along with enhanced cell uptake. Then a decline was observed due to the increased cytotoxicity resulting from the superfluous PEI 25K. It is thought that the different optimum N/P ratios in the transfection of polymer/DNA complexes were associated with the difference of cytotoxicity as well as charge density.

The high gene transfection efficiency of PAE was further checked by the observation of the expression of green fluorescent protein. Therefore, HeLa cells were transfected with PAE/pEGFP-N2 complexes at N/P ratio 40. PEI 25K/pEGFP-N2 complexes with N/P ratio 10 and PEI 423/pEGFP-N2 complexes with N/P ratio 40 were used as controls. As shown in Fig. 8, PEI 423/DNA complexes showed low gene expression under an inverted fluorescent microscopy, whereas, compared with PEI 25K/pEGFP-N2 complexes, more EGFP expression was observed with the PAE/pEGFP-N2 complexes, indicating the great potential of PAE as an effective gene carrier.

We also checked the effect of serum on the gene transfection efficiency of PAE/DNA complexes. As well known, it is a key issue to develop non-viral gene delivery systems that are stable in serum, especially for *in vivo* use (31). As shown in Fig. 9(a), at the same N/P ratio, compared with the transfection efficiency of PEI 25K/DNA complexes without serum, the transfection efficiency of PEI 25K/DNA complexes in the presence of 10% serum showed a tendency to remarkably decrease, whereas serum had slight effects on the transfection efficiency of PAE/DNA complexes.

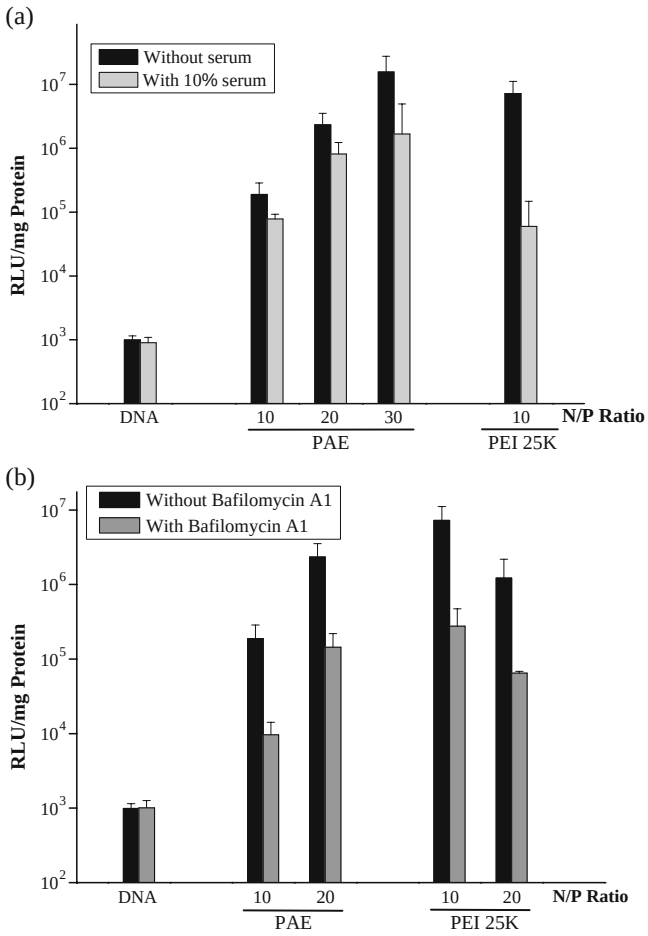

Fig. 9. Effect of (**a**) serum and (**b**) bafilomycin A1 on gene transfection efficiency in HeLa cell lines (mean ± SD, N=3).

At N/P ratio 10, PEI 25K showed high transfection efficiency and low toxicity, whereas the transfection efficiency of the PEI was significantly decreased in the presence of serum. Also, it has been known that the PEI 25K has no degradation or excretion pathway, which caused accumulation in body and resulted in potential cytotoxicity (32). On the other hand, the gene expression of PAE at N/P ratio of 40 is a little higher than PEI 25K at N/P ratio 10. Furthermore, even in the presence of serum, the PAE showed rather good gene transfection efficiency because the PAE/DNA complexes with hydrophilic shell structure enhanced their serum-resistant ability. And as shown in Fig. 6, the PAE showed low cytotoxicity compared to PEI 25K due to the low charge density and degradable peptide backbone.

To explore the mechanism of PAE-mediated gene transfection, treatment of bafilomycin A1 was performed. As a specific inhibitor of vacuolar type proton ATPase, bafilomycin A1 inhibits the endo-/lysosomal proton pump, resulting in the decrease of PEI-mediated gene transfection (33). As shown in Fig. 9(b), transfection of PAE/DNA complexes at N/P ratios of 10 and 20 were drastically decreased when the HeLa cells were treated with 200 nM of bafilomycin A1, just similar to that of PEI, suggesting that the mechanism of PAE-mediated gene transfection is based on the "proton sponge effect" due to the presence of low molecular weight PEI in the PAE.

In Vivo Gene Expression

As well known, it is a bottleneck to improve the gene transfection efficiency of a polymer-mediated non-viral gene vector (34,35). To know the potential application of PAE as gene carrier *in vivo*, we also investigated its *in vivo* EGFP gene delivery efficiency.

As illustrated in Fig. 10, naked DNA and PBS showed the same level of EGFP gene expression *in vivo*. In comparison with naked DNA, PAE showed greatly enhanced gene expression, just similar to that of PEI, suggesting its potential as a gene carrier *in vivo.*

Biodistribution Assay

The biodistribution of PAE/DNA complexes was investigated after i.v. administration using polymer labeled with ^{99m}Tc.

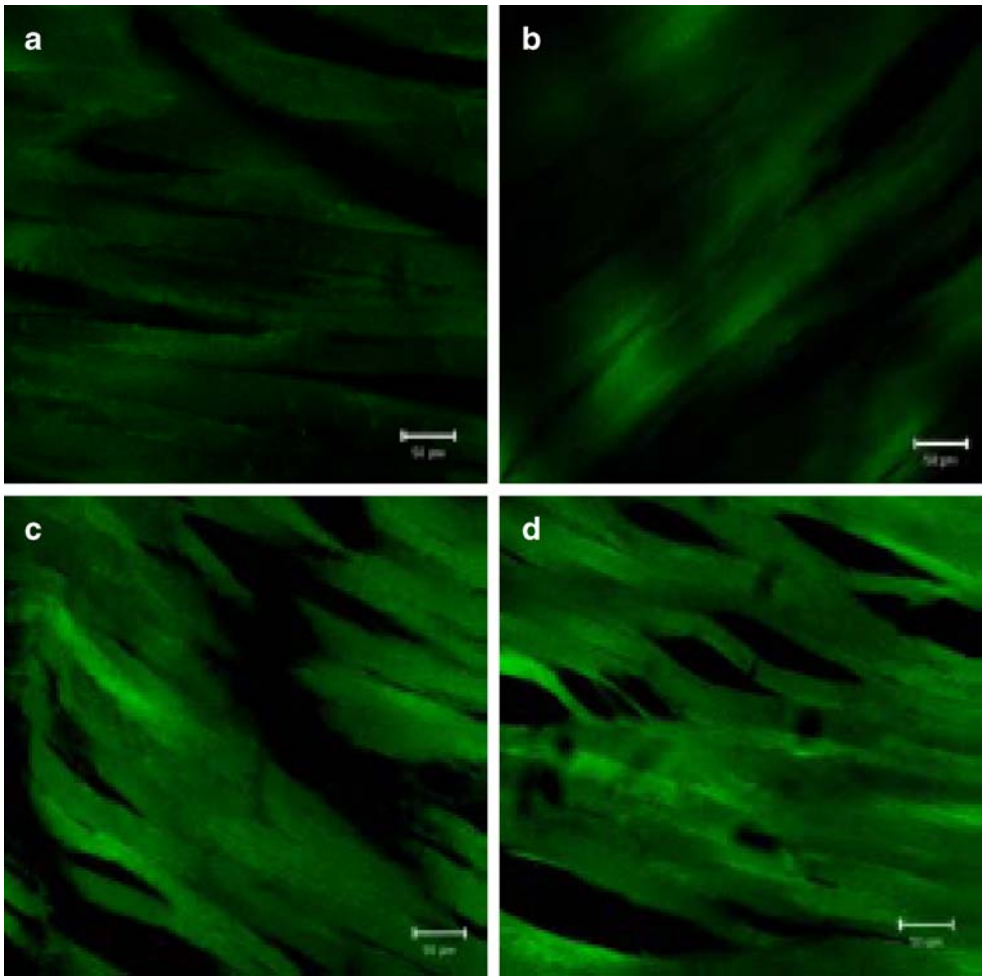

Fig. 10. EGFP expression observation by fluorescent microscopy after muscle injection to BALB/c mice (bar = 50μm). (**a**) PBS; (**b**) pEGFP-N2 plasmid DNA; (**c**) PEI/ pEGFP-N2 nanoparticles; and (**d**) PAE/ pEGFP-N2 nanoparticles.

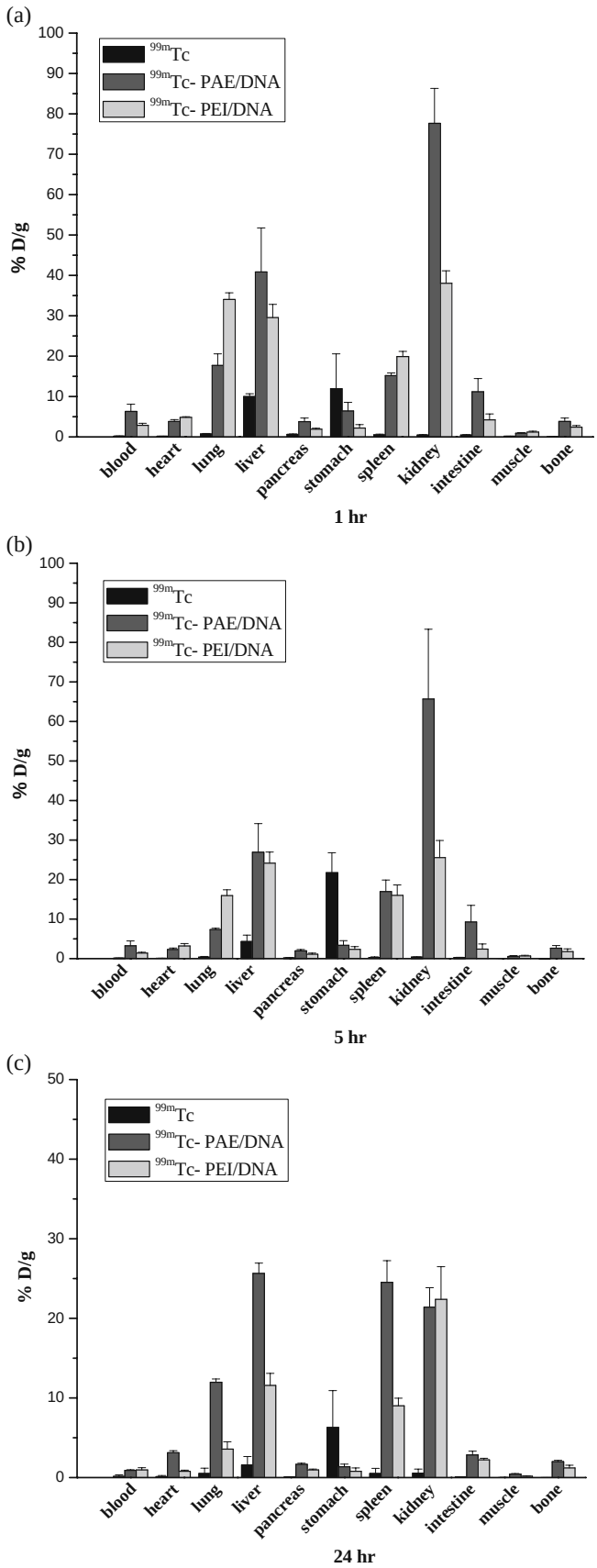

Fig. 11. Biodistribution of ^{99m}Tc-PAE/DNA and ^{99m}Tc-PEI/DNA complexes at a dose of 4 ug DNA and N/P ratio of 10 for PEI, 40 for PAE at (**a**) 1 h, (**b**) 5 h and (**c**) 24 h after i.v. injection to BALB/c mice. Values are expressed as a percent injected dose per gram (% ID/g) (n=4).

As shown in Fig. 11(a), the PAE/DNA and PEI/DNA complexes were rapidly cleared from the blood. One hour after i. v. injection, only 6.3±1.8% ID/g of ^{99m}Tc-PAE was left, which is higher than that of ^{99m}Tc-PEI (2.7±0.5% ID/g). It is considered that the hydrophilic parts in the shell of the polymer/DNA complexes reduce opsonization and lead to a slower mononuclear phagocytic system (MPS) uptake, consequently prolonging the circulation time (36). The core shell structure of PAE/DNA complexes reduced plasma interactions and aggregation, accordingly increasing the blood circulation time.

Both the ^{99m}Tc-PAE/DNA and ^{99m}Tc-PEI/DNA were more highly distributed in the lungs, liver, spleen, and kidneys than other organs after i.v. injection into BALB/c mice (Fig. 11). The highest accumulation of the PAE was found in the kidneys. The percents injected dose per gram of ^{99m}Tc-PAE in the kidney at 1 h and 5 h after administration were 77.6±8.6 and 65.7±17.6, respectively, which is much higher than that of ^{99m}Tc-PEI (38.0±3.1, 25.6±4.3). This is in agreement with the PAE/pEGFP-N2 gene expression experiment (data not shown), after systemic injection of the PAE/pEGFP-N2 complexes to the BALB/c mice, where they showed higher gene expression in the kidney than PEI/pEGFP-N2. The percent injected dose per gram of ^{99m}Tc-PAE was significantly decreased to 21.4±2.5% ID/g after 24 h, indicating that ^{99m}Tc-PAE might be excreted from kidney.

Initially, the accumulation of ^{99m}Tc-PAE in the spleen was found lower than that of ^{99m}Tc-PEI, with a slow increase in the time, suggesting that the ^{99m}Tc-PAE in the other organs redistributed to the spleen. It might be also considered that the PAE reduced the opsonization, resulting in slow uptake by MPS system. The liver accumulation of ^{99m}Tc-PAE was also higher than that of PEI, due to the different particle sizes of PAE/DNA complexes and PEI/DNA ones. Kunath *et al.* (36) reported similar results with the PEGylation of PEI; when compared with PEI, the accumulation of PEI(PEG)$_{50}$ and PEI(PEG)$_6$ was decreased in the spleen, but increased in the liver 2 h after systemic administration of the complexes.

Uptake of ^{99m}Tc-PAE/DNA complexes by the lungs was lower than ^{99m}Tc-PEI/DNA complexes because the core-shell structure of the PAE/DNA complexes increased the stability of the complexes and the particle sizes are not large enough to lodge in the lungs, leading to substantial avoidance of the lung uptake, consequently leading to uptake by the liver and spleen (37).

CONCLUSIONS

In this paper, the PAE, a brush-like polycation was synthesized and then was used to fabricate a novel core-shell gene delivery system. The PAE showed a good ability to condense and protect DNA in the form of nanoparticles self-assembled from polymer and DNA. The positive zeta potential and appropriate size of nanoparticles, additionally essential low-toxicity of PAE, and rather high gene transfec tion efficiency both in *in vitro* and *in vivo* demonstrated the potential use of PAE as an effective gene carrier.

ACKNOWLEDGEMENTS

This work was financially supported by Korea Science and Engineering Foundation (ROI-2005-000-10087-0) and Natural Science Foundation of China (No. 20504010). We also acknowledge the National Instrumentation Center for Environmental Management (NICEM) for permission to take NMR measurements. J. H. Yu was supported by KOSEF as a post-doctor under Korea-China Young Scientist Exchange Program, and J.S. Quan was supported by BK21 program.

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