Degradable Poly(ester amine) Based on Poly(ethylene glycol) Dimethacrylate and Polyethylenimine as a Gene Carrier

Jia-Hui Yu,[1,2] Jin Huang,[2] Jae-Woon Nah,[3] Myung-Haing Cho,[4] Chong-Su Cho[1]

[1]*Department of Agricultural Biotechnology, Seoul National University, Seoul 151-921, South Korea*
[2]*College of New Drug Innovation Research and Development, East China Normal University, Shanghai 200062, People's Republic of China*
[3]*Department of Polymer Science and Engineering, Sunchon 540-742, South Korea*
[4]*College of Veterinary Medicine, Seoul National University, Seoul 151-742, South Korea*

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ABSTRACT: Degradable poly(ester amine) (PEA) based on poly(ethylene glycol) dimethacrylate (PMEG) and polyethylenimine (PEI) were synthesized by Michael addition reaction. The ratios of PEI to PMEG in PEAs were 0.99, 1.02, and 1.07 with corresponding number-average molecular weight of 1.3×10^4, 1.2×10^4, and 0.9×10^4, respectively. Degradation rate of PEA at pH 7.4 was higher than that at pH 5.6. Good plasmid condensation and protection ability was shown when N/P molar ratio of PEA to DNA was above 15 (N: nitrogen element in PEA, P: phosphate in DNA). PEA/DNA complexes had positive zeta potential, narrow size distribution, good dispersity, and spheric shape with size below 250 nm when N/P ratio was above 30, suggestion of their endocytosis potential. Compared with PEI 25 KDa, the PEAs showed essential nontoxic to HeLa, HepG2 and 293T cells. With an increase in the molecular weight of PMEG, the transfection efficiency of PEAs in HeLa, HepG2 and 293T showed a tendency to decrease as well as the percent decrease of gene transfection efficiency with serum. The mechanism of PEA-mediated gene transfection was attributed to "proton sponge effect" of PEI in the PEA. J Appl Polym Sci 115: 1189–1198, 2010

Key words: gene delivery; degradable; cytotoxocity; poly(ester amine); polyethylenimine

INTRODUCTION

Interest in gene therapy has been renewed because of its exciting results in clinical trials. In recent years, gene therapy clinical trials for cancer are moving rapidly from phase I to III, and the first anti-cancer gene therapy drug has been licensed in China.[1] In the protocol of clinical gene therapy, the therapeutic gene was designed to introduce into patents' cells to cure the acquired or inherited disease in genetic level by adding, correcting, or replacing gene.[2] Since gene is prone to be rapidly degraded by nuclease and exhibit poor cellular uptake when delivered in aqueous solution, the development of effective delivery platforms that prevent their degradation and facilitate cellular uptake is a key issue.[3] Viral vectors are mostly used as gene carriers in current clinical and preclinical trials. Besides the relatively high gene expression of viral gene vectors, there are concerns over endogenous recombination, oncogenic effects and immunological reactions leading to potentially serious complications,[4,5] which have resulted in parallel efforts to develop nonviral alternatives due to the improved safety profile, easy to preparation and manipulation compared with viral vectors.[6]

As non-viral gene carriers, cationic polymers, such as poly-L-lysine, polyethylenimine (PEI), and poly (amido amine) dendrimer have attracted an increasing interest, which are contributed to their positive charge nature at physiological pH.[7–9] Gene materials can be condensed into nanoparticles, and protected from enzymatic degradation via complexes formed between gene and polycation, and facilitating the cell uptake and endolysosomal escape.[10]

Of all the cationic polymers, PEI is known as the one of the most effective gene carriers *in vitro* because of its "proton sponge effect" in endolysosome.[11] Previous reports showed that high transfection efficiency of PEI, along with its cytotoxicity,

Correspondence to: C.-S. Cho (chocs@plaza.snu.ac.kr).
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greatly depended on its molecular weight.[12,13] It is generally believed that PEI with a molecular weight higher than 25 KDa displays high transfection efficiency and cytotoxicity due to its nondegradability and high charge density, while PEI with a molecular weight less than 1.8 KDa shows low transfection but is less toxic.[14]

It was also well-known that cationic polymer, including PEI-mediated gene transfection *in vitro* was most efficient at an excess of cationic polymer leading to positively charged polyplexes which bind to anionic cell surfaces, facilitateing the cell uptake. Unfortunately, positively charged polyplexes interact unnecessarily with serum components along with precipitation and reduction of the blood circulation time.[15] Additionally, the polyplexes showed a tendency to aggregate due to the decreased water solubility induced by charge neutralization through the electrostatic interaction between polycationic vectors and polyanionic DNA.[14] To overcome the inherent barriers of polyplexes, such as solubility, cytotoxicity, and half-life in blood stream, various nonionic water-soluble polymers, such as poly(*N*-vinyl pyrrolidone), poly(ethylene glycol) (PEG), poly[*N*-(2-hydroxypropyl)-methacrylamide] have been grafted onto PEI.[15–18]

In our group, another strategy has been proposed to improve the gene transfection efficiency, biocompatibility, stability and half-life of polyplexes in blood. Initially, poly(ester amine) (PEA258) based on PEG diacrylate (PEGDA, M_n: 258) and low molecular weight PEI was synthesized. It was found that PEA258-mediated gene expression in the lung and liver was higher than that of the conventional PEI carrier, and noninvasive aerosol delivery induced higher gene expression in all organs compared to intravenous method in an *in vivo* mice study.[19] However, the effects of PEG molecular weights on the biological performance of PEA as gene carriers were not detailed. Herein, PEA were synthesized via Michael addition reaction between low molecular weight PEI and PEG dimethacrylate (PMEG) with different molecular weights. The degradability in different pH medium, DNA protection ability, cytotoxicity, transfection mechanism and the effects of PMEG molecular weight on the biological performance, such as degradation half-lives and DNA condensation ability of PEA, the gene transfection efficiency and zeta potentials of PEA/DNA complexes were detailed.

EXPERIMENTAL

Materials

PEG dimethacrylate (PMEG, M_n: 330, 550, and 875 Da), polyethylenimine (PEI, M_n: 423 Da), anhydrous dichloromethane, agarose, ethidium bromide (EtBr) and calf thymus DNA were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). 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 1000 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). Plasmid pEGFP-N2, which has the early promoter of CMV and enhanced green fluorescent protein (EGFP) gene, were obtained from Clontech (Palo Alto, CA).

Cell lines and culture

HeLa (human cervix epithelial carcinoma cells), 293T (human kidney cells), and HepG2 (human hepatoblastoma cells) were thawed and cultured in (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.

Plasmid amplification and purification

Plasmid pEGFP-N2 and pGL3-control were amplified with a competent *Escherichia coli* bacterial strain DH5α and their purification was performed using a QIAGEN (Chatsworth, CA) kit according to the manufacturer's protocol. Plasmid DNA concentration was calculated according to its absorbance at 260 nm, and plasmid DNA purity was determined by the ratio of absorbance at 260 and 280 nm. All samples showing an A_{260}/A_{280} ratios of 1.9–2.0 were stored at −20°C before use.

Preparations of PEAs

PEAs were synthesized by Michael addition reaction.[20–23] In a typical reaction procedure, PEI (M_n: 423 Da) and PMEG (M_n: 330, 550, and 875 Da) were separately dissolved in 20 mL of anhydrous dichloromethane in equal molar ratio of PEI to PMEG. The solutions of PEI and PMEG were mixed, and maintained at room temperature under dark with constant stirring for 12 h. After the occurrence of gel, the dichloromethane was removed in vacuum at room temperature. The residue was thoroughly mixed with 40 mL of ice cold water by vortex, and dialyzed immediately using Spectra/Pro membrane (MWCO = 3500) against deionized water at 4°C for

24 h. After removal of the little water-insoluble gel by filteration, the lyophilized products were labeled as EM1, EM2 and EM3 according to PMEG molecular weight of 330, 550 and 875, respectively, and stored at −20°C before use.

Degradation of copolymers

Degradation of PEA in different pH buffer was characterized by measurement of molecular weight of the degraded PEA. Briefly, PEAs were dissolved in different buffer (0.5 g/mL) with pH 5.6 (0.2 M HAc/NaAc) and 7.4 (0.2 M PBS), respectively, and incubated at 37°C with constant shaking at 100 RPM. The molecular weights of the degraded PEAs were measured with Agilent 1200 gel permeation chromatography (Agilent Technologies, Shanghai Branch) at a determined time point. Agilent 1200 refractive index detector and aqueous SECstart-up kit were used. Chromatography columns (2 × PL aquagel-OH 30, 8 μm, Polymer Laboratories, Amherst, MA) were calibrated with PEG kit. The column temperature was maintained at 25°C. Mobile phase was 0.2 M $NaNO_3$ and 0.01 M NaH_2PO_4 (pH 7.0), and the flow rate was 1 mL/min.

To know better the chemical structure of the degraded PEAs, PEAs were dissolved in PBS (0.2 M, pH7.4) prepared with D_2O and phosphate. The solutions (15 g/mL) were incubated at 37°C for 18 days with constant shaking at 100 RPM. The chemical structures of the degraded PEAs were characterized by ^{1}H-NMR (Bruker Avarice™ 500).

Agarose gel electrophoresis

Complexes formation of the PEAs and plasmid DNA were examined by the electrophoretic mobility of the complexes. Briefly, polymer/DNA complexes with various N/P ratios from 1 to 30 were prepared freshly before use by gently vortexing a mixture of pGL3-control (0.1 μg/well) and polymer solution (10 μL) (N: nitrogen element in PEA, P: phosphate in DNA). The complexes were incubated at room temperature for 20 min and then followed by addition of 2 μL of 6× agarose loading dye mixture (Biosesang, 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 4 μL of PEA/DNA complexes with various N/P ratios from 12 to 30 and naked plasmid DNA (pGL3-control, 0.1 μg) were separately incubated with 1 μL of DNase–I (1unit) in DNase/Mg^{2+} digestion buffer (50 mM Tris–Cl, pH 7.6 and 10 m*M* $MgCl_2$) at 37°C with shaking at 100 RPM for 30 min as the similar method previously described by Gebhart et al.[24] For DNase inactivation and DNA release, all samples were treated with 4 μL of 250 m*M* EDTA for 10 min and mixed with 9 μL of sodium dodecyl sulfate dissolved in 0.1 M NaOH (pH 7.2). Finally, samples were incubated at room temperature for 2 h and then followed by addition of 3 μL of 6× agarose loading dye mixture (Biosesang, 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 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 zeta potential, particle sizes and size distributions of polymer/DNA complexes were measured by dynamic light scattering (Zetasizer Nano ZS, Malvern Instruments, UK). Polymer/DNA complexes were prepared in water at N/P ratios of 10, 20, 30, and 45, respectively, and allowed to incubate at room temperature for at least 25 min. The volume of each sample was 2 mL, containing 80 μg of calf thymus DNA. Data were fit using Gaussian intensity distribution.

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 30 was carefully dropped onto clean copper grids and negatively stained with 1.5 wt % phosphotungstic acid (pH7.4) for 5 sec. The copper grids 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 tests were 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 and 293T) 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 (1, 5, 10, 20, and 30 μg/mL). Cells were incubated for 24 h and then followed by addition 20 μL of Cell Titer 96 A_{queous} One Solution Reagent. After further incubation for 2 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 (%) = $(OD_{sample}/OD_{control}) \times 100$, where OD_{sample} represents a OD value from a well treated with polymer and $OD_{control}$ from a well treated with PBS buffer only.

In vitro luciferase activity assay

Cells were seeded in 24 well plate 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 (10, 20, 30, and 45) 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 manufacture'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.[25] Each transfection experiment was carried out in triplicate, and transfection activity was expressed as relative light units.

To deduce the gene transfection mechanism of PEA, 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 20 and 45.

RESULTS AND DISCUSSION

Preparation and characterization of PEAs

As mentioned in introduction, low molecular weight PEI showed low toxicity as well as low gene transfection efficiency. A commercially available PEI with molecular weight of 423 is unable to mediate gene transfection, because it cann't efficiently buffer the low pH found in the secondary lysosome after endocytosis.[26] To improve the gene transfection efficiency, PEAs based on PEI (M_n: 423) and PMEG with different molecular weights (M_n: 330, 550, and 875) were successfully synthesized through Michael addition reaction (Fig. 1). Anhydrous dichloromethane was used as reaction medium due to the hydrolizable ester bonds in PMEG. Since the autocatalystic nature of Michael addition, nucleophilic reaction is easy to be finished in several hours at

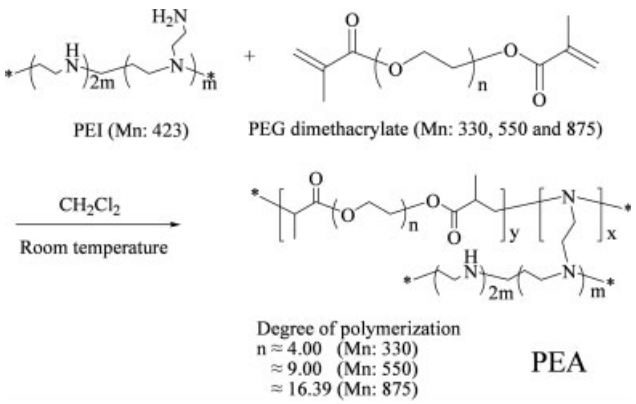

Figure 1 The proposed synthesis scheme of PEAs.

room temperature between amino groups of PEI and acrylate groups of PMEG. After dialysis in ice-cold water, and removal of the little water-insoluble gel by filteration, the lyophilized PEAs were ointment-like solid with light yellow color, and easy to be dissolved in water. ^{1}H-NMR spectra of purified PEA and the simple mixture of PEI and PMEG were shown in Figure 2. The disappearance of the peak at 5.57 and 6.12 ppm assigned to $CH_2C(CH_3)COOCH_2$— in PMEG clearly indicated the Michael addition reaction between amine groups of PEI and acrylate groups of PMEG, which was confirmed by the chemical shift movement of —CH_3 from 1.95 (in PMEG) to 1.16-1.17 ppm (in PEA). The appearance of IR absorption peaks at 3416, 2938, 1716, 1455 and 1107 cm^{-1} assigned to the stretching vibrations of —NH, —CH_2, —COO, C—N—C and C—O—C in PEA, respectively, further identified the chemical structure of PEA (Fig. 3). Molar ratios of PEI to PMEG in PEAs calculated from the integral area ratio of methylene peak at 2.69–3.04 ppm (—$NHCH_2$) to 3.49-3.85 ppm (—OCH_2) in ^{1}H-NMR were from 0.99 to 1.07, just around 1, very similar to the feed molar ratios of PEI to PMEG, indicating the Michael addition reaction mainly occurred between primary amines of PEI and acrylate groups of PMEG. The characteristics of PEAs labeled as EM1, EM2 and EM3, according to the molecular weight of PMEG of 330, 550, and 875, respectively, were shown in Table I. The number mean molecular weights of PEAs reached around 1×10^4, which were high enough for polycations as gene carriers. Additionally, with an increase in the molecular weight of PMEG, the yield of PEA showed a tendency to decrease, which resulted from the low reaction activity of PMEG with high molecular weight.

As an ideal gene carrier in therapeutic application *in vivo*, it is expected that cationic polymers show good gene transfection efficiency and low cytotoxicity, especially long-term biocompatibility. The cytotoxicity of current polycations mainly results from

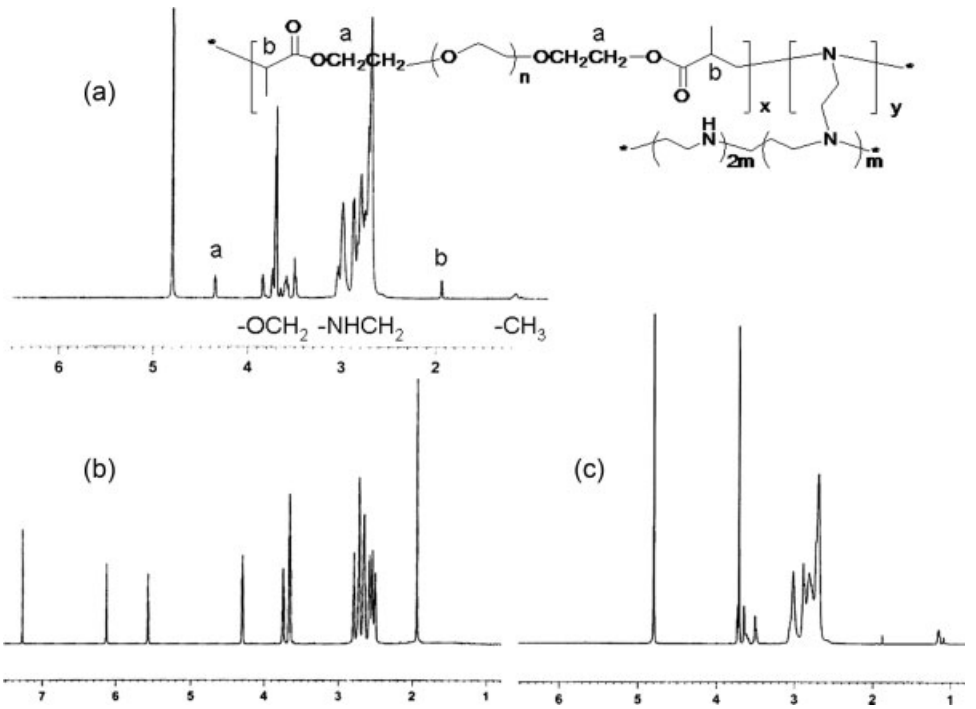

Figure 2 ^{1}H-NMR spectra of the polymers. (a) EM1 in D_2O, 1.16–1.17 (—CH_3); 1.94 ($OCOCH(CH_3)CH_2NH$); 2.69–3.04 (—$NHCH_2$); 3.49–3.85 (—OCH_2); 4.34–4.36 ($COOCH_2$); (b) the simple mixture of PEI and PMEG (M_n: 330) in $CDCl_3$, 1.95 ($CH_2C(CH_3)COOCH_2$—), 2.50–2.81 (—$NHCH_2$), 3.64–3.75 (—OCH_2), 4.29–4.30 ($CH_2C(CH_3)COOCH_2$—), 5.57 and 6.12 ($CH_2C(CH_3)COOCH_2$—); and (c) EM1 after degradation in PBS at 37°C for 18 days. The signal at 4.34–4.36 ppm assigned to ester bonds ($COOCH_2$) disappeared.

their nondegradability, thus, the hydrolyzable moieties should be introduced into new generation of cationic polymers such that the polymers readily degrade into nontoxic byproducts. In particular, it is also expected that the cationic polymer is stable in serum (pH 7.4), and degradable in lysosome (pH 5.6), which facilitates strong DNA condensation capacity outside cells and good DNA unpackage ability inside cells, leading to improved gene transfection efficiency as well as reduced cytotoxicity. To check the degradability of EM1, EM2 and EM3, the molecular weights of the degraded PEAs were monitored by GPC. As shown in Figure 4, it was found that the PEAs degraded rapidly in PBS at pH 7.4, especially at early times. Representative ^{1}H-NMR of EM1 after degradation was shown in Figure 2(C). After 18 days, the disappearance of the signal at 4.34–4.36 ppm assigned to ester bonds (—$COOCH_2CH_2O$—) clearly indicated the complete degradation of EM1 at pH 7.4. The degradation half-lives of EM1, EM2 and EM3 were 20, 7.2, and 5.3 h, respectively, at pH 7.4, which showed an order of EM1 > EM2 > EM3 due to the increased hydrophilicity of used PMEG in corresponding PEA. In addition, the half-life of EM3 at pH 5.6 was 24.2 h, longer than that at pH 7.4, suggested that the acidic pH was unbeneficial to the degradation of PEA.

Characterization of PEA/DNA complexes

The polyanionic nature and large hydrodynamic size of naked DNA resulted in low cellular uptake efficiency along with inefficient gene transfection. Therefore, DNA condensation ability was one of the prerequisite for a polymeric gene carrier. It was an efficient approach that packing of DNA into a

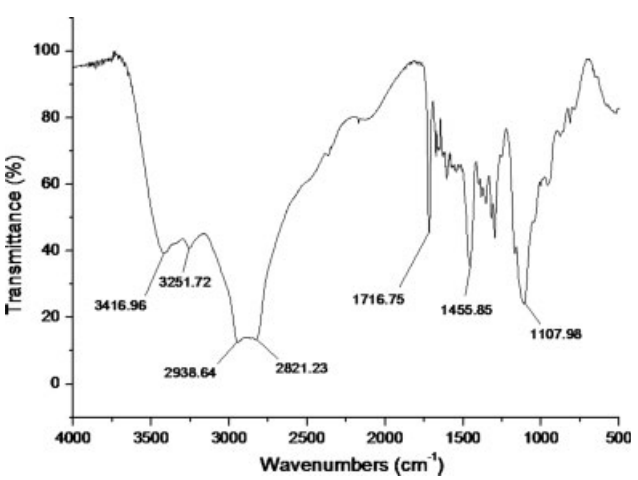

Figure 3 FTIR spectrum of EM1. Absorption peaks at 3416, 2938, 1716, 1455, and 1107 cm^{-1} are assigned to the stretching vibrations of —NH, —CH_2, —COO, C—N—C and C—O—C in EM1, respectively.

TABLE I
Characteristics of PEAs

Sample No.	Molecular weight (M_n) PEI	Molecular weight (M_n) PMEG	Feed molar ratio of PEI to PMEG	Molar ratio of PEI to PMEG[a]	Yield of PEAs (%)	Polydispersity of PEAs	Molecular weight of PEAs ($M_n \times 10^{-4}$)[b]
EM1	423	330	1 : 1	1.07 : 1	71	1.5	0.9
EM2	423	550	1 : 1	1.02 : 1	65	1.7	1.2
EM3	423	875	1 : 1	0.99 : 1	49	2.4	1.3

[a] Determined by ^{1}H-NMR.
[b] Determined by Agilent 1200 GPC, PEG as standard samples.

condensed structure by overcoming the Coulombic barrier related to the negatively charged phosphates on the DNA. To overcome the electrostatic repulsion within the DNA molecule, its negative charges must be sufficiently neutralized. Polycations, including those synthesized in this work, are able to interact with the negatively charged phosphate groups of DNA, which results in the formation of neutral polyelectrolyte complexes unable to migrate under the influence of electric field in agarose gel. The condensation profiles are shown in Figure 5. The results showed that with the increase of N/P ratio, the migration of polymer/DNA complexes showed a tendency to decrease. When the N/P ratio of polymer/DNA complex was around 15, the migration of DNA was completely retarded, indicating that the DNA charge is almost neutralized, and the condensation of DNA occurs.[27]

After the formation of complexes between DNA and polycations, the DNA should be protected from degradation by nucleases, which could be confirmed via DNase-I protection evaluation by using agarose gel electrophoresis. Figure 6 showed that all the polymers were able to protect DNA from enzymatic hydrolysis when N/P ratio was above 15, which confirmed the formation of complexes between polymers and DNA.

To know better polymer/DNA complexes, the particle sizes and size distributions of complexes were measured by using dynamic light scattering. As shown in Figure 7(a), when N/P ratio was 10, DNA couldn't be packed efficiently into compact structures. Particles with size above 400 nm were obtained. However, the particle sizes of complexes decreased sharply to less than 250 nm with increasing N/P ratios above 20, suggestion of their effective endocytosis potential.[28]

The morphology of polymer/DNA complexes was observed by EF-TEM. Representative EF-TEM images of EM1/DNA complexes at N/P ratio 30 demonstrated the relatively homogenous complex particles with spherical shape, compact structure and good dispersity [Fig. 7(b)]. Sizes observed from EF-TEM images were very similar to those measured by dynamic light scattering.

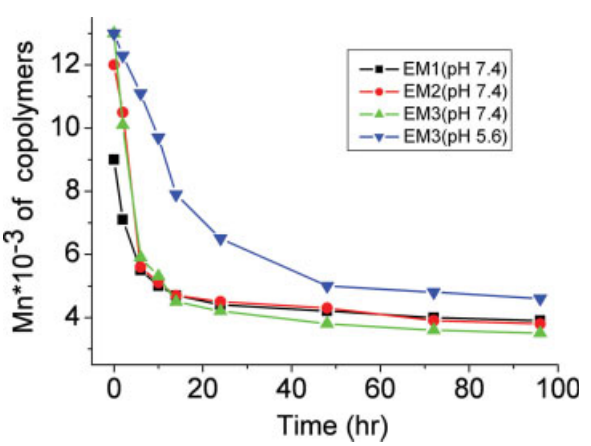

Figure 4 Degradation of PEAs. Copolymers were dissolved in different buffer (0.5 g/mL) with pH 5.6 (0.2 M HAc/NaAc) and 7.4 (0.2 M PBS), respectively, and incubated at 37°C with constant shaking at 100 RPM. The molecular weights of the degraded copolymers were measured with GPC at a determined time point. [Color figure can be viewed in the online issue, which is available at www.intersience.wiley.com.]

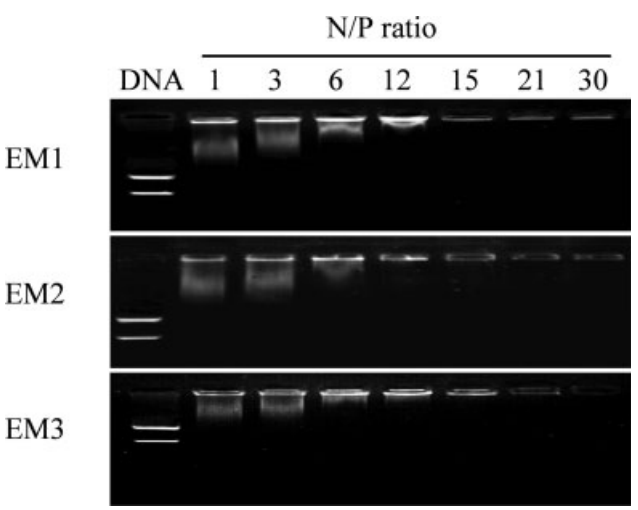

Figure 5 Agarose gel electrophoresis of polymer/DNA (pGL3-control) complexes at various N/P ratios.

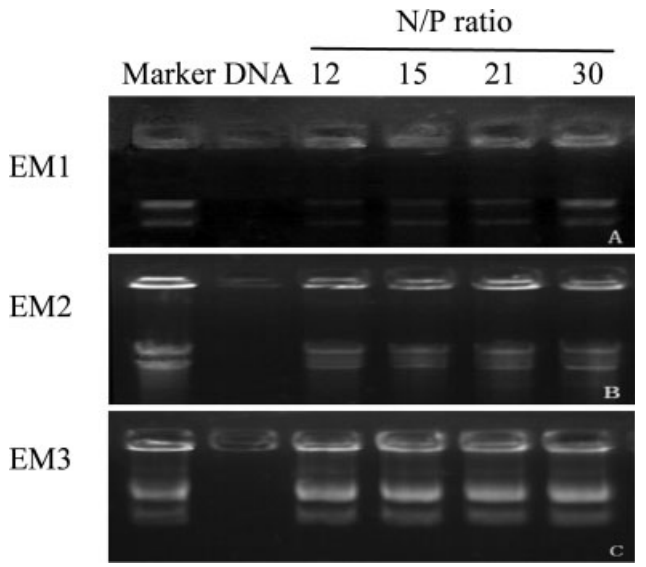

Figure 6 Electrophoretic mobility analysis of polymer/DNA (pGL3-control) complexes at various N/P ratios treated with DNase-I enzyme.

As mentioned above, a positive surface charge of untargeted polyplex is necessary to assure its attachment to negatively charged cell surface. The self-assembly of cationic polymer and DNA resulted in neutralization of negative charge of DNA. As illustrated in Figure 7(c), all the polycation/DNA complexes showed positive surface charge. With increasing N/P ratios from 10 to 45, the zeta potentials of the complexes gradually increased from 12 to 20 mV.

The strong positive charge repulsive forces as well as the existence of PEG chain prevented aggregation among complexes, which resulted in good dispersity and narrow size distributions of complexes [Fig. 7(d)].

Cell viability assay

To evaluate the cytotixicity of copolymer EM1, EM2, and EM3, cell viability was measured by Cell Titer 96 A_{queous} One Solution Reagent at various concentrations of PEAs. As illustrated in Figure 8, the cell viability of PEI 25K decreased sharply from 98 to 10% with increasing polymer concentration from 1 to 30 μg/mL in HeLa, HepG2 and 293T cell lines, suggestion of high cytotoxicity of PEI25K. However, EM1, EM2 and EM3 showed about 80–100% of cell viability in three different cell lines, even at 30 μg/mL of high polymer dose. It was thought that the reduced cytotoxicity of PEAs was contributed to their good degradability.

In vitro transfection efficiency assay of PEAs

In vitro transfection efficiencies in HeLa, Hep2, and 293T cells with PEAs are shown in Figure 9. Since

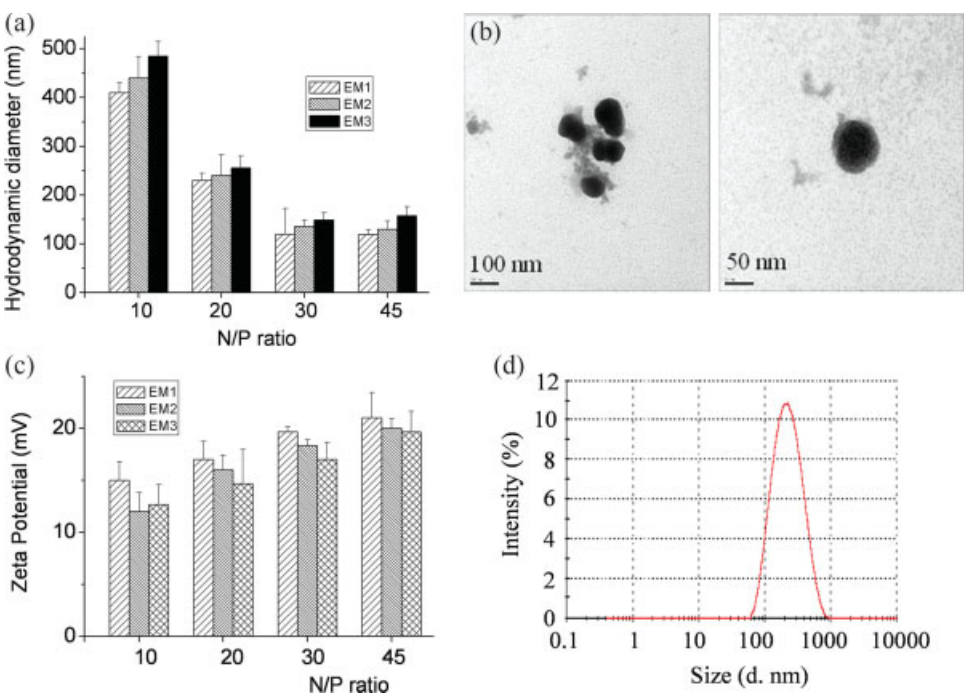

Figure 7 (a) Particle sizes of polymer/DNA complexes in distilled water at various N/P ratios (mean ± SD $n = 3$), (b) EF-TEM imagines of EM1/DNA complexes at N/P ratio 30 (about 110 nm), phosphotungstic acid was used as negative staining agent, (c) Zeta potential of polymer/DNA complexes in distilled water at various N/P ratios (mean ± SD $n = 3$), (d) Size distribution of EM1/DNA complexes in distilled water at N/P ratio 30 (about 120 ± 30 nm). [Color figure can be viewed in the online issue, which is available at www.intersience.wiley.com.]

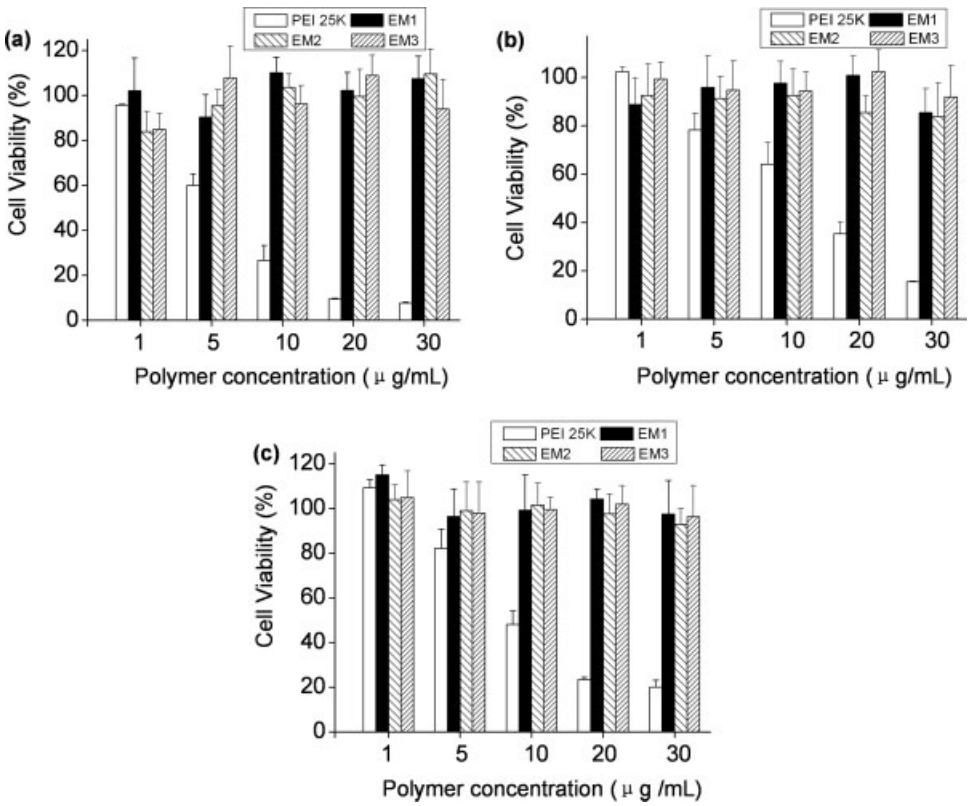

Figure 8 Cell viability of copolymers at various concentrations in different cell lines (mean ± SD $n = 3$) (a) HeLa, (b) HepG2, and (c) 293T.

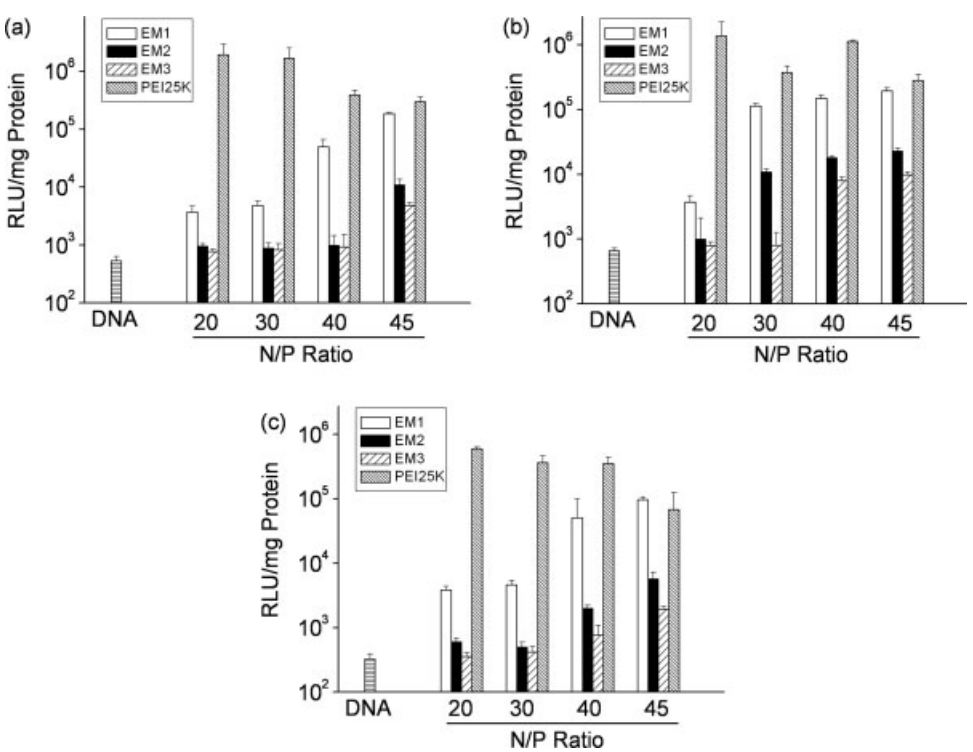

Figure 9 Transfection efficiency of copolymer/pGL3-control at various N/P ratios and in different cell lines (mean ± SD $n = 3$), (a) HeLa, (b) HepG2, and (c) 293T.

the gene transfection efficiency of PEA is rather lower when the N/P ratios of polymer/DNA complexes are less than 20, the complexes with N/P ratios ranging from 20 to 45 were used in the following evaluation of transfection study. It was found that the transfection efficiency of PEI 25K showed a tendency to decrease with increasing the N/P ratios from 20 to 45, suggesting that its remarkable cytotoxicity reduced its gene transfection efficiency at high N/P ratios. On the other hand, the gene transfection efficiency of PEA increased with an increase of N/P ratio, and the most efficient gene expression occurred at N/P ratio 45. Good gene transfection of PEA at high N/P ratios may be associated with their good degradability, which resulted in reduced cytotoxicity and enhanced release of plasmid DNA from polymer/DNA complexes.

From Figure 9, it was also found that in all three cell lines, the gene transfection efficiency decreased dramatically as the molecular weight of PMEG increased, and followed the order of EM1 > EM2 > EM3. It is suggested that the PEG shielding effects in the copolymer probably increased as the molecular weight of PMEG increased, which resulted in reduced attachment of polymer/DNA complexes to cell surface along with reduced cell uptake as well as gene transfection efficiency.

To deduce the gene transfection mechanism of PEA, treatment of bafilomycin A1 was performed. As a specific inhibior of vacuolar type proton ATPase, bafilomycin A1 inhibits the endo-/lysosomal proton pump, which results in the decrease of PEI-mediated gene transfection.[29] As shown in Figure 10, transfection of EM1/DNA complexes at N/P ratios of 20 and 45 were drastically decreased when the Hela cells were treated with 200 nM of bafilomycin A1, suggesting that the mechanism of PEA-midiated gene transfection is based on the "proton sponge effect", just similar to that of PEI.

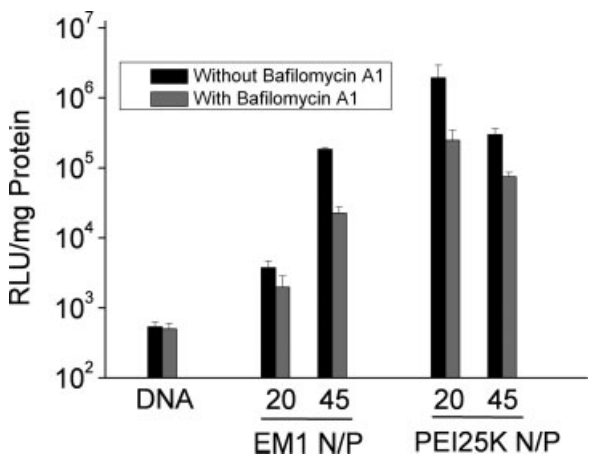

Figure 10 Effect of bafilomycin A1 on gene transfection efficiency in HeLa cell line (mean ± SD, $N = 3$).

TABLE II
The Percent Decrease of Gene Transfection Efficiency of PEA in the Presence of Serum

N/P	EM1/%	EM2/%	EM3/%
20	58.8	12.5	0.8
30	22.9	9.2	3.6
40	79.9	20.9	3.8
45	46.6	8.7	17.2

The data were calculated from the equation: (1-b/a) %, in which a and b the gene transfection efficiency of the same polymer at same N/P ratio without or with serum, respectively.

As mentioned in introduction, one of the practical problems for *in vivo* gene delivery by non-viral carrier is that gene delivery efficiency is retarded by serum, leading to low gene transfection efficiency. Thus, it is a key issue to develop gene delivery systems that are stable even in serum for *in vivo* use of non-viral delivery systems. We evaluated the effect of serum on the gene transfection efficiency of PEA/DNA complexes. The percent decrease of transfection efficiency at diffenent N/P ratios were shown in Table II. At the same N/P ratio, the percent decrease of transfection efficiency of PEAs showed a tendency to increase with increasing the molecular weight of PMEG in the PEA. The results indicated that PEA with the longer PEG chain could prevent serum protein adsorption from cell culture medium, and its gene transfection efficiency was less affected by the presence of serum.

CONCLUSIONS

In this work, a new strategy has been proposed to improve the gene transfection efficiency, biocompatibility, stability, and half-life of polyplexes in blood by copolymerization of PEI423 and PMEG as gene carriers. The prepared copolymers exhibited nontoxic, degradability and good DNA condensation and protection ability. With increase of the molecular weights of PMEG, the transfection efficiency of the PEA without serum decreased, whereas, the percent decrease of transfection efficiency of the PEA with serum showed a tendency to increase. Therefore, it is our next work to screen a PEA with ideal gene transfection efficiency *in vivo* by balancing the molecular weights of PMEG and PEI.

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