Hyperbranched PEI Grafted by Hydrophilic Amino Acid Segment Poly[*N*-(2-hydroxyethyl)-L-glutamine] as an Efficient Nonviral Gene Carrier

Jie Chen,[1] Huayu Tian,[1] Zhaopei Guo,[1] Lin Lin,[1] Xuan Dong,[1] Xiaojuan Zhu,[2] Xuesi Chen[1]

[1]*Key Laboratory of Polymer Ecomaterials, Changchun Institute of Applied Chemistry, Chinese Academy of Sciences, Changchun 130022, China*
[2]*Institute of Cytology and Genetics, Key Laboratory of Molecular Epigenetics of Ministry of Education, Northeast Normal University, Changchun, 130022, China*

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ABSTRACT: A polyethylenimine-poly(hydroxyethyl glutamine) copolymer (PEI-PHEG) was designed and synthesized as a gene delivery system. The molecular structure of PEI-PHEG was characterized using nuclear magnetic resonance. Moreover, PEI-PHEG/*p*DNA complexes were fabricated and characterized by gel retardation assay, particle size analysis, and zeta potential analysis. The transfection efficiency and cytotoxicity of PEI-PHEG were evaluated using human cervical carcinoma (HeLa), human embryonic kidney (HEK293), and murine colorectal adenocarcinoma (CT26) cells *in vitro*. The results show that PEI-PHEG could effectively form positively charged nanosized particles with *p*DNA; the particle size was in a range of 130.2 to 173.0 nm and the zeta potential was in a range of 27.6 to 41.0 mV. PEI-PHEG exhibited much lower cytotoxicity and higher gene transfection efficiency than PEI-25K with different cell lines *in vitro*. An animal test was also conducted on a Lewis Lung Carcinoma tumor model in C57/BL6 mice by using subcutaneous intratumoral administration. The results show that *in vivo* transfection efficiency of PEI-PHEG was improved greatly compared with that of commercial PEI-25K. These results demonstrate that PEI-PHEG can be a potential nonviral vector for gene delivery systems both *in vitro* and *in vivo*. © 2011 Wiley Periodicals, Inc. J Appl Polym Sci 123: 2257–2265, 2012

Key words: hydrophilic polymers; nanoparticle; graft copolymers; gene transfection; low cytotoxicity

INTRODUCTION

Gene therapy has attracted more and more attention for the treatment of numerous genetic and acquired diseases that are incurable currently.[1] In gene therapy, exogenous genes, gene segments, or oligonucleotides need to be introduced into the targeted tissues or cells. However, this introduction is challenging because genes can be easily degraded by nucleases, and they are difficult to be internalized as a result of their polyanionic nature.[2] Therefore, the lack of efficient gene-delivery systems is an obstacle to the practical clinical application of gene therapy.[3–5]

Viral vectors have been proved to be highly potent gene delivery systems, but their practical application is limited by safety concerns such as immunogenicity, virus replication, and inflammatory reactions.[6–8] Therefore, nonviral carriers have been proposed as much safer alternatives for gene therapy, which may offer advantages and solve some of the current problems associated with viral gene carriers.[9,10]

Among nonviral systems, polyethylenimine (PEI) is one of the most popular polycationic gene carriers because of its high transfection efficiency resulting from a unique proton sponge effect.[11,12] However, the major limitation of PEI as a gene carrier is its high cytotoxicity.[13] To reduce the toxicity of PEI and to enhance its gene transfection efficiency, scientists have attempted to make a further modification of PEI. Asp-*g*-PEI was synthesized by the ring-opening reaction of poly(L-succinimide) with low molecular weight branched PEI.[14] Hydrophilic polyethylene glycol (PEG) was also introduced into the PEI moiety to shield the high positive charges.[12,15–17] Recently, some other biodegradable gene carriers based on PEI,

Correspondence to: H. Tian (thy@ciac.jl.cn).
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such as poloxamer,[18] polycaprolactone,[19] glycerol dimethacrylate,[20] multiarmed poly(L-glutamic acid)-graft-OEI (MP-*g*-OEI),[21] ternary PEI-*p*DNA-INS,[22] PEI-grafted polycarbonates (PMAC-*g*-PEIx),[23] and PEI-modified silica nanoparticles,[24] have proved to have remarkable transfection efficiency and prominently reduced cytotoxicity.

Recently, many studies proved that hydrophobic moieties can improve transfection efficiency of cationic polymers.[25,26] In our previous work, we investigated polyethylenimine-poly(*g*-benzyl L-glutamate) (PEI-PBLG) as a gene delivery system, which was prepared from hyperbranched PEI grafted by poly(*g*-benzyl L-glutamate) (PBLG) segments at the hyperbranched chain ends. The grafting of biocompatible PBLG onto PEI shielded the toxicity of PEI and condensed the DNA into small particles of about 100 nm in diameter. This new nonviral gene carrier has been one of the most efficient DNA delivery systems based on our previous work.[27,28] Moreover, PEI-PBLG was used successfully for siRNA-mediated gene silencing, which could greatly improve the siRNA silence activity. Thereby, PEI-PBLG has the potential to be used as a siRNA transfection reagent.[29]

In this work, PEI-PBLG was further modified via aminolysis using 2-aminoethanol to get a totally hydrophilic copolymer, polyethylenimine-poly[*N*-(2-hydroxyethyl)-L-glutamine] (PEI-PHEG). Biological characterizations such as the MTT assay, *in vitro* transfection and *in vivo* transfection were carried out on PEI-PHEG to study the influence of the hydrophilic structure on the gene carrier transfection properties.

EXPERIMENTAL

Materials

Hyperbranched PEI with an average molecular weight of 25 kDa (PEI-25K) was purchased from Aldrich (St. Louis, MO). *N*-Carboxyanhydride of *g*-benzyl-L-glutamate (BLG-NCA) was prepared according to the reported method;[30] 3-[4,5-dimethylthiazol-2-yl]–2,5-diphenyltetrazolium bromide (MTT) was purchased from Amresco (Solon, OH). Chloroform and *N*,*N*-dimethylformamide (DMF) were treated with CaH_2 and distilled before use. Dulbecco's modified Eagle medium (DMEM) and fetal bovine serum (FBS) were from Gibco (Grand Island). Fluorescein isothiocyanate (FITC) was purchased from Dingguo Bio-Technology Company (Beijing, China). Plasmid DNA (*p*EGFP-N1) was purchased from Clontech (Palo Alto, CA), and the plasmid encoding firefly luciferase (*p*GL3-control), luciferin substrate, and lysis buffer were obtained from Promega (Madison, WI). Calf-thymus DNA, 4,6-diamidino-2-phenylindole dihydrochloride (DAPI), and other chemicals were purchased from ς-Aldrich (Munich, Germany).

Synthesis of PEI-PHEG

The PEI-PBLG copolymer was prepared according to our previous report.[28] The molecular weight of PEI was 25 kDa and the content of PEI was 55.1%. The PEI-PBLG molecular weight was 45 kDa.

Lyophilized PEI-PBLG (500 mg, 6.7 mmol) was dissolved in distilled water (4 mL), and an equal volume of DMF (4 mL) was added. Then the reaction system was warmed up to 60°C, followed by addition of 2-aminoethanol (2.5 mL, 0.042 mmol). The mixture was stirred for 18 h at 60°C to allow the reaction to proceed completely. The resulting solution was then dialyzed (with a molecular weight cut-off of 7000 Da) with purified water (500 mL; with four changes over 48 h). The dialysate was finally dried under a vacuum. ^{1}H-NMR spectra were recorded in D_2O at 25°C on a Bruker AV-400 spectrometer (Bruker, Ettlingen, Germany). Fourier transform infrared (FTIR) spectra of the polymers were measured on a Vertex 70 FT-IR spectrometer (Bruker, Germany) using KBr disks.

Gel retardation assay

A gel retardation assay was performed as follows. Both the polymer and *p*EGFP-N1 were diluted to 0.1 mg/mL. The polymer solution was then added to the *p*EGFP-N1 solution (5 μL) with the same volume at various weight ratios of the polymer to *p*EGFP-N1 and the mixture was briefly vortexed. After 20 min incubation at room temperature, 2 μL of 6× DNA loading buffer and 10 μL of each of the complex solutions were analyzed by agarose gel (1%, w/v) electrophoresis at 100 V for 45 min. The locations of the *p*EGFP-N1 bands were visualized with an ultraviolet lamp using a UVP EC3 bioimaging system (UVP Inc., Upland, CA).

Particle size and zeta potential analysis

The calf-thymus DNA was used to form complexes with the cationic carriers for particle size and ζ-potential measurements. The complexation solutions were prepared at various weight ratios of the carrier to the DNA and vortexed. After 20 min incubation at room temperature, the particle size and zeta potential of the complexes were measured by using a Zetasizer Nano ZS90 (Malvern Instruments, Malvern, UK).

Cell culture

Human cervical carcinoma (HeLa), human embryonic kidney (HEK293), and murine colorectal adenocarcinoma (CT26) cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with high glucose, supplemented with 10% (v/v) heat-inactivated FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin in a 5% CO_2 incubator at 37°C under 95% humidity.

Cytotoxicity assay

The cytotoxicity of the as-synthesized cationic polymer PEI-PHEG was assessed in comparison with PEI-25K by the MTT assay. Generally, the HeLa cells were seeded in 96-well plates at 1.0×10^4 cells/well, and then cultured for 24 h. Various concentrations of PEI-25K and PEI-PHEG were prepared in the OPTI-MEM solution, respectively. DMEM (180 μL) and a polymer (20 μL) were added to each well and the plate was returned to the incubator for an additional 24 h. Then 20 μL of the MTT solution (5 mg/mL in PBS) was added to each well. The plate was again returned to the incubator for 4 h, and then the MTT solution was carefully removed from each well; subsequently 200 μL of dimethylsulfoxide (DMSO) was added to dissolve the MTT formazan crystals formed by proliferating cells. The plate was incubated for an additional 10 min before the absorbance at 492 nm was recorded by an ELISA microplate reader (Bio-Rad). The cell viability (%) was calculated according to the following equation:

$$\text{Cell viability}(\%) = (A_{\text{sample}}/A_{\text{control}}) \times 100$$

where A_{sample} is the absorbance of the polymer-treated cells and A_{control} is the absorbance of the untreated cells. Each experiment was performed as the average values of four runs and repeated a minimum of three times.

In vitro transfection

The *p*GL3-control and *p*EGFP-N1 plasmid DNA were both used to study the transfection activity of the cationic carriers in this experiment, HeLa cells, HEK293 cells, and CT26 cells were seeded in 96-well plates at an initial density of 1.0×10^4 cells/well in 200 μL DMEM containing 10% FBS, respectively. After 24 h incubation, the culture medium was aspirated from each well and replaced with 180 μL of DMEM before the addition of [carrier]/[*p*DNA] (0.2 μg *p*DNA) complexes at various weight ratios of the carrier to the DNA. The cells were then incubated for 48 h.

The relative levels of EGFP expression were characterized using fluorescence microscopy directly. For a luciferase assay, the medium was removed and the cells were washed with PBS gently. A cell lysis buffer (Promega; 50 μL/well) was added, and then the mixture was frozen in a −80°C freezer for 1 h. The luciferase activity was determined by detecting the light emission from an aliquot of 20 μL of the cell lysate incubated with 100 μL of a luciferase assay reagent (Promega) in a luminometer (GloMax 20/20; Promega). The total protein was measured by using a BCA protein assay kit (Pierce, Rockford, IL) and the luciferase activity was reported in terms of RLU/mg protein. All the experiments were performed in triplicate to ascertain the reproducibility.

A quantitative transfection efficiency study was conducted by flow cytometry at the optimal weight ratios of the carrier to *p*EGFP for both PEI-PHEG and PEI determined by fluorescence microscopy observation. The cells were seeded in 6-well plates at 1.0×10^5 cells/well. After an initial 24 h incubation period, the medium was replaced with 10% serum DMEM containing a carrier/*p*DNA (3 μg *p*DNA) complex. The cells were incubated for an additional 48 h. Then the transfected culture was washed once with PBS. The cells were detached with 0.25% (w/v) trypsinase. Finally, the cells were resuspended in 600 μL of PBS (pH 7.4). The transfection efficiency was evaluated as the percentage of cells expressing green fluorescent protein (EGFP) using a FACS Calibur system from Becton-Dickinson (San Joes, CA).

Confocal laser scanning microscopy (CLSM) observation

Both PEI-PHEG and PEI were labeled with FITC to observe intracellular trafficking. Glass coverslips were placed at the bottom of wells in 6-well plates. HeLa cells were seeded on glass coverslips at an initial density of 2.0×10^5 cells. The cells were incubated for 24 h, and then the medium was replaced with fresh DMEM. An FITC-labeled carrier/*p*DNA complex was added at an optimal ratio for transfection. The cellular locations of the FITC labeled carriers were analyzed at 1, 2.5, and 5 h post-transfection by using a confocal laser scanning microscope ((Leica TCS SP2; Leica Microsystems, Wetzlar, Germany). Before CLSM observation, cells were immobilized with 3.7% paraformaldehyde (FDA). After 10 min immobilization, coverslips were washed five times with PBS, and then the nuclei were stained with DAPI for 10 min. The coverslips were then washed several times with PBS and enclosed in glycerol and visualized by CLSM.

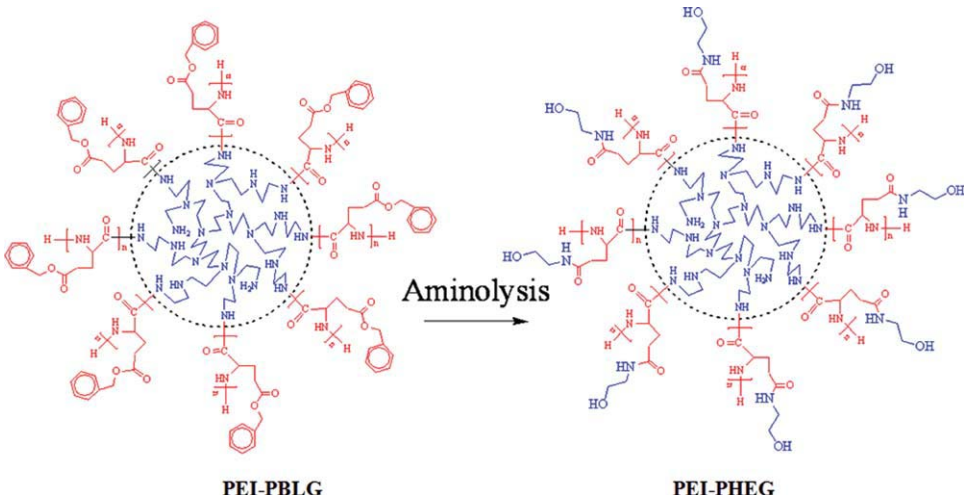

Figure 1 Schematic representation of PEI-PHEG synthesis and structure. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

In vivo transfection

Female C57/BL6 mice of 6 to 8 weeks were purchased from the Institute of Experimental Animal of Jilin University. The mice were inoculated subcutaneously in the right flank leg with 1×10^6 Lewis Lung Carcinoma (LLC) tumor cells 2 weeks before treatment. A cationic carrier containing 30 μg *p*GL3-control in a 100-μL injection volume was injected intratumorally. Noninjected mice with similar-sized tumors were used as negative controls. The C57/BL6 mice were sacrificed 48 h later by cervical dislocation, and stored at −80°C. The tumors were removed and homogenized in a lysis buffer (Promega, Mannheim), and subsequently centrifuged at 5000 × *g*, 4°C for 10 min to separate insoluble cell components.[31–33] After centrifugation, the supernatant was determined as described above. All animal procedures were approved and controlled by the local ethics committee and carried out according to the guidelines of the Chinese law of protection of animal life.

RESULTS AND DISCUSSION

Synthesis of PEI-PHEG

In our previous study, a copolymer PEI-PBLG was designed and synthesized by introducing hydrophobic poly(γ-benzyl L-glutamate) segments (PBLG) into hyperbranched polyethylenimine (PEI). Polyethylenimine-poly-[*N*-(2-hydroxyethyl)-L-glutamine] (PEI-PHEG) was then obtained by aminolysis of PEI-PBLG with 2-aminoethanol. The schematic structure and synthesis procedures of the PEI-PHEG are presented in Figure 1.

PEI-PBLG has exhibited much better transfection efficiency, and lower cytotoxicity than commercial PEI-25K. However, due to the existence of hydrophobic amino acids fragment PBLG in PEI-PBLG, when in *in vivo* experimentation, large doses of PEI-PBLG in the body are prone to precipitation. Therefore, hydrophilic improvement for PEI-PBLG seemed both appealing and practical. Polyethylenimine-poly-[*N*-(2-hydroxyethyl)-L-glutamine] (PEI-PHEG) was obtained by aminolysis of PEI-PBLG with 2-aminoethanol. PEI-PHEG carrier is predicted to have the following advantages: Firstly, good biocompatibility; secondly, biodegradation; thirdly, good dispersity in a large dose, without forming precipitation when complexed with *p*DNA in *in vivo*.

PEI-PHEG Characterization

The structure of PEI-PHEG was characterized by ^{1}H-NMR spectra and compared with that of PEI-PBLG (Fig. 2). In Figure 2(A), signal e corresponded to the phenyl group (CH_2-C_6H_6) and signal d was from the ethyl group (CH_2-C_6H_6) in copolymer PEI-PBLG. After aminolysis, these two signals disappeared in the ^{1}H-NMR spectra for copolymer PEI-PHEG [Fig. 2(B)]. Both of them were aminolysized by 2-aminoethanol. In copolymer PEI-PBLG, the PBLG segments are hydrophobic and the PEI segments are hydrophilic, so PEI-PBLG is an amphiphilic copolymer. After aminolysis, hydrophobic benzyl groups were replaced by hydrophilic aminoethanol groups, so PEI-PHEG became a totally hydrophilic copolymer. The FTIR spectra of PEI-25K, PEI-PBLG, and PEI-PHEG copolymers are shown in Figure 3. IR results show that the benzyl groups are lost (700 cm^{-1} and 750 cm^{-1}), while the characteristic absorption band of amide linkage is still existent (1665 cm^{-1}). All of the evidences can claim that the desired polymer has been successfully synthesized. Because the PEI-PBLG molecular weight was known as 45

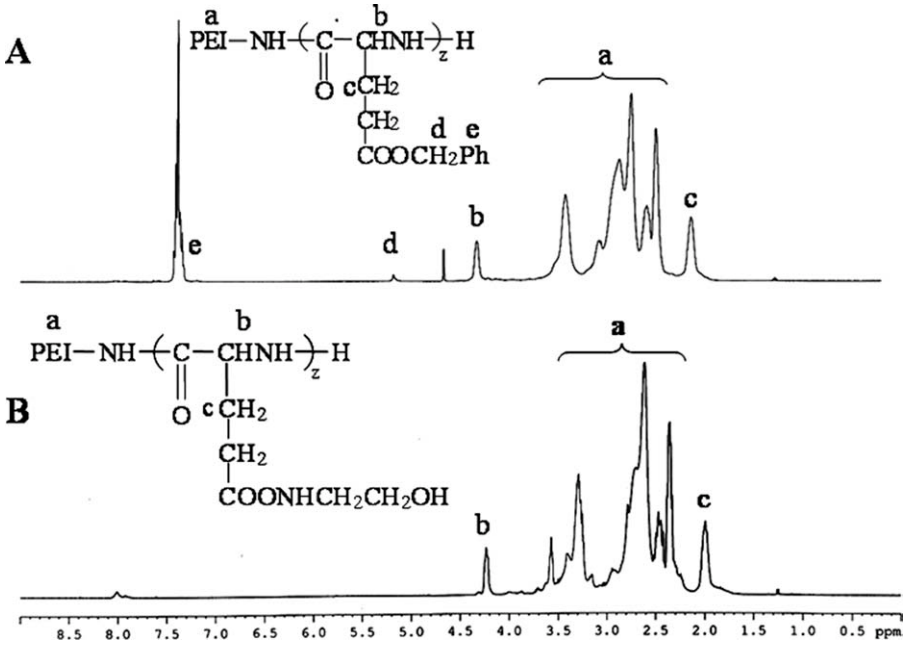

Figure 2 ^{1}H-NMR spectra of PEI-PBLG and PEI-PHEG in D_2O.

kDa and the content of PEI was 55.1%, the molecular weight of PEI-PHEG can be calculated as 41 kDa.

Particle size analysis

Condensing *p*DNA into nanoparticles is an essential process of the efficient endocytosis of [carrier]/[DNA] complexes.[22] PEI-PHEG/DNA complexes of various sizes were prepared at various [carrier]/[DNA] ratios (wt/wt). As shown in Figure 4, PEI-PHEG could efficiently condense DNA and the diameters of the complexes were in the range of 130.2 to 173.0 nm at various weight ratios of [carrier]/[DNA]. The particle size of these complexes decreased with increasing the [carrier]/[DNA] (wt/wt) ratio, which could be explained by the negatively charged DNA partially neutralizing the positively charged PEI shell. The results show that the PEI-PHEG copolymer had a similar property to that of PEI-25K in condensing DNA into nanoparticles. The diameters of PEI-PHEG/DNA complexes were stably kept in the range 130.2 to 173.0 nm, which meets the requirements of efficient endocytosis of these complex particles.

Zeta potentials

Zeta potentials of [carrier]/[DNA] complexes were closely related to cellular uptake according to our previous study.[28] The zeta potentials of the

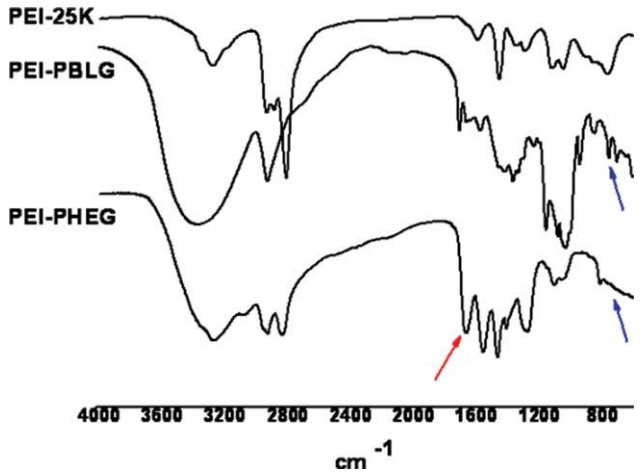

Figure 3 FTIR spectra of PEI-25K, PEI-PBLG, and PEI-PHEG copolymers. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

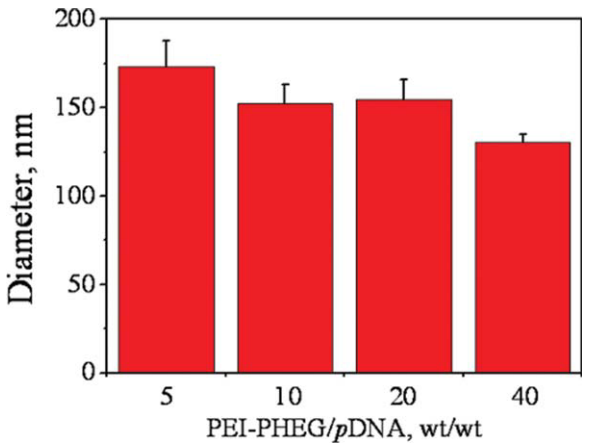

Figure 4 Particle sizes of [carrier]/[DNA] complexes at various [carrier]/[DNA] (wt/wt) ratios. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

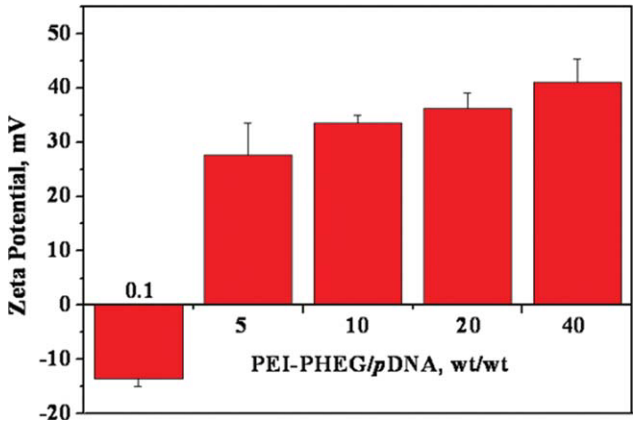

Figure 5 Zeta potentials of the [PEI-PHEG]/[DNA] complexes. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

complexes at various weight ratios are shown in Figure 5. At a [carrier]/[DNA] (wt/wt) ratio of 0.1, the zeta potential of the PEI-PHEG/DNA complex was negative. With an increasing [carrier]/[DNA] (wt/wt) ratio, the zeta potential of the complexes rapidly increased to a positive value at a [carrier]/[DNA] (wt/wt) ratio of 5, and then the PEI-PHEG/DNA complexes were saturated and the surface charge was maintained at around 27.6 to 41.0 mV in a weight ratio range from 10 : 1 to 40 : 1.

Gel retardation assay

Efficient DNA complexation is one important prerequisite for a gene carrier.[30] The gel retardation assay confirmed the formation of PEI-PHEG/DNA complexes by using PEI-25K as the control. As shown in Figure 6, PEI-PHEG could efficiently bind to *p*DNA, and the critical weight ratio for complete retardation was about 0.3. The result was similar to that of the control (PEI-25K), which completely retarded *p*DNA at a weight ratio of 0.2. However, PEI-PHEG required a slightly higher weight ratio for complete retardation, possibly due to the introduction of the PHEG segments as well as the increase in the molecular weight of the polymer.

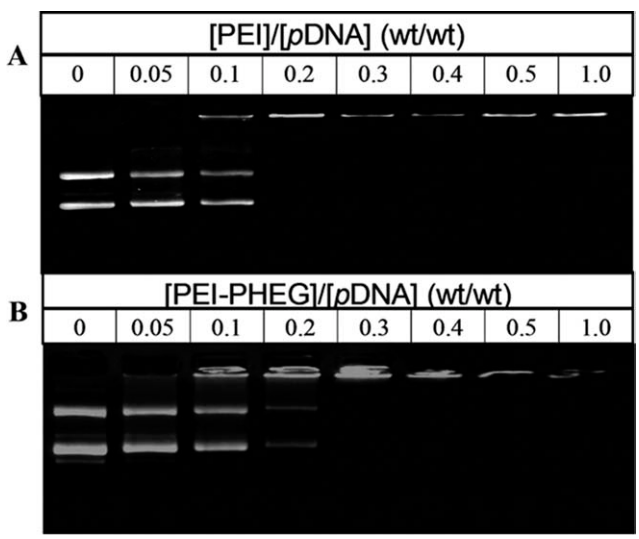

Figure 6 Gel retardation assay results for PEI-25K (A) and PEI-PHEG (B). Carriers/pDNA (pEGFP) complexes were made at various weight ratios and incubated for 20 min at room temperature. Then the complexation solution was applied to the agarose gel and electrophoresed at 100 V for 45 min.

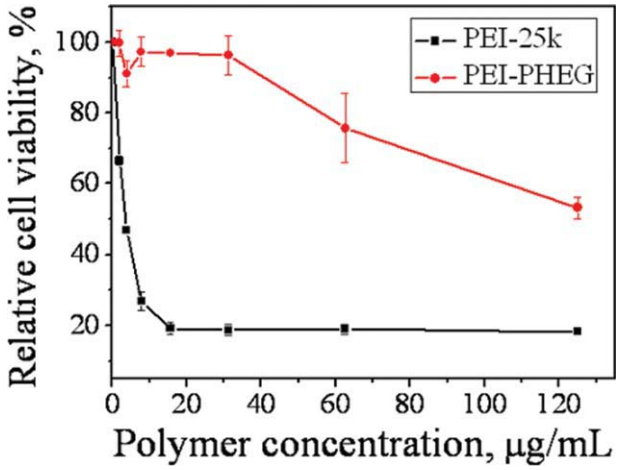

Figure 7 Viability of HeLa cells exposed to PEI-25K and PEI-PHEG at various polymer concentrations. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Cytotoxicity

The cytotoxicity of the polycationic carriers was assessed by the MTT assay. Figure 7 presents the cell viability of HeLa cells upon incubation for 24 h with PEI-25K and PEI-PHEG at various concentrations. PEI-PHEG exhibited obviously improved cytocompatibility compared with PEI-25K. The reduced cytotoxicity of the PEI-PHEG copolymer may be a result of the introduction of the biocompatible PHEG moiety, in which the hydrophilic PHEG block could shield the surface positive charge on PEI to a degree.

Transfection activity *in vitro*

Transfection activity of PEI-PHEG/*p*DNA complexes *in vitro* was assessed in HeLa cells by comparing the green fluorescence protein assay results in fluorescence images. PEI-25K was used as the control at different [carrier]/[*p*DNA] (wt/wt) ratios. As shown in Figure 8, the optimized [carrier]/[*p*DNA] (wt/wt) ratios for PEI-25K and PEI-PHEG were 1 : 1 and 20 : 1, respectively.

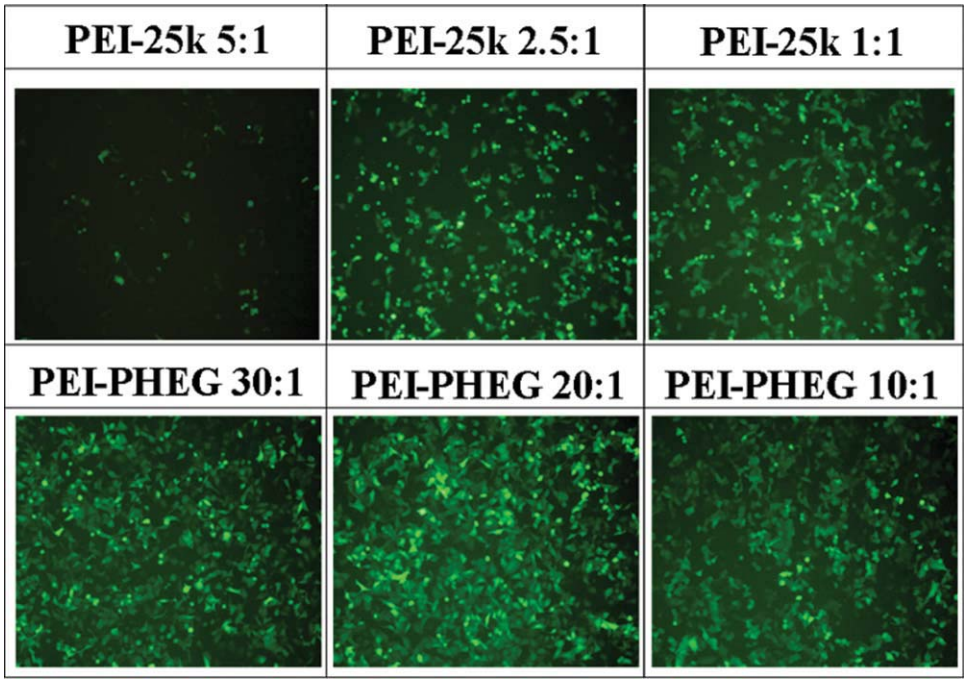

Figure 8 The transfection efficiency shown by green fluorescent protein of HeLa cells expressed by PEI-25K at [PEI]/[*p*DNA] (wt/wt) ratios of 5 : 1, 2.5 : 1, and 1 : 1, and by PEI-PHEG at [PEI-PHEG]/[*p*DNA] (wt/wt) ratios of 30 : 1, 20 : 1, and 10 : 1. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

The transfection efficiencies of PEI-25K and PEI-PHEG for HeLa cells, HEK293 cells and CT26 cells were quantitatively determined by flow cytometry at their optimal [carrier]/[*p*DNA] (wt/wt) ratios. As seen in Figure 9(A–C), the maximal transfection efficiency of PEI-PHEG was improved significantly as compared with PEI-25K in different cell lines; the maximal transfection efficiencies of PEI-PHEG in HeLa cells, HEK293 cells and CT26 cells were 50.9%, 94.6%, and 74.4%, respectively. It should be pointed out that PEI-25K was a common commercial transfection reagent. The higher transfection and the lower cytotoxicity of PEI-PHEG can be attributed to the introduction of hydrophilic PHEG segments onto the PEI surface.

Furthermore, the transfection properties of the PEI-PHEG copolymer and PEI-25K were also assessed by a luciferase assay in HeLa cells. The plasmid DNA (*p*GL3-control) was used as the reporter gene. As shown in Figure 10, the [PEI-PHEG]/[DNA] complexes showed significantly higher transfection efficiencies than that of PEI-25K at the optimized weight ratio of the carrier to the DNA. The result indicates again that the transfection efficiency of PEI-PHEG had significant improved compared with commercial PEI-25K. PEI-PHEG has also exhibited comparable behavior than other good copolymers we have designed. More importantly, the cytotoxicity of PEI-PHEG is greatly reduced, which may due to the introduction of hydrophilic

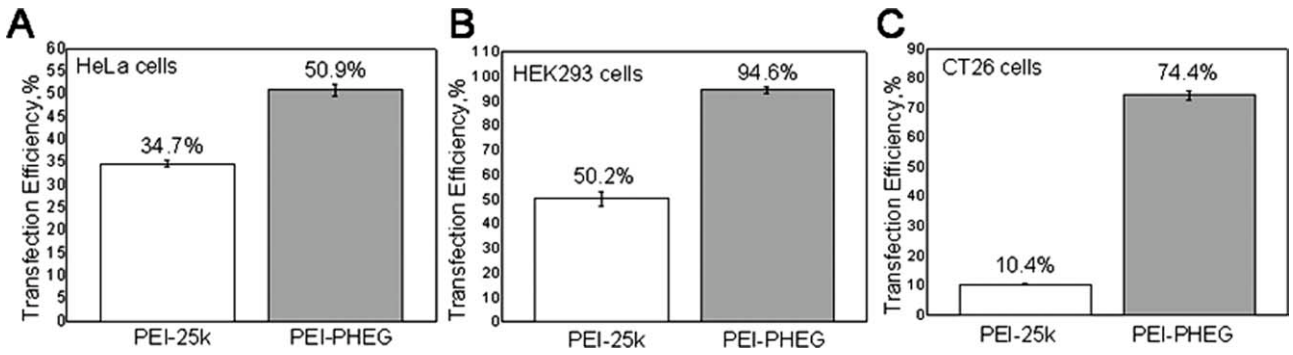

Figure 9 Optimal transfection efficiencies of PEI-25K and PEI-PHEG for HeLa cells (A), HEK293 cells (B), and CT26 cells (C) as assessed by flow cytometry.

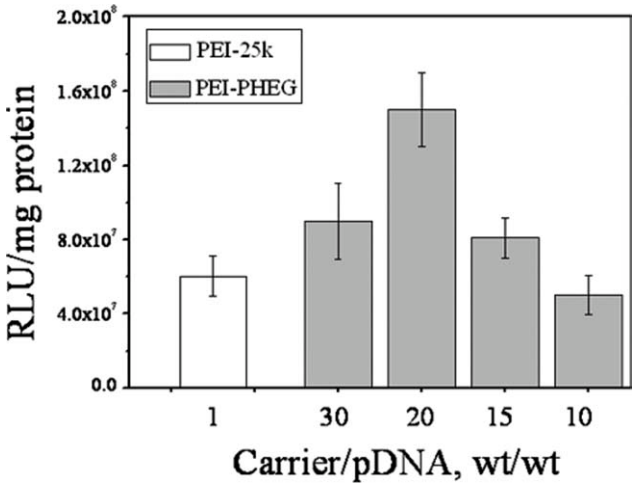

Figure 10 Transfection quantitative analysis results for the complexes of carriers/*p*GL3-control in comparison with that of PEI-25K. Data represent the mean ± SD ($n = 3$).

segment. Therefore, PEI-PHEG could be a promising nonviral gene delivery carrier.

Intracellular uptake of carrier/*p*GL3-control complexes

The intracellular trafficking of the PEI-PHEG/*p*DNA complex was observed by confocal laser scanning microscopy and compared with that of PEI-25K/*p*DNA. Figure 11 illustrates the uptake of the complexes at 1, 2.5, and 5 h post-transfection. At 1 h post-transfection, no significant uptake of the complexes into the cells was observed, and only a small number of FITC-labeled particles were localized around the boundaries of cells. Plenty of green fluorescence aggregated in cytoplasm was observed at 2.5 h post-transfection. At 5 h post-transfection, most of the labeled particles appeared in the perinuclear area. These images revealed that both the PEI-PHEG copolymer and PEI-25K could mediate *p*DNA into cells efficiently, and the cellular entry was also a

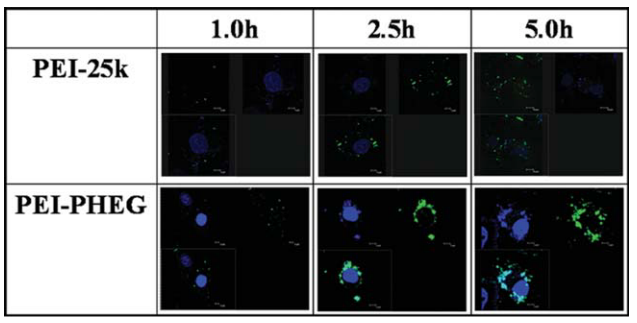

Figure 11 CLSM was performed at three time points (1 h, 2.5 h, and 5 h post-transfection) in HeLa cells transfected by PEI-25K and PEI-PHEG. Gene carriers were labeled with FITC (green), and nuclei were stained with DAPI (blue). Scale bars: 5 μm. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

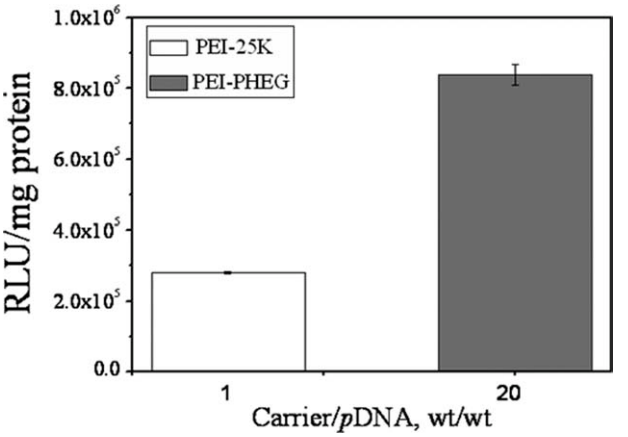

Figure 12 Transfection efficiency of the carriers/*p*DNA complexes in C57/BL6 mouse tumor models prepared by the subcutaneous injection of Lewis Lung Carcinoma cells (LLC cells). The luciferase gene (*p*GL3-control) expression levels were obtained in LLC tumors when they were treated with PEI-25K and PEI-PHEG by intratumoral injection.

dynamic process. We could see much more fluorescence of PEI-PHEG than PEI-25K, which means that PEI-PHEG could be a more efficient carrier than PEI-25K.

Transfection *in vivo*

Investigation of *in vivo* transfection efficiency was carried out using highly transfection efficient PEI-PHEG (based on the *in vitro* studies described above) by intratumoral injection into 6-week-old female C57/BL6 mice. Subcutaneous injection of LLC cells into the C57/BL6 mice produced tumors. When the tumors were about 6 mm in diameter, both PEI-25K and PEI-PHEG with weight ratios of 1 : 1 and 20 : 1 to *p*DNA were injected into the tumors, respectively. Then the luciferase reporter gene expression levels within the tumors were evaluated after 48 h post-transfection. The tumors treated with the PEI-PHEG/*p*GL3-control complex exhibited approximately a three-old higher luciferase gene expression level than those treated with the PEI-25K/*p*GL3-control complex (Fig. 12). The result indicates that PEI-PHEG will be suitable as a novel clinical gene therapy carrier.

CONCLUSIONS

In this work, we have successfully prepared and evaluated a novel PEI-PHEG copolymer as a gene carrier. The results show that the obtained cationic copolymer had a strong capability to bind and condense *p*DNA proved by gel retardation, particles size, and zeta potential analyses. This copolymer had lower cytotoxicity than PEI-25K *in vitro*. The reduced cytotoxicity of the PEI-PHEG copolymer may be a result of the introduction of the biocompatible PHEG moiety, in which

the hydrophilic PHEG block could shield the surface positive charge on PEI to a degree. PEI-PHEG exhibited much higher transfection efficiency than PEI-25K both *in vitro* and *in vivo*. Observations made by confocal laser scanning microscopy also showed that PEI-PHEG copolymer could deliver *p*DNA into cells much more efficiently than PEI-25K. Therefore, the PEI-PHEG copolymer has a potential to be used as a low toxic, highly effective gene delivery carrier.

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