Nonviral Gene Carriers Based on Diblock Copolymers of Poly(2-ethyl-2-oxazoline) and Linear Polyethylenimine

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Diblock copolymers that consist of poly(2-ethyl-2-oxazoline) (PEOz) and linear polyethylenimine (LPEI) were prepared for use as nonviral gene carriers. The PEOz-*b*-LPEI copolymers were synthesized by coupling PEOz with LPEI in a thiol−disulfide exchange reaction between the sulfhydryl and pyridyl disulfide terminal groups. A polymer/DNA weight ratio (P/D) of over 12 was required to enable PEOz-*b*-LPEI to condense DNA completely. The DNA-condensing capability of the diblock copolymers was increased with increasing the hydrolytic degrees of the LPEI segment. The PEOz-*b*-LPEI polyplexes were stable in 150 mM NaCl aqueous solution and had a mean diameter around 190 nm, whereas BPEI and LPEI polyplexes formed large aggregates in the range 300−500 nm. In addition, these polyplexes exhibited the sensitivity to solution pH and were dissociated in the acidic buffers (pH ≤ 5.5). The results of in vitro cell viability and luciferase assay indicated that PEOz-*b*-LPEI showed not only low cytotoxicity but also high transfection efficiency in gene expression.

INTRODUCTION

Nonviral vectors, such as liposomes and cationic polymers, have recently attracted increasing interest. They are usually considered to be safer and able to be designed with more flexible structures and chemical properties (*1*−*3*). Among the various types of polymer gene carriers, branched polyethyleneimine (BPEI) is the most effective vector for delivering genes because the BPEI/DNA polyplexes exhibit great transgene expression both in vitro and in vivo (*4*, *5*). The high transfection efficiency of BPEI is probably attributed to its large buffering capacity, which disrupts the endosomal membrane, resulting in the release of the polyplex into the cytoplasm (*6*). Additionally, linear polyethylenimine (LPEI) which can be obtained by hydrolyzing poly(2-ethyl-2-oxazoline) (PEOz) has indicated that the in vitro transfection activity of LPEI is as high as that of BPEI, but its cytotoxicity is lower (*7*).

However, the application of cationic homopolymers is hampered by their poor solubility due to charge neutralization and aggregation under physiological conditions, such as in salt or bovine serum albumin (*8*, *9*). A potential solution to these problems is to conjugate a hydrophilic polymer to the polycations. Poly(ethylene glycol) (PEG) is the polymer that is most often used for this purpose (*10*−*14*). Following complexation with plasmid DNA, the PEGylated polycation/DNA complexes exhibit neutral surface charge, low cytotoxicity, and high solubility in water. PEOz is a biocompatible and hydrophilic polymer that is ionized at acidic pH and leads to the formation of intra- and intermolecular hydrogen bond (*15*, *16*). Hence, PEOz has been investigated for use in the controlled release drug delivery systems (*16*, *17*). However, it has never been exploited as a vector in gene therapy.

The authors' previous work studied the ability of amphiphilic block copolymers that consist of LPEI and poly(L-lactide) (PLLA) to modulate the delivery of plasmid DNA (*18*). In this work, gene carriers were prepared by coupling PEOz and LPEI with various percentages of hydrolysis. The resultant diblock copolymers condensed plasmid DNA and formed micelles with core−shell structure. The physicochemical and biological characteristics of the copolymer/DNA micelles were evaluated herein.

EXPERIMENTAL SECTION

Materials. 2-Ethyl-2-oxazoline (Acros) and methyl *p*-toluenesulfonate (Aldrich) were purified by vacuum distillation over CaH_2. Acetonitrile (Tedia) was dried over CaH_2 and distilled under dry nitrogen. Potassium thioacetate, 2,2′-dithiodipyridine, and ammonia in methanol solution were purchased from Acros. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from ICN Biomedicals, Inc. pUHC 13-3 encoding for luciferase as a reporter gene was received as a gift from Prof. Jia-Ling Yang at National Tsing Hua University, Taiwan. Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), and trypsin−EDTA were the products of Gibco-BRL. HeLa human adenocarcinoma cells were provided by Prof. Yu-Chen Hu of National Tsing Hua University, Taiwan. Dialysis tubing was obtained from Spectrum Medical Industries, Inc.

Synthesis of Poly(2-ethyl-2-oxazoline) Thioacetate (PEOz-SAc). A solution of methyl *p*-toluenesulfonate (370 mg, 1.98 mmol) and 2-ethyl-2-oxazoline (20 mL, 197.72 mmol) in dry acetonitrile (60 mL) was heated to 100 °C and stirred for 24 h in an atmosphere of nitrogen. The living PEOz polymer chains were terminated by adding potassium thioacetate (0.7 g) at 0 °C; the solution was then stirred for 24 h. The polymer solution was filtered through the silica gel and purified by precipitation in diethyl ether. After complete drying, a brown powdery product was obtained. The molecular weight and polydispersity of PEOz-SAc were determined by gel permeation chromatography (GPC). ^{1}H NMR ($CDCl_3$): $\delta = 0.98$ ppm ($N(COCH_2CH_3)$), 2.35 ($N(COCH_2CH_3)$), 3.42 ($N(COCH_2CH_3)CH_2CH_2$), 3.00 ($CH_3N(COCH_2CH_3)$), 2.92 ($CH_2CH_2S(CO)CH_3$). Elemental

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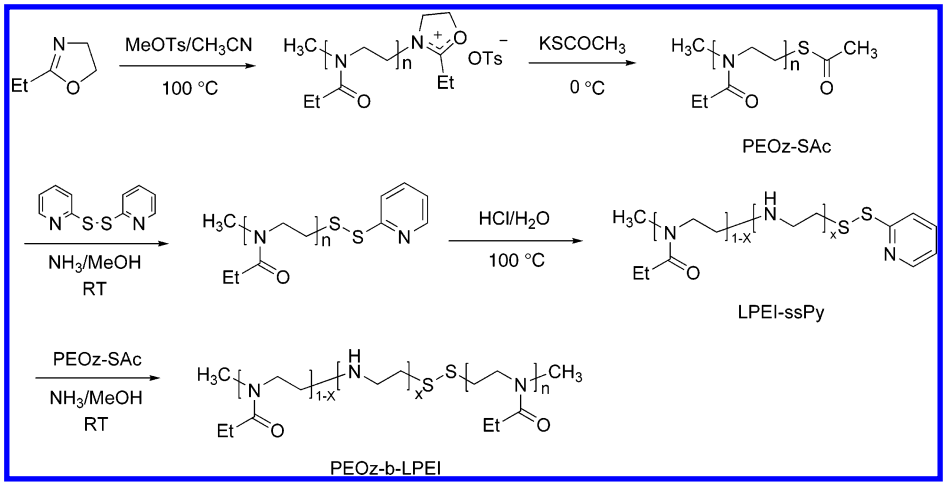
Scheme 1. Synthetic Route of the PEOz-*b*-LPEI



anal. (mass fraction): (C: 60.42, H: 9.08, N: 14.01, S: 0.36). Calculated: (C: 60.39, H: 9.13, N: 14.00, S: 0.32).

Synthesis of Linear Polyethylenimine Pyridyl Disulfide (LPEI-ssPy). PEOz-SAc (1 g, 0.1 mmol) and 2,2′-dithiodipyridine (111 mg, 0.5 mmol) were dissolved in ammonia/methanol solution (7 N, 20 mL). The mixture was stirred in a nitrogen atmosphere at room temperature for 24 h. After the solvent had been removed, the solid residue was redissolved in dichloromethane and then precipitated in diethyl ether several times. The purified product was vacuum-dried to yield a yellow powder. PEOz-ssPy (1 g) was heated with a mixture of concentrated hydrochloric acid (10 mL, 35%) and water (8 mL) at 100 °C for 3 h. The pH of the polymer solution was adjusted to 9−10 by adding NaOH pellets. The product was then purified by dialysis method for 3 days using cutoff dialysis tubing with a molecular weight of 3500. After completely drying in a vacuum, the viscous semisolid product was obtaied. ^{1}H NMR ($CDCl_3$): δ = 2.77 ppm ($NHCH_2CH_2$), 7.094, 7.601, 8.41 (pyridyl ring). Elemental anal. (mass fraction): (C: 57.54, H: 10.48, N: 24.31, S: 1.03). Calculated: (C: 57.47, H: 10.46, N: 24.35, S: 1.10).

Synthesis of Diblock Copolymer Poly(2-ethyl-2-oxazoline)-*block*-Linear Polyethylenimine (PEOz-*b*-LPEI). LPEI-ssPy (1 g, 0.196 mmol) and PEOz-SAc (235 mg, 0.065 mmol) were dissolved in ammonia/methanol solution (7 N, 15 mL). The reaction was carried out in a nitrogen atmosphere at room temperature for 24 h. The unreacted LPEI-ssPy and byproducts were purified by dialysis (with a molecular weight cutoff of 6000−8000) against methanol and distilled water for 3 days, respectively. After evaporation, the obtained diblock copolymer was then dried in vacuo and stored at −20 °C before use. Elemental anal. (mass fraction): (C: 58.51, H: 10.11, N: 20.31, S: 0.73). Calculated: (C: 58.56, H: 10.01, N: 20.38, S: 0.68).

Preparation of Polyplexes. An amount of 4 μg of Luciferase encoding plasmid pUHC-13−3 and the desired amount of polymer were each diluted in 100 μL of distilled water and gently mixed. The polyplex formulation was incubated at room temperature for 30 min before it was used.

Gel Retardation Assay. Various formulations of polyplexes were prepared as described above before they were loaded into the gel. The samples were electrophoresed on 1% agarose gel in 1× Tris−boric acid−EDTA (TBE) buffer at 100 mV until the 1× loading dye ran through 80% of the gel. A 1 kb DNA marker (Violet), used to determine the size of DNA, was also run. The gel was stained with 0.5 μg/mL ethidium bromide for 45 min and analyzed using an UV transilluminator.

Measurement of Particle Size and Zeta Potential. The size of the particles of the polyplexes prepared in distilled water or 150 mM NaCl was measured at 25 °C by dynamic light scattering using a Zetasizer 3000 HS from Malvern Instruments, Worcestershire, U.K. The viscosity (0.89 mPa/s) and the refractive index (1.333) of water at 25 °C were used to analyze the data. The zeta potential of the polyplexes prepared in distilled water was determined by laser Doppler anemometry in the standard capillary electrophoresis cell of the Zetasizer 3000 HS from Malvern Instruments at 25 °C. The mean values of the zeta potential were calculated from the data obtained in five runs.

pH-Sensitivity Testing. The polyplexes formed by mixing plasmid DNA and diblock copolymers were incubated in the phosphate buffer (pH 7 and 6.5) and succinate buffer (pH 6, 5.5, 5, 4.5) solutions at room temperature for 1 h. Then, the aliquots obtained from the buffer solutions were analyzed with 1% agarose gel electrophoresis.

In Vitro Cell Viability. Human cervix carcinoma HeLa cells (1×10^4 cells/well) were cultured onto a plate with 96 wells in DMEM media supplemented with 10% fetal bovine serum (FBS) in a humid atmosphere of 5% CO_2 at 37 °C for 24 h. The growth medium was replaced with a medium that contained the desired amount of polymers. Cells were incubated for 24 h, and cell viability was assayed by adding 100 μL of medium that contained 10 μL of MTT PBS solution (5 mg/mL). After incubation for 4 h, the formazan crystals were dissolved in 100 μL of DMSO. The absorbance of each well was measured using a microplate reader (Stat Fax 2100, Awareness) at a test wavelength of 570 nm and a reference wavelength of 630 nm.

In Vitro Transfection and Total Protein Assay. Human cervix carcinoma HeLa cells (2×10^5 cells/well) plated in six-well plates were incubated in DMEM media supplemented with 10% FBS in a humid atmosphere of 5% CO_2 at 37 °C for 24 h. Transfection was conducted after the cells had become approximately 80% confluent. A 2 mL volume of growth medium that contained DNA or polyplexes that contained finally 4 μg of plasmid was incubated for 48 h at 37 °C. Following transfection, the cells were washed with cold PBS and then lysed using 400 μL of 1× cell lysis buffer. The cell lysate was then transferred into eppendorf tubes and centrifuged for 15 s at 12 000 rpm. Luciferase activity was measured in relative light units (RLU), using a 96-well plate luminometer (Wallac 1420 Multilabel Counter, Perkin-Elmer) and a luciferase assay kit (Promega). The total amount of protein was measured at 595 nm using a BioRad protein assay.

RESULTS AND DISCUSSION

Synthesis of Diblock Copolymer PEOz-*b*-LPEI. Scheme 1 presents the synthesis of PEOz-*b*-LPEI. Cationic ring-opening polymerization of 2-ethyl-2-oxazoline using the monofunctional

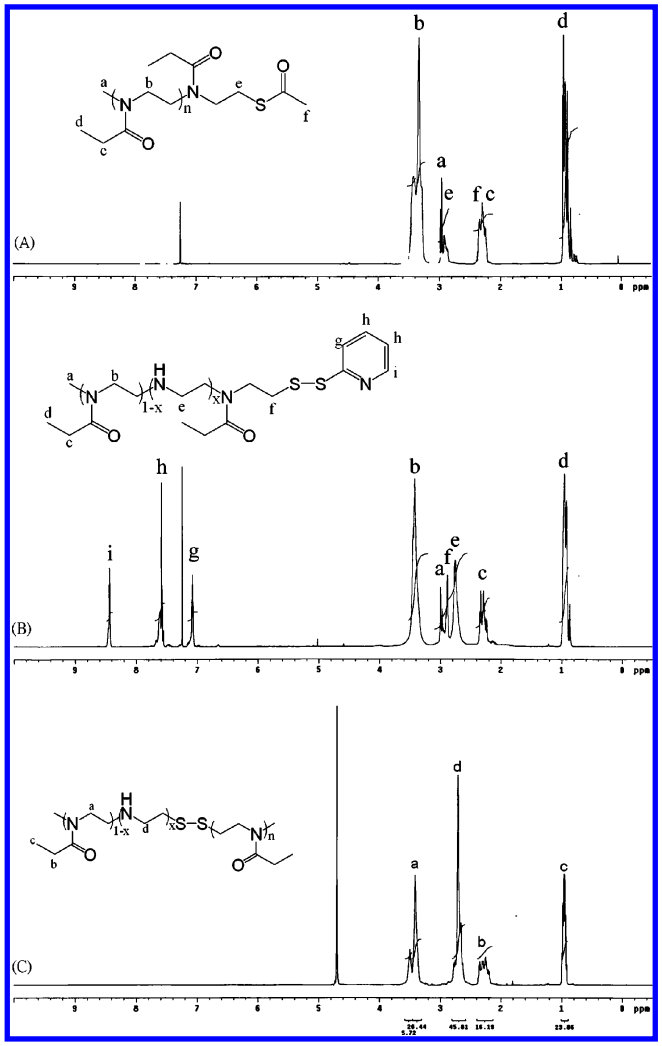


Figure 1. ^{1}H NMR spectra of (A) PEOz-SAc in $CDCl_3$, (B) LPEI-ssPy in $CDCl_3$, and (C) PEOz-*b*-LPEI in D_2O.

initiator methyl *p*-toluenesulfonate is a typical method for synthesizing PEOz (*19*, *20*). The oxazolinium salt structure of the growing polymer can readily react with nucleophiles, such as amines or water to introduce functional groups into the end of polymer chain (*21*). A thiol is used herein to couple the PEOz and LPEI via disulfide bond. However, the thiol was susceptible to oxidation to the formation of dimer; thus, the more stable form of thioacetate was desired. Adding potassium thioacetate to terminate the reaction capped the end of PEOz with the thioacetate functional group. The number average molecular weight (M_n) and polydispersity index (PDI) of PEOz-Sac determined by GPC were 10000 and 1.12, respectively. Figure 1A shows the ^{1}H NMR spectrum of PEOz-SAc dissolved in $CDCl_3$.

LPEI was prepared by the acid hydrolysis of PEOz. Because the terminal thioacetate group could be hydrolyzed as well, it was first deacetylated by treating with ammonia/methanol solution and then converted to pyridyl disulfide group by thiol−disulfide exchange reaction with 2,2′-dithiodipyridine before performing hydrolysis reaction. Figure 1B shows the ^{1}H NMR spectrum of LPEI-ssPy in $CDCl_3$. The percentage of LPEI-ssPy hydrolyzed was calculated by comparing the integral peak area of the ethylenimine groups to the *N*-propionylethylenimine groups from the ^{1}H NMR spectrum. The molecular weight of the LPEI-ssPy was estimated from the percentage of hydrolysis.

Diblock copolymers were synthesized by coupling the PEOz-SAc and LPEI-ssPy via a disulfide exchange reaction of the deprotected sulfhydryl goup of the PEOz and the pyridyl

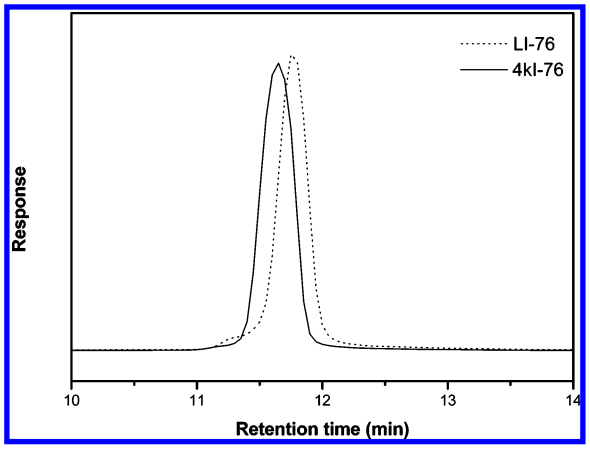


Figure 2. GPC chromatograms before and after LPEI conjugation.

Table 1. Compositions of LPEI and PEOz-*b*-LPEI

polymer	M_n (PEOz)	M_n (LPEI)	EI content (%)
L-76	-	5700	76
L-89	-	5000	89
4k-76	3600	5700	56
4k-89	3600	5000	66

disulfide group of LPEI. The disulfide bond of the diblock copolymer is cleavable intracellularly, but it is relatively stable in the extracellular milieu (*22*). Figure 1C shows the ^{1}H NMR spectrum of PEOz-*b*-LPEI in D_2O. The Mn and PDI of PEOz-SAc used in this step were 3600 and 1.18, respectively. The GPC chromatogram before and after LPEI conjugation was shown in Figure 2. The molecular weight was increased after successfully coupling PEOz and LPEI, and the unreacted polymers were completely removed. Table 1 presents the compositions of the LPEI and PEOz-*b*-LPEI. For comparison, a commercially available BPEI of 25 kDa was also evaluated in this work.

Gel Retardation Assay. Agarose gel electrophoresis was conducted to evaluate the capacity of BPEI, LPEI and PEOz-*b*-LPEI to interact with plasmid DNA. Figure 3 shows the result of gel retardation assay for polycations/DNA complexes. As the concentration of the polycation increased, the mobility of the plasmid DNA was reduced by increasing DNA condensation and compaction. BPEI was the most efficient that it could completely retard DNA migration at a polymer/DNA (P/D) ratio (w/w) of 1 while it required P/D ratios of 8 and 6 for the L-76 and L-89 to complex DNA, respectively (Figure 2A). The 4k-76 began to form polyplexes at a P/D ratio of 14, and a P/D ratio of 12 was required for 4k-89 to complex DNA completely (Figure 2B). These results demonstrated that the DNA binding capacity of LPEI increased with the hydrolytic degrees.

Particle Size and Zeta Potential. After determining the amount of the different polycations for polyplexes formation, the particle size of the polyplexes was estimated by laser light scattering. The polyplexes prepared in either an aqueous solution of physiological salt (150 mM NaCl) or in distilled water were used to measure the size of polyplex to elucidate whether the ionic strength of the media influences the stability of the polyplexes. During the measurement, we found the lowest P/D ratio for stable polyplexes formation of each polymer. The polyplexes that formed below this P/D ratio usually led to aggregation. Figure 4 presents the particle sizes of BPEI, LPEI, and PEOz-*b*-LPEI polyplexes at the lowest P/D ratio for the formation of stable polyplexes. The BPEI polyplex had a mean diameter of 100 nm in water that was reasonably consistent with the findings of Tang and Szoka (*2*). The sizes of LPEI and PEOz-*b*-LPEI polyplexes were around 190 nm. When the polyplexes were dispersed in 150 mM NaCl solution, BPEI and

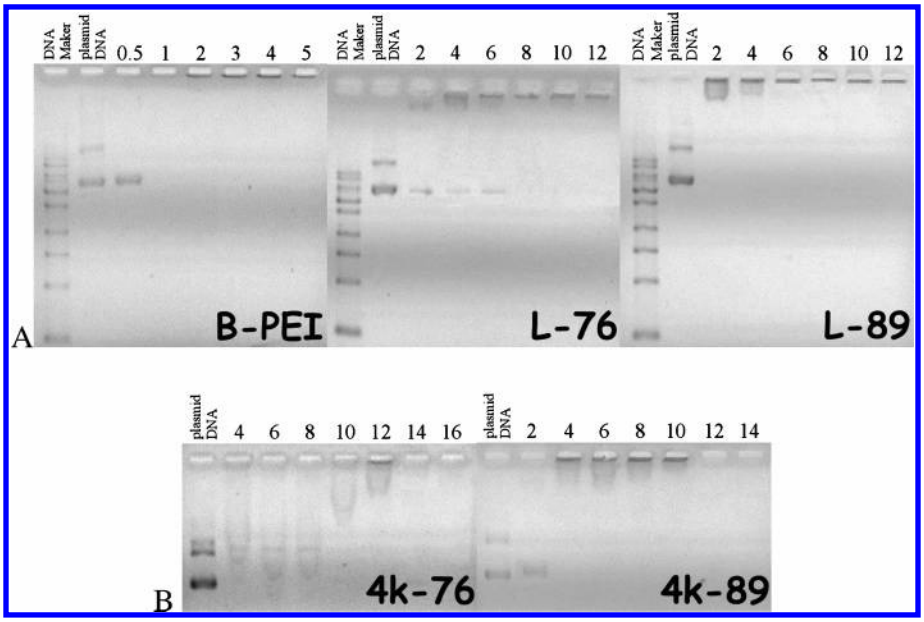


Figure 3. Gel retardation assay of the polyplexes: (A) BPEI and LPEI, (B) PEOz-*b*-LPEI. The numbers in the first row above each panel noted the P/D ratios of the complexes.

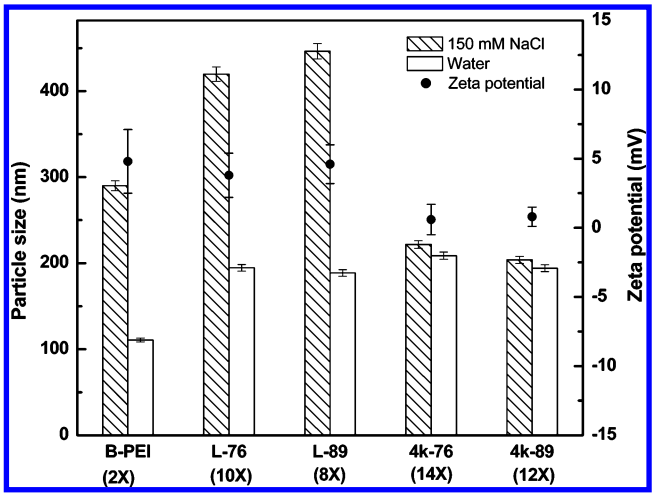


Figure 4. Particle size and zeta potential of BPEI, LPEI, and PEOz-*b*-LPEI polyplexes at the optimum P/D ratios.

LPEI polyplexes all aggregated as larger particles. However, the PEOz-*b*-LPEI polyplexes did not show any aggregation in salt-containing media.

The zeta potential of the polyplexes wasmeasured by mixing the polyplexes at the lowest P/D ratio to stabilize them. Figure 4 shows that BPEI and LPEI polyplexes all had moderate positive surface charges, but the zeta potentials of PEOz-*b*-LPEI polyplexes were almost zero. When BPEI and LPEI were mixed with DNA at a P/D ratio that was slightly less than the lowest P/D ratio for the formation of stable polyplexes, the polyplexes also had a neutral surface charge (data not shown). This result indicates that the neutral BPEI and LPEI polyplexes were prone to aggregate due to the lack of electrostatic repulsion, so the polyplexes with a positive surface charge were stable. However, the PEOz-*b*-LPEI polyplexes were stable and had a neutral surface charge probably because they possessed a core−shell structure with a hydrophobic LPEI/DNA core and a hydrophilic PEOz shell, which prevented the polyplexes from aggregating.

pH-Sensitivity Testing. The recent study conducted in our laboratory showed that the PEOz was a pH-sensitive polymer that would become a hydrogen-bonded complex at acidic buffer solutions, because of the ionization of the amide groups (*18*).

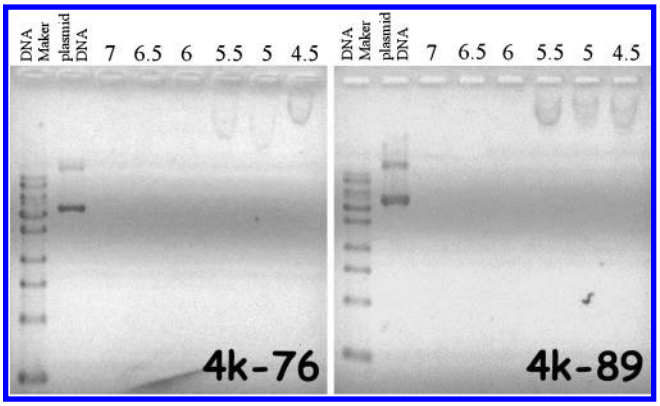


Figure 5. The pH-sensitivity of PEOz-*b*-LPEI polyplexes in various buffer solutions. The numbers in the first row above each panel noted the pH values of the buffer solutions.

The PEOz-*b*-LPEI polyplexes were incubated in buffer solutions at pH values from 7 to 4.5, to determine whether the introduction of PEOz to L-PEI made the polyplexes sensitive to pH. The integrity of the polyplexes was consistent with the analysis made by agarose gel electrophoresis. Figure 5 shows the gel electrophoresis results for PEOz-*b*-LPEI polyplexes. The 4k-76 polyplex was stable at pH $\geq$ 6, but began to dissociate at pH $\leq$ 5.5. The 4k-89 polyplex also had the same behavior as 4k-76. The LPEI polyplexes were also examined. They remained intact in all tested buffer solutions (Data not shown). These results demonstrate that the sensitivity of PEOz-*b*-LPEI polyplexes to pH does stem from the tendency of PEOz and cause the polyplexes to dissociate in acidic environments.

In Vitro Cell Viability. The in vitro cytotoxicities of the different polycations at various concentrations were quantitated by MTT assay using HeLa human cervix carcinoma cells. The wells that contained only media without polycation were treated as positive controls, with a cell viability of 100%. The relative cell viability was calculated as $[Abs]_{sample}/[Abs]_{control} \times 100$. Figure 6 compares the cell viability of HeLa cells at various polymer concentrations of BPEI, LPEI, and PEOz-*b*-LPEI, revealing that BPEI was the most toxic polycation and the cell viability was below 10% as the polymer concentration $\geq$ 0.1 mg/mL. The cell viability of LPEI declined as the hydrolyzed percentage increased, as it did in the case of PEOz-*b*-LPEI.

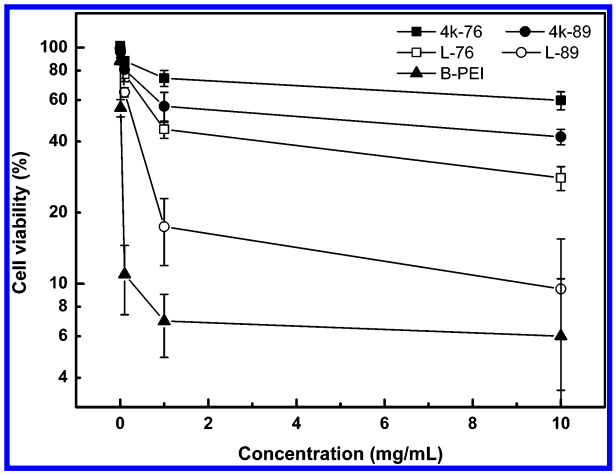


Figure 6. The cell viability of HeLa cells at various polymer concentrations ($n = 6$).

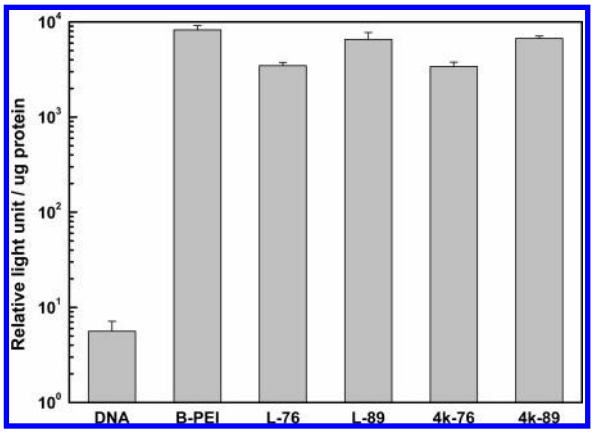


Figure 7. Luciferase gene expressions of the naked DNA, BPEI, LPEI, and PEOz-*b*-LPEI at the optimum P/D ratios ($n = 3$).

Furthermore, the PEOz-*b*-LPEI was less toxic than the corresponding LPEI. These results demonstrated that the charge density in the LPEI strongly affected cell viability. Therefore, PEOz-coupled LPEI further reduced the cationic charge density and the cytotoxicity.

In Vitro Transfection and Total Protein Assay. The total protein production, which is an indirect measure of induced toxicity, was analyzed and normalized to be expressed in relative light unit (RLU). Figure 7 presents the luciferase gene expressions of the naked DNA, BPEI, LPEI, and PEOz-*b*-LPEI at optimum P/D ratios. The results showed that all the polycationic gene carriers exhibited greater transfection efficiency than naked DNA. BPEI polyplex mediated the strongest gene expression of all polycations. The transfection efficiency of PEOz-*b*-LPEI was as high as that of LPEI. PEOz-coupling did not affect the transfection efficiency of the LPEI. Moreover, the protein production of PEOz-*b*-LPEI exceeded that of BPEI and LPEI, indicating that PEOz-*b*-LPEI polyplexes had lower cytotoxicity than others.

CONCLUSION

In this study, the pH-sensitive diblock copolymers PEOz-*b*-LPEI were synthesized by coupling PEOz with LPEI, and the physicochemical and biological characteristics were evaluated. The polyplexes of the PEOz-*b*-LPEI were shown to have neutral surface charge and stably sized particles, unlike LPEI and BPEI. These diblock copolymer polyplexes exhibited a pH-sensitivity that caused them to dissociate at pH values lower than 5.5, suggesting that they may release DNA intracellularly. The results also demonstrated that the PEOz-*b*-LPEI had low cytotoxicity and high transfection efficiency. Accordingly, the diblock copolymers have potential for application in nonviral gene therapy.

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