Linear polyethylenimine produced by partial acid hydrolysis of poly(2-ethyl-2-oxazoline) for DNA and siRNA delivery in vitro

Abstract: Polyethylenimines (PEIs) are the most efficient synthetic vectors for gene delivery available to date. With its high charge density and strong proton-buffering effect, PEI has an ability to condense DNA and small interfering RNA at physiologic pH. However, the polymer suffers from the disadvantage of high cellular toxicity. To reduce its cellular toxicity, we synthesized linear PEIs by partial hydrolysis of poly(2-ethyl-2-oxazoline). Three linear PEIs with different hydrolysis percentages (30%, 70%, and 96%, respectively) were produced as PEI30, PEI70, and PEI96. PEI30 and PEI96 cannot be considered as suitable transfection agents because of low transfection efficiency (PEI30) or high cellular toxicity (PEI96). PEI70 displayed very weak cell toxicity. The charge density of this polymer (PEI70) was strong enough to condense DNA and small interfering RNA at a physiologic pH of 7.4. Our results also show that PEI70 was highly efficient in DNA delivery and small interfering RNA-mediated knockdown of target genes. Thus, polymers such as PEI70 appear to be very promising vectors for gene delivery.

Keywords: nonviral vector, polyethylenimine, gene delivery, DNA, small interfering RNA

Introduction

Gene transfer has been used widely as a research tool to study gene regulation and control. Gene therapy, ie, the treatment of human diseases by gene transfer, is becoming a promising application against various inherited or acquired diseases. It offers controlled, specific delivery of nucleic acid (DNA and RNA) to targeted cells, leading to treatments that are less invasive, more effective, and less expensive than existing modalities. However, a large size and negative surface charge density most likely limit DNA uptake by negatively charged cell membranes via nonspecific endocytosis. Small interfering RNA (siRNA) alone cannot penetrate the cell membrane because of its highly charged backbone. It is more susceptible than DNA to nuclease destruction. Therefore, the primary challenge for gene therapy is to develop carrier (vectors) capable of efficiently protecting and delivering genes to targeted cells with minimal toxicity. Different vector systems have been tested in gene transfer studies for potential clinical use. Viral gene therapy consists of using viral vectors which, given their structure and mechanisms of action, are good models to carry therapeutic genes efficiently, leading to long-term expression. However, viral vectors can cause several problems for patients, ie, toxicity, oncogenic effects, and immune and inflammatory responses. Synthetic and natural cationic (positively charged) polymers have been used widely to carry negatively charged DNA and condense it into small particles, facilitating cellular internalization via endocytosis through charge-charge interactions with anionic sites on cell surfaces.

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Certain nonviral vectors are limited as gene carriers by typically low transfection efficiency and transient gene expression (eg, chitosan). Polyethylenimines (PEIs), a series of synthetic polymers, are well known to be effective cationic polymers, demonstrating high transfection efficiency in vitro and in vivo. PEIs exist as either branched or linear structures. While branched PEI is normally synthesized via acid-catalyzed polymerization of aziridine, linear PEI is produced in two steps. Poly(2-ethyl-2-oxazoline) (PETOx) is obtained by living polymerization of 2-ethyl-2-oxazoline, followed by acid hydrolysis. It has been demonstrated that the transfection level is closely related to polymer molecular weight, the charge ratio of polymer to DNA/siRNA, particle size, and zeta potential. The transfection efficiency of PEIs increases with increasing molecular weight. High molecular weight PEI results in high cytotoxicity. The optimal molecular weight of a PEI-DNA formation is 5–25 kDa. Most of the PEIs used for gene transfer are highly branched. A previous study demonstrated the effective delivery of DNA and siRNA with linear PEI in vivo. Linear PEI has lower cytotoxicity, but is also less effective in condensing DNA than branched PEI. PEI-based gene transfer technology is starting to reach the market as commercial transfection agents, including the DNA transfection agent jet-PEI® (Polyplus-transfection Inc, Illkirch, France), using a linear derivative of PEI. DNA transfection with linear PEI, obtained by acid hydrolysis of PETOx with varying acid volume, has been demonstrated. However, most studies of linear PEI-siRNA were performed with commercial PEI products, which contained significant amounts of N-acyl residues that limited gene transfection.

In the present investigation, we aimed to synthesize low molecular weight linear PEI (10 kDa) containing different amounts of ethylenimine units. Partial hydrolysis of PETOx was achieved, by varying acid hydrolysis duration, for reducing the cellular toxicity of PEI. The potential of such linear PEIs as DNA and siRNA delivery vectors was explored in vitro.

Materials and methods

Materials

2-Ethyl-2-oxazoline (99%), p-toluenesulfonyl chloride (99+%), the β-galactosidase reporter gene activity detection kit, and other chemicals were sourced from Sigma-Aldrich Chemical Co (St Louis, MO, USA), if not stated otherwise. Dulbecco’s Modified Eagle’s Medium, fetal bovine serum, 0.25% trypsin-ethylenediaminetetraacetic acid (EDTA) solution, penicillin-streptomycin mixture, Lennox L Broth Base®, agarose, ethidium bromide, Lipofectamine™ 2000, and oligofectamine reagent were obtained from Invitrogen Canada Inc (Burlington, ON, Canada). The plasmid DNA VR1412 encoding the β-galactosidase reporter gene with a cytomegalovirus promoter was obtained from Vical Inc (San Diego, CA, USA). QIAfilter plasmid mega kits were supplied by Qiagen (Mississauga, ON, Canada). Sjögren syndrome antigen- (SSB, GenBank accession number NM_009278) targeted siRNA was provided by Merck & Co., Inc. (West Point, PA, USA). SSB-siRNA was selected as validated siRNA to evaluate our delivery strategies.

PETOx preparation

2-Ethyl-2-oxazoline (99%) and p-toluenesulfonyl chloride (99+%) were dried and vacuum-distilled over calcium hydride before use. Acetonitrile was purified in a solvent system via two packed columns of activated alumina provided by Innovative Technology Inc (Cartersville, GA, USA). Water was deionized in a MilliQ system (EMD Millipore, Bedford, MA, USA).

Polymerization was undertaken according to a reported procedure with slight modification. Typically, a round-bottom flask equipped with N₂-filled condenser and rubber stopper was charged with 2-ethyl-2-oxazoline (6 mL, 60 mmol), acetonitrile (20 mL), and methyl p-tosylate (90 µL, 0.6 mmol) via an oxygen-free syringe at room temperature. After mixing, the flask was immersed in an oil bath preheated to 65°C. Polymerization was continued for 60 hours with stirring. Next, 5 mL of KOH methanol solution (1.0 M) was added to quench poly(2-isopropyl-2-oxazoline) oxazolinium living end groups. The termination reaction was continued for 12 hours at 65°C. Thereafter, the polymerization solution was cooled to room temperature, and diluted with deionized water to 80 mL, then dialyzed against deionized water for 3 days with a 3,500 Da molecular weight cutoff membrane. The purified polymer was recovered by freeze-drying. The yield was 4.8 g, 80%. Gel permeation chromatography: Mn = 1.05 × 10⁴. ¹H-nuclear magnetic resonance (NMR) (CDCl₃, δ) ppm: 0.96 (br, −CH₂CH₂), 2.25 (br, −CH₂CH₂), 3.45 (br, −N(C=O)CH₂−). Fourier transform infrared spectroscopy (KBr, cm⁻¹), ν 2,976, 2,934, 2,873, 1,646, 1,474, 1,431, 1,205, 1,160, 1,089, and 755 cm⁻¹.

Partial acid hydrolysis of PETOx

Hydrolysis was performed in a round-bottom flask equipped with a magnetic stirrer and condenser. Typically, 1.0 g of PETOx was dissolved in 5 mL of deionized water. After dissolution, 10 mL of HCl solution (10 w/w%) was added. The solution was refluxed at 100°C for a few hours. It was then
cooled to room temperature and neutralized to pH 9–10 by 5 N NaOH solution. The neutralized polymer solution was dialyzed against deionized water for 3 days with a 5,000 Da molecular weight cutoff membrane. The polymer was recovered by freeze-drying. Yield was 0.4–0.6 g. Polymer characteristics were analyzed by 1H-NMR spectroscopy (ARX-400 400 MHz, Bruker Corporation, Billerica, MA, USA). The solvent was deuterium oxide (D2O). Ultraviolet-visible spectra were measured in an 8452A photodiode array spectrometer (Hewlett Packard, Palo Alto, CA, USA). Polymer concentration was 10 wt% in 1 M NaCl. The degree of hydrolysis was evaluated by 1H-NMR, by comparing the integration of resonances at 2.90 (−NHCH2−) and 3.45 (−N(C=O)CH2−) ppm, and was found to be 31, 70, and 96 mol% for polymers hydrolyzed for 3, 5, and 7 hours.

Plasmid DNA amplification and purification
Plasmid DNA VR1412 was purified using a Qiagen QIAfilter plasmid mega kit, according to the manufacturer’s instructions, and resuspended in water. Purity was confirmed by 0.8% agarose gel electrophoresis and DNA absorption at 260 nm.

Linear PEI-DNA or PEI-siRNA complex preparation
PEIs of different hydrolysis percentages were dissolved in NaCl buffer (150 mM, pH 7.4) to form solutions of 10 mg/mL and passed through a 0.22 μm filter. PEI-DNA and PEI-siRNA complexes were produced by mixing 5 μg DNA or siRNA in 50 μL of 150 mM NaCl with various amounts of PEI stock solutions in 150 mM NaCl, resulting in various N/P ratios in a final volume of 100 μL. The nanoparticles were incubated for 30 minutes at room temperature and tested without further modifications.

Particle size
Particle size was measured with a Nano ZS90 Zetasizer (Malvern Instruments Ltd, Malvern, UK). First, 100 μL of PEI-DNA or PEI-siRNA complexes at different N/P ratios were obtained as indicated earlier. The final volume of 100 μL was processed with disposable, low-volume polystyrene (100 μL) cuvettes. Particle size was measured in triplicate.

Zeta potential
The zeta potential of PEI-DNA and PEI-siRNA complexes was quantified by Nano ZS90 Zetasizer based on the principle of phase analysis light scattering. First, 100 μL of PEI-DNA or PEI-siRNA complexes at different N/P ratios were generated, as indicated earlier. The solution was then diluted to 1 mL with NaCl buffer. Measurements were in triplicate in disposable zeta potential capillary cells.

Evaluation of siRNA integrity by agarose gel electrophoresis after synthesis
DNA and siRNA binding with PEI was measured by 0.8% or 2% agarose gel electrophoresis in Tris-borate EDTA buffer (4.45 mM Tris-base, 1 mM sodium EDTA, 4.45 mM boric acid) at pH 8.0. A series of different PEI to DNA or siRNA N/P ratios was loaded (20 μL of sample containing 1 μg of DNA or siRNA) on gel, and electrophoresis was carried out at 55 V for 120 minutes. The gel was stained with ethidium bromide (0.5 μg/mL) and photographed in a Multilmage™ light cabinet (Alpha Innotech Corporation, San Leandro, CA, USA).

Cell culture and transfection protocol in vitro
HeLa cervical carcinoma cells were obtained from the American Type Culture Collection (Rockville, MD, USA) and grown in Dulbecco’s Modified Eagle’s Medium supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin. Cultured in a 5%–95% CO2−O2 atmosphere at 37°C, they were seeded, 24 hours prior to transfection, on 24-well plates at a density of 50,000 cells/well in 1 mL of their usual culture medium supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin. On the day of transfection, the culture medium was replaced by 900 μL of serum-free Dulbecco’s Modified Eagle’s Medium and 100 μL of PEI-DNA or PEI-siRNA complexes containing either free DNA or siRNA, polymer-DNA or polymer-siRNA complexes with DNA or siRNA equivalent to 5 μg. After a 6-hour incubation, the cells received 1 mL of complete medium and were incubated for 60 hours post-transfection.

Cell viability
The cells were seeded and transfected with free DNA or siRNA, PEI-DNA or PEI-siRNA complexes, then incubated for 24 hours at a temperature of 37°C. Cell viability was evaluated by the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT, Sigma-Aldrich Chemical Co) colorimetric assay based on reduction of MTT to water-insoluble formazan by mitochondria in viable cells. Absorbance was measured at 570 nm with a universal
microplate reader (EL800, Molecular Devices Corporation, Menlo Park, CA, USA).

**Determination of transfection efficiency**
The VR1412 gene codes for β-galactosidase, an enzyme that catalyzes the hydrolysis of β-galactosides. Expression of β-galactosidase activity was analyzed using a β-galactosidase reporter gene activity detection kit (Sigma-Aldrich Chemical Co) according to the manufacturer’s instructions. Briefly, the cells were lysed for 60 hours after transfection with lysis buffer included in the kit, and the cell extracts were centrifuged at maximum speed for 15 minutes at 4°C to remove any debris. The reaction was quantified in a microplate reader (Molecular Devices Corporation). Total protein content of the samples was measured using a bicinchoninic acid protein assay (Pierce, Rockford, IL, USA).

**Evaluation of transfection efficiency by rTPCR**
Total RNA was extracted from cells with TRIzol reagent (Invitrogen Canada Inc) according to the manufacturer’s specifications. RNA was quantified with a RiboGreen RNA quantitation kit (Molecular Probes, Eugene, OR, USA). For real-time reverse transcription polymerase chain reaction (RT-PCR) analysis, 1 µg of total RNA was reverse-transcribed with reverse transcription kits (Qiagen), as detailed in the manufacturer’s guidelines. One-fifth of the reverse transcriptase reaction was analyzed by RT-PCR. The following primers were used: SSB, forward 5′-CCA GCC TTC ATC AAA TTG AGT ATT-3′, reverse 5′-AGC CTC GCC TTT GCC GA-3′, forward 5′-CTG GTG CCT GGG GCG-3′. SSB mRNA expression in cultured cells was expressed as fold changes relative to control conditions (unstimulated cells) according to the ∆∆CT method detailed in the manufacturer’s guidelines (Stratagene, La Jolla, CA, USA). A ∆CT value was calculated first by subtracting the CT value for the β-actin housekeeping gene from each sample. A ∆∆CT value was then calculated by subtracting the ∆CT value of the control from the ∆CT value of each treatment. Fold changes relative to the controls (unstimulated cells) were then determined by raising 2 to the power of ∆∆CT. Each PCR was performed in triplicate on two separate occasions in at least three independent experiments.

**Statistical analysis**
The data are expressed as the mean ± standard error of the mean of at least three independent experiments. All statistics were generated by Prism software (GraphPad Software, San Diego, CA, USA). Statistical significance was analyzed by unpaired Student’s t-test, and P < 0.05 was considered to be statistically significant.

**Results**
**PETOx polymerization and hydrolysis**
Linear PEI was synthesized in two steps (Figure 1). First, PETOx oligomers, which are precursors of linear PEIs, were synthesized by living cationic polymerization of 2-ethyl-2-oxazoline (Figure 1A). The reaction was initiated with
p-toluenesulfonyl chloride in acetonitrile at 65°C, followed by termination with KOH solution. Such polymerization achieves the low molecular weight and narrow distributions required for PEtOx application as a gene delivery vector.

Second, linear PEIs were prepared by acid hydrolysis of PEtOx with an excess of hydrochloric acid (Figure 1B). Three linear PEI derivatives were obtained by sampling a single hydrolysis mixture at various time intervals. As hydrolysis time increased, the extent of hydrolysis grew, as reported in Table 1. Hydrolysis percentages of PEtOx molecular weight 10,000 at various hydrolysis times of 3, 5, and 7 hours were 30%, 70%, and 96%, respectively. Figure 2 shows the 'H-NMR spectrum of linear PEI derivatives in D$_2$O. The hydrolysis percentage of linear PEI was evaluated by 'H-NMR by comparing the integration of resonances at 2.90 (–NHCH$_2$–, peak d) and 3.45 (–N(C=O)CH$_2$–, peak c) ppm. The peak of various methylene groups (peak d) corresponded to ethyleneimine units (2.9 ppm) and ethyleneimine units substituted by (–N(C=O)CH$_2$–) side groups (3.45 ppm, peak c). The ratio of the areas of these peaks allowed the determination of ethyleneimine unit content.

**PEI cytotoxicity**

The cytotoxicity of acid hydrolysis of PEtOx samples was examined by MTT assay of human HeLa cells. Figure 3A presents the polymer composition dependence of cell viability after a 24-hour incubation of cells with 1 mg/mL polymer solution. PEI30 (30% hydrolyzed PEtOx) and PEI70 (70% hydrolyzed PEtOx) were nontoxic compared with the controls, ie, nontreated cells. Viability decreased dramatically by 8.7% ± 0.54% (n = 3, P < 0.001) when the cells were incubated with PEI96 (96% hydrolyzed PEtOx), in comparison with nontreated cells. Figure 3B reports on cell viability after a 24-hour incubation with different concentrations (0 to 3 mg/mL) of polymer solutions. IC$_{50}$ values for PEIs of different acid hydrolysis were 14.29 mg/mL (PEI30 of 30% hydrolyzed PEtOx), 7.08 mg/mL (PEI70 of 70% hydrolyzed PEtOx), and 0.025 mg/mL (PEI96 of 96% hydrolyzed PEtOx), respectively.

**DNA inclusion and integrity**

We assessed plasmid DNA VR1412 containing β-galactosidase gene condensation and the integrity of these three PEIs by gel electrophoresis analysis. Linear

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<th>Table 1</th>
<th>Acid hydrolysis of PEtOx at 100°C and compositions of linear PEI</th>
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<td>Polymer</td>
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Note: *Determined using 'H nuclear magnetic resonance spectra.

**Abbreviations:** EL, ethylenimine; PEI, polyethylenimine; EtOx, ethyl-2-oxazoline; PEI30, 30% hydrolyzed PEtOx; PEI70, 70% hydrolyzed PEtOx; PEI96, 96% hydrolyzed PEtOx; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide.
PEI-DNA complexes were synthesized at different N/P ratios of linear PEI/DNA. N/P ratios were calculated on the basis of linear PEI nitrogen per DNA phosphate. Figure 4 shows the gel retardation result of linear PEI-DNA complexes with increasing N/P ratios for samples of 30% hydrolyzed PEtOx (PEI30, Figure 4A), 70% hydrolyzed PEtOx (PEI70, Figure 4B), and 96% hydrolyzed PEtOx (PEI96, Figure 4C). Intact β-galactosidase plasmid DNAs, before nanoparticle synthesis, are seen in lane 2 (Figure 4A–C). Complete retardation of PEI30/DNA was apparent at the N/P ratio of 10 (Figure 4A). However, both PEI70 and PEI96 started to form stable complexes at the N/P ratio of 3. Taken together, these results show that the efficiency of complexation is related to the fraction of $-\text{N(C=O) CH}_2-$ groups. The higher the hydrolysis of ethyleneimine units, the more efficient is neutralization of the DNA phosphate groups.

Figure 4 Electrophoresis analysis on 0.8% agarose gel. (A) PEI30-DNA, (B) PEI70-DNA, and (C) PEI96-DNA. Effect of different percentages of acid hydrolysis of poly(2-ethyl-2-oxazoline) on DNA binding efficiency at N/P ratios (polymer to DNA) from 0.5:1 to 100:1. Lane 1, ladder 1 Kb; lane 2, free naked DNA (1 µg/lane).

**Abbreviations:** PEI, polyethylenimine; PEI30, 30% hydrolyzed PETox; PEI70, 70% hydrolyzed PETox; PEI96, 96% hydrolyzed PETox.

**Zeta potential**

Solutions of different N/P ratios of PEI30-DNA, PEI70-DNA, and PEI96-DNA were prepared to measure their zeta potential. Zeta potential allows the measurement of surface charge of polymeric complexes, representing a critical factor in their interaction with cellular membranes. As shown in Figure 5A, zeta potential was directly proportional to N/P ratio. The zeta potentials of PEI30-DNA synthesized at any N/P ratios were negative. For PEI70 and PEI96, when the N/P ratio of all these polymeric/DNA complexes was around 1, the zeta potential was below zero, which could be attributed to the negatively charged phosphate groups in plasmid DNA and suggests plasmid DNA not bound by polymer. When PEI70 and PEI96 were mixed with plasmid DNA at the N/P ratio of 5, the zeta potential changed from negative to positive. At N/P ratios between 5 and 100, the zeta potential increased slightly. PEI70-DNA and PEI96-DNA complexes all had moderate positive surface charges.

**Particle size**

Particle size is a crucial factor in cellular uptake of polymeric complexes. The influence of different hydrolysis percentages...
Figure 5 Characterization of polymer-DNA complexes. The zeta potential (mV) (A) and size (nm) (B).

and N/P ratios on polymer and DNA complex sizes was monitored by dynamic light scattering assay. The particle size of the PEI-DNA complexes was measured (Figure 5B). The particle size of PEI30-DNA varied between 87.20 ± 8.69 nm in diameter (n = 3, N/P ratio = 1) and 470.33 ± 101.3 nm in diameter (n = 3, N/P ratio = 5). When the N/P ratio of PEI70-DNA nanoparticles was 1, the nanoparticle size was around 279.67 ± 7.86 nm in diameter (n = 3, Figure 5B) and zeta potential was close to zero (Figure 5A). When the N/P ratio increased, the PEI70-DNA nanoparticle size decreased to 116.83 ± 4.04 nm (n = 3, N/P ratio = 50) and the zeta potential increased past 6 mV. For PEI96 (96% hydrolyzed PEOx), larger particles (1,000 nm) were generated at N/P ratios of 1, 5, and 10. Compact complexes of PEI96-DNA formed at the N/P ratio of 50 (217.33 ± 30.94 nm in diameter, n = 3) and 100 (86.77 ± 7.12 nm in diameter, n = 3, Figure 5B).

Cytotoxicity of PEI-DNA complexes
To investigate the cytotoxicity of PEI/DNA complexes, cell viability was examined by MTT assay after a 24-hour incubation (Figure 6A). Cells without PEI/DNA complex treatment were considered as controls, with cell viability of 100%. Figure 6A shows the effects of PEIs of different acid hydrolysis to DNA N/P ratio on cell viability. Viable cells decreased slightly upon contact with free DNA (5 µg/well in 24-well plates, 89.5%) in comparison with untreated cells. DNA-Lipofectamine 2000 5 µg drastically reduced cell viability to below 49.0%. The results revealed over 65% average cell viability for PEI30-DNA and PEI70-DNA complexes formulated at various N/P ratios of 1:1, 5:1, 10:1, 50:1, or 100:1 (containing 5 µg of DNA). Viability declined significantly when the cells were incubated with PEI96-DNA at N/P ratios of 50:1 (60.9%) and 100:1 (44.3%).

In vitro transfection
The transfection efficiency of the different polymer series was tested on human HeLa cervical carcinoma cells. Increased amounts of polymer were complexed with 5 µg of plasmid encoding for β-galactosidase, an enzyme that catalyzes β-galactoside hydrolysis, and applied to cells for 6 hours in the absence of serum. β-galactosidase reporter gene activity and protein content were measured for 60 hours thereafter, according to the manufacturer’s instructions. For comparison, we included the commercial transfection agent Lipofectamine 2000 as a positive control in our assays because it is known to be among the most efficient in vitro transfection agents. Figure 6B presents the β-galactosidase activity expression of naked DNA, PEI30-DNA, PEI70-DNA, and PEI96-DNA at different N/P ratios.

HeLa cells treated with free β-galactosidase plasmid DNA (Figure 6B) achieved β-galactosidase activity expression of 2.84 ± 0.18 mU/mg protein (n = 6). PEI30-plasmid β-galactosidase DNA gave similar β-galactosidase activity expression for all N/P ratios (4.45 ± 0.46 mU/mg protein at N/P of 1; 4.26 ± 0.54 µg/mg protein at N/P of 5; 3.14 ± 0.40 mU/mg protein at N/P of 10; 3.81 ± 0.23 mU/mg protein at N/P of 50; and 3.21 ± 0.31 mU/mg protein at N/P of 100, respectively). At an N/P of 1, 5, and 10, PEI70-plasmid β-galactosidase DNA showed β-galactosidase expression similar to that of PEI30/DNA. However, PEI70-plasmid β-galactosidase DNA at an N/P of 50 and 100 significantly increased β-galactosidase activity expression levels by 7.98 ± 0.08 mU/mg protein (n = 6, P < 0.001 compared with PEI30/DNA-treated cells) and 13.17 ± 2.29 mU/mg protein (n = 6, P < 0.05 compared with PEI30/DNA-treated cells), respectively. HeLa cells transfected with PEI96-plasmid β-galactosidase DNA nanoparticles generated N/P ratios of PEI to DNA-dependent response: 3.03 ± 0.08 mU/mg protein at N/P of 1;
6.54 ± 0.83 mU/mg protein at N/P of 5; 11.12 ± 0.86 mU/mg protein at N/P of 10; 21.46 ± 1.19 mU/mg protein at N/P of 50; and 22.57 ± 2.41 mU/mg protein at N/P of 100, respectively. PEI-DNA complexes containing mismatched control plasmid DNA presented no β-galactosidase expression, confirming gene transfection efficiency (data not included).

Taken together, 30% hydrolyzed PEI30 exhibited similarly low efficiencies at all N/P ratios tested. PEI70 and PEI96 exhibited greater transfection efficiency than naked DNA. The transfection efficiency of PEI70 at N/P ratio of 100 was similar to that of Lipofectamine 2000. PEI96 mediated the strongest gene expression at N/P ratios of 50 and 100.

**siRNA delivery**

We tested SSB-targeted siRNA provided by Merck & Co., Inc., as validated siRNA to investigate the delivery ability of linear PEI prepared by partial acid hydrolysis of PEtOx. The ability of PEI to interact with siRNA was studied by agarose gel
electrophoresis (Figure 7). PEI30 did not bind siRNA completely at any weight ratio (Figure 7). However, complete siRNA binding to PEI70 and PEI96 at N/P ratios of 50:1, 100, and 150 could be observed. Only PEI-siRNA at a N/P ratio of 50 was selected for size and zeta potential measurement. Twenty-four hours after complex formation, nanoparticle size was 210.6 ± 21.9 nm, 95.10 ± 31.3 nm, and 88.94 ± 43.0 nm for PEI30-siRNA, PEI70-siRNA, and PEI96-siRNA, respectively. Their zeta potential (24 hours after complex formation) was 9.47 ± 0.67 mV (PEI30-siRNA), 6.48 ± 1.14 mV (PEI70-siRNA), and 13.17 ± 0.50 mV (PEI96-siRNA).

Cell viability after a 24-hour incubation with PEI-siRNA was tested by MTT assay. As illustrated in Figure 8A, an average HeLa cell viability of over 83% was noted for free siRNA-treated cells in comparison with nontreated cells (considered as 100%). Cell viability with PEI30-DNA, PEI70-DNA, or PEI96-siRNA complexes was not significantly different from that of free siRNA-treated cells; ie, 74%–88%.

The transfection efficiency of polymer-siRNA complexes was analyzed relative to SSB mRNA expression by real-time RT-PCR. HeLa cells (Figure 8B) treated with free SSB siRNA achieved approximately 15% knockdown of SSB mRNA expression compared with nontreated cells. No change in SSB mRNA expression was evident with PEI30-siRNA compared with nontreated cells (negative controls). However, silencing efficiency was dependent on and increased with acid hydrolysis percentage of PEtOx. PEI70-siRNA nanoparticles exerted a 28% gene silencing effect (Figure 8B), whereas HeLa cells transfected with PEI96-siRNA nanoparticles generated 57% gene silencing. PEI-siRNA complexes containing mismatched control siRNA presented no SSB inhibition, confirming gene silencing efficiency (data not included).

**Discussion**

Nonviral vectors such as PEI are attractive gene delivery systems for tumors and nonlethal diseases such as arthritis.
Compared with viral vectors, they are noninfectious, relatively nonimmunogenic, can accommodate large DNA plasmids or RNA, and may be produced on a large scale. However, the main drawback with PEIs as nonviral gene carriers is their typically high toxicity. To reduce PEI cell toxicity, we synthesized linear PEI by partial hydrolyzation of PEtOx. In our study, linear PEI was synthesized in two steps. First, PEtOx oligomers were synthesized by living cationic polymerization of 2-ethyl-2-oxazoline. Second, linear PEI was prepared by acid hydrolysis of PEtOx with excess hydrochloric acid. Three linear PEI derivatives were obtained by sampling a single polymerization mixture at various times. Our study showed that PEtOx with up to 70% ethylenimine segments (hydrolyzed EtOx) displayed very weak cell toxicity. In addition, the charge densities of these polymers were strong enough to condense DNA/siRNA at physiologic pH of 7.4. The transfection efficiency of PEI70 at an N/P ratio of 100 was similar to Lipofectamine 2000.

The results of our study are in good agreement with those in the literature. Poly(oxazolines) have been a subject of research since 1960, with special focus on the polymerization of 2-substituted oxazolines. Their applications include nanoscale systems such as membranes and nanoparticles, drug and gene delivery, as well as stimuli-responsive systems. Poly(oxazolines) are emerging as advanced synthetic biomaterials for DNA and RNA delivery. Their biocompatibility and cytotoxicity have been widely studied in vitro, showing that they do not exert any immunosuppressive effect or toxic behavior in cells.

Research over the years has revealed that branched PEI is one of the desirable polymeric carriers of DNA-siRNA because of its superior transfection efficiency and high cytotoxicity. The high transfection efficiency of branched PEI is attributed to its large “proton sponge” effect which permits DNA-siRNA condensation under physiologic pH conditions. Its large buffering capacity also allows complexes of PEI-DNA and PEI-siRNA to escape from endosomes, resulting in polyplex release into the cytoplasm. Simple modification of branched PEI leads to highly efficient siRNA carriers with low toxicity by altering amines with ethyl acrylate, acetylation of primary amines, or the introduction of negatively charged propionic acid or succinic acid groups to the polymer structure.

Linear PEI has been reported to be an effective gene carrier in vitro and in vivo, with lower cytotoxicity than branched PEI. Linear PEI is known to have toxicity as low as that of polyethylene glycol. In the present study, PEI30 (1 mg/mL) and PEI70 (1 mg/mL) were nontoxic compared with controls (nontreated cells). Viability decreased dramatically by 8.7% ± 0.54% (n = 3, P < 0.001) when the cells were incubated with PEI96 (1 mg/mL) in comparison with nontreated cells. IC_{50} values for PEIs of different acid hydrolysis were 14.29 mg/mL (PEI30), 7.08 mg/mL (PEI70), and 0.025 mg/mL (PEI96), respectively. These results suggest that the extent of PEtOx hydrolysis can be important for cell viability. Toxicity is mainly associated with the strong positive charge of this polycation. Our findings show that PEI30-DNA and PEI70-DNA nanoparticles have lower cytotoxicity than commercial transfection agents, such as Lipofectamine 2000, even at a high N/P ratio of 100:1. Average cell viability with PEI70-DNA at N/P ratios of 50 and 100 was between 65.7% and 67.6% compared with nontreated cells. The cytotoxicity observed for PEI96 with a high percentage of acid hydrolysis was similar to that of Lipofectamine 2000, which is considered to have high cell toxicity.

In general, polyplexes are toxic because of their strong interaction with negatively charged cell membranes. However, toxicity has to be balanced with transfection efficiency. The ability of PEI to deliver DNA and siRNA both in vitro and in vivo was reported recently. Our transfection experiment with PEI70-DNA demonstrated that similar transfection efficiency as with Lipofectamine 2000 can be obtained while lowering cytotoxicity. The influence of N/P ratio of PEI to DNA and varying hydrolysis percentages of PEtOx was explored. Thirty percent hydrolyzed PEI30 exhibited similarly low efficiencies at all N/P ratios tested. Seventy percent hydrolyzed PEI70 and 96% hydrolyzed PEI96 exhibited greater transfection efficiency than naked DNA, depending on their N/P ratios. Higher N/P ratios of PEI70 and PEI96 provided better transfection efficiency. The N/P ratio seems to play a critical role in cell transfection. However, PEI30 and PEI96 cannot be considered as suitable transfection agents because of low transfection efficiency (PEI30) and high cellular toxicity (PEI96). Only 70% hydrolyzed PEI70 showed higher transfection and lower cytotoxicity than the two other polymers. Taken together, it is expected that PEI70 can serve as a favorable candidate for gene delivery with both in vitro and in vivo applications.

siRNA silencing technology has been exploited in a wide range of biological studies, but has also become one of the most challenging therapeutic strategies. However, because of its poor delivery and susceptibility to nuclease degradation, siRNA-based approaches need a protective transport system. Unprotected, naked siRNAs are relatively unstable in blood and serum and have short half-lives in vivo.
siRNAs do not freely cross cellular membranes because of their large molecular weight (approximately 13 kDa) and strong anionic charge. They are rapidly degraded by nuclease.

A variety of polymer formulations have been proposed as potential carriers in the literature. Research over the years has revealed that PEI is one of the desirable polymeric carriers of DNA because of its ability to form stable complexes by electrostatic interactions with nucleic acids. It has been shown to be less effective in siRNA delivery. It is toxic at high doses. Toxicity is mainly associated with its strong positive charge, which leads to strong interaction with cell membrane surfaces. Our results show that PEI-siRNA toxicity was greatly reduced by the polymer molecular weight, charge density, and ratio of protonatable polymer amine groups to nucleic acid phosphate groups, while the ionic strength of the medium can affect electrostatic binding between siRNA and cationic polymers. In comparison with DNA delivery, binding requires higher polymer to siRNA N/P ratios. Such differences are caused by the fact that short length siRNA (21–25 base pairs) and linearity bind to PEI in a manner different to that observed with plasmid DNA. PEIs, which have known efficiency in DNA delivery, were found to be far less effective for application with siRNA. PEI30 did not show any gene knockdown. In the current study, 70% hydrolyzed PEI70 had a 30% gene-silencing effect. PEI96 was able to induce more than 50% silencing of the SSB gene at an siRNA of 5 µg in HeLa cells (relative to untreated cells).

**Conclusion**

In conclusion, linear PEIs were prepared by partial acid hydrolysis of PEtOx, which showed high efficiency in DNA delivery. In particular, PEI70 resulted in lower polymer toxicity in comparison with PEI96 (high hydrolysis percentage). PEI70 completely condensed DNA and formed nanoparticles (120 nm) at a PEI70 to DNA N/P ratio of 50:1. Compared with PEI30 (low hydrolysis percentage), PEI70 improved transfection efficiency. Formulation of siRNA with PEI70 induced remarkable target gene knockdown. These findings may encourage further exploration of the utility of PEI as a nonviral gene delivery vector.

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**Disclosure**

The authors report no conflicts of interest in this work.

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