
Research Paper

Biophysical and Structural Characterization of Polyethylenimine-Mediated siRNA Delivery *in Vitro*

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Purpose. The goals of this study were as follows: 1) to evaluate the efficacy of different polyethylenimine (PEI) structures for siRNA delivery in a model system, and 2) to determine the biophysical and structural characteristics of PEI that relate to siRNA delivery.

Materials and Methods. Biophysical characterization (effective diameter and zeta potential), cytotoxicities, relative binding affinities and *in vitro* transfection efficiencies were determined using nano-complexes formed from PEI's of 800, 25,000, (both branched) and 22,000 (linear) molecular weights at varying N:P ratios and siRNA concentrations. The HR5-CL11 cell line stably expressing luciferase was used as a model system *in vitro*.

Results. Successful siRNA delivery was observed within a very narrow window of conditions, and only with the 25,000 branched PEI at an N:P ratio of 6:1 and 8:1 and with 200 nM siRNA. While the zeta potential and size of PEI:siRNA complexes correlated to transfection efficacy in some cases, complex stability may also affect transfection efficacy.

Conclusions. The ability of PEI to transfer functionally active siRNA to cells in culture is surprisingly dependent on its biophysical and structural characteristics when compared to its relative success and ease of use for DNA delivery.

KEY WORDS: luciferases; polyethylenimine; RNA interference; small interfering RNA; transfection.

INTRODUCTION

The discovery that duplex RNA can silence genes with high specificity (1,2), termed RNA interference, created a potentially new therapeutic class that reduces or eliminates undesirable protein expression. The RNA interference mechanism can be mediated in several ways, including small interfering RNA (siRNA, 21–25 nucleotide duplex RNA) (2–4), short hairpin RNA (shRNA) (5–10), and siRNA expression cassettes (11,12). Herein we focus our attention on siRNA.

Two biophysical characteristics that hinder the intracellular delivery of siRNA are that it is polyanionic and macromolecular. The polyanionic character of siRNA may limit its interaction with the cell membrane to internalization via adsorptive endocytosis in regions of the cell membrane having positive charge, while the macromolecular nature of siRNA restricts its passive diffusion through the cell membrane. It is generally accepted that delivery reagents for

siRNA must overcome both of these barriers to enable cellular uptake and transport to the cytosol. One common approach to facilitating the transport of siRNA to the cytosol is to use reagents originally used for DNA delivery. Our interests lie in the use of polyethylenimine (PEI) to better understand the structural and biophysical characteristics of delivery reagents that relate to siRNA transfection efficiency.

PEI is a polycation that is widely used to deliver nucleic acids, including DNA (13), siRNA (14–17), ribozymes (18,19), and oligonucleotides (13,20). The prevailing hypothesis is that the positively charged amine groups of PEI electrostatically complex with the negatively charged phosphate groups of nucleic acids to produce a neutral or positively charged complex with sufficient stability to allow intracellular delivery of the genetic material. PEI is also believed to facilitate nucleic acid delivery through a proton sponge mechanism wherein the osmotic pressure within an acidified vesicle is enhanced leading to destabilization and rupture (13,21,22). Polycations, and PEI in particular, are therefore widely believed to be useful siRNA delivery reagents since siRNA, like DNA, has negatively charged phosphate groups that allow electrostatic complexation with the polycation of interest. Further, the proton sponge mechanism of PEI hypothetically will lead to release of intact siRNA into the cytosol where it can initiate the RNA interference pathway. Recent reports in the literature show that PEI can deliver siRNA both *in vitro* and *in vivo* (14–17). However, a systematic study of different PEI structures to elucidate the chemical and biophysical characteristics related

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ABBREVIATIONS: HEPES, 4-(2-hydroxyethyl) piperazine-1-ethanesulfonic acid; PEI, polyethylenimine; siRNA, small interfering RNA; 22 K L-PEI, 22,000 molecular weight linear PEI; 25 K B-PEI, 25,000 molecular weight branched PEI; 800 B-PEI, 800 molecular weight branched PEI.

to siRNA delivery efficacy has yet to be reported. The goals of the work reported here are 1) to evaluate the efficacy of different PEI structures for siRNA delivery in a model system, and 2) to determine the biophysical and structural characteristics of PEI that relate to successful delivery of siRNA.

MATERIALS AND METHODS

Polyethylenimine Polymers

Branched polyethylenimine (PEI) of 25,000 and 800 molecular weights (25 K B-PEI and 800 B-PEI, respectively) were obtained from Sigma-Aldrich (St. Louis, MO). Linear PEI of 22,000 molecular weight (22 K L-PEI) was obtained from Polysciences (Warrington, PA). These molecular weights and structures of PEI were chosen based on transfection studies reported in the literature for plasmid DNA (13,24–32) and siRNA delivery (14–17). ^1H NMR analysis of the polymers were consistent with those previously reported elsewhere (17,48). Detailed ^1H NMR analysis of the linear PEI showed that less than 1% of the *N*-propionyl protecting group remained on the polymer, an issue outlined in a recent manuscript (17).

siRNA Sequences

Duplex siRNA targeted to positions 153–171 relative to the first nucleotide of the start codon of the GL2 luciferase gene (GenBank accession no. X65324, sense sequence 5'-CGU ACG CGG AAU ACU UCG A dTdT-3') and non-specific control duplex siRNA (sense sequence 5'-AUG UAU UGG CCU GUA UUA G UU-3'), 20 nmol each, were purchased from Dharmacon, Inc. (Lafayette, CO).

Effective Diameter and Zeta Potential

All buffers were sterilized by autoclave and/or 0.22 μm filtration prior to use. Nanocomplexes of anti-luciferase GL2 duplex siRNA and PEI's were formed in a manner similar to a previously published procedure (33). A 20 μM stock solution of siRNA was first diluted in 10 mM sterile 4-(2-hydroxyethyl) piperazine-1-ethanesulfonic acid (HEPES) buffer of pH 7.2 to give siRNA aliquots with a final volume of 50 μl in sterile microcentrifuge tubes. The volume of siRNA stock solution used, V_{siRNA} , was calculated as follows:

$$V_{\text{siRNA}} = (C_{\text{siRNA,final}} \times V_{\text{final}}) / (C_{\text{siRNA,stock}}) \quad (1)$$

where $C_{\text{siRNA,final}}$ = final desired concentration of siRNA in the sample (200 or 750 nM for size or zeta potential measurements, respectively), V_{final} = final sample volume (0.75 or 0.80 ml for the size or zeta potential measurements, respectively), and $C_{\text{siRNA,stock}}$ = concentration of siRNA stock solution (20 μM). The different PEI structures were diluted in HEPES buffer of pH 7.2 to make stock solutions having concentrations of 0.1 mg/ml (800 and 25 K B-PEI) or 0.5 mg/ml (22 K L-PEI). Aliquots of PEI dilutions with a total volume of 50 μl were prepared by adding the appropriate volume of PEI stock solutions and HEPES to microcentrifuge tubes. The volume of PEI stock solution

required (V_{PEI}) for a given sample was determined as follows:

$$V_{\text{PEI}} = (C_{\text{siRNA,final}} \times M_{\text{w,siRNA}} / M_{\text{w,P}} \times \Phi_{\text{N:P}} \times \Phi V_{\text{final}}) / (C_{\text{PEI,stock}} / M_{\text{w,N}}) \quad (2)$$

where $M_{\text{w,siRNA}}$ = molecular weight of siRNA duplex, $M_{\text{w,P}}$ = molecular weight per phosphate group of siRNA, $\Phi_{\text{N:P}} = \text{N:P}$ ratio (the molar ratio of nitrogens from the PEI to the ratio of phosphates from the siRNA), $C_{\text{PEI,stock}}$ = concentration of PEI in the stock solution (0.1 or 0.5 mg/ml), and $M_{\text{w,N}}$ = molecular weight per nitrogen of PEI (43 g/mol).

The 50 μl siRNA aliquots were added to the microcentrifuge tubes containing the 50 μl diluted PEI aliquots. The tubes were then vortexed for 5 s each, and incubated at room temperature for at least 20 min to allow complex formation.

Effective diameters and zeta potentials of nanocomplexes were characterized on a Malvern Zetasizer Nano-ZS using Dispersion Technology Software 4.00. Size measurements were performed on samples prepared by diluting 100 μl of the PEI:siRNA complexes with an additional 0.65 ml of 10 mM sterile HEPES buffer, pH 7.2, in Sarstedt microcuvettes to a final siRNA concentration of 200 nM and volume of 0.75 ml. Three measurements, each consisting of three runs of 10 s each, were performed on each sample using a fixed position of 4.65 mm and attenuator setting of 11. Zeta potential measurements were performed on samples prepared by diluting 100 μl of PEI:siRNA complexes with an additional 0.7 ml of 10 mM sterile HEPES buffer, pH 7.2, to a final siRNA concentration of 750 nM and volume of 0.8 ml. More concentrated samples were required for zeta potential measurements than for sizing measurements in order to obtain a detectable count rate. Three measurements, each consisting of 20 runs, were performed on each sample. The Smoluchowski model was used to calculate zeta potential values.

Gel Retardation

Stock 20 μM anti-luciferase GL2 duplex siRNA solution was diluted in sterile 10 mM HEPES pH 7.2 to a concentration of 2 μM . The diluted siRNA was then added to sterile microcentrifuge tubes containing varying volumes of HEPES and PEI solutions to produce samples with a final siRNA concentration of 1 μM and N:P ratios of 1:1, 3:1, 6:1, 8:1, and 10:1. The tubes were vortexed for 5 s each and incubated at room temperature for at least 20 min. Control complexes of RNAiFect™ (Qiagen, Valencia, CA) and siRNA were formed in OPTI-MEM® I (Gibco® Cell Culture, a division of Invitrogen Corporation, Carlsbad, CA) according to the manufacturer's protocol. Aliquots of 20 μl of each sample were combined in fresh sterile microcentrifuge tubes with 4 μl of Blue/Orange Loading Dye, 6X (Promega, Madison, WI) and mixed by pipetting. Aliquots of 20 μl of the prepared samples were then loaded on a 4% agarose gel (Invitrogen Corporation, Carlsbad, CA) containing 0.5 $\mu\text{g/ml}$ ethidium bromide (Fisher Scientific, Agawam, MA). The gel was run in Tris-Acetate-EDTA (TAE) buffer in a Bio-Rad

Wide Mini Sub-Cell GT System at 100 V for 20 min. Digital imaging was performed on a Bio-Rad ChemiDoc system using Quantity One v4.4.1 software. Image contrast was enhanced using Adobe® Photoshop®.

Relative Binding Affinity

The relative strength of electrostatic binding between GL2 anti-luciferase siRNA or pCMV-luc plasmid DNA (Elim Biopharmaceuticals, Hayward, CA) and PEI was measured using ethidium bromide (EB) fluorescence quenching, a technique previously reported in the literature for use with DNA (34). Stock solutions of siRNA, EB, and PEI were combined in sterile 25 mM HEPES buffer, pH 7.2, in 96-well plates. The final concentration of EB was approximately 0.0047 mM, and a 2:1 ratio of phosphate groups to EB monomer units was used. Plates were incubated at room temperature for 20 min, and then read on a Molecular Devices SpectraMax Gemini EM at $\lambda_{\text{exc}} = 535$ nm and $\lambda_{\text{emi}} = 595$ nm.

siRNA Transfection

The HR5-CL11 cell line, a HeLa derivative, was purchased from ECACC (the European Collection of Cell Cultures, Health Protection Agency, Salisbury, U.K.). Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) with 10% v/v fetal bovine serum (FBS), 100 U/ml of penicillin and 100 µg/ml of streptomycin (all cell culture reagents were obtained from Gibco®), and the equivalent of 1 µg/ml doxycycline (prepared from doxycycline hyclate, Sigma-Aldrich). Cells were maintained at 37°C and 5% CO₂, and passaged every 3 to 4 days.

One day before siRNA transfection, 8,000 cells/well of the HR5-CL11 cell line were plated into sterile opaque and clear 96-well plates. Cells were plated in medium without phenol red and incubated at 37°C and 5% CO₂ overnight. Just prior to transfection, the culture medium was removed from the wells and replaced with 100 µl of OPTI-MEM® I (Gibco® Cell Culture, a division of Invitrogen Corporation, Carlsbad, CA). Aliquots of 25 µl of PEI:siRNA complexes were formed in 10 mM pH 7.2 sterile HEPES buffer, by adding appropriate volumes of PEI stock solutions (0.1 or 0.5 mg/ml) to HEPES in sterile microcentrifuge tubes. siRNA was briefly vortexed before being added to the tubes containing PEI and HEPES. The volumes of PEI, siRNA, and HEPES required were calculated using Eqs. (1) and (2), with $V_{\text{final}} = 125$ µl (100 µl of OPTI-MEM® I plus 25 µl of complexes) and $C_{\text{siRNA,final}} = 200$ nM. Tubes were vortexed for 5 s to mix the solutions, and incubated at room temperature for at least 20 min to allow complex formation. To each well of the plates were then added 25 µl of complexes. Experiments were performed in sextuplicate: three wells were treated with each condition in opaque 96-well plates for luciferase determination, and three wells were treated with each condition in clear 96-well plates for total protein determination. Plates were incubated at 37°C and 5% CO₂ for 24 h, at which time the OPTI-MEM® I with complexes was removed from the wells and replaced with 100 µl of culture medium per well. Plates were incubated for a further 24 h at 37°C and 5% CO₂ before assaying for

luciferase and total protein using the Bright-Glo™ Luciferase Assay (Promega) and BCA™ Protein Quantitation Assay (Pierce Biotechnology).

The following positive control was used to ensure that the HR5-CL11 cell system was an accurate model to study siRNA activity. Cells that were transfected with RNAiFect™ as a control were plated at a density of 4,000 cells/well and received 50 nM siRNA. Transfection medium for these cells consisted of approximately 0.2 µl of RNAiFect™, 0.25 µl of siRNA, 75 µl of cell culture medium, and 24.55 µl of OPTI-MEM® I according to the manufacturer's directions for a total transfection volume of 100 µl. These cells were incubated with the transfection medium for a total of 48 h before assaying for luciferase and total protein using the Bright-Glo™ Luciferase Assay and BCA™ Protein Quantitation Assay.

Bright-Glo™ Luciferase Assay

After a total incubation time of 48 h since transfection, 100 µl of Bright-Glo™ working solution, prepared according to the manufacturer's directions (Promega), were added to each well of the opaque 96-well plates containing treated cells. Plates were incubated at room temperature for approximately 3 min and were read on a Molecular Devices SpectraMax Gemini EM (automatic calibration, 5 s of mixing prior to reading, automatic sensitivity of PMT detector, ten readings per well in luminescence top-read mode, and 96 well standard opaque assay plate).

BCA™ Protein Quantitation Assay

After a total incubation time of approximately 48 h since transfection, culture medium was removed from the clear 96-well plates and each well was rinsed with 200 µl of phosphate buffered saline. Aliquots of 30 µl of RIPA buffer (150 mM NaCl, 1% Igepal CA-630, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate in 50 mM pH 8.0 Tris buffer, and 0.5% protease inhibitor cocktail, cat.# P8340, all chemicals from Sigma-Aldrich) were then added to each well. Plates were incubated at 4°C for at least 12 min, and 10 µl of lysate from each well were pipetted into a new clear 96-well plate. To each well were then added 200 µl of BCA™ working solution, prepared according to the manufacturer's directions (Pierce Biotechnology). Plates were incubated at 37°C and 5% CO₂ for 30 min before being read at 562 nm on a Molecular Devices SpectraMax Plus³⁸⁴.

Cytotoxicity

HR5-CL11 cells were plated in 200 µl of culture medium at a density of 8,000 cells/well in clear 96-well plates. Twenty-four hours after plating, PEI solutions were diluted in cell culture medium in sterile microcentrifuge tubes to various concentrations. The tubes were vortexed for 5 s each to ensure solution uniformity. Cell culture medium was removed from the 96-well plates and replaced with 75 µl of cell culture medium without doxycycline and 75 µl of the PEI dilutions per well. Final concentrations of each PEI were 0, 0.01, 0.07, 0.13, 0.34, 0.67, 1.01, 1.35, 2.02, 2.70, 5, 10, 16.87, 20, and 50 µg/ml. Plates were incubated at 37°C and 5% CO₂

for 48 h after addition of the PEI to the wells. The CellTiter 96[®] AQueous One Solution Cell Proliferation Assay (Promega) was performed according to the manufacturer's instructions. Plates were then incubated at 37°C and 5% CO₂ for two and one-half hours before the absorbance was read at 490 nm on a Molecular Devices SpectraMax Plus³⁸⁴. Sigmoidal Boltzmann fits were performed to the data points using the XLfit4 add-in (ID Business Solutions, Inc., Emeryville, CA) for Microsoft Excel. IC₅₀ values were calculated as the concentration of PEI, in micrograms per milliliter, at which the calculated absorbance was one-half the absorbance measured at a PEI concentration of 0 µg/ml.

Statistical Analysis

Statistical significance of results was evaluated using two-tailed heteroscedastic Student's *t*-tests according to the TTEST function in Microsoft Excel.

RESULTS

Effective Diameter and Zeta Potential

The measured effective diameters of nanocomplexes formed from siRNA designed to inhibit the GL2 version of the luciferase gene (GL2 siRNA) and the three PEI's of interest are shown in Fig. 1. Complexes were made with varying N:P ratios. With the exception of complexes having an N:P ratio of 3:1, the complexes formed from 25 K B-PEI were always smaller than those formed from either 800 B-PEI or 22 K L-PEI at a given N:P ratio. The 25 K B-PEI complexes at 1:1, 6:1, and 8:1 N:P ratios were smaller than 150 nm, which is considered the size limit for nonspecific endocytosis via clathrin-coated pits (35). The smallest 22 K L-PEI complexes, at an N:P ratio of 8:1, were 155 nm in diameter. The 800 B-PEI complexes did not form structures smaller than 195 nm (at an N:P ratio of 1:1).

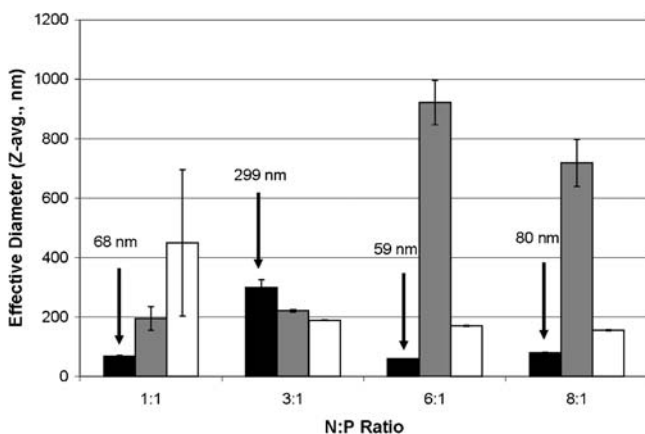


Fig. 1. Effective diameters of complexes formed from 200 nM anti-luciferase GL2 duplex siRNA and PEI's of various structures at different N:P ratios. *Solid bars:* 25 K B-PEI; *shaded bars:* 800 B-PEI; *open bars:* 22 K L-PEI. *Error bars* are the standard deviation (SD) of three measurements performed on the same sample. Results shown are typical of two separate experiments.

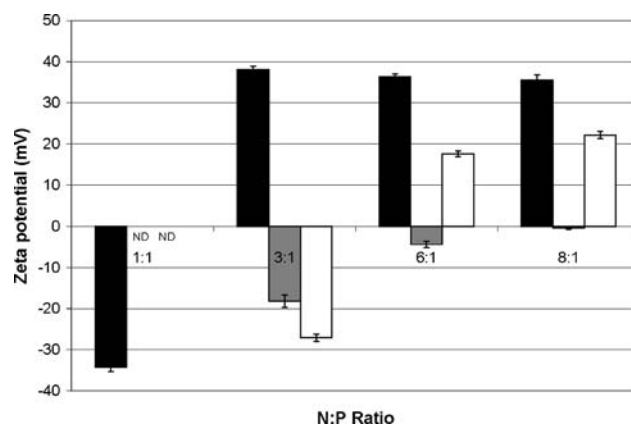


Fig. 2. Zeta potentials of complexes formed from 750 nM anti-luciferase GL2 duplex siRNA and PEI's of various structures at different N:P ratios. *Solid bars:* 25 K B-PEI; *shaded bars:* 800 B-PEI; *open bars:* 22 K L-PEI. *Error bars* are the standard deviation of three measurements performed on the same sample. *ND* Not determinable.

The measured zeta potentials of complexes formed from GL2 siRNA and the three PEI's are shown in Fig. 2. Although zeta potentials were not determinable for the 800 B-PEI or 22 K L-PEI complexes at an N:P ratio of 1:1, the 25 K B-PEI complexes at this N:P ratio had a large negative zeta potential (-34 mV). Increasing the N:P ratio to 3:1 caused the 25 K B-PEI complexes to have a large positive zeta potential (+38 mV), while both the 800 B-PEI and 22 K L-PEI complexes at this same N:P ratio remained negatively charged. It seems likely that the complexes formed from the 800 B-PEI and 22 K L-PEI at an N:P ratio of 1:1 also have negative zeta potentials, since they have less positively charged PEI than the complexes at an N:P ratio of 3:1. Complexes having N:P ratios of 6:1 and 8:1 and consisting of 800 B-PEI remained negatively charged, while 25 K B-PEI and 22 K L-PEI complexes at these N:P ratios were positively charged.

Gel Retardation

Gel retardation of the siRNA:polymer complexes (results not shown) was performed to corroborate the zeta potential data. Lanes including siRNA only, buffer only, and complexes formed from RNAiFect[™] commercial transfection reagent were included as controls. Negatively charged samples are expected to migrate into the gel, while neutral or positively charged samples are expected to remain at the top of the gel.

The observed behavior of the PEI complexes in the gel is consistent with the measured and expected zeta potentials of the complexes. The 25 K B-PEI complexes at an N:P ratio of 1:1 migrated into the gel, indicating the presence of either negatively charged complexes or uncomplexed siRNA. The remainder of the 25 K B-PEI complexes at higher N:P ratios did not migrate, suggesting the siRNA is fully complexed at these N:P ratios. The 22 K L-PEI complexes at N:P ratios of 1:1 and 3:1 also migrated into the gel. At N:P ratios of 6:1 or larger, no migrated bands were seen for 22 K L-PEI complexes, implying they were neutral or positively charged. These results are consistent with the measured zeta potentials. The 800 B-PEI complexes migrated to the bottom of the

gel at all tested N:P ratios, in agreement with the measured negative zeta potentials for these complexes.

Relative Binding Affinity

Figure 3a shows the relative binding affinity, as measured by ethidium bromide fluorescence quenching, for 25 K B-PEI, 800 B-PEI, and 22 K L-PEI complexed with GL2 siRNA. The interaction of these PEI's with plasmid DNA (pCMV-luc) was also characterized for comparison (Fig. 3b). Ethidium bromide fluoresces when it is intercalated between double stranded nucleic acids. Electrostatic complexation of the nucleic acid with a polycation, however, excludes the ethidium bromide from the nucleic acid and causes a reduction in the fluorescence. The reduction in fluorescence is therefore correlated with the relative binding affinity of the polycation and nucleic acid for one another (36).

At N:P ratios less than one, the binding affinities of all three PEI's for siRNA were statistically similar. Between N:P ratios of 1 and 2, the 800 B-PEI had the greatest affinity for siRNA, as indicated by the lowest level of fluorescence. This result was statistically significant compared to that of the 25 K B-PEI at N:P ratios of 1:1 and 1.5:1, and from that of the 22 K L-PEI at an N:P ratio of 1:1 ($p < 0.001$). The 25 K B-PEI had intermediate affinity for siRNA in the same range of N:P

ratios, while the 22 K L-PEI had the lowest affinity for siRNA. At $3:1 \leq N:P \leq 6:1$, the relative affinities of the 800 and 25 K B-PEI for siRNA were statistically identical. At N:P ratios of 8:1 and 10:1 the difference in binding affinities of these two polymers was statistically significant ($p < 0.001$), with the 25 K B-PEI exhibiting stronger binding affinity than the 800 B-PEI for the siRNA (0.89 ± 1.1 and 0.44 ± 1.08 RFU for 25 K B-PEI at 8:1 and 10:1 compared to 3.62 ± 0.72 and 2.74 ± 0.67 RFU for 800 B-PEI at 8:1 and 10:1). The 22 K L-PEI did not achieve a statistically similar binding affinity as the 800 B-PEI until the N:P ratio was 6:1 or larger, and only attained a similar binding affinity to that of the 25 K B-PEI at an N:P ratio of 10:1 ($p > 0.001$).

In contrast, the affinity of the three PEI structures for DNA was quite different. The 25 K B-PEI had the highest affinity for DNA over the entire range of N:P ratios tested. The binding affinity of the 25 K B-PEI was statistically different ($p < 0.001$) from that of the 800 B-PEI at an N:P ratio of 1.5, and from that of the 22 K L-PEI at N:P ratios of 1.5:1, 8:1, and 10:1. Although the 800 B-PEI had intermediate affinity for DNA when the N:P ratio ranged from 1:1 to 2:1, and the lowest affinity of all three PEI structures when the N:P ratio was less than 1:1, these results were not statistically significant ($p > 0.001$).

siRNA Transfection

Wide ranges of N:P ratios and siRNA concentrations were used for transfection to explore the effect of these two parameters on transfection efficacy. The HR5-CL11 cell line, a HeLa cell line derivative that stably expresses luciferase via a tetracycline-controlled transcriptional transactivator, was used as a model system (37). Our results will be particularly relevant for the molecular therapy field, as the HeLa cell line is a popular model system for molecular medicine studies. Figure 4a shows the reduction in luciferase expression in HR5-CL11 cells treated with PEI:siRNA complexes when the siRNA concentration was kept constant at 200 nM and the N:P ratio was varied from 0:1 to 10:1, corresponding to final PEI concentrations in the wells ranging from 0.0–3.37 $\mu\text{g/ml}$. Luciferase expression (relative light units, RLU) was normalized by the total protein content (absorbance at 562 nm, A_{562}) to decouple cytotoxicity from luciferase knock-down. Cells transfected with RNAiFect™, a commercially available siRNA transfection reagent, are shown for comparison. No difference in luciferase expression was observed between cells that received anti-luciferase GL2 or nonspecific control duplex siRNA without PEI (N:P ratio of 0:1), indicating that the anti-luciferase siRNA does not mediate luciferase knockdown in the absence of a delivery reagent. At N:P ratios of 1:1 and 3:1 no reduction in luciferase expression was observed for cells treated with anti-luciferase GL2 siRNA and any of the PEI structures compared to cells that received nonspecific control siRNA with PEI. Cells that received 25 K B-PEI with anti-luciferase siRNA (solid bars) at an N:P ratio of 6:1 had reduced luciferase expression compared to cells that received the 25 K B-PEI with nonspecific control siRNA (diagonally striped bars), although this result was not statistically significant ($p \approx 0.035$). At N:P ratios of 8:1 and 10:1 the reduction in luciferase expression for cells that received the 25 K B-PEI with anti-

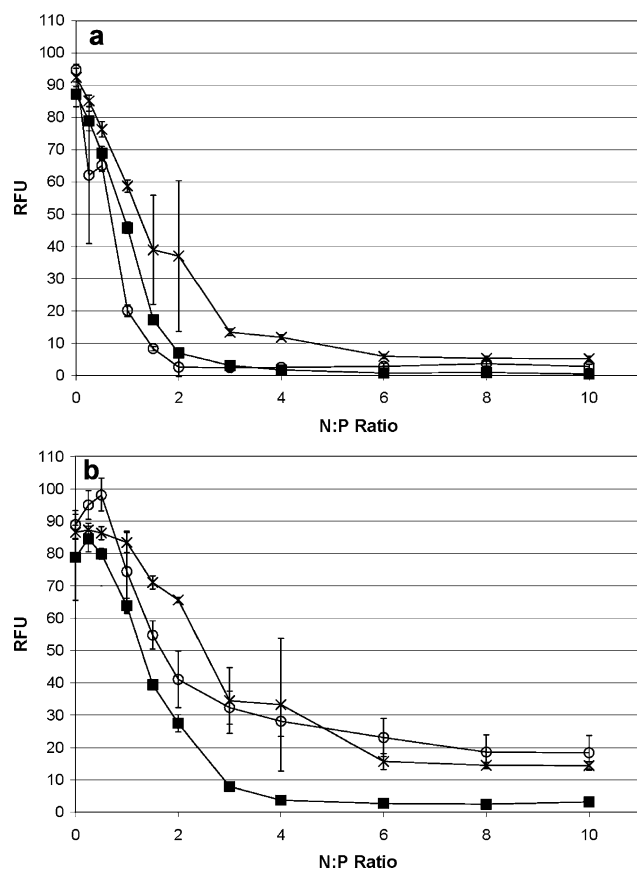


Fig. 3. Relative binding affinity of 25 K B-PEI (■), 800 B-PEI (○), and 22 K L-PEI (×) for nucleic acids as measured by ethidium bromide fluorescence quenching. (a) PEI with anti-luciferase GL2 duplex siRNA. (b) PEI with pCMV-luc luciferase plasmid DNA. Data are the average \pm SD ($n = 3$).

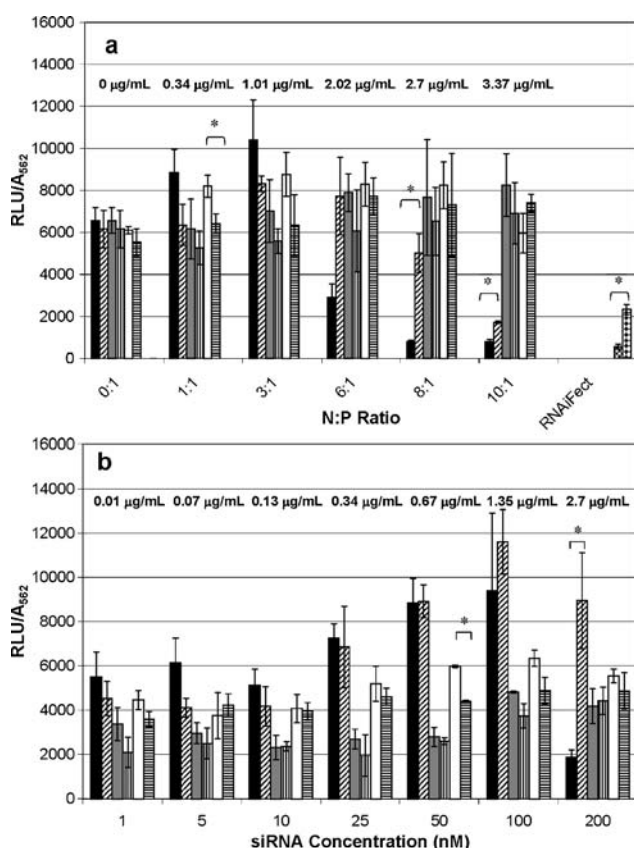


Fig. 4. Luciferase knockdown 48 h after transfection with varying N:P ratios and siRNA concentrations. Experiments for each N:P ratio and siRNA concentration were conducted with both anti-luciferase GL2 siRNA and nonspecific control siRNA to control for potential off-target effects. Native luciferase expression levels were comparable to or less than the 0:1 sample in 4A. *Solid bars*: 25 K B-PEI with anti-luciferase GL2 siRNA; *diagonally striped bars*: 25 K B-PEI with nonspecific control duplex siRNA; *shaded bars*: 800 B-PEI with anti-luciferase GL2 siRNA; *vertically striped bars*: 800 B-PEI with nonspecific control duplex siRNA; *open bars*: 22 K L-PEI with anti-luciferase GL2 siRNA; *horizontally striped bars*: 22 K L-PEI with nonspecific control duplex siRNA. Final concentration of PEI ($\mu\text{g/ml}$) in wells after transfection indicated for each sample group at top of panel. Data are the average \pm SD ($n = 3$). (a) 200 nM siRNA complexed with PEI's at varying N:P ratios. $p < 0.015$ indicated by * symbol; RNAiFect control experiments performed with 4,000 cells/well, 50 nM siRNA, and 0.2 μl /well of RNAiFect. *Checkered bars*: RNAiFect with GL2 anti-luciferase siRNA; *diagonally checkered bars*: RNAiFect with nonspecific control duplex siRNA. (b) Varying concentrations of siRNA complexed with PEI at 8:1 N:P ratio. $p < 0.03$ indicated by * symbol.

luciferase GL2 siRNA was statistically significant ($p < 0.015$ and 0.001, respectively) compared to cells that received the nonspecific control siRNA with 25 K B-PEI (diagonally striped bars). At an N:P ratio of 10:1 there was a marked reduction in luciferase expression in cells that received the nonspecific control siRNA, suggesting the possibility of sequence non-specific effects on luciferase expression. Neither the 800 B-PEI nor 22 K L-PEI appeared to mediate a statistically significant reduction in luciferase expression.

Figure 4b shows the luciferase expression in HR5-CL11 cells treated with PEI:siRNA complexes when the N:P ratio was kept constant and the siRNA concentration was varied

from 1 to 200 nM. An N:P ratio of 8:1 was selected for these experiments as this was the lowest N:P ratio that produced a statistically significant reduction in luciferase expression in Fig. 4a. The final PEI concentration in the wells ranged from 0.01–2.7 $\mu\text{g/ml}$. A statistically significant reduction in luciferase expression was only observed when 25 K B-PEI was complexed with anti-luciferase siRNA at a concentration of 200 nM. No reduction in luciferase expression was observed when 25 K B-PEI was used with lower concentrations of siRNA, or when 800 B-PEI or 22 K L-PEI were used at any siRNA concentration. These data were consistent over a series of similar experiments (data not shown).

Cytotoxicity

To ensure that polymer cytotoxicity was not a significant factor in these studies, the IC_{50} values for the 25 K B-PEI, 800 B-PEI, and 22 K L-PEI were calculated and are summarized in Table I. Figure 5 shows representative data and a fitted curve for HR5-CL11 survival as a function of 25 K B-PEI concentration. The concentration of 25 K B-PEI that was found to mediate RNA interference in Fig. 4 (2.7 $\mu\text{g/ml}$ at an siRNA concentration of 200 nM and N:P ratio of 8:1) is indicated on Fig. 5 for comparison. The observed relative cytotoxicities of the three PEI structures, in order from least to most cytotoxic, are 800 B-PEI < 22 K L-PEI < 25 K B-PEI.

DISCUSSION

The results of the siRNA transfection studies indicate that only the 25 K B-PEI complexed with siRNA at a concentration of 200 nM and at an N:P ratio of 6:1 or 8:1 was capable of mediating functional siRNA delivery while avoiding sequence non-specific effects. Other researchers have recently reported success using PEGylated 25 K B-PEI for both *in vitro* and *in vivo* siRNA delivery (23). It is not surprising that 25 K B-PEI complexes in the present studies were able to deliver siRNA, as the 25 K B-PEI complexes at N:P ratios of 6:1 or larger had consistently small sizes and positive zeta potentials. The poor transfection efficacy of 25 K B-PEI complexes at N:P ratios of 1:1 and 3:1 are consistent with their negative zeta potentials and large sizes, respectively. The fact that gene knockdown was only observed at a siRNA concentration of 200 nM is somewhat surprising. In other work from our lab, the inhibition of luciferase in the same cell model system has been shown using lower concentrations of siRNA when cationic lipids and other commercial transfection reagents are used (manuscript submitted). However, the delivery of siRNA using cationic lipids has been shown to stimulate interferon response, both

Table I. Calculated IC_{50} Values of Various PEI Structures After 48 h Incubation in 96-Well Plates Containing 8,000 HR5-CL11 Cells per Well

PEI		Calculated IC_{50} ($\mu\text{g/ml}$)
Molecular weight	Structure	
25,000	Branched	19
800	Branched	>50
22,000	Linear	37

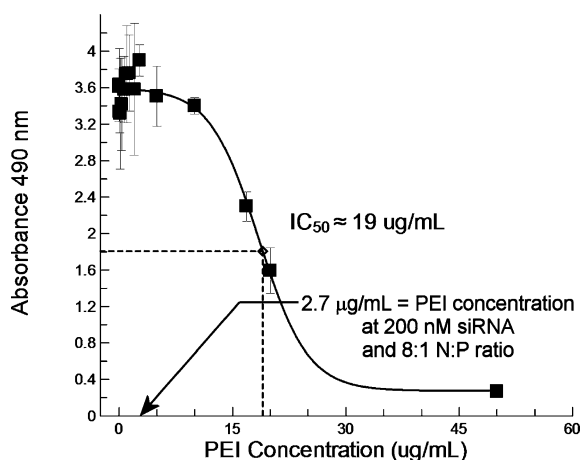


Fig. 5. Cytotoxicity of 25 K B-PEI in HR5-CL11 cell line as determined by cell survival after 48 h incubation in 96-well plates containing 8,000 cells per well.

in a sequence- and delivery method-dependent manner (38–42). The higher concentrations of siRNA required in the present studies to achieve gene knockdown may therefore not be disadvantageous provided that immunostimulation by PEI can be avoided. The potential of PEI for stimulating immune response when complexed with siRNA has not yet been studied, but low levels of pro-inflammatory cytokines have been measured in mice treated with 750 kDa PEI complexed with albumin and plasmid DNA (43).

No reduction in luciferase expression was observed for cells that were treated with the 800 B-PEI under any of the studied conditions. Although the binding affinity results suggest that complexes made from 800 B-PEI may be sufficiently stable to act as siRNA transfection reagents, the large sizes and negative zeta potentials of these complexes observed at all the tested N:P ratios may impede their internalization by endocytosis. The relatively weaker binding affinity of the 800 B-PEI for DNA compared to the 25 K B-PEI is similar to results obtained by others who characterized the binding affinity of branched PEI's of high and low molecular weights (44). Other researchers have also reported poor DNA transfection efficacy when using low molecular weight ($\leq 1,800$) B-PEI (24,25).

Our data suggest that the 22 K L-PEI complexes at 6:1 and 8:1 N:P ratios would facilitate siRNA delivery, given their sizes and zeta potentials. Further, the relative binding affinity data indicate that the 22 K L-PEI is able to successfully complex with siRNA at N:P ratios of 6:1 and 8:1. However, these complexes did not reduce luciferase expression (Fig. 4). One possible explanation for the poor transfection efficacy of these complexes might be complex instability. Polycations that condense DNA at lower N:P ratios form complexes that are less susceptible to disruption by competing polyanions (45), and this may also be the case for siRNA complexes. The facts that 1) the 22 K L-PEI did not appear to form small complexes until an N:P ratio of 3:1 was used, 2) the size of the 22 K L-PEI complexes steadily decreased as the N:P ratio was increased, and 3) the 22 K L-PEI had overall the lowest binding affinity for siRNA over the range of tested N:P ratios, all suggest that the 22 K L-PEI complexes may be less stable under the investigated con-

ditions than the 25 K B-PEI complexes and could therefore dissociate at unfavorable cellular locales during the transfection process.

Although successful DNA delivery has been reported *in vitro* and *in vivo* with linear PEI of comparable molecular weights (26–32), mixed results have been reported for siRNA delivery *in vitro* and *in vivo*. One study showed a 60% reduction in target gene expression 96 h after SKOV-3 cells were treated in 24-well plates with jetPEI™ and 10 pmol of siRNA. In the same study, a 50% reduction in HER-2 mRNA levels after 2 weeks was observed *in vivo* when athymic nude mice were treated with 0.6 nmol of PEI-complexed HER-2 siRNAs every 2 to 3 days (14). Estimating an average mouse blood volume of 1.5 ml, 0.6 nmol siRNA translates to ~ 400 nM, twice the maximum concentration used in the present studies. Another study reported an 83% reduction in GFP expression when jetPEI™ was used to deliver 50 nM of anti-GFP siRNA to PC-3 cells (15). A separate study, however, reported only a 20% inhibition of a target gene when 1 pmol of siRNA was transfected with *in vivo* jetPEI™ into the brains of newborn mice, and no knockdown when 0.2 pmol siRNA was used under the same conditions (16). Although the backbones of both jetPEI™ and *in vivo* jetPEI™ are linear PEI, the manufacturer does not provide any information about the molecular weight or possible chemical modification of either structure, making direct comparison with the present studies difficult. A recent report in the literature also found that complexes of siRNA and deacylated 25 K linear PEI at N:P ratios of 5:1 and 7.5:1 were effective in suppressing target gene activity *in vivo* by 76 ± 5 and $77 \pm 3\%$, respectively, in the mouse lung (17). Again, however, a direct comparison with the present studies cannot be made due to the difficulty of calculating the effective siRNA and PEI concentrations *in vivo*. Moreover, the better delivery efficacy reported could be due to the deacylation of the 25 K linear PEI, which increases the number of protonatable nitrogens and presumably allows better binding (i.e., more stable complexes) of the siRNA with the PEI. The inconsistent results found in the literature with respect to linear PEI suggest that predictability of siRNA transfection efficacy is not straightforward and that variable transfection results might also occur with other PEI architectures.

The observed relative cytotoxicities of the three PEI structures (800 B-PEI < 22 K L-PEI < 25 K B-PEI) are comparable to results reported by others, where low molecular weight branched PEI was shown to be less cytotoxic than high molecular weight branched PEI (46), and linear PEI (750 K) had comparable or slightly lower cytotoxicity than branched PEI (25 K) (47).

CONCLUSION

In summary, only one of the three PEI structures that were investigated (25 K B-PEI) was found to successfully mediate siRNA delivery *in vitro*, and successful delivery occurred only when an N:P ratio of 6:1 or 8:1 was used with an siRNA concentration of 200 nM. The zeta potential and size of the complexes correlated to transfection efficacy for complexes formed from the 25 K B-PEI and 800 B-PEI, but not the 22 K L-PEI. Other factors, such as the relative

stability of the complexes, may also play a role in determining the transfection efficacy of a given PEI structure. The ability of PEI to transfer functionally active siRNA to cells in culture is surprisingly dependent on its biophysical and structural characteristics when compared to the relative success and ease of use for DNA delivery, and it is apparent that siRNA transfection efficacy cannot be directly predicted based on DNA transfection studies.

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REFERENCES

1. A. Fire, S. Xu, M. K. Montgomery, S. A. Kostas, S. E. Driver, and C. C. Mello. Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. *Nature* **391**:806–811 (1998).
2. S. M. Elbashir, J. Harborth, W. Lendeckel, A. Yalcin, K. Weber, and T. Tuschl. Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. *Nature* **411**:494–498 (2001).
3. S. M. Hammond, E. Bernstein, D. Beach, and G. J. Hannon. An RNA-directed nuclease mediates post-transcriptional gene silencing in *Drosophila* cells. *Nature* **404**:293–296 (2000).
4. P. Zamore, T. Tuschl, P. Sharp, and D. Bartel. RNAi: double-stranded RNA directs the ATP-dependent cleavage of mRNA at 21 to 23 nucleotide intervals. *Cell* **101**:25–33 (2000).
5. T. R. Brummelkamp, R. Bernards, and R. Agami. A system for stable expression of short interfering RNAs in mammalian cells. *Science* **296**:550–553 (2002).
6. G. Sui, C. Soohoo, E. B. Affar, F. Gay, Y. Shi, W. C. Forrester, and Y. Shi. A DNA vector-based RNAi technology to suppress gene expression in mammalian cells. *Proc. Natl. Acad. Sci. USA* **99**:5515–5520 (2002).
7. C. R. Paul, P. D. Good, I. Winer, and D. R. Engelke. Effective expression of small interfering RNA in human cells. *Nat. Biotechnol.* **20**:505–508 (2002).
8. D. Gou, N. Jin, and L. Liu. Gene silencing in mammalian cells by PCR-based short hairpin RNA. *FEBS Lett.* **539**:113–118 (2003).
9. S. Q. Harper and B. L. Davidson. Plasmid-based RNA interference: construction of small-hairpin RNA expression vectors. *Methods Mol. Biol.* **309**:219–236 (2005).
10. Y. Guo, J. Liu, Y.-H. Li, T.-B. Song, J. Wu, C.-X. Zheng, and C.-F. Xue. Effect of vector-expressed shRNAs on hTERT expression. *World J. Gastroenterol.* **11**:2912–2915 (2005).
11. N. Miyagishi and K. Taira. U6 promoter-driven siRNAs with four uridine 3' overhangs efficiently suppress targeted gene expression in mammalian cells. *Nat. Biotechnol.* **20**:497–500 (2002).
12. N. S. Lee, T. Dohjima, G. Bauer, H. Li, M.-J. Li, A. Ehsani, P. Salvaterra, and J. Rossi. Expression of small interfering RNAs targeted against HIV-1 *rev* transcripts in human cells. *Nat. Biotechnol.* **20**:500–505 (2002).
13. O. Boussif, P. Lezoualc'h, M. A. Zanta, M. D. Mergny, D. Scherman, B. Demeneix, and J.-P. Behr. A versatile vector for gene and oligonucleotide transfer into cells in culture and *in vivo*: polyethylenimine. *Proc. Natl. Acad. Sci. USA* **92**:7297–7301 (1995).
14. B. Urban-Klein, S. Werth, S. Abuharbid, F. Czubayko, and A. Aigner. RNAi-mediated gene-targeting through systemic application of polyethylenimine (PEI) complexed siRNA *in vivo*. *Gene Ther.* **12**:461–466 (2005).
15. M. L. Read, S. Singh, Z. Ahmed, M. Stevenson, S. S. Briggs, D. Oupicky, L. B. Barrett, R. Spice, M. Kendall, M. Berry, J. A. Preece, A. Logan, and L. W. Seymour. A versatile reducible polycation-based system for efficient delivery of a broad range of nucleic acids. *Nucleic Acids Res.* **33**:e86 (2005).
16. Z. Hassani, G. F. Lemkine, P. Erbacher, K. Palmier, G. Alfama, C. Giovannangeli, J.-P. Behr, and B. A. Demeneix. Lipid-mediated siRNA delivery down-regulates exogenous gene expression in the mouse brain at picomolar levels. *J. Gene Med.* **7**:198–207 (2005).
17. M. Thomas, J. J. Lu, Q. Ge, C. Zhang, J. Chen, and A. M. Klibanov. Full deacylation of polyethylenimine dramatically boosts its gene delivery efficiency and specificity to mouse lung. *Proc. Natl. Acad. Sci. USA* **102**:5679–5684 (2005).
18. T. Merdan, K. Kunath, D. Fischer, J. Kopecek, and T. Kissel. Intracellular processing of poly(ethylene imine)/ribozyme complexes can be observed in living cells by using confocal laser scanning microscopy and inhibitor experiments. *Pharm. Res.* **19**:140–146 (2002).
19. A. Aigner, D. Fischer, T. Merdan, C. Brus, T. Kissel, and F. Czubayko. Delivery of unmodified bioactive ribozymes by an RNA-stabilizing polyethylenimine (LMW-PEI) efficiently down-regulates gene expression. *Gene Ther.* **9**:1700–1707 (2002).
20. S. Dheur, N. Dias, A. van Aerschot, P. Herdewijn, T. Bettinger, J.-S. Rémy, C. Hélène, and E. T. Saison-Behmoaras. Polyethylenimine but not cationic lipid improves antisense activity of 3'-capped phosphodiester oligonucleotides. *Antisense Nucleic Acid Drug Dev.* **9**:515–525 (1999).
21. J.-P. Behr. The proton sponge: a trick to enter cells the viruses did not exploit. *Chimia* **51**:34–36 (1997).
22. A. Akinc, M. Thomas, A. M. Klibanov, and R. Langer. Exploring polyethylenimine-mediated DNA transfection and the proton sponge hypothesis. *J. Gene Med.* **7**:657–663 (2005).
23. R. M. Schiffelers, A. Ansari, J. Xu, Q. Zhou, Q. Tang, G. Storm, G. Molema, P. Y. Lu, P. V. Scaria, and M. C. Woodle. Cancer siRNA therapy by tumor selective delivery with ligand-targeted sterically stabilized nanoparticle. *Nucleic Acids Res.* **32**:e149 (2004).
24. W. T. Godbey, K. K. Wu, and A. G. Mikos. Size matters: molecular weight affects the efficiency of poly(ethyleneimine) as a gene delivery vehicle. *J. Biomed. Mater. Res.* **45**:268–275 (1999).
25. C.-H. Lee, Y.-H. Ni, C.-C. Chen, C.-K. Chou, and F.-H. Chang. Synergistic effect of polyethylenimine and cationic liposomes in nucleic acid delivery to human cancer cells. *Biochim. Biophys. Acta* **1611**:55–62 (2003).
26. S. Choosakoonkriang, B. A. Lobo, G. S. Koe, J. G. Koe, and C. R. Middaugh. Biophysical characterization of PEI/DNA complexes. *J. Pharm. Sci.* **92**:1710–1722 (2003).
27. B. Brissault, A. Kichler, C. Guis, C. Leborgne, O. Danos, and H. Cheradame. Synthesis of linear polyethylenimine derivatives for DNA delivery. *Bioconjug. Chem.* **14**:581–587 (2003).
28. S. Ferrari, E. Moro, A. Pettenazo, J. P. Behr, F. Zacchello, and M. Scarpa. ExGen 500 is an efficient vector for gene delivery to lung epithelial cells *in vitro* and *in vivo*. *Gene Ther.* **2**:1100–1106 (1997).
29. D. Goula, C. Benoist, S. Mantero, G. Merlo, G. Levi, and B. A. Demeneix. Polyethylenimine-based intravenous delivery of transgenes to mouse lung. *Gene Ther.* **5**:1291–1295 (1998).
30. D. Goula, J. S. Remy, P. Erbacher, M. Wasowicz, G. Levi, B. Abdallah, and B. A. Demeneix. Size, diffusibility, and transfection performance of linear PEI/DNA complexes in the mouse central nervous system. *Gene Ther.* **5**:712–717 (1998).
31. S.-M. Zou, P. Erbacher, J.-S. Remy, and J.-P. Behr. Systemic linear polyethylenimine (L-PEI)-mediated gene delivery in the mouse. *J. Gene Med.* **2**:128–134 (2000).
32. G. F. Lemkine and B. A. Demeneix. Polyethylenimines for *in vivo* gene delivery. *Curr. Opin. Mol. Ther.* **3**:178–182 (2001).
33. D. Putnam, C. A. Gentry, D. W. Pack, and R. Langer. Polymer-based gene delivery with low cytotoxicity by a unique balance of side-chain termini. *Proc. Natl. Acad. Sci. USA* **98**:1200–1205 (2001).

34. A. N. Zelikin, E. S. Trukhanova, D. A. Putnam, V. A. Izumrudov, and A. A. Litmanovich. Selectivity of binding of polycations to DNA and synthetic polyanions. *Polymer Preprints* **44**:519–520 (2003).
35. N. E. Bishop. An update on non-clathrin-coated endocytosis. *Rev. Med. Virol.* **7**:199–209 (1997).
36. U. Rungsardthong, T. Ehtezazi, L. Bailey, S. P. Armes, M. C. Garnett, and S. Stolnik. Effect of polymer ionization on the interaction with DNA in nonviral gene delivery systems. *Biomacromolecules* **4**:683–690 (2003).
37. M. Gossen, S. Freundlieb, G. Bender, G. Müller, W. Hillen, and H. Bujard. Transcriptional activation by tetracyclines in mammalian cells. *Science* **268**:1766–1769 (1995).
38. Z. Ma, J. Li, F. He, A. Wilson, B. Pitt, and S. Li. Cationic lipids enhance siRNA-mediated interferon response in mice. *Biochem. Biophys. Res. Commun.* **330**:755–759 (2005).
39. V. Hornung, M. Guenther-Biller, C. Bourquin, A. Ablasser, M. Schlee, S. Uematsu, A. Noronha, M. Manoharan, S. Akira, A. Fougerollesde, S. Endres, and G. Hartmann. Sequence-specific potent induction of IFN- α by short interfering RNA in plasmacytoid dendritic cells through TLR7. *Nat. Med.* **11**:263–270 (2005).
40. A. D. Judge, V. Sood, J. R. Shaw, D. Fang, K. McClintock, and I. MacLachlan. Sequence-dependent stimulation of the mammalian innate immune response by synthetic siRNA. *Nat. Biotechnol.* **23**:457–462 (2005).
41. M. Sioud. Induction of inflammatory cytokines and interferon responses by double-stranded and single-stranded siRNAs is sequence-dependent and requires endosomal localization. *J. Mol. Biol.* **348**:1079–1090 (2005).
42. Y. Federov, A. King, E. Anderson, J. Karpilow, D. Ilsley, W. Marshall, and A. Khvorova. Different delivery methods—different expression profiles. *Nat. Methods* **2**:283–287 (2005).
43. R. Smolarczyk, T. Cichoń, A. Sochanik, and S. Szala. Negligible induction of IFN- γ , IL-12, and TNF- α by DNA-PEI 750 kDa/albumin complexes. *Cytokine* **29**:283–287 (2005).
44. K. Kunath, A. von Harpe, D. Fischer, H. Petersen, U. Bickel, K. Voigt, and T. Kissel. Low-molecular-weight polyethylenimine as a non-viral vector for DNA delivery: comparison of physicochemical properties, transfection efficiency and *in vivo* distribution with high-molecular-weight polyethylenimine. *J. Control. Release* **89**:113–125 (2003).
45. E. Ramsay, J. Hadgraft, J. Birchall, and M. Gumbleton. Examination of the biophysical interaction between plasmid DNA and the polycations, polylysine and polyornithine, as a basis for their differential gene transfection *in-vitro*. *Int. J. Pharm.* **210**:97–107 (2000).
46. D. Fischer, T. Bieber, Y. Li, H.-P. Elsässer, and T. Kissel. A novel non-viral vector for DNA delivery based on low molecular weight, branched polyethylenimine: effect of molecular weight on transfection efficiency and cytotoxicity. *Pharm. Res.* **16**:1273–1279 (1999).
47. S. M. Moghimi, P. Symonds, J. C. Murray, A. C. Hunter, G. Debska, and A. Szewczyk. A two-stage poly(ethylenimine)-mediated cytotoxicity: implications for gene transfer/therapy. *Mol. Ther.* **11**:990–995 (2005).
48. D. Wang, A. S. Narang, M. Kotb, A. O. Gaber, D. D. Miller, S. W. Kim, and R. I. Mahato. Novel branched poly(ethylenimine)-cholesterol water-soluble lipopolymers for gene delivery. *Biomacromolecules* **3**:1197–1207 (2002).