Acetylation of Polyethylenimine Enhances Gene Delivery via Weakened Polymer/DNA Interactions

Nathan P. Gabrielson[†] and Daniel W. Pack*[,†,‡]

Department of Chemical and Biomolecular Engineering and Department of Bioengineering, University of Illinois, Urbana, Illinois 61801

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We previously reported that gene delivery efficiency of 25-kDa, branched polyethylenimine (PEI) increased upon acetylation of up to 43% of the primary amines with acetic anhydride. In the present work, we investigated the effects of further increasing the degree of acetylation and elucidated the source of the higher gene delivery efficiency. Despite reduced buffering capacity, gene delivery activity continued to increase (up to 58-fold in HEK293) with acetylation of up to 57% of primary amines but decreased at higher degrees of acetylation. Characterization of polymer−DNA interactions showed that acetylated polymers bind less strongly to DNA. Further, a fluorescence resonance energy transfer assay showed that increasing acetylation causes polyplexes to unpackage inside cells to a higher degree than polyplexes formed with unmodified PEI. Overall, the data suggest that the increased gene delivery activity may be attributable to an appropriate balance between polymer buffering capacity and strength of polymer/DNA interactions.

Introduction

Safe and efficient delivery of genetic material remains the most challenging aspect of human gene therapy.[1−3] Gene carriers must traverse multiple extra- and intracellular obstacles to reach the target cell nucleus, including passage across the plasma membrane, escape from acidic and potentially degradative endocytic vesicles, migration toward the nucleus, transport across the nuclear membrane, and release of genetic material at the appropriate point (currently unknown) in this pathway. Most delivery strategies can be placed in one of two categories: viral or nonviral.[4] The majority of clinical trials to date have employed viral vectors due to their innate ability to surmount many of the existing barriers and, therefore, to function with high efficiency. However, safety concerns limit the utility of engineered viruses.[5,6]

Nonviral vectors include cationic peptides, lipids, and polymers that are capable of electrostatically condensing DNA into nanoscale complexes. Polymers in particular are the subject of intense research due to their flexible properties, facile synthesis, robustness, and proven gene delivery potential.[7] Unfortunately, most polymeric vectors fail at one or more of the intracellular barriers described above. Much of the polymer gene delivery literature focuses on off-the-shelf materials, i.e., polymers not originally intended for gene delivery, and there is no reason to expect the properties of such materials to be optimal. Furthermore, a major difficulty facing design of new polymers is poor understanding of their intracellular trafficking and of polymer structure/activity relationships. As a result, most polymeric gene delivery vectors are orders of magnitude less efficient than viruses.

Polyethylenimine (PEI) is one of the most effective commercially available gene delivery polymers and has been used both in vitro and in vivo.[8,9] As such, PEI is a useful material for studies of polymer-mediated gene delivery mechanisms.[10,11] The prevailing explanation for the relatively high gene delivery activity of PEI is known as the "proton-sponge" hypothesis.[12] According to this hypothesis, polymers with pK_a values between neutral and endosomal pH, such as PEI, are able to buffer the ATPase-mediated acidification of endocytic vesicles. Because of the strong buffering capacity of these polymers, every third atom of PEI is a potentially protonatable nitrogen, a relatively large number of protons may accumulate in the endocytic vesicles. The positive charge must be balanced by an influx of counterions (primarily chloride), which causes the endosome to osmotically swell and burst, releasing polyplexes into the cytosol.

The proton sponge hypothesis has been a subject of debate, speculation and corresponding research. Evidence supporting as well as refuting the hypothesis has been offered.[10,13−15] Sonawane et al. used a chloride-sensitive fluorophore to monitor the chloride accumulation and swelling in endosomes.[16] Their work revealed that the enhanced endosomal buffering of PEI relative to polylysine resulted in increased chloride retention and endosomal swelling. Likewise, it has been shown that the addition of the buffering agent chloroquine to cells transfected using nonbuffering polymers has a positive effect on transfection efficiency but no effect when added to known proton-sponge polymers.[17,18,19]

Imaging of fluorescently labeled polyplexes with confocal microscopy reveals that a significant fraction of polyplexes inside the cell remain sequestered in vesicles adjacent to the perinuclear region of the cell.[20] Also, data obtained with pH-sensitive dyes have shown that DNA polyplexes containing PEI are trafficked to acidic environments.[15] However, a similar pH-based assay developed by Akinc and Langer to measure the ability of nonviral vectors to avoid lysosomal trafficking did not indicate PEI trafficking to acidic regions.[21] Their work suggested that nonbuffering agents such as poly-L-lysine showed low gene delivery efficiency because these vectors were unable to avoid trafficking to acidic lysosomes, whereas proton sponge polymers that were able to escape endosomes prior to acidification showed a higher efficiency. In a separate study, Kulkarni

* Corresponding author. E-mail: dpack@uiuc.edu. Phone: 217-244-2816. Fax: 217-333-5052.

[†] Department of Chemical and Biomolecular Engineering.

[‡] Department of Bioengineering.

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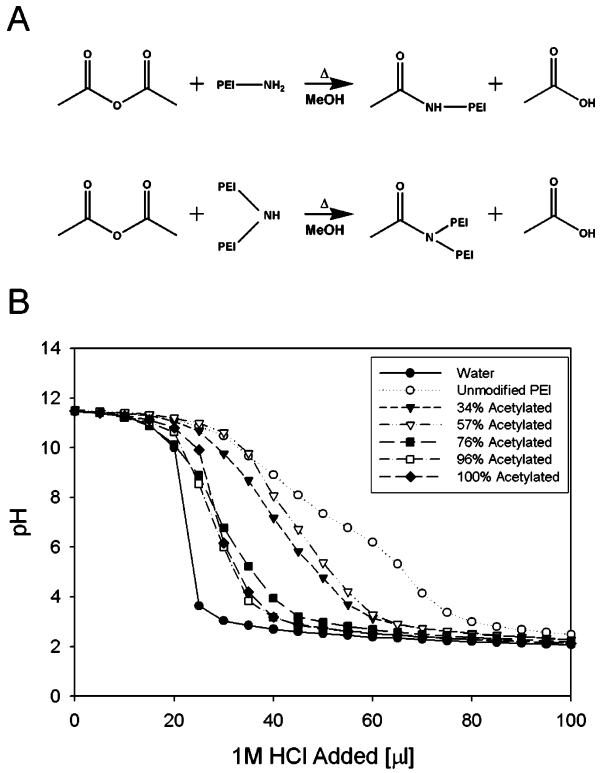

Figure 1. (A) Derivatization of PEI to generate acetylated PEI. Primary amines react with acetic anhydride to generate secondary amides and secondary amines react with acetic anhydride to generate tertiary amides. The polymers are named PEI−Ac_x, where x is the percentage of primary amines converted to amides, as determined by ^{1}H NMR spectroscopy. (B) Titration of aqueous polymer solutions (0.4 mg/mL) with 1 M HCl from pH 11.5 to ~2. Solutions were adjusted to pH 11.5 with 1 M NaOH, and 5 μL aliquots of 1 M HCl were subsequently added and the pH measured.

et al. used buffering vectors (e.g., PEI and β-cyclodextrin-containing polymers possessing an imidazole group) as well as nonbuffering vectors (e.g., poly-L-lysine and β-cyclodextrin-containing polymers lacking imidazole) to deliver an oligonucleotide labeled with a fluorophore possessing dual pH sensitive emission lines.[13] Coupled with live, single cell spatiotemporal imaging techniques, their work demonstrated that no correlation existed between gene delivery efficiency and buffering capacity.

We previously reported acetylation of branched 25-kDa PEI to probe the effects of reduced endosomal buffering capacity on transfection efficiency.[22] To generate the polymers, acetic anhydride reacts with a fraction of the amines of PEI, transforming primary amines into secondary amides and secondary amines into tertiary amides (Figure 1A), thereby reducing the buffering capacity of the polymer. Somewhat surprisingly, we found that gene delivery activity increased upon acetylation as much as 21-fold at 43% modification of primary amines. These data and others, including those discussed above, suggest that buffering capacity does not correlate directly with gene delivery activity.[23] We report acetylation of PEI at higher degrees of modification than those described in our previous study. Acetylation of PEI increased transfection efficiency 20- to 60-fold in three model cell lines, and an optimal acetylation ratio was found. Further characterization of the polymer−DNA interactions shows that acetylation decreases polymer−DNA binding and suggests, therefore, that increased transfection may be attributed to enhanced dissociation of the polyplexes inside of the cell.

Materials and Methods

Cells and Plasmids. MDA-MB-231 human breast carcinoma and HEK293 human embryonic kidney cell lines were purchased from the American Type Culture Collection. The C2C12 murine myoblast cell line was a gift from Prof. Stephen Kaufman (University of Illinois, Urbana, IL). All cell lines were cultured according to their ATCC protocols at 37 °C and 5% CO_2 but were adapted from fetal bovine serum to 10% horse serum. The growth medium also contained 1% penicillin-streptomycin. The 5.3-kilobase expression vector pGL3 (Promega, Madison, WI) coding for the luciferase gene driven by the SV40 promoter and enhancer, was grown in DH5α *E. coli* (Gibco BRL, Rockville, MD) and purified with a commercial plasmid purification kit (Bio-Rad, Hercules, CA).

Acetylation of Polyethylenimine. Acetic anhydride was purchased from Sigma-Aldrich (St. Louis, MO) and used without further purification. Branched 25-kDa PEI (0.5 g) was transferred to a 20 mL scintillation vial and dissolved in 3 mL of freshly distilled methanol. Sufficient acetic anhydride to achieve the desired degree of acetylation was added, and the vial was sealed. The reaction was carried out at 60 °C with stirring for 4 h. The reaction was quenched with double distilled water (0.5 mL). The solvent was removed under reduced pressure. The remaining solution was purified by gel filtration chromatography (PD-10 columns, Pharmacia) and eluted with double distilled water. The purified polymers were then concentrated and stored at −80 °C.

To determine the extent of acetylation, each polymer was dissolved in D_2O and ^{1}H NMR spectra were acquired on a Varian Unity 400 with a 5-mm probe. The extent of primary and secondary amine acetylation was determined by peak integration using the following formula:

$$\text{degree of primary amine acetylation (\%)} = \left(\frac{E_{bb}}{4}\frac{3}{M'}N'\right)^{-1} \times 100\ \%$$

$$\text{degree of secondary amine acetylation (\%)} = \left(\frac{E_{bb}}{4}\frac{3}{M''}N''\right)^{-1} \times 100\ \%$$

where M' is the integration of δ 1.7−1.75 peaks (R−NHCOC**H**$_3$, acetylated primary amines), M'' is the integration of δ 1.8−1.85 peaks (R_2−NCOC**H**$_3$, acetylated secondary amines), and E_{bb} is the integration of δ 2.3−2.8 peaks ([C**H**$_2$C**H**$_2$N]$_x$[C**H**$_2$C**H**$_2$NH]$_y$[C**H**$_2$C**H**$_2$NH$_2$]$_z$, ethylene backbone). N' and N'' are mole fractions of primary and secondary amines of the PEI starting material, respectively, as reported previously.[22] Briefly, following the technique reported by vonHarpe, the starting material was dissolved in D_2O and the ^{13}C NMR spectrum was acquired using a Varian Unity Inova 750 MHz spectrometer with correction for nuclear Overhauser effects.[24] All integration was performed using the WinNuts software package (Acorn NMR, Livermore, CA). NMR scans and integration were performed in triplicate.

Gel Retardation Studies. A solution of DNA (1 μg/10 μL) was prepared in 150 mM NaCl, 20 mM PIPES at pH 7.2. Appropriate amounts of each polymer dissolved in double distilled water were added to 10 μL of the DNA solution to achieve the desired polymer:DNA weight ratio. Polyplexes were incubated at 4 °C for 30 min, after which loading dye was added, and 10 μL of the solution was run on a 0.75% agarose gel (60 V, 70 min). DNA was visualized with ethidium bromide.

Ethidium Bromide Exclusion. A solution of DNA (2 μg/250 μL) was prepared in 150 mM NaCl, 20 mM PIPES at pH 7.2. Appropriate amounts of each polymer dissolved in double distilled water were added to 250 μL of the DNA solution to achieve the desired weight ratio. Polyplexes were incubated in wells of a 96-well microplate at 4 °C for 15 min, after which 1 μg of ethidium bromide was added to each 250 μL aliquot of polyplex solution. The polyplexes and ethidium bromide were incubated at room temperature for another 10 min before exciting polyplexes at 510 nm and reading the fluorescence at 595 nm (Cary

Eclipse, Palo Alto, CA). The fluorescence values were normalized to wells containing only DNA and ethidium bromide. Experiments were performed in triplicate.

Heparan Sulfate Displacement. Polyplexes of 1 μg of DNA and acetylated or unmodified PEI were formed in 15 μL of 150 mM NaCl, 20 mM PIPES at pH 7.2. The amount of polymer added depended on the optimum transfection ratio for the given polymer. Polyplexes were incubated at 4 °C for 30 min before heparan sulfate was added at various weight ratios relative to the DNA in the polyplexes. The resulting mixture was incubated at room temperature for another 15 min after which loading dye was added and 15 μL of polyplex solution was run on a 0.75% agarose gel (60 V, 60 min). DNA was visualized with ethidium bromide staining.

Transfection. DNA/polymer complexes were prepared at room temperature by dissolving 1.5 μg of DNA in 150 mM NaCl, 20 mM PIPES, pH 7.2. An equal volume of polymer solution was added to achieve the desired polymer:DNA weight ratio. Polyplexes were then incubated at 4 °C for 30 min. Cells (MDA-MB-231, HEK293 or C2C12) were cultured in DMEM supplemented with 10% horse serum and 1% penicillin-streptomycin according to ATCC protocols and plated in 12-well plates at 4×10^4 cells/well 24 h prior to transfection. Immediately before transfection, the growth medium was replaced with fresh serum-free medium and 50 μL of polyplex solution was added to each well (0.5 μg plasmid/well). Transfection medium was replaced with serum-supplemented growth medium 4 h post-transfection. Luciferase expression was quantified 24 h post-transfection using the Promega luciferase assay system (Promega, Madison, WI). Luciferase activity was measured in relative light units (RLU) using a Lumat LB 9507 luminometer (Berthold, GmbH, Germany). Results were normalized to total cell protein using the Pierce BCA protein assay kit (Pierce, Rockford, IL).

Intracellular FRET Analysis. PEI samples were reacted with either 5,6-tetramethylrhodamine or Alexa Fluor 488 (Molecular Probes, Eugene, OR) in 0.1 M sodium bicarbonate buffer, pH 8.3 for 1 h. Both dyes were functionalized with amine reactive NHS-ester functional groups. Labeled PEI was purified from unreacted dye by gel filtration chromatography (PD-10 columns, Pharmacia). Polyplexes with the various acetylated PEI samples were formed as mentioned previously at their respective optimum weight ratios using equal ratios of TAMRA-labeled, Alexa Fluor 488-labeled, and unlabeled polymer. MDA-MB-231 cells, seeded at 2×10^5 cells/well and grown overnight in 6-well plates containing a coverslip in each well, were incubated with serum free DMEM containing polyplexes for 30 min before the polyplexes were removed and the media was replaced with DMEM supplemented with 10% horse serum and 1% penicillin-streptomycin. At 30 min, 1 h, 2 h, and 4 h, the cells were fixed with 3.7% formaldehyde and coverslips were mounted on microscope slides for confocal analysis.

Mounted cells were visualized with an Olympus model BX60 confocal microscope equipped with a 100× oil immersion lens as well as argon and Krypton lasers for visualizing the Alexa Fluor 488 (λ_{ex} = 488 nm; λ_{em} = 510−550 nm) and TAMRA (λ_{ex} = 568 nm λ_{em} = >605 nm) signals, respectively. Separate, representative images of each dye were captured at each time point. Image analysis was performed using Image J software (NIH). Briefly, the background-subtracted pixel intensity of easily identifiable polyplexes (minimum of 10 polyplexes per time point) was separately determined in the Alexa Fluor 488 and TAMRA channels and individually ratioed to construct the plot in Figure 6A.

Flow Cytometry. DNA complexes were formed with both the modified and unmodified polymers at their respective optimum weight ratios. The intercalating dye YOYO-1 was added according to the ratio 15 nl YOYO-1 per 1 μg of DNA. The complexes were then incubated at 4 °C for approximately 30 min. Cells (MDA-MB-231, HEK293 or C2C12) were cultured in DMEM supplemented with 10% horse serum and 1% penicillin-streptomycin according to ATCC protocols and plated in 12-well plates at 1×10^5 cells/well 24 h prior to transfection. Immediately before transfection, the growth medium was replaced with fresh serum-free medium and 100 μL of polyplex solution was added to each well (0.5 μg plasmid/well). Two hours post-transfection, the cells were rinsed twice with 0.001% SDS in PBS and PBS, respectively, to remove surface-bound complexes. Next, 500 μL of 0.25% trypsin in PBS was added to each well. The cells and trypsin were allowed to incubate for 5 min before 50 μL of horse serum was added to each well. The cells were then collected and stored on ice. FACS analyses were performed on a Coulter EPICS XL-MCL flow cytometer (Beckman-Coulter, Fullerton, CA). Data were analyzed using the Summit software package (Cytomation, Ft. Collins, CO).

Table 1. Percentage of Acetylation Determined by ^{1}H NMR Spectra (Average ± Standard Deviation)

polymer	1° amines acetylated (%)	2° amines acetylated (%)
PEI−Ac_{34}	33.6 ± 0.4	10.1 ± 0.3
PEI−Ac_{57}	57.4 ± 1.4	34.8 ± 2.2
PEI−Ac_{76}	76.3 ± 3.5	38.1 ± 3.7
PEI−Ac_{96}	95.9 ± 2.5	43.2 ± 3.1
PEI−Ac_{100}	100 ± 0.0	79.7 ± 10.1

Cytotoxicity Measurements. The cytotoxicity of the polymer on the three model cell lines was characterized using the CellTiter-Blue cell viability assay (Promega, Madison, WI). The assay is based on the ability of living cells to convert the redox dye resazurin into the fluorescent end product, resorufin. Briefly, cells (MDA-MB-231, HEK293, or C2C12) were seeded in 96-well microplates at 2×10^4 cells/well and grown overnight at 37 °C, 5% CO_2 in medium containing 10% horse serum and 1% penicillin-streptomycin. Approximately 24 h after seeding, the medium was replaced with serum-free DMEM and the free, uncomplexed polymer was added to the cells at final concentrations between 0 and 50 μg/mL. After 4 h of incubation, the medium was replaced with serum-containing medium and grown for another 20 h, after which 20 μL of the CellTiter-Blue reagent was added to each well. Following a 4 h incubation at 37 °C, 5% CO_2 the cells and CellTiter-Blue reagent were excited with a 560-nm laser and the fluorescence was read at 590 nm (Cary Eclipse, Palo Alto, CA). The background fluorescence of cells killed with ethanol was subtracted from the viable cell fluorescence and normalized to cells containing no polymer. Each experiment was repeated 8 times at each polymer concentration.

pH Titrations. A total of 2 mg of each polymer was dissolved in 5 mL of double distilled water and adjusted to a pH of approximately 11.5 with 1 M NaOH. Aliquots (5 μL) of 1 M HCl were added, and the solution pH was measured with a pH meter (Accumet AB15, Hudson, MA) after each addition.

Dynamic Light Scattering. Polymer/DNA complexes were formed in PIPES buffer (20 mM PIPES, 150 mM NaCl) at the optimum weight ratio for each of the modified and unmodified polymers. Following incubation at 4 °C, the polyplexes were diluted in DMEM and subjected to size measurement on a Brookhaven Instruments Corporation 90 Plus Particle Size Analyzer (Holtsville, NY) immediately after dilution and again after incubation for 4 h at 37 °C. Measurements were performed in triplicate.

Results

Acetylation of Polyethylenimine. We previously reported that 15−43% acetylated PEI exhibits enhanced gene delivery activity compared to unmodified PEI, and the gene delivery activity increased with the degree of acetylation.[22] To further explore the effects of acetylation on gene delivery and polymer−DNA interactions, we more extensively modified 25-kDa branched PEI with acetic anhydride. Thus, the polymer was reacted with differing amounts of acetic anhydride in methanol at 60 °C for 4 h (Figure 1A). The extent of primary and secondary amine acetylation was determined by NMR spectroscopy (Table 1). The reactions produced PEI with 34%, 57%,

76%, 96%, and 100% of their primary amines acetylated (the modified polymers are labeled PEI−Ac_X, where the subscript indicates the percentage of acetylated primary amines).

According to the proton sponge hypothesis, a polymer's buffering capacity is essential for swelling of endocytic vesicles, escape into the cytoplasm and overall gene delivery efficiency.[12] We therefore titrated solutions of unmodified and acetylated PEI from pH 11.5 to ~2 with addition of HCl. Because the secondary and tertiary amides on acetylated PEI exhibit lower pK_a values than the corresponding primary and secondary amines, the protonation profiles (e.g., buffering capacity) of the polymers were markedly affected (Figure 1B). The buffering capacity of the various acetylated polymers was defined here as the reciprocal slope of the titration plots over the pH range of 4 to 10 (with units of μL of 1 M HCl/pH unit, or simply μL). PEI is a strong buffer over this pH range, exhibiting a slope of 6.7 μL. Generally, an increase in the degree of acetylation resulted in a reduction of the polymer buffering capacity. Slopes of 4.3, 3.8, 3.1, 2.2, and 1.8 μL were measured for PEI−Ac_{34}, PEI−Ac_{57}, PEI−Ac_{76}, PEI−Ac_{96}, and PEI−Ac_{100}, respectively.

In Vitro Transfection Efficiency. The transfection efficiency of the acetylated polymers was tested on three model cell lines: MDA-MB-231 human breast carcinoma cells, HEK293 human embryonic kidney cells, and C2C12 mouse myocytes (Figure 2). Cells transfected with the luciferase-encoding plasmid (pGL3), in the absence of any polymer, exhibited negligible luciferase expression. Unmodified PEI mediated relatively efficient gene delivery in each of the cell lines. The highest luciferase expression consistently occurred using 2:1 (w:w) PEI:DNA in all three cell lines, and higher mass ratios had little effect (1:1 and lower mass ratios exhibited statistically significant reduction of gene delivery activity; not shown). In a previous paper, we reported that gene delivery efficiency increased with the degree of acetylation up to 43%.[22] That trend appears to continue up to 57% acetylation, but gene delivery decreased with further acetylation in all three cell lines. Specifically, the transfection efficiency of PEI−Ac_{57} (3:1 w:w) was approximately 47-, 58-, and 30-fold higher than unmodified PEI (2:1 w:w) in MDA-MB-231, HEK293, and C2C12 cells, respectively. At higher degrees of acetylation, gene delivery efficiency decreased, but the optimal polymer/DNA ratio increased (e.g., 4:1 w:w was optimal for PEI−Ac_{96} in all three cell lines and 5:1 w:w was the best ratio tested for PEI−Ac_{100} in MDA-MB-231 and C2C12 cells). The optimal degree of acetylation suggests that there may be a balance between two competing effects of acetylation. This possibility was addressed by physicochemical characterization of polyplexes and investigation of their intracellular trafficking, as described below.

Agarose Gel Retardation. Polymeric vectors must condense DNA into nanoscale structures that can be taken up by cells, often through nonreceptor-mediated endocytosis. We therefore investigated the ability of the polymers to condense plasmid DNA and as a result inhibit its migration in agarose gel electrophoresis. For each polymer, polyplexes were formed at various polymer:DNA weight ratios and electrophoresed on an agarose gel to reveal the polymer:DNA ratio necessary to generate charge-neutral polyplexes (Figure 3). Unmodified PEI was able to completely inhibit migration of plasmid DNA with as little as 0.1 μg polymer/μg DNA. As the degree of acetylation increased, larger amounts of polymer were required to prevent DNA migration. Specifically, 0.5 μg of polymer was required for PEI−Ac_{34}, whereas 0.7, 0.9, 1.0, and 1.7 μg of polymer were required for PEI−Ac_{57}, PEI−Ac_{76}, PEI−Ac_{96}, and PEI−Ac_{100}, respectively. The reduction in the number of protonated nitrogens per mass of polymer is likely to be the reason for the increased polymer needed to form polyplexes and retard gel migration.

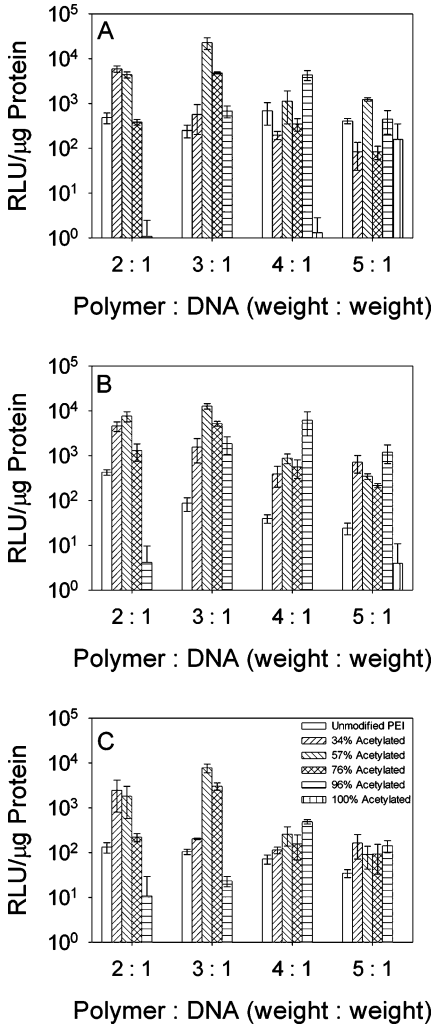

Figure 2. In vitro transfection efficiency. Polyplexes of plasmid DNA (pGL3) with unmodified or acetylated PEI were used to transfect (A) MDA-MB-231, (B) C2C12, and (C) HEK293 cells. Luciferase activity in the cell lysates is reported as relative light units (RLU) normalized by the mass of total protein in the lysate. ($N = 3$; error bars represent standard deviation.)

Polyplex Size Measurement. The effective diameters of the various polyplexes formed at their optimum transfection weight ratios were measured by dynamic light scattering (Table 2). Polyplexes composed of low to moderately acetylated PEI showed effective diameters in the range of roughly 250−270 nm, whereas PEI−Ac_{96} and PEI−Ac_{100} produced polyplexes with diameters of roughly 450 and 750 nm, respectively. There are two explanations for the increased size of the PEI−Ac_{96} and PEI−Ac_{100} polyplexes. First, these polyplexes are formed with 50−100% more polymer per weight of plasmid, compared to the other polyplexes, and thus should be larger. In addition, positive charge density is low at these high percentages of acetylation, and the polyplexes are significantly "looser" (see below). It is also noteworthy that upon incubation in DMEM for 4 h (mimicking transfection conditions) the polyplex size increased 2- to 3-fold with most of the polymers. This increase is most likely due to aggregation of polyplexes.

Ethidium Bromide Exclusion Analysis. Another measure of polyplex formation is the ability of the polymers to block

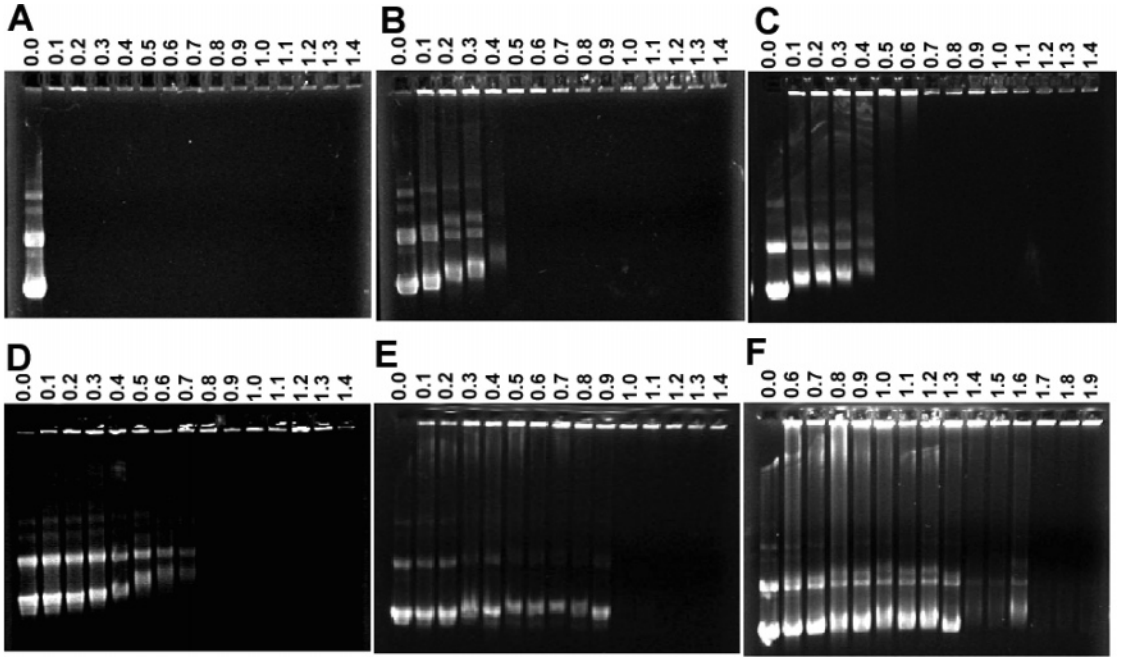

Figure 3. Agarose gel electrophoresis of polymer/DNA complexes. (A) Unmodified PEI, (B) PEI−Ac_{34}, (C) PEI−Ac_{57}, (D) PEI−Ac_{76}, (E) PEI−Ac_{96}, and (F) PEI−Ac_{100}. The polymer to DNA weight ratio used to form each polyplex is given above the corresponding lane.

Table 2. Diameter of Polymer/DNA Polyplexes, at the Optimum Transfection Polymer:DNA Ratios, Formed in PIPES Buffer and Measured with Dynamic Light Scattering in DMEM (Average ± Standard Deviation)

	polymer:DNA	effective diameter (nm)	
polymer	(w:w)	0 hr	4 hr
unmodified PEI	2:1	243.5 ± 7.5	699.2 ± 17.6
PEI−Ac_{34}	2:1	243.6 ± 5.1	769.7 ± 21.0
PEI−Ac_{57}	3:1	268.1 ± 3.3	738.3 ± 17.9
PEI−Ac_{76}	3:1	244.6 ± 4.3	637.5 ± 31.0
PEI−Ac_{96}	4:1	465.4 ± 8.7	853.9 ± 24.9
PEI−Ac_{100}	4:1	729.4 ± 45.6	668.7 ± 35.8

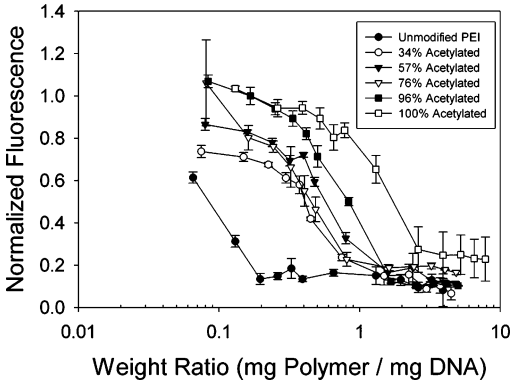

Figure 4. Ethidium bromide exclusion. Polyplexes were formed with unmodified and acetylated PEI at varying weight ratios. Normalized fluorescence is calculated as $(F - F_0)/(F_{DNA} - F_0)$. F, fluorescence from solution containing ethidium bromide and the complexes; F_0, fluorescence from solution of ethidium bromide only; and F_{DNA}, fluorescence from solution of DNA and ethidium bromide ($N = 3$, error bars represent standard deviation).

binding of intercalators such as ethidium bromide. Well-condensed, compact polyplexes may provide better protection of the DNA from the action of degradative enzymes. An ethidium bromide exclusion analysis was therefore performed to quantitatively evaluate the degree of condensation, characterized by the exclusion of ethidium bromide from the condensed DNA resulting in reduced fluorescence intensity of the intercalator (Figure 4).

Unmodified PEI complexes are tightly condensed at polymer/DNA ratios of as little as 0.2:1 (w:w). However, increasing amounts of polymer were required to condense DNA as the degree of acetylation increased. PEI−Ac_{34}, PEI−Ac_{57}, PEI−Ac_{76}, and PEI−Ac_{96} are able to tightly condense DNA (normalized fluorescence ~0.1) at polymer/DNA ratios of 1−2:1 (w:w). PEI−Ac_{100} required >2:1 (w:w) to reach a minimum in the normalized fluorescence of only ~0.2. Regardless of the degree of modification, both modified and unmodified PEI condensed DNA to roughly the same degree at high mass ratios, whereas at polymer/DNA ratios below 1:1 (w:w), the polyplexes formed of acetylated PEI were "looser." Transfections were performed with polyplexes of polymer/DNA ratios ≥ 2:1 (w:w), at which point the various polymers all showed similar degrees of condensation.

Polyethylenimine Displacement with Heparan Sulfate. It is essential that the polymer condensing the DNA be shed from the polyplex to allow transcription and perhaps to permit DNA entry into the nucleus. Anionic species such as vesicle membranes, proteins in the cytosol, or nucleic acids may compete with DNA for binding to positively charged polymers. In a similar fashion, the anionic proteoglycan heparan sulfate (HS) is known to competitively displace polycations from DNA, and the amount of HS required is related to the strength of the polymer−DNA binding.[25,26,27] To assess the relative strength of the acetylated polymers' binding to DNA, varying amounts of heparan sulfate were incubated along with polyplexes at their respective optimum polymer/DNA ratios for transfection, and the resulting mixtures electrophoresed on an agarose gel (Figure 5).

As acetylation of the PEI increased, less heparan sulfate was required to disrupt the polyplexes. Polyplexes containing unmodified PEI required the addition of 20 μg HS/μg DNA. For the modified PEI, 8, 15, 8, 6, and 4 μg HS/μg DNA were required for PEI−Ac_{34}, PEI−Ac_{57}, PEI−Ac_{76}, PEI−Ac_{96}, and PEI−Ac_{100}, respectively. Although it appears that less heparan sulfate is required to displace PEI−Ac_{34} than PEI−Ac_{57}, it

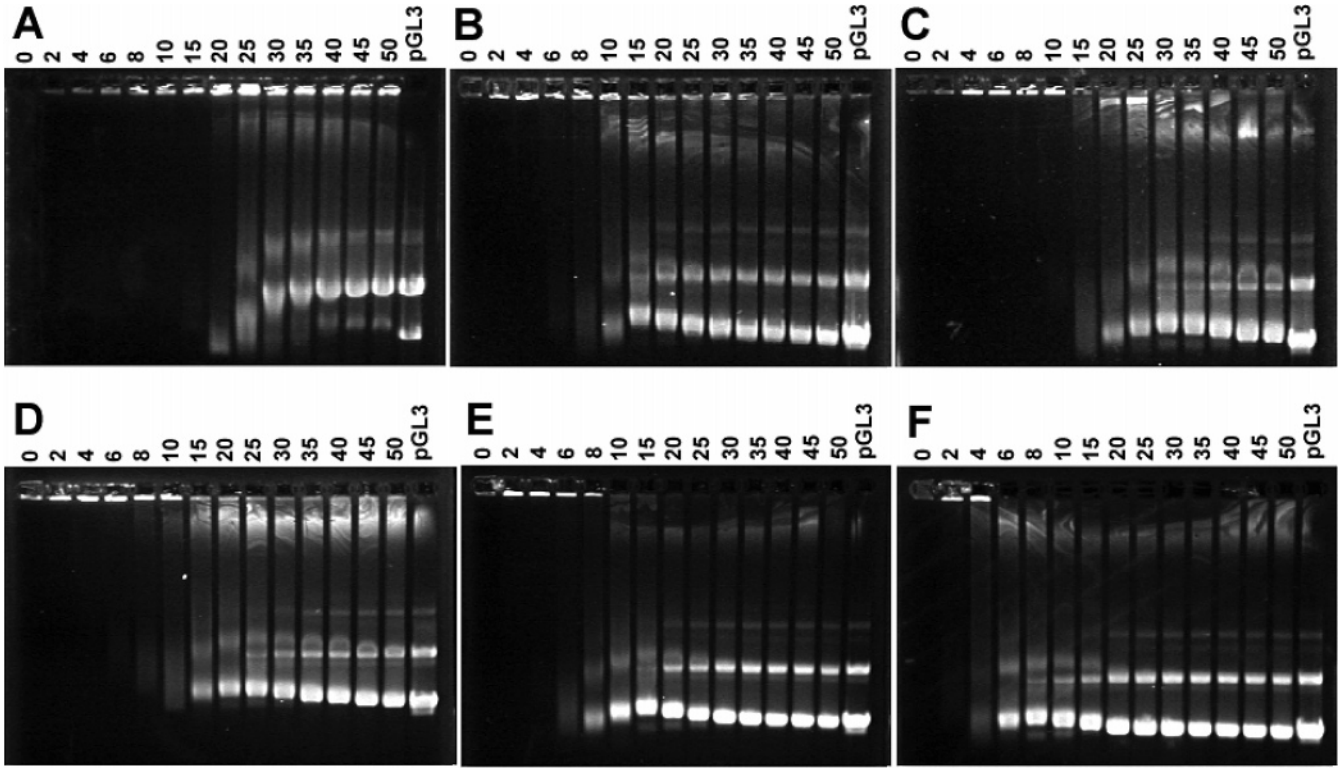

Figure 5. Agarose gel electrophoresis of polyplexes in the presence of various amounts of heparan sulfate. The polyplexes were formed at the polymer/DNA ratio found to be optimal for transfection. (A) Unmodified PEI, 2:1 (w:w); (B) PEI−Ac_{34}, 2:1 (w:w); (C) PEI−Ac_{57}, 3:1 (w:w); (D) PEI−Ac_{76}, 3:1 (w:w); (E) PEI−Ac_{96}, 4:1 (w:w); and (F) PEI−Ac_{100}, 4:1 (w:w). The number above each lane represents the mass of HS per mass of DNA. The right-most lane in each gel contains uncomplexed plasmid (pGL3).

should be noted that the polyplexes made with PEI−Ac_{34} were formed at a 2:1 mass ratio (polymer:DNA), whereas the polyplexes made with PEI−Ac_{57} were formed at a 3:1 mass ratio. The trend of easier polymer displacement with increased acetylation is likely due to the reduced positive charge per mass of the modified PEI.

Intracellular Polyplex Unpackaging. Clearly, at an as yet unknown step within the cell, the polymer and DNA must either disassociate or at least loosen to allow transcription factors access to the DNA. Fluorescence resonance energy transfer (FRET) between donor and acceptor dyes attached to the polymers was employed along with confocal microscopy to monitor the dissociation of the polyplexes. A similar approach has been used previously to investigate unpackaging of lipofectAMINE, poly-L-lysine, and PEI in vitro and inside cells.[28,29] Here, 5,6-tetramethylrhodamine (TAMRA) acted as the acceptor and Alexa Fluor 488 (AF488) was the donor. The dyes were present on separate polymer chains such that, in free polymer solutions, little energy transfer could occur. In polyplexes, however, the TAMRA- and AF488-labeled polymers were brought into close proximity such that a strong FRET signal (i.e., excitation of AF488 and emission from TAMRA) was observed. Thus, the ratio of AF488 and TAMRA emission intensities, upon excitation at 488 nm, is characteristic of the degree of unpackaging (or dissociation) of the polyplexes.

MDA-MB-231 cells were transfected with dual-labeled polyplexes, and the FRET signal was determined from confocal fluorescence micrographs at 30 min, 1 h, 2 h, and 4 h post-transfection (Figure 6A). Because each polymer exhibited slightly varying amounts of AF488 and TAMRA, the FRET signal corresponding to fully condensed polyplexes was different for each polymer. Therefore, the FRET signals are normalized to the value measured at 30 min, which is assumed to be sufficiently early that very little unpackaging has occurred. With unmodified PEI/DNA polyplexes, the AF488/TAMRA ratio decreased slightly between 30 min and 1 h post-transfection. A decrease in the FRET signal implies "tighter" polyplexes. However, it is more likely that the observed decrease is due to an anomalous effect such as photobleaching or quenching of one of the dyes due to a change in the physicochemical environment of the polyplexes as they are trafficked through the cell. Nevertheless, the most important result is the comparison of FRET signals with the acetylated polymer complexes relative to unmodified PEI and to one another. PEI−Ac_{34} showed no change in AF488/TAMRA ratio through 4 h post-transfection but was slightly higher than unmodified PEI at 2 and 4 h. However, PEI−Ac_{57} and PEI−Ac_{76} show increased AF488/TAMRA ratio with time after transfection. Furthermore, PEI−Ac_{76} showed significantly higher ($p < 0.01$) AF488/TAMRA ratios than PEI−Ac_{57} at 1 and 4 h. Thus, there appears to be a correlation between the degree of PEI acetylation and the relative rate and extent of unpackaging inside cells.

Cellular Uptake of Polyplexes. The endocytosis of cationic polyplexes lacking a targeting ligand is a nonspecific process. The positively charged polyplexes most likely bind to syndecans, a type of transmembrane heparan sulfate proteoglycan, through an electrostatic interaction and are taken into the cell via endocytosis.[30] Although acetylation of PEI has been shown to reduce the charge density of the polymer, the polyplexes retain a net positive charge at the polymer/DNA ratios used for transfection.[22] Thus, nonspecific endocytic processes are most likely responsible for internalization of acetylated PEI-containing polyplexes. To determine if increased transfection could be attributed to increased endocytosis, the uptake of fluorescently labeled polyplexes was measured with fluorescence-activated cell sorting (FACS).

For each cell line, it appears that unmodified PEI is endocytosed less than its acetylated counterparts (Figure 6B).

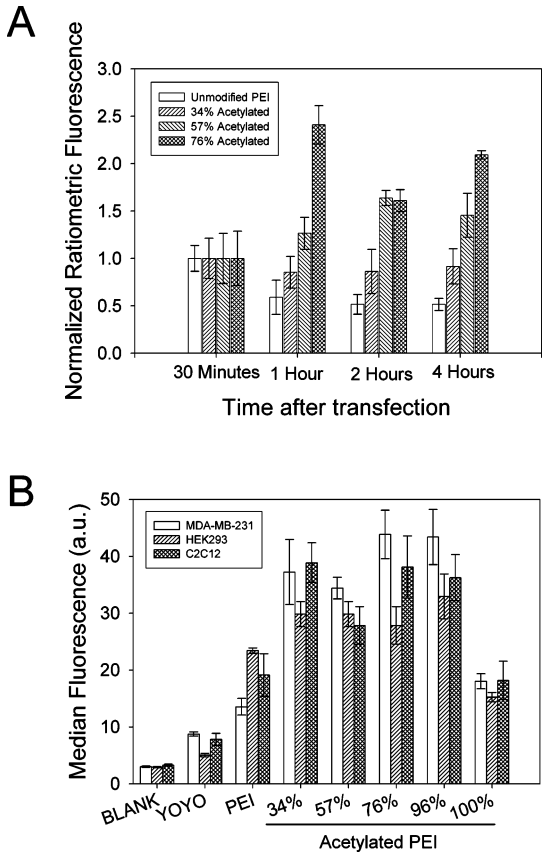

Figure 6. (A) FRET disassociation assay of polyplexes formed with TAMRA-labeled and Alexa Fluor 488-labeled polyplexes transfected into MDA-MB-231 cells. Cells were imaged with a confocal microscope at indicated times after transfection. From the images, the average pixel intensity ratios of TAMRA to Alexa Fluor 488 for individual polyplexes were plotted at various time points post-transfection to gauge polyplex disassociation for various acetylated polymers. (B) Uptake of fluorescently labeled polyplexes. Polyplexes incubated with the fluorescent intercalator YOYO-1 were added to cells for 2 h and subjected to FACS analysis to gauge the effect of acetylation on endocytosis ($N = 3$, error bars represent standard deviation).

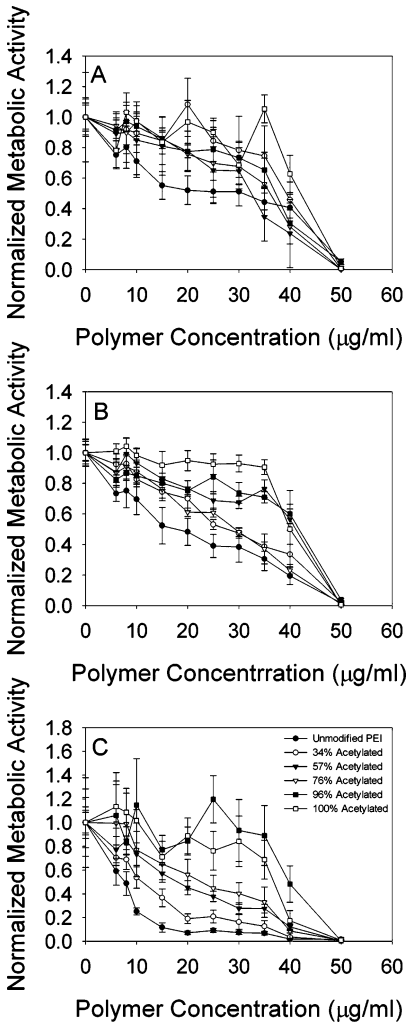

Figure 7. Cytotoxicity of PEI and acetylated derivatives was determined as the normalized metabolic activity of (A) MDA-MB-231, (B) C2C12, and (C) HEK293 cell lines in the presence varying amounts of polymers. The normalized standard deviation is shown for each data point ($N = 8$, error bars represent standard deviation).

It is possible that the addition of acetyl groups to the PEI enhances the lipophilicity of the complexes, thereby increasing polyplex association with the cell membrane and enhancing uptake. Regardless of the mechanism, enhanced uptake is partially responsible for the increased transfection efficiency. Uptake increases by a factor of at most 4, whereas transfection efficiency is seen to increase between 30- and 58-fold, however. Further, polyplexes containing PEI−Ac_{76} and PEI−Ac_{96} were internalized equal to or more than PEI−Ac_{57}, but gene delivery activity was poorer with the former two polymers, suggesting that increased uptake is not solely responsible for increased transfection activity.

Cytotoxicity. PEI is toxic to many types of cells.[31] The cytotoxicity of the various acetylated polymers in each of the three cell lines was therefore compared with unmodified PEI. Generally, the same electrostatic interactions that draw polycations to negatively charged membranes also induce a hemolytic event in cells.[32,33] Acetylating the amines of PEI reduces their surface charge and thus may reduce their lytic ability. Each cell line was incubated with polymers at concentrations up to 50 μg/mL for 4 h, and metabolic activity was determined 20 h later (Figure 7). Cytotoxicity decreased with increasing acetylation, as expected. Regardless, both acetylated and unmodified polymers were toxic to these cells at high concentrations.

Discussion

Development in nonviral gene delivery vectors has focused on mimicking viruses. Viruses efficiently escort their genetic payload over critical obstacles including binding to the cell surface, escape from the endosomal pathway and localization to the nucleus. To mimic these functions, nonviral vectors have been derivatized with targeting ligands,[34,35,36] proton sponge polymers have been designed to escape endosomes,[37] and nuclear localization peptides have been attached to DNA[38,39] and polymers.[40] Viruses have also evolved specific mechanisms for shedding their coat within cells. Thus, mimicking the innate ability of viruses to decomplex from their genetic cargo and to deliver free and uncomplexed DNA to the nucleus is an important avenue to pursue in the construction of an "artificial virus." Little attention has been devoted to understanding and controlling polymer−DNA disassociation, however.

We have modified commercially available, branched, 25-kDa PEI through the addition of acetyl groups to amines in the polymer backbone. Polyplexes formed with acetylated PEI present many of the same characteristics as those containing unmodified PEI. Although slightly larger, low to moderately acetylated polyplexes have diameters in the range of 250−270 nm, allowing them to enter the cell through nonreceptor-

mediated endocytosis. Likewise, despite having a reduced surface charge, acetylated PEI still forms polyplexes with a positive zeta potential (not shown). The positive charge facilitates nonspecific association with the negatively charged membranes of cells. The similarity in polyplex size and charge between unmodified and acetylated PEI suggests that little difference should be expected in the endocytosis of both types of polyplexes. While it appears that partial acetylation does increase uptake by as much as 4-fold (Figure 6B), the increase in the number of polyplexes is unlikely to fully explain the 30- to 58-fold increase in transfection efficiency.

Importantly, the polyplexes formed with acetylated PEI are more easily dissociated into free polymer and DNA than unmodified PEI. The heparan sulfate displacement assay (Figure 5) suggests that the reduced positive charge of acetylated complexes weakens the electrostatic interaction between polymer and DNA. According to the ethidium bromide exclusion analysis (Figure 4), polyplexes formed with acetylated PEI are also looser, or less tightly condensed, than those formed with unmodified PEI at low weight ratios. It is plausible therefore that cellular proteins or negatively charged cellular or endosomal membranes could pull the acetylated polymers away from the negatively charged DNA more easily, leaving a partially uncomplexed DNA free in the cytosol. The FRET results (Figure 6A) support this contention.

Increased transfection efficiency through reduction of positive charge density has been reported elsewhere. The addition of dextran to PEI increased transfection efficiency between 1.3 and 3 times compared to transfection using unmodified PEI.[41] The gluconoylation of polylysine was reported to increase transfection, as well.[42] Although not directly addressed in these previous studies, the reduced charge density may have resulted in more efficient polyplex unpackaging in these cases. A variety of degradable polymers have also shown increased transfection compared to nondegradable counterparts. For example, the addition of biodegradable cross-linkers to 800-Da PEI to create higher molecular weight, hydrolyzable vectors also show transfection increases between 2- and 16-fold.[43] Presumably, as the cross-linkers degrade, the 800-Da PEI segments exhibit decreased binding strength that ultimately results in more facile dissociation.

Although a net positive charge has benefits with regard to increased nonreceptor-mediated endocytosis, the reduced charge also has advantages. For one, the reduced surface charge means the polymer is less likely to be toxic. This trend was observed with all of the acetylated PEIs (Figure 7). However, the least toxic polymer is not the most efficient gene delivery vehicle. This indicates that the increased transfection efficiency is not strictly the result of reduced vector toxicity. The reduced surface charge may also mean that covalently attached ligands would be more effective.[44]

Finally, the acetylated polymers have a reduced buffering capacity (Figure 1B). According to the proton-sponge hypothesis, a reduction in buffering capacity may be expected to lead to reduced transfection efficiency. However, this trend was not observed, except when the degree of acetylation was greater than 57%. Data questioning the effect of buffering capacity on transfection have been presented elsewhere. For example, the pH environment of polyplexes has been shown to reach lysosomal conditions. It has also been shown that chloroquine, a buffering agent capable of disassociating lactosylated polylysine from DNA, increases transfection efficiency.[43] However, the addition of ammonium chloride and methylamine, both buffering agents incapable of disassociating polyplexes, does not increase transfection efficiency.[45]

In summary, acetylated PEI is a highly efficient in vitro nonviral vector. Characterization of the formation, disassociation, and uptake of polyplexes formed with acetylated PEI allows the source of its efficiency to be probed. The acetylated polyplexes maintained the size, charge, and similar level of uptake as polyplexes containing unmodified PEI. Further, polymer buffering capacity decreased with acetylation. Finally, acetylation appears to decrease polymer/DNA interactions and may lead to more facile unpackaging of polyplexes. The optimal degree of acetylation is likely due to a trade off between escape from endocytic vesicles due to polymer buffering capacity, which decreases with acetylation, and efficient unpackaging, which increases with acetylation. Thus, design of materials for gene delivery should consider unpackaging as an important design criterion.

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