Partial Acetylation of Polyethylenimine Enhances *In Vitro* Gene Delivery

M. Laird Forrest,[1] Glenna E. Meister,[1] James T. Koerber,[1] and Daniel W. Pack[1,2]

Received July 18, 2003; accepted October 14, 2003

Purpose. Polyethylenimine (PEI) is a highly effective gene delivery vector, but because it is an off-the shelf material, its properties may not be optimal. To investigate the effects of the protonation properties of the polymer, we generated PEI derivatives by acetylating varying fractions of the primary and secondary amines to form secondary and tertiary amides, respectively.

Methods. Reaction of PEI with increasing amounts of acetic anhydride at 60°C for 4.5 h yielded polymers with 15%, 27%, and 43% of the primary amines modified with acetyl groups. Polymer–DNA complexes were characterized by dynamic light scattering and ζ potential measurements. Cytotoxicity of the polymers was assessed by XTT assay for metabolic activity, and gene delivery efficiency was determined as the relative expression of a luciferase gene in MDA-MB-231 and C2C12 cell lines.

Results. Acetylation of PEI decreased the "physiological buffering capacity," defined as the moles of protons absorbed per mole of nitrogen on titration from pH 7.5 to 4.5, from 0.29 mol H^+/mol N to 0.17 mol H^+/mol N, 0.12 mol H^+/mol N, and 0.090 mol H^+/mol N for PEI-Ac_{15}, PEI-Ac_{27}, and PEI-Ac_{43}, respectively. In addition, acetylation decreased the ζ potential of polyplexes from 14 mV to 8–11 mV and increased the polyplex diameter by two- to threefold. Surprisingly, acetylation had a negligible effect on cytotoxicity of the polymers and increased gene delivery effectiveness by up to 21-fold compared to unmodified PEI, both in the presence and absence of serum.

Conclusions. Reduction of the buffering capacity of PEI greatly enhanced the gene delivery activity of the polymer. The mechanism is not yet understood, but the enhancement may be caused by more effective polyplex unpackaging, altered endocytic trafficking, and/or increased lipophilicity of acetylated PEI–DNA complexes. Future studies will address these possibilities in more detail.

KEY WORDS: gene delivery; polyethylenimine; proton-sponge; buffering capacity.

INTRODUCTION

Human gene therapy promises to prevent, treat, or cure a variety of conditions including genetic, acquired, and infectious diseases. However, gene delivery—the process of transferring therapeutic genetic material to specific human cells safely and efficiently—remains a limiting challenge (1). Recombinant viruses are extremely efficient (2) but are limited by small gene-carrying capacity, immunogenicity, pathogenicity, and difficult and expensive production. Nonviral materials, including cationic lipids and polymers, are orders of magnitude less efficient than viruses. As a result, only about 12% of gene therapy clinical trials have used liposomes, and none has used polymer-mediated gene delivery (3).

Because of their important advantages, especially in terms of safety and robustness, many types of cationic polymers have nevertheless been studied for gene delivery (4–7). Although several types of polymers have been designed and synthesized expressly for gene delivery, much of the literature is devoted to off-the-shelf polymers. For example, poly-L-lysine (PLL) was one of the first nonviral gene delivery materials reported (8,9). Although much was learned about polyplex gene delivery from these and subsequent studies, PLL is not efficient enough to be generally useful as a gene therapy agent. Polyethylenimine (PEI), another off-the-shelf polymer, has been used extensively for delivery of plasmid DNA *in vitro* and *in vivo* (10,11) and is one of the most efficient polymers reported to date. Only a few polymers that are more efficient than branched, 25-kDa PEI have been reported (12,13).

The mechanisms by which PEI mediates relatively efficient gene delivery have been widely investigated (14–16). It is generally accepted that its efficiency derives from its ability to escape from endocytic vesicles by the "proton-sponge" mechanism. According to the proton-sponge hypothesis, partially protonated polymers can absorb protons brought into endocytic vesicles by the ATPase proton pump, causing osmotic swelling and ultimately rupture of the vesicles (17). PEI makes an excellent proton sponge because of its very high density of amines.

A focus of recent work is to quantitatively understand the structure–activity relationships of gene delivery polymers in order to facilitate the design of new materials (14,15,18,19). For example, the molecular weight of the PEI is known to be an important factor in gene delivery efficiency. Low-molecular-weight PEI, less than 2000 Da (20), is essentially ineffective. There are varying reports of the effects of increasing PEI size, but polymers > 10 kDa appear to be generally effective gene delivery agents (21). Similarly, recent studies have compared linear vs. branched PEI and found that the linear polymer can be as effective as the branched PEI for transfection *in vitro* and *in vivo* (22,23).

We have recently reported quantitative kinetic measurements of the endocytic trafficking of PEI–DNA polyplexes (15). We found that, in contrast to some of the conventional wisdom (24), PEI-containing polyplexes do not escape from endosomes but are trafficked to the acidic environment of the lysosomes. A previous study by Lecocq *et al.* (18), reporting subcellular localization of PEI polyplexes on *in vivo* administration, supports these results.

Because PEI is an off-the-shelf polymer, not designed specifically for gene delivery, there is no reason to expect that its properties should be optimal. Although PEI is a rather effective gene delivery agent, relatively simple changes in the polymer structure may be expected to yield improvements. For example, there appears to be some discrepancy in the literature regarding the effect of polymer buffering capacity. Several studies with PEI and other polymers have found no clear correlation between buffer capacity and transfection efficiency, but others have implied that increased buffering capacity leads to more efficient transfection (25,26). In the present study, we have investigated the effects of systematically

[1] Department of Chemical and Biomolecular Engineering, University of Illinois, Urbana, IL 61801.

[2] To whom correspondence should be addressed (email: dpack@uiuc.edu).

0724-8741/04/0200-0365/0 © 2004 Plenum Publishing Corporation

varying the buffering capacity of branched, 25-kDa PEI. We have acetylated varying fractions of the primary and secondary amines of PEI by reaction with acetic anhydride. We show that these polymers are up to 26-fold more efficient than the unmodified PEI. In addition, the acetylated PEI is similarly or less toxic than unmodified PEI in the cell lines studied.

MATERIALS AND METHODS

Cells and Plasmid DNA

The MDA-MB-231 human breast carcinoma cell line was purchased from the American Type Culture Collection, and the C2C12 murine myoblast cell line was a gift from Prof. Stephen Kaufman (University of Illinois, Urbana, IL). All cell lines were maintained according to their respective ATCC protocols, at 37°C and 5% CO_2, but were adapted from fetal bovine serum to 10% horse serum. The 5.3-kilobase pair expression vector pGL3 (Promega, Madison, WI), containing 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). Plasmids were further purified by ethanol precipitation; the ratio of absorbances at 260 and 280 nm was 1.8 or greater.

Acetylation of PEI

All reagents were purchased from Sigma-Aldrich (St. Louis, MO) and used without further purification unless noted otherwise. Branched, 25-kDa PEI (0.5 g) was transferred to a 1-oz scintillation vial and dissolved in 3 ml of freshly distilled methanol (1.19 M primary amines). Sufficient acetic anhydride to achieve the desired degree of acetylation was added (e.g., 0.595 M acetic anhydride for 50% acetylation, assuming 50% yield), and the vial was sealed with a solvent-resistant cap. The reaction was carried out at 60°C with shaking for 4.5 h. The reaction was then quenched with 0.5 ml of double-distilled H_2O, and most of the methanol was removed under vacuum. The remaining solution was purified by gel filtration chromatography (PD-10 columns, Pharmacia) eluted with double-distilled H_2O, and the polymers were lyophilized and stored at −80°C.

Determination of Extent of Acetylation

The ratio of primary, secondary, and tertiary amines in the 25-kDa PEI starting material was determined following the technique reported by von Harpe (27), with modifications as indicated. Briefly, PEI was dissolved in D_2O (180 mg/ml), and the ^{13}C-NMR spectrum was acquired using a Varian Unity Inova 750 MHz spectrometer (Palo Alto, CA) (188.56 MHz, 10-mm probe at 30°C, 2500 scans, recycle delay 5 s) with correction for nuclear Overhauser effects. Integration was performed using the WinNuts 2D software package (Acorn NMR, Livermore, CA); the signal-to-noise ratios on integrated peaks were at least 600.

Acetylated polymers were dissolved in D_2O (100 mg/ml), and ^{1}H-NMR spectra were acquired on a Varian Unity Inova 500NB with a 5-mm probe (500.08 MHz, probe temperature 25°C, 200 scans). The extent of secondary and tertiary amide formation in PEI was determined by peak integration using the following formulas:

$$\text{acetylated secondary amines (\%)} = \left(\frac{E_{bb}}{4}\cdot\frac{3}{M'}\cdot N'\right)^{-1}\cdot 100\%$$

$$\text{acetylated tertiary amines (\%)} = \left(\frac{E_{bb}}{4}\cdot\frac{3}{M''}\cdot N''\right)^{-1}\cdot 100\%$$

where M' is the integration of δ 1.70–1.75 peaks (R–NHCOC**H**$_3$, acetylated secondary amines), M'' is the integration of δ 1.80–1.85 peaks (R_2–NCOC**H**$_3$, acetylated tertiary amines), E_{bb} is the integration of δ 2.4–2.9 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), and N' and N'' are mole fractions of primary and secondary amines, respectively, based on the above analysis of the PEI starting material.

Polyplex Formation and Transfection

DNA–polymer complexes (polyplexes) were prepared at room temperature in 150 mM NaCl (pH 7.3, buffered with 20 mM PIPES) by addition of 150 μL of polymer solution to an equal volume of 3 μg plasmid to achieve the desired polymer/DNA ratio. Polyplexes were then incubated at 4°C for 15 min. Cells were cultured in DMEM supplemented according to ATCC protocols and plated in 6-well plates at 1×10^5 cells/well 24 h before transfection. Immediately before transfection, the growth medium was replaced with fresh medium (with 10% serum or serum-free, as appropriate), and 100 μL of polyplexes (1 μg plasmid/well) was added to each well. Transfection medium was replaced with growth medium 4 h posttransfection. Luciferase expression was quantified 24 h later using a Promega luciferase assay system ($n \geq 6$). Luciferase activity was measured in relative light units (RLU) using a Lumat LB 9507 luminometer (Berthold, GmbH, Germany) and converted to luciferase concentration by comparison to recombinant luciferase standards (Promega). Results were normalized to total cell protein as determined using a Bio-Rad Protein Assay Kit.

Cytotoxicity Determination

Cytotoxicity was characterized as a decrease in metabolic activity measured using the XTT assay (28). Cells were plated in 96-well plates at an initial density of 50,000 cells per well in 100 μl of growth medium for 24 h. Afterwards, the growth medium was replaced with fresh, serum-free medium containing the polymer of interest. Cells were incubated with polymers for 4 h, and the medium was replaced with complete growth medium for 24 h. Fresh XTT (1 mg/ml) and coenzyme Q_0 (80 μg/ml) stock were prepared each day in PBS and filter sterilized (0.22-μm syringe filter). Both components were diluted in PBS (0.5 μg/μl XTT and 0.04 μg/μl coenzyme Q_0), and 10-μl aliquots were added to each well. The samples were incubated for 4 h at 37°C, and the absorbance was read at 450 nm relative to blank wells prepared without cells ($n = 8$).

Polymer Titrations

Polymers (5.0 mg) were dissolved in 2.0 ml of 10 mM NaCl and dialyzed against 10 mM NaCl for 3 days using 3000 MW cutoff Slide-A-Lyzer cassettes (Pierce, Rockford, IL). Polymers were then adjusted to pH 11.5 using 1 M NaOH and titrated with 0.05 M HCl to pH 3.0. The "physiological buffering capacity" was calculated as:

$$\text{capacity} = \frac{\Delta H^+_{\text{titrated}} - \Delta H^+_{\text{observed}}}{NH_x}$$

where $\Delta H^+_{\text{titrated}}$ is the moles H^+ added during titration with HCl from pH 7.5 to 4.5, $\Delta H^+_{\text{observed}}$ is calculated as $10^{-7.5} \cdot$ (volume at pH 7.5) – $10^{-4.5} \cdot$ (volume at pH 4.5), and NH_x is the total moles of amines (primary, secondary, and tertiary) in the titrated polymer sample.

Gel Retardation Studies

Appropriate amounts of each polymer, in 10 μl of 150 mM NaCl (pH 7.3, 20 mM PIPES as buffer) were added to an equal volume of DNA solution (500 ng/10 μl) to achieve the desired polymer/DNA ratio. Polyplexes were incubated at 4°C for 15 min, after which 10 μl was run on a 0.75% agarose gel (70 V, 1 h). DNA was visualized with Vista Green staining (Molecular Probes, Eugene, OR).

Polyplex Size and ζ Potential Determination

Polyplexes were prepared as in the above transfection procedure. Polyplexes were then diluted with 0.45-μm filtered PBS to a final concentration of 1 μg DNA/ml. Polyplex size determination was performed by dynamic light scattering on a DynaPro-MS800 particle sizer (Protein Solutions, Lakewood, NJ), and the data were analyzed with Dynamics v6.3 software. The ζ potential measurements were performed on a Zetasizer 3000HS (Malvern, Worcestershire, UK). Samples were run in triplicate.

RESULTS

Acetylation of Polyethylenimine

To investigate the effects of polymer buffering capacity on gene delivery efficiency, we modified commercially available 25-kDa PEI with various amounts of acetic anhydride to acetylate the primary and secondary amines. PEI was mixed with acetic anhydride in methanol at molar ratios of 0.2, 0.32, 0.47 (acetic anhydride/amine), and the reaction proceeded at 65°C for 4.5 h. The extent of primary and secondary amine acetylation was determined from the relative methyl and methylene signals in the ^{1}H-NMR spectra (Table I).

To characterize the effects of acetylation on the buffering capacity of PEI, we measured protonation profiles of the polymers by titrating aqueous polymer solutions (5 mg/ml) with HCl from pH 11.5 to 3.0. Unmodified PEI exhibits a "physiological buffering capacity," defined as the change in protonation per amine between pH 7.5 and 4.5, of 0.29 mol H^+/mol N. The buffering capacities of the polymers decreased with increasing amount of acetylation: PEI-Ac_{15}, 0.17 mol H^+/mol N; PEI-Ac_{27}, 0.12 mol H^+/mol N; and PEI-Ac_{43}, 0.090 mol H^+/mol N.

Table I. Properties of the Acetylated PEI Derivatives

	% NH_2 acetylated[a]	% NH acetylated[a]	Mole fraction of amines[b] 1°	2°	3°	M_w
PEI	—	—	0.307	0.395	0.297	25,000
PEI-Ac_{15}	15%	9.6%	0.262	0.403	0.335	27,000
PEI-Ac_{27}	27%	16%	0.223	0.415	0.362	28,600
PEI-Ac_{43}	43%	23%	0.176	0.435	0.389	30,500

[a] Percentage of acetylation determined from ^{1}H-NMR spectra.
[b] Mole fractions calculated from percentage acetylation.
[c] Molecular weight calculated assuming M_w of unmodified polymer = 25,000 and adding molecular weight of the required number of acetyl groups.

Characterization of Acetylated PEI–DNA Complexes

It is important for efficient gene delivery that polymer bind to and condense plasmid DNA to form relatively tight complexes a few hundred nanometers in size. Converting primary and secondary amines to secondary and tertiary amides, respectively, may be expected to affect the electrostatic interaction between polymer and DNA. Altered binding could have effects on the polyplex size, density, or surface charge, which could in turn impact uptake of the polyplexes by target cells. In addition, weaker binding might lead to easier "unpackaging" of the polyplexes inside cells.

PEI forms compact particles on mixing with plasmid DNA through electrostatic attraction. The formation of these polyplexes can be observed as a reduction of mobility of the plasmid in agarose gel electrophoresis. We mixed DNA with increasing amounts of unmodified and acetylated PEI to determine the ability of the polymers to form polyplexes with DNA. Unmodified PEI completely retarded DNA migration at a ratio of 0.175:1 (w:w), indicating the formation of charge-neutral complexes. The amount of polymer required to completely retard DNA migration increased with the extent of acetylation: PEI-Ac_{15}, 0.25:1; PEI-Ac_{27}, 0.30:1; and PEI-Ac_{43}, 0.40:1 (w:w) (Table II). This result was expected because of the increased molecular weight after acetylation (Table I) and the decreased percentage of protonated amines at pH 7.5.

Polyplexes containing unmodified PEI exhibited ζ potentials of 14–15 mV at PEI/DNA ratios from 2:1 to 10:1 (w:w). As expected because of the decrease in the average pK_a of the polymers on acetylation, ζ potentials of the modified polymers decreased with increasing acetylation to 8–11 mV over the same range of polymer/DNA (Fig. 1). The decreased ζ potential of the modified polyplexes indicated that acetylation decreased the surface charge of the polyplexes, consistent with the decreased percentage of charged nitrogens on the polymers, independent of nitrogen:phosphate (N:P) ratio.

Table II. Polymer/DNA Ratios Used in Formation of Polyplexes, Given in Terms of Weight Ratios, Nitrogen-to-Phosphate Ratios, and Positive to Negative Charge Ratios for Neutral Polyplexes and Polyplexes that Mediate the Highest Transfection of Both Cell Lines (i.e., "Optimal Transfection")

	Charge neutral[a]			Optimal transfection		
	PEI:DNA (w:w)	N:P	+/–	PEI:DNA (w:w)	N:P	+/–
PEI	0.175	1.35	1	2	15	11
PEI-Ac_{15}	0.25	1.8	1	2	14	8
PEI-Ac_{27}	0.30	2.0	1	3	20	10
PEI-Ac_{43}	0.40	2.5	1	3	19	8

[a] Charge–neutral ratio determined as the smallest polymer/DNA ratio that completely retarded migration of DNA in gel electrophoresis (not shown).

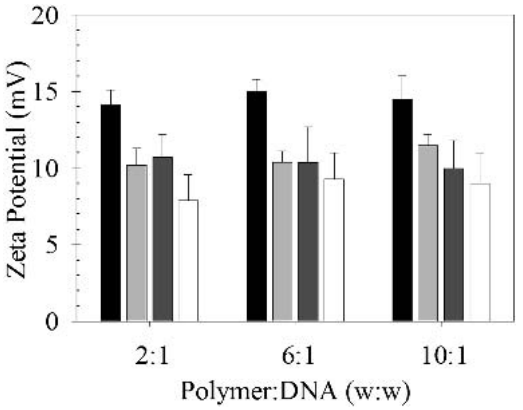

Fig. 1. The ζ potential of polyplexes at various polymer/DNA ratios. Black bars, unmodified 25-kDa PEI; light gray bars, PEI-Ac_{15}; dark gray bars, PEI-Ac_{27}; white bars, PEI-Ac_{43}. (n = 3, error bars represent standard deviation).

Because polyplex size can be important for efficient cell uptake, we investigated the effect of PEI acetylation on polyplex size. Polyplexes with polymer/DNA ratios of 2:1 to 10:1 (w:w), typical of transfection experiments, were formed, and dynamic light scattering was used to measure polyplex size. We found that unmodified PEI produced polyplexes with diameters of 40–60 nm over the observed range of polymer/DNA ratios. Acetylated PEI formed slightly larger polyplexes. The largest polyplexes (at 6:1 w:w) were between 80 and 120 nm in diameter (Fig. 2). Previous reports have indicated that complexes in this size range are efficiently endocytosed during transfection (29,30).

Cytotoxicity of Acetylated PEI

Polycationic polymers have been reported to damage cell membranes as a rsult of the electrostatic attraction of polymers to the plasma membrane; however, neutral and polyanionic polymers showed minimal damage to cellular membranes (31). Because acetylation decreased the ζ potential of the polyplexes, we expected that the cytotoxicity of the modified PEI may be reduced. We assayed cytotoxicity as reduced metabolic activity using an XTT assay. Human breast carcinoma cells (MDA-MB-231) and murine myoblasts (C2C12) were exposed to various concentrations of polymers for 4 h, and metabolic activity was assayed 20 h later (Fig. 3). MDA-MB-231 cells were resistant to 25-kDa PEI; metabolic activities remained near control levels over the concentration range investigated. Metabolic activity increased slightly above baseline levels for the derivatized polymers, indicating that the polymers were essentially nontoxic at concentrations normally used in gene delivery. The 25-kDa PEI is toxic to C2C12 cells, reducing metabolic activity by 50% at 15 μg/ml, the concentration typically used in transfection studies. No statistically significant decrease was observed with the acetylated polymers. Overall, the cytotoxicity of acetylated PEI remained very similar to that of unmodified PEI over the observed concentration ranges in both cell lines.

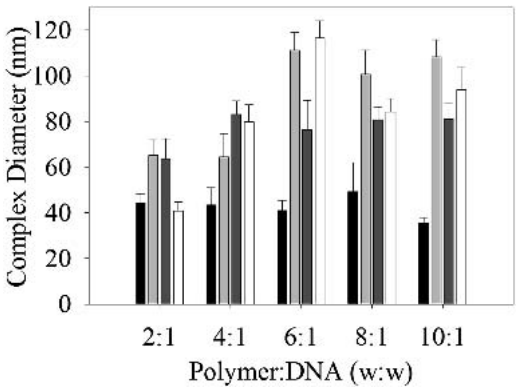

Fig. 2. Polyplex size, determined from dynamic light scattering, of polyplexes at various polymer/DNA ratios. Black bars, unmodified 25-kDa PEI; light gray bars, PEI-Ac_{15}; dark gray bars, PEI-Ac_{27}; white bars, PEI-Ac_{43} (n = 3, error bars represent standard deviation).

In Vitro Transfection of Model Cell Lines

We investigated the efficacy of the acetylated PEI polymers in gene delivery to MDA-MB-231 and C2C12. These model cell lines were chosen because of our interest in gene therapy for treatment of cancer and muscular dystrophy. Both cell lines were transfected *in vitro* with 1 μg of plasmid DNA per 100,000 cells, complexed with varying amounts of each polymer. Gene transfection efficiency was measured as luciferase enzyme activity, normalized to total cell protein (Fig. 4). Although not shown, transfection with plasmid DNA only (no polymer) routinely resulted in undetectable gene expression in both of these cell lines. The optimal polymer/DNA ratio for the starting material, 25-kDa PEI, was 2:1

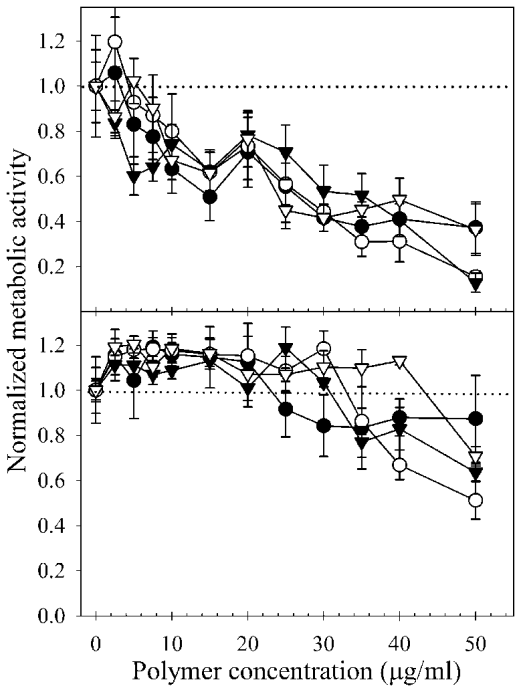

Fig. 3. Cytotoxicity of PEI and acetylated PEI derivatives. Metabolic activity of (A) C2C12 and (B) MDA-MB-231 cell lines in the presence of varying amounts of unmodified 25-kDa PEI (●), PEI-Ac_{15} (○), PEI-Ac_{27} (▼), and PEI-Ac_{43} (∇). Metabolic activity was normalized to controls with no polymer (n = 8, error bars represent standard deviation).

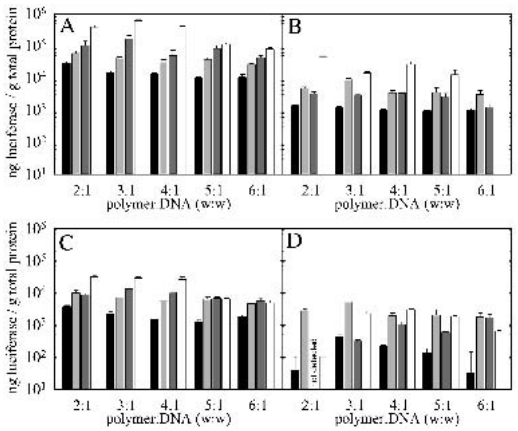

Fig. 4. Gene delivery activity of acetylated PEI derivatives. Luciferase expression in C2C12 cells (A and B) and MDA-MB-231 cells (C and D) upon transfection in the absence (A and C) and presence (B and D) of 10% serum. Luciferase expression was normalized by total cellular protein in cell lysates. Black bars, unmodified 25-kDa PEI; light gray bars, PEI-Ac_{15}; dark gray bars, PEI-Ac_{27}; white bars, PEI-Ac_{43} (n = 6, error bars represent standard deviation).

(w:w) in both cell lines (transfection data for polyplexes at < 2:1 w:w are not shown).

Acetylation was found to increase the transfection efficiency of PEI in both cell lines (Fig. 4A,C). In the C2C12 cell line, PEI-Ac_{43} induced up to a 21-fold enhancement in transfection efficiency at polymer/DNA ratio of 3:1 (w:w) compared to unmodified PEI at 2:1 (w:w) polymer/DNA. PEI-Ac_{27} (3:1 w:w) and PEI-Ac_{15} (2:1 w:w) enhanced transfection six- and twofold, respectively, compared to unmodified PEI. It should be noted that for the two most acetylated polymers, the optimal polymer/DNA ratio was higher than with unmodified PEI, although there was no statistically significant difference in polymer toxicity between the acetylated and unmodified polymers. Transfection of the MDA-MB-231 cell line was also enhanced by acetylation of PEI. PEI-Ac_{43} induced up to an eightfold increase in transfection at polymer/DNA ratio of 2:1 (w:w), compared to unmodified PEI. PEI-Ac_{27} (3:1 w:w) and PEI-Ac_{15} (2:1 w:w) were both about threefold more effective than unmodified PEI. The MDA-MB-231 cell line was less sensitive to the polymer content of polyplexes than the C2C12 cell line. Transfection efficiency did not vary significantly with polymer/DNA ratio from 2:1 to 4:1 (w:w) for all three acetylated polymers.

The addition of serum to the media more closely approximates the *in vivo* environment and more effectively maintains viability of the cells during the transfection experiment, so we tested the polymers in transfection media supplemented with 10% serum (Fig. 4B,D). Acetylation of PEI enhanced gene delivery to both cell lines in the presence of serum. Serum reduced the transfection efficiency of unmodified PEI by 20-fold in the C2C12 cell line. The transfection activity of PEI-Ac_{43} was reduced 13-fold by the addition of serum, but even at this reduced level, PEI-Ac_{43} with serum was twofold better than unmodified PEI without serum (compare Figs. 4A and 4B). In the MDA-MB-231 cells, the addition of serum reduced the efficiency of unmodified PEI by 10-fold. In the presence of serum, the most effective polymer was PEI-Ac_{15} (3:1 w:w), which was nearly twofold more efficient than unmodified PEI without serum. In summary, although the efficiencies of the acetylated and unmodified PEI were all reduced by the addition of serum to the transfection media, some of the acetylated polymers exhibited higher transfection efficiencies in the presence of serum than unmodified PEI in the absence of serum.

DISCUSSION

In the past 15 years, numerous polymers have been used as gene delivery agents. Although a number of new polymers have been synthesized, many studies have relied on commercially available materials. One such polymer is PEI, available in several different molecular weights and in branched and linear forms. PEI is one of the most effective nontargeted polycations used for gene delivery. Its relatively high efficiency is believed to stem from its high amine density and buffering capacity (17).

However, there is little reason to assume that the protonation properties of PEI are optimal. Thus, we chose to investigate the effects of systematically altering the polymer protonation. We acetylated the primary and secondary nitrogens of PEI to varying extents by reaction with acetic anhydride to form secondary and tertiary amides. With conversion of primary amines to secondary amides and secondary amines to tertiary amides, the average protonation constant of the polymer became more acidic. The result is polymers with fewer positive charges at neutral pH and a decreased buffering capacity in the pH range of interest.

Thomas and Klibanov recently reported a similar approach for enhancing PEI-mediated gene delivery (12). These authors varied polymer charge, protonation properties, and hydrophobic–hydrophilic balance by modifying all of the amino groups on 25-kDa PEI with a variety of chemical groups including permethylation and perethylation to generate all quaternized amines on the polymer, choline, amino acids, and long-chain alkyl groups. Most of the modifications reduced gene delivery activity of PEI, although addition of alanine marginally increased gene delivery efficiency. Further, dodecylation and hexadecylation of low-molecular-weight, 2000-Da PEI enhanced gene delivery in the presence of serum by five- to sixfold in comparison to unmodified 25-kDa PEI. Unfortunately, it is difficult to directly compare these results to the effects of acetyl modification reported here because the degree of modification was much higher in Klibanov's polymers, and the effects of their modification on complexation and buffering were not reported.

Previous studies have also emphasized the importance of the polymer lipophilicity, which may impact the interaction between polymer and DNA as well as the interactions of the polyplexes with cell membranes. Notably, a series of papers has recently appeared reporting synthesis and characterization of a water-soluble lipopolymer (WSLP) consisting of PEI (1.8, 10, and 25 kDa) derivatized with cholesterol on its primary or secondary amines (25,32–34). These polymers showed dramatically increased gene delivery activity compared to the analogous unmodified PEI. The proposed benefits of the WSLP include altered buffering capacity and increased uptake due to the presence of the cholesterol.

Acetylation effected relatively minor changes in the properties of the polyplexes. The polyplexes formed with modified polymers were, in general, larger than polyplexes containing unmodified PEI, but even the largest polyplexes

observed (~120 nm) are within the size range needed for efficient uptake via endocytic mechanisms. The increase in size may be reflective of less compact complexes because of potentially weaker binding of the modified polymers to DNA. Alternatively, a larger number of plasmids (and associated polymer) may be present in the polyplexes. Further, acetylation decreased the ζ potential of the polyplexes. This may be expected because of the decreased fraction of protonated nitrogens on the modified PEI and the larger "surface area" over which the positive charge is dispersed.

We found that, in general, acetylation of the polymers increased the gene delivery activity of PEI in the absence of serum. Further, the amount of enhancement increased with the degree of acetylation up to the maximum studied, corresponding to 43% of the primary amines and 23% of the secondary amines being converted into the corresponding amides. Indeed, PEI-Ac_{43} mediated luciferase expression more than one order of magnitude more effectively than unmodified PEI, making this one of the most efficient polycation gene delivery agents reported to date.

Acetylated PEI was also more effective than unmodified PEI in the presence of serum. In fact, the gene delivery activity of modified PEI in the presence of serum can exceed that of unmodified PEI in the absence of serum. Cationic polyplexes are known to interact with anionic proteins in serum (35), resulting in reduced endocytosis. Reduction of protein adsorption by several means has been shown to enhance gene delivery (36,37). Acetylation may result in a similar decrease in protein interactions by decreasing the ζ potential of the polyplexes. Regardless of the mechanism, such strong transfection in the presence of serum may be extremely useful for gene delivery to fragile cell lines or primary cells.

Acetylation of PEI had little effect on the apparent toxicity of the polymers to either cell line, measured as reduced metabolic activity of cells in culture (Fig. 3). MDA-MB-231 cells are equally resistant to the unmodified and acetylated PEI at concentration up to ~25 μg/ml, whereas over the same concentration range, the metabolic activity of C2C12 cells was reduced approximately equally in the presence of any of the polymers tested. One might expect the acetylated PEI to be less toxic than unmodified PEI based on the reduced number of positive charges at a given concentration. However, the toxicity increased with acetylation at any given concentration of protonated nitrogens. For example, at pH 7.5, 15.0 μg/ml PEI corresponds to 0.220 μmol/ml of protonated amine (N^+); to reach the same 0.220 μmol N^+/ml requires 18.4 μg/ml PEI-Ac_{16}, 33.8 μg/ml PEI-Ac_{26}, or 65.5 μg/ml PEI-Ac_{43}. Previous studies have shown that polycation toxicity correlates not only with the number of positively charged groups but also with other factors including pK_a, charge density, and polymer structure (38). These other factors likely play a major role in determining toxicity of the modified polymers. Nevertheless, when the toxicities of the polymers are compared at approximately the concentrations used in optimal transfection experiments (equivalent to 3–8 μg/ml of polymer), there is little difference.

Because acetylation had little effect on cytotoxicity of the polymers, other factors must be responsible for the enhanced gene delivery activity. Several observations suggest possible sources for the enhanced gene delivery activity. Acetylation reduced the number of positively charged amines on the polymer at a given pH. As a result, we may expect weaker binding between the modified polymers and DNA, which in turn may provide more efficient unpackaging—separation of polymer and DNA—inside cells. Previous studies have shown a correlation between decreasing polymer/DNA binding and increased gene expression (21,39,40). Other effects of acetylation are the reductions of the average pK_a and buffering capacities of the polymers. Such changes in polymer protonation may influence the endocytic trafficking of the polyplexes and their escape into the cytosol. Finally, acetylation may impact the lipophilicity of the PEI, which has been shown to be beneficial for gene delivery mediated by PEI and other polymers. More thorough investigation of the mechanism of the enhancement will be reported elsewhere.

In summary, we have found that altering the protonation properties of PEI through conversion of primary and secondary amines to secondary and tertiary amides, respectively, greatly enhances gene delivery activity of the polymer. The mechanism of the enhancement is not yet fully understood, but we believe it is a combination of more effective polyplex unpackaging and altered endocytic trafficking. Further studies are aimed at elucidating the specific effects of acetylation on these intracellular processes. In any case, this simple modification of PEI produced one of the most effective gene delivery polymers yet reported, both in the absence and presence of serum.

ACKNOWLEDGMENT

The work was supported by awards from the American Heart Association and the National Science Foundation (BES-0120101 and BES-0134163). In addition, acknowledgment is made to the donors of The Petroleum Research Fund, administered by the ACS, for partial support of this research. We thank Steven C. Zimmerman and Jennifer A. Lewis (University of Illinois, Urbana, IL) for the generous use of equipment and facilities.

REFERENCES

1. I. M. Verma and N. Somia. Gene therapy—promises, problems and prospects. *Nature* **389**:239–242 (1997).
2. R. C. Mulligan. The basic science of gene therapy. *Science* **260**: 926–932 (1993).
3. Protocols by Vector. *J. Gene Med. (accessed Apr. 7, 2003)* http://www.wiley.co.uk/genetherapy/clinical/
4. A. Rolland and P. L. Felgner. Non-viral gene delivery systems. *Adv. Drug Deliv. Rev.* **30**:1–3 (1998).
5. D. W. Pack. Gene delivery polymers. In: J. I. Kroschwitz (ed.), *Encyclopedia of Polymer Science and Technology.* John Wiley & Sons, New York, 2001.
6. D. Luo and W. M. Saltzman. Synthetic DNA delivery systems. *Nat. Biotechnol.* **18**:33–37 (2000).
7. M. E. Davis. Non-viral gene delivery systems. *Curr. Opin. Biotechnol.* **13**:128–131 (2002).
8. G. Y. Wu and C. H. Wu. Receptor-mediated gene delivery and expression *in vivo. J. Biol. Chem.* **263**:14621–14624 (1988).
9. E. Wagner, M. Zenke, M. Cotten, H. Beug, and M. L. Birnstiel. Transferrin-polycation conjugates as carriers for DNA uptake into cells. *Proc. Natl. Acad. Sci. USA* **87**:3410–3414 (1990).
10. O. Boussif. F. 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).
11. B. Abdallah, A. Hassan, C. Benoist, D. Goula, J.-P. Behr, and B. A. Demeneix. A powerful nonviral vector of *in vivo* gene transfer into the adult mammalian brain: polyethylenimine. *Hum. Gene Ther.* **7**:1947–1954 (1996).
12. M. Thomas and A. M. Klibanov. Enhancing polyethylenimine's

delivery of plasmid DNA into mammalian cells. *Proc. Natl. Acad. Sci. USA* **99**:14640–14645 (2002).
13. D. M. Lynn, D. G. Anderson, D. Putnam, and R. Langer. Accelerated discovery of synthetic transfection vectors: parallel synthesis and screening of a degradable polymer library. *J. Am. Chem. Soc.* **123**:8155–8156 (2001).
14. A. Kichler, C. Leborgne, E. Coeytaux, and O. Danos. Polyethylenimine-mediated gene delivery: a mechanistic study. *J. Gene Med.* **3**:135–144 (2001).
15. M. L. Forrest and D. W. Pack. On the kinetics of polyplex endocytic trafficking: implications for gene delivery vector design. *Mol. Ther.* **6**:57–66 (2002).
16. A. Akinc and R. Langer. Measuring the pH environment of DNA delivered using nonviral vectors: implications for lysosomal trafficking. *Biotechnol. Bioeng.* **78**:503–508 (2002).
17. J.-P. Behr. The proton sponge: A trick to enter cells the viruses did not exploit. *Chimia* **51**:34–36 (1997).
18. M. Lecocq, S. W.-D. Coninck, N. Laurent, R. Wattiaux, and M. Jadot. Uptake and intracellular fate of polyethylenimine *in vivo*. *Biochem. Biophys. Res. Commun.* **278**:414–418 (2000).
19. M. Tang and J. F. C. Szoka. The influence of polymer structure on the interactions of cationic polymers with DNA and morphology of the resulting complexes. *Gene Ther.* **4**:823–832 (1997).
20. W. T. Godbey, M. A. Barry, P. Saggau, K. K. Wu, and A. G. Mikos. Poly(ethylenimine)-mediated transfection: a new paradigm for gene delivery. *J. Biomed. Mater. Res.* **51**:321–328 (2000).
21. 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).
22. B. Brissault, A. Kichler, C. Guis, C. Leborgne, O. Danos, and H. Cheradame. Synthesis of linear polyethylenimine derivatives for DNA transfection. *Bioconj. Chem.* **14**:581–587 (2003).
23. 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).
24. W. T. Godbey, K. K. Wu, and A. G. Mikos. Tracking the intracellular path of poly(ethylenimine)/DNA complexes for gene delivery. *Proc. Natl. Acad. Sci. USA* **96**:5177–5181 (1999).
25. 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).
26. D. Fischer, A. von Harpe, K. Kunath, H. Petersen, Y. Li, and T. Kissel. Copolymers of ethylene imine and N-(2-hydroxyethyl)-ethylene imine as tools to study effects of polymer structure on physicochemical and biological properties of DNA complexes. *Bioconj. Chem.* **13**:1124–1133 (2002).
27. A. von Harpe, H. Petersen, Y. Li, and T. Kissel. Characterization of commercially available and synthesized polyethylenimines for gene delivery. *J. Control. Rel.* **69**:309–322 (2000).
28. M. G. Stevens and S. Olsen. Comparative analysis of using MTT and XTT in colorimetric assays for quantitating bovine neutrophil bactericidal activity. *J. Immunol. Methods* **157**:225–231 (1993).
29. D. D. Dunlap, A. Maggi, M. R. Soria, and L. Monaco. Nanoscopic structure of DNA condensed for gene delivery. *Nucleic Acids Res.* **25**:3095–3101 (1997).
30. G. Liu, M. Molas, G. A. Grossmann, M. Pasumarthy, J. C. Perales, M. J. Cooper, and R. W. Hanson. Biological properties of poly-L-lysine-DNA complexes generated by cooperative binding of the polycation. *J. Biol. Chem.* **276**:34379–34387 (2001).
31. S. Choksakulnimitr, S. Masuda, H. Tokuda, Y. Takakura, and M. Hashida. *In vitro* cytotoxicity of macromolecules in different cell culture systems. *J. Control. Rel.* **34**:233–241 (1995).
32. S.-O. Han, R. I. Mahato, and S. W. Kim. Water-soluble lipopolymer for gene delivery. *Bioconj. Chem.* **12**:337–345 (2001).
33. D. Y. Furgeson, W. S. Chan, J. W. Yockman, and S. W. Kim. Modified linear polyethylenimine-cholesterol conjugates for DNA complexation. *Bioconj. Chem.* **14**:840–847 (2003).
34. M. Lee, J. Rentz, S.-O. Han, D. A. Bull, and S. W. Kim. Water-soluble lipopolymer as an efficient carrier for gene delivery to myocardium. *Gene Ther.* **10**:585–593 (2003).
35. M. B. Roufai and P. Midoux. Histidylated polylysine as DNA vector: elevation of the imidazole protonation and reduced cellular uptake without change in the polyfection efficiency of serum stabilized negative polyplexes. *Bioconj. Chem.* **12**:92–99 (2001).
36. M. Ogris, S. Brunner, S. Schüller, R. Kircheis, and E. Wagner. PEGylated DNA/transferrin–PEI complexes: reduced interaction with blood components, extended circulation in blood and potential for systemic gene delivery. *Gene Ther.* **6**:595–605 (1999).
37. P. R. Dash, M. L. Read, K. D. Fisher, K. A. Howard, M. Wolfert, D. Oupicky, V. Subr, J. Strohalm, K. Ulbrich, and L. W. Seymour. Decreased binding to proteins and cells of polymeric gene delivery vectors surface modified with a multivalent hydrophilic polymer and retargeting through attachment of transferrin. *J. Biol. Chem.* **275**:3793–3802 (2000).
38. I. R. Hill, M. C. Garnett, F. Bignotti, and S. S. Davis. *In vitro* cytotoxicity of poly(amidoamine)s: relevance to DNA delivery. *Biochim. Biophys. Acta* **1427**:161–174 (1999).
39. P. Erbacher, A. C. Roche, M. Monsigny, and P. Midoux. The reduction of the positive charges of polylysine by partial gluconoylation increases the transfection efficiency of polylysine/DNA complexes. *Biochim. Biophys. Acta* **1324**:27–36 (1997).
40. M. G. Banaszczyk, C. P. Lollo, D. Y. Kwoh, A. T. Phillips, A. Amini, D. P. Wu, P. M. Mullen, C. C. Coffin, S. W. Brostoff, and D. J. Carlo. Poly-L-lysine-graft-PEG-comb-type polycation copolymers for gene delivery, *J. Macromol. Sci. Pure Appl. Chem.* **36**:1061–1084 (1999).