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Polyethylene glycol–grafted polyethylenimine used to enhance adenovirus gene delivery

Kumar Singarapu, Ivy Pal, Joshua D. Ramsey

School of Chemical Engineering, Oklahoma State University, Stillwater, Oklahoma

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Abstract: An improved adenoviral-based gene delivery vector was developed by complexing adenovirus (Ad) with a biocompatible, grafted copolymer PEG-g-PEI composed of polyethylene glycol (PEG) and polyethylenimine (PEI). Although an Ad-based gene vector is considered relatively safe, its native tropism, tendency to elicit an immune response, and susceptibility to inactivating antibodies makes the virus less than ideal. The goal of the current study was to determine whether Ad could be complexed with a PEG-g-PEI copolymer that would enable the virus to transduce cells lacking the Ad receptor, while avoiding the issues commonly associated with PEI. A copolymer library was synthesized using 2 kDa PEG and either linear or branched PEI (25 kDa) with a PEG to PEI grafting ratio of 10, 20, or 30. The results of the study indicate that PEG-g-PEI/Ad complexes are indeed able to transduce CAR-negative NIH 3T3 cells. The results also demonstrate that the PEG-g-PEI/Ad complexes are less toxic, less hemolytic, and more appropriately sized than PEI/Ad complexes. © 2012 Wiley Periodicals, Inc. J Biomed Mater Res Part A: 101A: 1857–1864, 2013.

Key Words: gene delivery, hybrid vector, adenovirus, polyethylenimine

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INTRODUCTION

Gene therapy, which is emerging as a feasible method to treat diseases and has broad potential,[1–3] is the treatment of human diseases by transfer of genetic material into a patient's cells. The field had been hindered significantly because of difficulty in delivering genetic material in both a safe and efficient manner. Traditional gene delivery vectors are classified generally as either viral or nonviral vectors. Viral vectors are efficient, often capable of infecting dividing and quiescent cells, and can provide long-term gene expression; viral vectors, however, possess drawbacks such as immunogenicity, pathogenicity, a natural tropism that makes targeting difficult, and a high cost of production and purification. In contrast, nonviral vectors are relatively inefficient, transfect only dividing cells, and are limited to transient expression; nonviral vectors, however, possess advantages such as low toxicity, immunogenicity, and pathogenicity. Nonviral vectors are also relatively easy and inexpensive to produce. Because of the above drawbacks, however, neither viral nor nonviral vectors are ideal.

The ideal gene delivery vector would possess advantages commonly associated with viral and nonviral vectors and none of the drawbacks. Improvements along these lines have been achieved by using both viral and synthetic materials. For example, other groups have worked with a combination of retrovirus and polymers, such as polyethylenimine (PEI),[4] poly-L-lysine[5] or Polybrene.[6,7] Researchers have also used polyethylene glycol (PEG) to PEGylate or modify the surface of adenovirus (Ad), which resulted in improvements to the delivery vector.[8–10]

The approach presented in this study explores the benefits of a hybrid PEI/Ad vector. Ad was chosen as the viral platform for the hybrid vector because the virus is highly efficient, easy to produce in high titers, and nononcogenic. The virus is also able to transduce dividing and quiescent cells.[11] Ad is used currently in more than 23% of the ongoing clinical trials.[12] Although Ad is promising, there are some serious drawbacks that limit widespread use of the virus. The major drawbacks are immunogenicity and issues associated with targeting.[13–18]

The polymer PEI, which is the most commonly used polymer in gene delivery,[19,20] assists in cellular uptake through electrostatic interactions and promotes endosomal escape.[21] Previous studies, however, have reported undesirable characteristics such as cytotoxicity and incompatibility with the circulatory system.[21,22] These studies demonstrated that PEI is not ideal for standalone gene delivery applications.[22–26] To overcome these drawbacks associated with PEI, researchers have synthesized PEI copolymers such as PEG-grafted-PEI (PEG-g-PEI).[22] One advantage of using a PEG-g-PEI is that the copolymer is less toxic.[21] In addition, a PEG-g-PEI/Ad complex will be almost neutrally charged,

Additional Supporting Information may be found in the online version of this article.

Correspondence to: J. D. Ramsey; e-mail: josh.ramsey@okstate.edu

thereby reducing interactions between the complex and proteins within the circulatory system. PEGylated copolymers also elicit less of an immune response.[22,27] On the basis of these previous studies, a PEG-g-PEI copolymer may be able to overcome drawbacks associated currently with the Ad/PEI vector while maintaining many of the advantages associated with the virus, such as high efficiency and infection of quiescent cells.

The long-term goal of this work is to evaluate the performance of a hybrid vector in terms of immunogenicity and targeting flexibility. The focus of the current study was on synthesis of the copolymer and optimization of transduction efficiency on a coxsackie and adenovirus receptor (CAR) deficient cell line. This work also focused on how grafting ratio (GR) and PEI (i.e., linear versus branched) affected the polymer characteristics, interactions with serum proteins, toxicity, hemolysis, particle size and charge, and buffer capacity.

MATERIALS AND METHODS

Cells

Human embryonic kidney (HEK 293) and mouse fibroblast (NIH 3T3) cell lines were purchased from American Type Culture Collection (ATCC, Manassas, VA). HEK 293 cells were cultured in Dulbecco's modified eagle's medium (DMEM) (Gibco-BRL, Grand Island, NY) with 10% fetal bovine serum (FBS) (Mediatech, Manassas, VA), and NIH 3T3 cells were cultured in DMEM with 10% calf serum (CS) (Mediatech). HEK 293 and NIH 3T3 cells were subcultured every 2–3 days and were stored in a humidified incubator at 37°C and 5% CO_2.

Production and Purification of Virus

Ad lacking E1 and E3 genes and encoding the *lacZ* reporter gene was amplified by infecting HEK 293 cells in 10-cm dishes. The infected cells were incubated until the cytopathic effect was observed after which three freeze/thaw cycles were performed to lyse the virus-producing cells. Ad contained in the cell lysate was isolated and purified using a Vivapure Adenopack purification kit (Sartorius Stedim GmbH, Heidelberg, Germany). The viral titer (cfu/mL) was determined by infecting HEK 293 cells with serial dilutions of the virus and staining the infected cells using X-gal.

Synthesis of PEG-g-PEI

One milliliter of 0.1 m*M* PEI (molecular weight [MW] 25 kDa) (Polysciences, Warrington, PA) was mixed with various volumes, ranging from 90 to 180 μL of 5 m*M* methoxy PEG succinimidyl carboxymethyl (mPEG-SCM, MW 2 kDa) (Laysan Bio, Huntsville, AL). The mixtures were stirred at room temperature for 45 min, after which 50 μL of 1*M* glycine was added to quench the reaction. The reaction mixture was dialyzed using a dialysis cassette with a MW cutoff of 3.5 kDa. Dialysis was performed in a phosphate-buffered saline (PBS) solution containing 5 m*M* ethylenediaminetetraacetic acid for 24 h and then in distilled water for 48 h. The dialysate was lyophilized and stored at −80°C. The MW, composition, and GR of the PEG-g-PEI copolymers were determined from the MW values of the homopolymers, PEI, and PEG, and H-NMR spectroscopy, where peak areas were determined for the $-CH_2CH_2NH-$ protons of PEI and the $-CH_2CH_2O-$ protons of PEG (calculations described in the Supporting Information section).

Formation of Polymer/Virus Complexes

Ad was diluted into serum-free DMEM to produce a desired multiplicity of infection of 10. The virus solution was vortexed while the polymer solution was added dropwise. The amount of polymer added to Ad was varied from 6 to 150 μg of equivalent amounts of PEI for each of the polymers synthesized. The polymer/Ad complexes were formed by incubating for 10 min at room temperature. The negatively charged Ad and positively charged polymer form electrostatic complexes.

Transduction Efficiency

The transduction efficiency of polymer/Ad complexes was studied on CAR-negative NIH 3T3 cells, which are not infected by Ad alone. The cells were seeded 24 h before transduction in 12-well plates, at a seeding density of 1×10^5 cells/cm^2. The cells were then transduced with the polymer/Ad complexes both in the presence and absence of serum. For transduction studies conducted in the absence of serum, the cell medium containing serum was replaced with serum-free DMEM immediately before the addition of the complexes. For transduction studies conducted in the presence of serum, the original DMEM containing serum was replaced with fresh medium containing 10% serum immediately before the addition of the complexes. In case of transduction studies conducted in the absence of serum, after 4–6 h of incubation, the serum-free medium was replaced with the medium containing serum. Quantitative levels of transduction (i.e., reporter gene expression) were measured 48 h post-transduction using the chemiluminescence-based, Beta-Glo assay (Promega, Madison, WI), which quantifies β-galactosidase protein expressed from the *lacZ* reporter gene encoded by the virus. The quantity of β-galactosidase was measured in terms of relative light units with a Lumat LB9507 luminometer (EG&G, Berthold, Bundoora, Australia). The bicinchoninic acid protein assay (Pierce, Rockford, IL) was performed to normalize gene expression to total cellular protein. The polymer concentration was varied from 6 to 150 μg PEI/10^6 virus. The mass of PEI content was maintained the same for all the polymers to find the effect of GR.

Cytotoxicity Assay

NIH 3T3 cells were seeded in 96-well plates at a seeding density of 1×10^5 cells/cm^2 and incubated at 37°C and 5% CO_2 for 24 h. The cells were transduced with polymer/Ad complexes and incubated for 10 h. Then the Celltiter-Blue Cell Viability assay reagent (Promega) was added to each well. Celltiter-Blue Cell Viability assay provided the information about the destructive impact of the polymers on cell membranes. In a reference experiment without polymer, samples with only DMEM and Ad were used as controls. The sample with only Ad was reported as zero polymer concentration in Figure 3 and shows that the cells were 100%

TABLE I. Characteristics of PEG-g–PEI Copolymers

Polymer Product	Fraction of Modified Amine Residues (%)	Molar Ratio PEG:PEI	Mass Fraction (%)		Mw (kDa)
			PEG	PEI	
Linear PEI					
GR 10	1.8	10.2	44.9	55.1	45
GR 20	3.4	19.8	61.3	38.7	65
GR 30	5.2	30.1	70.7	29.3	85
Branched PEI					
GR 10	1.8	10.3	45.2	54.8	46
GR 20	3.5	20.3	61.9	38.1	66
GR 30	5.2	30.1	70.7	29.3	85

viable. The cells were incubated further for 4 h, and fluorescence readings at 540 nm were taken using a Spectra Max Gemini spectrophotometer. The assay uses resazurin, a dark blue nonfluorescent dye. After adding resazurin to the cells, the dye is oxidized to highly fluorescent resorufin by oxygen present in viable cells. The fluorescence intensity is directly proportional to the number of viable cells, and thus, inversely proportional to the toxicity of the particles.

Hemolytic Activity

Fresh bovine red blood cells (RBCs; Innovative Research, Toledo, OH) were used to test the hemolytic activity of the polymers. Various concentrations of the polymers, ranging from 0.05 to 5 μg PEI/μL, were added to RBCs suspended in PBS. As a positive and negative control, the RBCs were suspended in equal volume of either PBS or water, respectively. PBS results in 0% hemolysis and water produces 100% hemolysis. The samples containing RBCs and polymer were incubated 1–4 h at 37°C. These samples were then centrifuged at 13,600*g* for 5 min, and the supernatant was transferred into 96-well plates. Fluorescence readings were taken at 540 nm using a Spectra Max Gemini XS multi-well plate fluorescence reader (Molecular Devices, Sunnyvale, CA) to quantify the amount of hemoglobin released. On the basis of the fluorescence reading, % hemolysis was calculated using the following equation:

$$\%\ \text{Hemolysis} = \frac{(\text{Abs of RBCs in polymer sol'n} - \text{Abs of RBCs in PBS})}{(\text{Abs of RBCs in water} - \text{Abs of RBCs in PBS})} \times 100$$

Size and Charge Measurement

The effective hydrodynamic diameter of the polymer/Ad complexes was measured using a 90Plus ZetaPALS particle sizer (Brookhaven Instruments Corporation [BIC], Holtsville, NY). The complexes were formed and diluted to a final volume of 800 μL in DMEM (pH 7.4). The sample was maintained at 25°C, and light scattering was measured at 632 nm and at a 90° angle relative to the laser source. The zeta-potential of the complexes was measured using a BIC PALS zeta-potential analyzer. The complexes were formed in PBS (pH 7.4), and the sample was diluted to a final volume of 1500 μL. The size and charge of each sample was calculated from 10 repeat measurements of three independent samples, where each measurement was performed over a 1-min interval. The reported size and zeta-potential measurements were performed at the optimum polymer concentration determined during the transduction studies.

Buffer Capacity

The buffer capacity of the polymers over the physiologically relevant pH range (i.e., ~7.5–4.5) was determined by acid–base titration as described by Zhong et al.[28] In brief, 6 mg of each polymer was dissolved in 30 mL of 0.1*M* NaCl to bring the final concentration of the polymer to 0.2 mg/mL. Using a 1*M* NaOH solution, the pH of the sample containing polymer was brought to 10, and the sample was then titrated with 50 μL aliquots of 0.1*M* HCl until the pH dropped below 3 at room temperature. The pH of the sample was measured 10 min after each addition of acid aliquots to ensure equilibrium had been reached, and the time period was the same for all the samples. The buffering capacity of the polymers between pH 7.5 and 4.5 was determined by using a trend line from linear regression to calculate the slope of the titration over this range.

Statistical Analysis

All the experiments were conducted in triplicates ($n = 3$) or for $n > 3$. Reported values were represented as mean ± SD. Significant difference between two groups was analyzed using a paired *t*-test with 95% confidence interval. Differences in the results were considered statistically significant when $p < 0.05$.

RESULTS

Polymer Synthesis

Cationic copolymers were synthesized by grafting various amounts of PEG to linear PEI (LPEI) or branched PEI (BPEI). The nomenclature and characteristics of the resulting PEG-g-PEI copolymers are shown in Table I (calculations described in the Supporting Information section). H-NMR was used to characterize the synthesized polymers. The results, presented in Table I, indicate that the desired polymers were successfully synthesized with PEG:PEI GRs of 10, 20, and 30.

Transduction Efficiency

The ability of the polymer to facilitate delivery and uptake of Ad in a CAR-independent manner was evaluated by

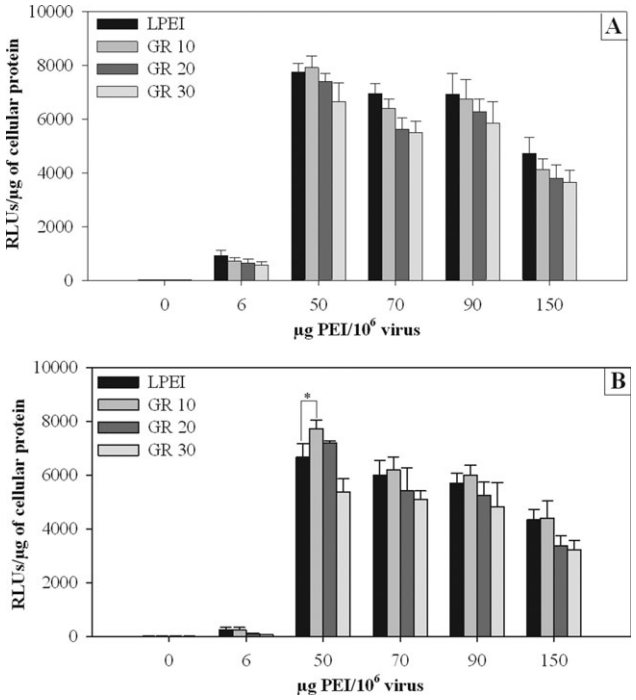

FIGURE 1. Transduction efficiency of LPEI/Ad and PEG-g-LPEI/Ad complexes in the (A) absence and (B) presence of calf serum. $*p < 0.05$.

forming polymer/Ad complexes that were added to NIH 3T3 cells. As shown in Figures 1 and 2, polymer/Ad complexes resulted in significantly higher levels of reporter

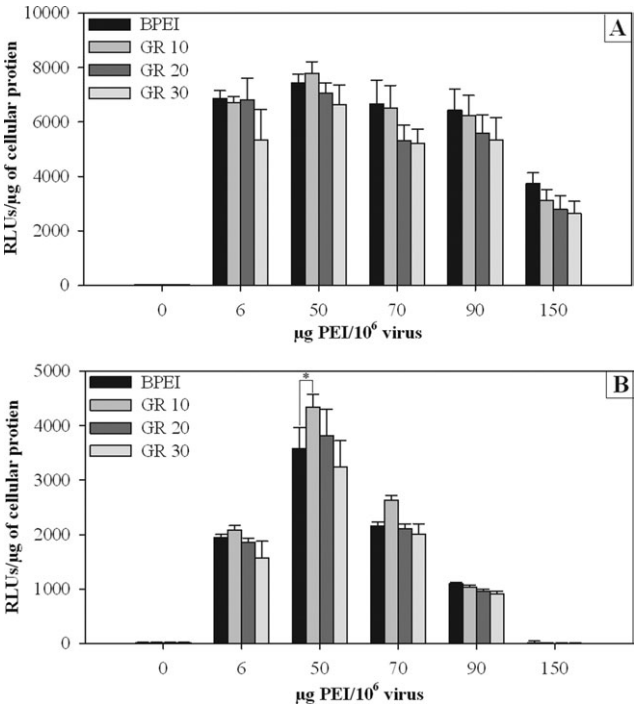

FIGURE 2. Transduction efficiency of BPEI/Ad and PEG-g-BPEI/Ad complexes in the (A) absence and (B) presence of calf serum. $*p < 0.05$.

TABLE II. Effect of Serum on Transduction Efficiency of Polymer/Ad Complexes at Different Polymer Concentrations

Effect of Serum on Transduction Efficiency			
	Polymer Concentration (μg PEI/10^6 virus)		
	50	70	90
LPEI	−14 ± 4%	−14 ± 5%	−18 ± 6%
GR 10	−2 ± 1%	−3 ± 1%	−11 ± 2%
GR 20	−3 ± 1%	−4 ± 1%	−16 ± 2%
GR 30	−19 ± 6%	−7 ± 4%	−18 ± 5%
BPEI	−52 ± 5%	−68 ± 8%	−84 ± 7%
GR 10	−44 ± 3%	−59 ± 9%	−83 ± 9%
GR 20	−46 ± 7%	−60 ± 9%	−83 ± 8%
GR 30	−51 ± 8%	−62 ± 10%	−84 ± 5%

gene expression than cells exposed to native Ad alone. When the polymer concentration was optimized for transduction in serum-free medium, LPEI improved the transduction efficiency of Ad the most (485-fold) and, although not significant, was outperformed slightly by PEG-g-PEI with a GR of 10 (495-fold).

Transduction studies were performed also in the presence of serum to determine the effect on transduction efficiency (Figs. 1 and 2). In general, copolymers formed from LPEI performed better than the BPEI copolymers in the presence of serum. Unmodified LPEI and BPEI improved the transduction efficiency by approximately 400- and 380-fold, respectively. Under optimized conditions, the PEG-g-PEI copolymer performed even better than the unmodified PEI. Copolymer formed from LPEI with a GR of 10 improved the transduction efficiency by nearly 500-fold, and copolymer formed from BPEI with a GR of 10 improved transduction efficiency by approximately 460-fold.

The relative performance of the polymers in the presence and absence of serum is important in understanding how the PEG-g-PEI/Ad complexes may perform *in vivo* relative to the *in vitro* studies performed here. The results demonstrate that polymer/Ad complexes formed from unmodified PEI were affected more by serum proteins than complexes formed from the PEG-g-PEI copolymers. Table II shows the reduction in transduction efficiency in the presence of serum compared with transduction in the absence of serum. At an optimum concentration of 50 μg PEI/10^6 virus, the complexes formed from LPEI showed a 14% decrease in gene expression. In comparison, complexes formed from PEG-g-LPEI copolymer with a GR of 10 had only a 2% decrease in gene expression. Similarly, at a concentration of 50 μg PEI/10^6 virus, the complexes formed from BPEI showed a 52% decrease in gene expression, and complexes formed from PEG-g-BPEI copolymer with a GR of 10 had only a 44% decrease in gene expression. Both LPEI and BPEI were affected more significantly at higher polymer concentrations. Surprisingly, increasing the GR from 10 to 30 did not have a significant improvement on the transduction efficiency in the presence of serum, and in some cases, increasing the GR actually had a negative impact on the transduction efficiency in the presence of serum.

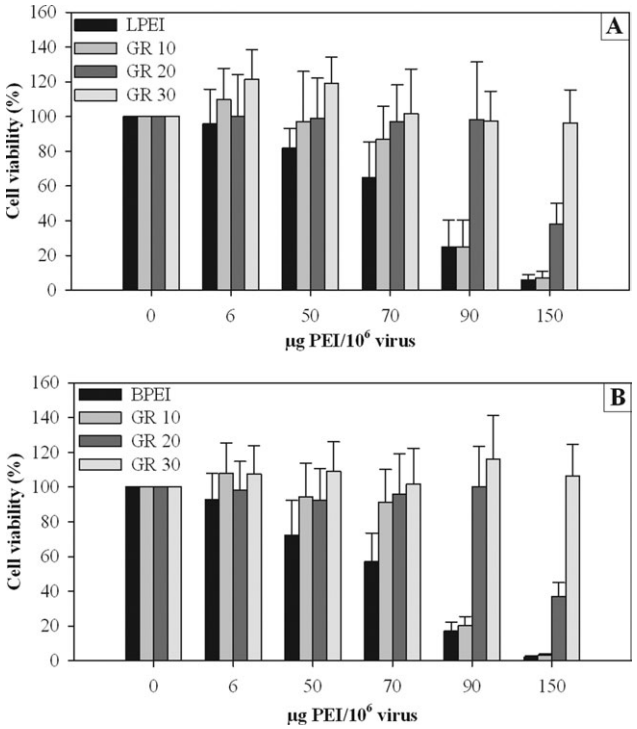

FIGURE 3. Cytotoxicity of (A) LPEI/Ad and PEG-g-LPEI/Ad complexes and (B) BPEI/Ad and PEG-g-BPEI/Ad complexes.

Cytotoxicity

The cytotoxicity of the polymers was studied on NIH 3T3 cells using the same polymer concentration range used for optimizing transduction efficiency. Figure 3 shows cell viability as a function of the amount of polymer used to form complexes. As the polymer/Ad ratio was increased for the copolymers, a slight increase in cell viability was observed at low polymer ratios. As the polymer/Ad ratio was increased further, there was a significant decrease in cell viability in the presence of LPEI and BPEI. For both types of polymer, the addition of PEG improved cell viability compared with the unmodified polymer, and greater GRs corresponded to higher levels of cell viability. At 50 μg PEI/10^6 virus, the polymer/Ad ratio that optimized transduction efficiency, LPEI has a cell viability of 84%. At the same optimum polymer concentration, PEG-g-LPEI had cell viabilities near or slightly above 100% for GRs of 10, 20, and 30. Similarly, BPEI had a cell viability of 72%, and PEGylated BPEI had cell viabilities of 93, 93, and 110% at GRs of 10, 20, and 30, respectively. These results demonstrate that the PEGylated PEI is significantly less toxic than the unmodified polymer.

Hemolytic Activity

An important requirement for a gene delivery vector intended to be administered *in vivo* is that the vector has low levels of interactions with RBCs, which might otherwise lead to cell lysis. A hemolytic activity assay was performed on the copolymers to determine how each polymer affects bovine RBCs. Hemolytic activity, which was measured and expressed in terms of percent hemolysis, was less than 2% for all of the copolymers, at all concentrations (data not shown). Unmodified LPEI and BPEI demonstrated only slightly higher levels of hemolysis with a minor increase with increasing concentration. On the basis of these results, polymer/Ad vectors composed of PEGylated PEI are expected to perform better *in vivo* because the vector is less likely to result in significant levels of hemolysis.

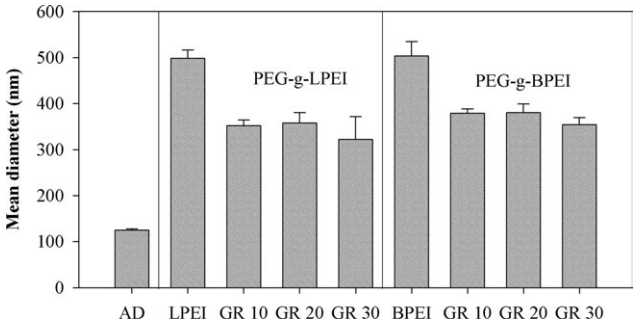

FIGURE 4. Effective diameter of polymer/Ad complexes.

Polymer/Ad Complex Size

Particle size has been shown to be a major factor in transduction efficiency and cellular trafficking.[29,30] The efficiency with which particles are internalized is strongly influenced by particle size, and if the size of a vector becomes too large, it may lead to a loss in transduction efficacy because of reduced internalization.[29,30] The effective hydrodynamic diameter of Ad was measured as 120 nm, which is within the range often reported for Ad.[31] Figure 4 shows that the size of the complexes formed from linear and branched copolymers were at the higher end of the desired range (i.e., less than 300 nm).[32,33] Compared with these complexes, polymer/Ad complexes formed from the grafted copolymer were significantly smaller, resulting in particles more likely to be compatible with *in vivo* gene delivery. The reported complex size values in Figure 4 were performed at the optimum polymer concentration.

Polymer/Ad zeta-Potential

Complexes with a net cationic surface charge tend to aggregate in the presence of negatively charged serum proteins.[21] Therefore, a slightly anionic complex is desired to overcome

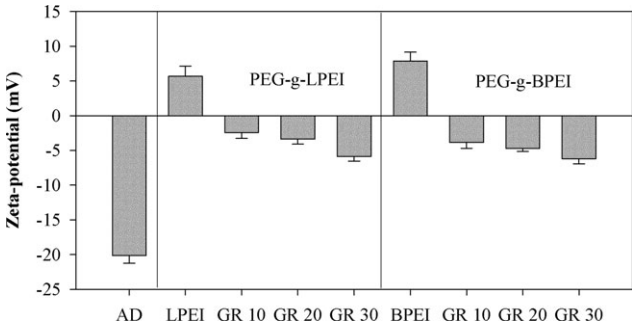

FIGURE 5. Zeta-potential of polymer/Ad complexes.

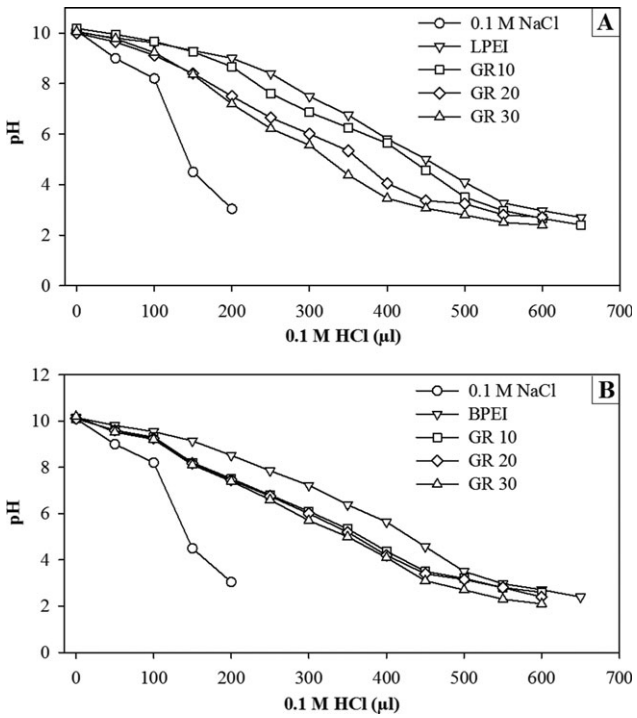

FIGURE 6. Titration curves of (A) LPEI and PEG-g-LPEI and (B) BPEI and PEG-g-BPEI. The slopes of the titration curves for linear PEI (A) between pH 7.5 and 4.5 are LPEI = −0.017, GR10 = −0.016, GR20 = −0.016, and GR30 = −0.018. The slopes of the titration curves for branched PEI (B) between pH 7.5 and 4.5 are BPEI = −0.017, GR10 = −0.015, GR20 = −0.016, and GR30 = −0.016.

aggregation, cytotoxicity, and undesired interactions with the proteins in the circulatory system.[21] Binding of the cationic polymer to the negatively charged Ad (−20 mV) changed the overall surface charge of the complex. As shown in Figure 5, the zeta-potential went from being negative to positive when unmodified LPEI or BPEI was complexed with the virus. The addition of the grafted copolymer increased the zeta-potential but did not result in positively charged particles. The zeta-potential of the complexes that used grafted copolymer made from LPEI was −2.4, −3.6, and −6.1 mV for GRs of 10, 20, and 30, respectively. The use of grafted copolymer made from BPEI produced similar results; the zeta-potentials were −3.8, −4.7, and −6.2 mV.

Buffer Capacity

According to the proton sponge hypothesis, "proton polymers," including PEI, are assumed to induce endosomal escape because of their high buffer effect between physiologic and lysosomal pH. This buffer effect causes an increase in osmotic pressure within endosomes, leading to the disruption of the endosomal membrane, which allows complex transport into the cytoplasm. Therefore, the buffer capacity of the polymers may directly affect endosomal escape. Buffer capacity of the polymers was determined by acid–base titration. Our intent was to show that at the GRs used in this study, grafting of PEG to PEI has little effect on the buffering capacity of PEI. As shown in Figure 6, for a given mass of polymer, slightly less acid is needed to reduce the pH of the solution containing PEI with higher GRs compared with unmodified PEI. This was expected as the amine groups of PEI (which are responsible for buffer capacity), reacted with the PEG chains, reducing the number of amine groups available to maintain pH. To look specifically at the buffering capacity over the physiologically relevant range, the slope of the titration curve between pH 7.5 and 4.5 was determined. The similarity of the slopes (Fig. 6) further supports that there was minor changes in the buffering capacity over the relevant pH range.

DISCUSSION

Forming complexes between polymers and virus particles is a feasible means of delivering genetic material to cells and imparting advantages that the virus alone would not possess. Just as LPEI or BPEI may be used to form electrostatic complexes with plasmid DNA, PEI polymers may be combined with negatively charged Ad particles to produce a hybrid gene delivery vector capable of transducing CAR-negative cells. Compared with virus alone, LPEI improves the transduction efficiency of CAR-negative, NIH 3T3 cells by 485-fold in the absence of serum. Similarly, BPEI improves the transduction efficiency by 464-fold.

Both BPEI and LPEI have certain drawbacks that may prevent their use *in vivo*, such as reduced efficiency in the presence of serum, high cellular toxicity, and lysis of RBCs. The current study showed that when combined with Ad, PEGylated PEI copolymer performs better than unmodified PEI with regard to several of these drawbacks. One of the main advantages of the copolymer was its ability to form complexes that retained their effectiveness in the presence of serum. When LPEI was used with Ad, the resulting complex retained only 84% of its transduction efficiency when the transduction was performed in the presence of serum. In contrast, the PEG-g-LPEI/Ad complex retained 98% of its transduction efficiency under the same conditions. These findings are consistent with what others have reported, where PEG-g-PEI improves the transduction efficiency of plasmid DNA complexes compared with PEI alone.[34,35] Also consistent with previous *in vivo* studies that showed that LPEI performed better than BPEI,[36–39] the current study showed that copolymers composed of LPEI performed better than copolymers composed of BPEI. The greater efficiency of LPEI was attributed previously to an inherent kinetic instability of LPEI in the presence of salt and serum that led to larger particles[40] and may explain why the copolymer composed of LPEI performed better in this situation as well.

Just as PEG reduces the interaction of the PEGylated material with serum proteins, PEG also inhibits the cellular uptake of PEGylated particles. In the current study, however, the PEG-g-PEI/Ad particles proved to be slightly more efficient than the PEI/Ad particles in the absence of serum. Increasing the GR of PEG:PEI reduced the efficiency of the PEGylated polymer but retained a level of transduction similar to the unmodified polymer.

Grafting PEG onto PEI also improved the cytotoxicity and hemolytic activity of the polymers. The linear and

branched PEG-g-PEI concentrations that produced the optimum transduction efficiency produced negligible toxicity. In comparison, the unmodified LPEI and BPEI resulted in 18 and 27% cell death, respectively. Reduced toxicity of PEGylated polymers has been attributed previously to steric hindrances from PEG side chains that prevent PEI from disrupting the cell membrane.[41,42] The reduction in toxicity observed in this case, however, is not attributed to reduced uptake of polymer/Ad particles because the results of the transduction assay actually indicate increased uptake and improved transduction efficiency. The reduction in toxicity may be due, in part, to the reduced amine content in the polymer[43], as indicated from the polymer buffering studies. Reducing slightly the buffering capacity of the PEI polymer will affect the ability of the polymer to escape the endolysosomal network, but the improvement in toxicity and particle stability looks to have offset the reduction in endosomal escape to result in an overall improvement in the level of transduction.

The size of the polymer/Ad complex may introduce a significant health risk if the size is not precisely controlled and maintained at less than 300 nm.[44] The LPEI and BPEI complex with Ad to produce particles that exceeds this size that is generally considered safe. The PEG-g-PEI copolymer, however, results in particles that are less than 370 nm. Although these particles are slightly larger than desired, the significant difference between unmodified PEI and PEG-g-PEI demonstrates the advantage that PEG imparts to the complex. Unlike the LPEI and BPEI, the grafted copolymers also maintain a negative zeta-potential, which prevents aggregation of the complexes and minimizes nonspecific binding with cells, a feature that will be advantageous when targeting ligands are introduced.

CONCLUSIONS

The results of this study demonstrate that a PEI-based hybrid vector can be formed with Ad to infect CAR-negative cells that are resistant to infection by Ad virus alone. Furthermore, the use of PEG to produce a PEG-g-PEI copolymer that is used in the formation of the complex imparts several advantages over the polymer alone. The PEG-g-PEI/Ad complexes are more efficient than the PEI/Ad complexes. The PEGylated polymer performs better in the presence of serum, has lower toxicity, and produces particles closer to the desired size. Although the effects of PEG on the immune response was not investigated in this study, PEG has been shown to reduce the immune response to Ad and limit the inactivation of Ad by antibodies[8,9] and is expected to be a benefit associated with the PEG-g-PEI/Ad vectors.

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