MODIFIED POLYETHYLENIMINE USED TO ENHANCE ADENOVIRUS GENE DELIVERY

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TABLE OF CONTENTS

CHAPTER 1. INTRODUCTION 7

CHAPTER 2. LITERATURE REVIEW 10

2.1 Gene therapy 10

2.2 Challenges to gene delivery 11

2.2.1 Extra cellular barriers 11

2.2.2 Cellular association 12

2.2.2 Endosomal escape 12

2.2.4 Intracellular barriers 12

2.3 Strategies to overcome barriers 13

2.4 Adenovirus 14

2.5 Polymers for gene delivery 15

2.5.1 PEI Polymer structure and molecular weight 15

2.5.2 PEI-g-PEG copolymer synthesis 16

2.5.3 Advantages of PEG 16

CHAPTER 3. MATERIALS AND METHODS 18

3.1 Cells 18

3.2 Production and purification of virus 18

3.3 Synthesis of PEG-g-PEI.....19
3.4 Formation of virus/polymer complexes.....19
3.5 Transduction efficiency.....20
3.6 Cytotoxicity assay.....21
3.7 Hemolytic activity.....21
3.8 Size and charge measurement.....22
3.9 Buffer capacity.....22
3.10 Statistical Analysis.....23

CHAPTER 4. RESULTS.....24
4.1 Polymer synthesis.....24
4.2 Transduction efficiency.....24
4.3 Cytotoxicity.....30
4.4 Hemolytic activity.....32
4.5 Polymer/Ad complex size.....34
4.6 Polymer/Ad Zeta-potential.....35
4.7 Buffer capacity.....36

CHAPTER 5. DISCUSSION.....38

CHAPTER 6. CONCLUSION AND FUTURE WORK.....41

REFERENCES.....43

APPENDIX A.....51

LIST OF TABLES

Table 2.1 Comparison of non-viral, viral and ideal gene delivery vectors.................11

Table 2.2 Strategies to overcome cellular barriers..12

Table 4.1 Characteristics of PEG-g-PEI copolymers 24

Table 4.2 Reduction in transduction efficiency of the polymer/Ad complexes in the presence and absence of the serum at different polymer concentrations....................29

LIST OF FIGURES

2.1 Barriers to gene delivery 13

2.2 Structure of adenovirus 15

4.1 Transduction efficiency of LPEI/Ad and PEG-g-LPEI/Ad complexes, (A) in the absence and (B) in the presence of calf serum 27

4.2 Transduction efficiency of BPEI/Ad and PEG-g-BPEI/Ad complexes (A) in the absence and (B) in the presence of calf serum. 28

4.3 Cytotoxicity of (A) LPEI/Ad and PEG-g-LPEI/Ad complexes and (B) BPEI/Ad and PEG-g-BPEI/Ad complexes 32

4.4 Hemolysis of (A) LPEI and PEG-g-LPEI polymers and (B) BPEI and PEG-g-BPEI polymers 33

4.5 Effective diameter of polymer/Ad complexes. 34

4.6 Zeta-potential of polymer/Ad complexes 35

4.7 Titration curves of LPEI and PEG-g-LPEI (A) and BPEI and PEG-g-BPEI (B) .. 37

CHAPTER I

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 patient's cell. The field had been hindered significantly due to 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 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 and limited to transient expression but possess advantages such as low toxicity, immunogenicity, and pathogenicity. Nonviral vectors are also relatively easy and inexpensive to produce. Due to these drawbacks, 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 (PLL) [5] or Polybrene [6, 7]. Researchers have also used polyethylene glycol (PEG) to PEGylate, or modify the surface of adenovirus (Ad) resulted in improvements to the delivery vector [8-10].

The approach presented in this study explores the benefits of a hybrid Ad/PEI vector. Adenovirus was chosen as the viral platform for the hybrid vector since 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]. Adenovirus is used currently in more than 28 percent of the ongoing clinical trials [12]. While adenovirus is promising there are some serious drawbacks that limit wide spread 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 stand-alone gene delivery applications [22-26]. To overcome these drawbacks associate 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 neutrally charged, thereby reducing interactions between the complex and proteins within the circulatory system. PEGylated copolymers also elicit

less of an immune response [22, 27]. Based on these previous studies, a PEG-g-PEI copolymer may be able to overcome drawbacks associated currently with the Ad/PEI vector.

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 present study was on synthesis of the copolymer and optimization of transduction efficiency on a CAR-negative 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.

CHAPTER 2

LITERATURE REVIEW

2.1 Gene therapy

The delivery of regulated genes or nucleic acids into affected cells is possibly an assuring strategy for improved drug delivery to treat acquired or hereditary diseases. Gene therapy is defined as the treatment of human diseases by the transfer of genetic materials into specific cells of the patient. Genes are a specific sequence of bases encoding instructions on how to make proteins that are found in the nucleus of a cell. A gene delivery vector is a system for protecting and transporting the therapeutic nucleic acids into targeted cells. Gene delivery vectors are broadly classified into, viral and nonviral vectors. Typical properties of viral, non-viral and ideal gene delivery vector were summarized in Table 2.1.

Table 2.1 Comparison of non-viral, viral and ideal gene delivery vectors.

Nonviral vectors	Viral vectors	Ideal vectors
✓ Flexible Targeting ✓ Typically nontoxic ✓ Nonimmunogenic ✓ Nonpathogenic ✓ Easy to produce ☒ Inefficient ☒ Limited transient expression	✓ Extremely efficient ✓ Long-term transgene expression ☒ Possesses a natural tropism ☒ Potential immunogenicity ☒ Potential pathogenicity ☒ Expensive to produce	✓ Flexible Targeting ✓ Nontoxic ✓ Non-immunogenic ✓ Nonpathogenic ✓ Reproducible Biodegradable ✓ Efficient ✓ Reliable and controllable ✓ Stable in blood and serum ✓ Cost effective ✓ High buffer capacity

2.2 Challenges to gene delivery

2.2.1 Extra cellular barriers

A gene delivery vector travels through an adverse environment within the circulation system before it reaches the nucleus of the cell. The gene delivery vector should be able to overcome attractive forces due to the charge difference among the vector, cells, and protein within the circulatory system. In addition the vector should be compatible within the circulatory system to avoid aggregate formation with blood and serum proteins. The size of the vector also plays a major role in avoiding these barriers [28]. A smaller size is effective in avoiding complex formation within the circulatory system. According to previous studies, an ideal gene delivery vector should be less than 300 nm [29].

2.2.2 Cellular association

After successfully avoiding extra cellular barriers, a gene delivery vector should associate with the targeted tissue's cells. Cellular association of adenovirus depends on whether or not the tissue's cells possess CAR receptors. If the cells do not possess the CAR, then adenovirus is not effective in delivering the genes to that type of tissue. Ideally, adenovirus should be modified in order to target any type of tissue. The size of the vector plays a major role in cellular association because larger size complexes cannot associate with cells in a tissue [29]. Targeting ligands play a major role in cellular association and can be used in directing the vector to specific tissue cells.

2.2.3 Endosomal escape

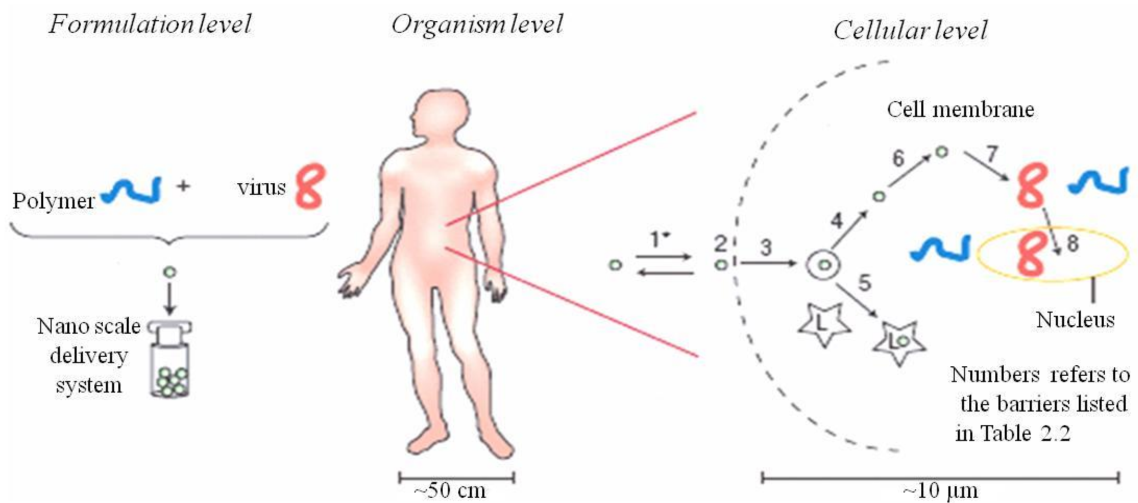
Next, there are barriers to gene delivery vectors inside the cell, and endosomal escape is the first in those internal cell barriers. A gene delivery vector should be able to pass through the acidic environment within lysosomes of the cell to deliver a gene to the nucleus of the cell. A gene delivery vector with high buffer capacity possesses the capability of endosomal escape. The buffer capacity will be discussed in greater detail in the subsequent chapters.

2.2.4 Intracellular barriers

The cell cytosol is a viscous fluid crowded with proteins, vesicles, and cytoskeletal structures. Studies have shown that macromolecules in the cell cytosol are diffusionally limited [30, 31]. These findings appear to indicate the necessity of an active transport method which helps the vector finally arrives at the nucleus [28].

2.3 Strategies to overcome barriers

Different levels of barriers to gene delivery as discussed earlier are presented in Figure 1.2 and the strategies to avoid those barriers are shown in Table 1.2.



Barriers/Challenges		
Formulation	*Organism*	*Cellular*
Cost effective	Less toxic	1: Extra cellular barriers
FDA approvable	Blood compatible	2: Cellular internalization
Long shelf life	Serum interaction	3, 4, 6, 7: Intra cellular transport
Reproducible	Site Specificity	5: Enzymatic degradation
	Long circulation	8: Nuclear entry

Figure 2.1 Barriers to gene delivery. The successful delivery of therapeutic DNA to target cells in the body faces a number of challenges. All three levels such as formulation, organism and cellular level barriers were represented using a nanoscale DNA delivery system (Adopted from Putnam [32]).

Table 2.2 Strategies to overcome cellular barriers of gene delivery system represented in Figure 2.1.

Cellular level barriers			
Barrier from Fig. 2	Barrier/Challenge	Approach	Material design
1, 2 and 3	Transport to cell surface, cellular association and internalization	Targeting ligands, blood and serum protein stability, and positive zeta-potential	Complex formation with cationic copolymer, and PEGylation
4 and 5	Endosomal escape	High buffer capacity to avoid enzymatic degradation	High molecular weight cationic polymers
6, 7, and 8	Transport through cell and nuclear entry	Minimal degradation through cell surface to nucleus and effective entry into of genes into nucleus	Polymers should be strong enough to bear acidic environment inside the cell and should degrade and nucleus of the cell.

2.4 Adenovirus

Many studies confirmed the significant role of adenovirus in gene delivery [33-36]. Adenovirus is considered one of the best options for gene delivery because it is highly efficient and non-oncogenic. The structure of adenovirus is as shown in Figure 1. Adenovirus interacts with tissues through its fiber protein. The fiber protein of adenovirus interacts with the cells possessing Coxsackievirus Adenovirus Receptor (CAR). Many types of cells contain CAR receptor such as liver and kidney, which resulted in an undesirable natural tropism of adenovirus [37, 38]. Major barriers in using adenovirus as a gene delivery vector are natural tropism and immunogenicity.

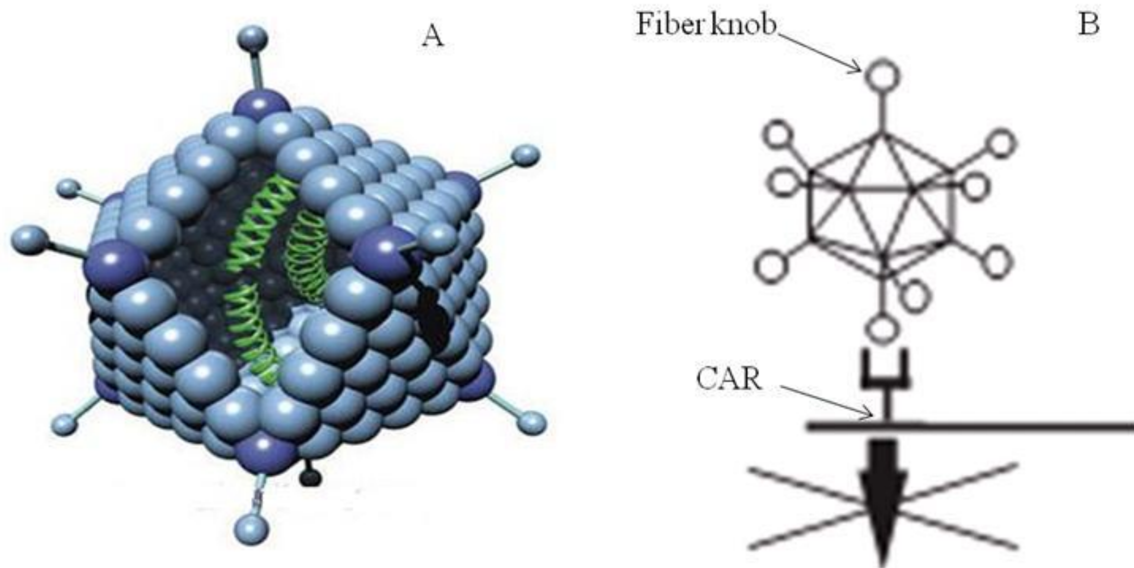


Figure 2.2 Structure of adenovirus, A) diagram of the icosahedral adenovirus capsid (three dimensional views) showing fiber knob proteins and B) Showing adenovirus interaction with the CAR receptor with the use of fiber knob proteins [39, 40].

2.5 Polymers for gene delivery

Cationic polymers play a major role in avoiding some of the barriers in gene delivery and in overcoming some of the problems associated with adenovirus, such as immunogenicity and native tropism. Some cationic polymers possess high buffer capacity, and improve gene delivery. Most cationic polymers are not toxic at low concentrations as they degrade into non-toxic monomers. Of all the cationic polymers available, polyethyleneimine (PEI) is the most commonly used polymer in gene delivery [19, 23, 25, 41-43].

2.5.1 PEI Polymer structure and molecular weight

A prominent feature of PEI is that, in an extracellular environment, PEI enhances the stability of gene delivery vector against degradation. PEI also assists in cellular

uptake of vector by electrostatic interaction of the positively charged PEI with the negatively charged cell surface and promotes endosomal release [44]. PEI is available in both linear and branched forms, and in a broad range of molecular weights, ranging from around 1,000 Da to 1,600 kDa. It has been observed that higher molecular weights of PEI are toxic due to aggregation on plasma membrane which induces necrosis [20]. On the other hand low molecular weight PEI has been shown to be less toxic, but also less effective in gene delivery applications [44]. Previous studies reported that some of the undesirable characteristics of PEI are cytotoxicity and incompatibility with circulatory system [44]. It is suggested that PEI are not recommended for stand-alone in gene delivery applications [19, 21, 45].

2.5.2 PEI-g-PEG copolymer synthesis

With the aim to develop an effective gene delivery carrier and to overcome some of the undesirable characteristics of PEI, researchers are driven towards the synthesis of PEI copolymers [46]. Covalent coupling of polyethylene glycol (PEG) to PEI resulting in a block or grafted copolymer has been investigated extensively [46] and called PEGylation.

2.5.3 Advantages of PEG

The main advantages of using a PEI-g-PEG are that the complex will be neutrally charged, which helps to avoid interactions with serum proteins and blood components. PEGylated copolymers are also less toxic than PEI [44], and PEG also plays a major role in avoiding immune response [22, 47, 48].

Based on these advantages of PEG, some of the problems associated with PEI can be overcome by synthesizing PEI-g-PEG copolymer. It was expected that because of the addition of PEG, PEI-g-PEG copolymer could overcome some of the disadvantages that PEI possesses and preserve the advantages of PEG. Different grafting ratios of PEI-g-PEG were synthesized, characterized, and its transduction efficiency studies were carried out. The ultimate aim of this research is to make this adenovirus and copolymer complex an ideal gene delivery vector by optimizing the copolymer and adenovirus proportions while forming complex.

CHAPTER 3

MATERIALS AND METHODS

3.1. 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, Inc., Manassas, VA), and NIH 3T3 cells were cultured in DMEM with 10% calf serum (CS) (Mediatech, Inc., Manassas, VA). HEK 293 and NIH 3T3 cells were subcultured every 2 to 3 days and were stored in a humidified incubator at 37 °C and 5% CO_2.

3.2. Production and purification of virus

Adenovirus (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. Adenovirus 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.

3.3. Synthesis of PEG-g-PEI

One milliliter of 0.1 mM PEI (molecular weight (MW) 25 kDa) (Polysciences, inc., Warrington PA) was mixed with various volumes, ranging from 90 – 180 µl of 5 mM methoxy PEG succinimidyl carboxymethyl (mPEG-SCM, MW 2 kDa) (Laysan Bio, Huntsville, AL). The mixtures were stirred at room temperature for 45 minutes, 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 MWCO of 3.5 kDa. Dialysis was carried out in a PBS solution containing 5 mM EDTA for 24 hours and then in distilled water for 48 hours. The dialysate was lyophilized and stored at -80 °C. The molecular weight, 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.

3.4. Formation of virus/polymer complexes

Adenovirus was diluted into serum-free DMEM to produce a desired multiplicity of infection (MOI) of 10. The virus solution was vortexed while the polymer solution was added drop wise. The amount of polymer added to adenovirus was varied from 0.03 – 0.75 µg of equivalent amounts of PEI for each of the polymer synthesized. The adenovirus/polymer complexes were formed by incubating for 10 minutes at room

temperature. The negatively charged adenovirus and positively charged polymer form electrostatic complexes and henceforth are referred to as polymer/Ad complexes.

3.5. Transduction efficiency

The transduction efficiency of polymer/Ad complexes was studied on CAR-negative NIH 3T3 cells, which are not infected by adenovirus alone. The cells were seeded 24 hours prior to 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 immediately before the addition of the complexes. In case of transduction studies conducted in the absence of serum, after $4 - 6$ hours 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 hours post transduction using the chemiluminescence-based, Beta-Glo assay (Promega Inc., 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 (RLUs) with a Lumat LB9507 luminometer (EG&G, Berthold, Bundoora, Australia). The bicinchoninic acid (BCA) protein assay (Pierce Inc., Rockford, IL) was carried out to normalize gene expression to total cellular protein. The polymer concentration was varied from 0.03 to

0.75 μg PEI/10^6 virus. The mass of PEI content was maintained the same for all the polymers to find the effect of GR.

3.6. 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 hours. The cells were transduced with polymer/Ad complexes and incubated for 10 hours. Then the Celltiter-Blue Cell Viability Assay Reagent (Promega Corporation, Madison, WI) was added to each well. Cell Titer 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 adenovirus were used as controls. The sample with only adenovirus was reported as zero polymer concentration in Figure 3.3 and shows that the cells were 100% viable. The cells were incubated further for 4 hours, and fluorescence readings at 540 nm were taken using a Spectra Max Gemini spectrophotometer. The assay utilizes resazurin, a dark blue non-fluorescent 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.

3.7. 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 – 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 hours at 37 °C. These samples were then centrifuged at 13,600 × g for 5 minutes, 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. Based on the fluorescence reading % hemolysis was calculated using the following equation:

$$\% \text{ Hemolysis} = \frac{(\text{Abs of complex} + \text{RBCs} - \text{Abs of RBCs in PBS})}{(\text{Abs of RBCs inWater} - \text{Abs of RBCs inPBS})} \text{ x } 100$$

3.8. 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 1,500 µl. The size and charge of each sample was calculated from ten repeat measurements of three independent samples where each measurement was performed over a 1-minute interval. The reported size and zeta-potential measurements were performed at the optimum polymer concentration.

3.9. Buffer capacity

The buffer capacity of the polymers was determined by acid-base titration as described by Zhong et al. [49]. Briefly, 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 minutes after each addition of acid aliquots to ensure equilibrium had been reached, and the time period was the same for all the samples.

3.10. 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 $p < 0.05$.

CHAPTER 4

RESULTS

4.1 Polymer synthesis

Cationic copolymers were synthesized by grafting various amounts of PEG to linear or branched PEI. The nomenclature and characteristics of the resulting PEG-g-PEI copolymers are shown in Table 4.1. Proton NMR was used to characterize the synthesized polymers. The results, presented in Table 4.1, indicate that the desired polymers were successfully synthesized with PEG:PEI GRs of 10, 20, and 30.

Table 4.1 Characteristics of PEG-g-PEI copolymers. The mass fractions of PEG and PEI are expressed at %PEG and %PEI, respectively.

Polymer Product	*%Modification*	*PEG/PEI Molar Ratio*	*%PEG*	*%PEI*	*Mw (kDa)*
Linear PEI					
GR 10	1.7	10.1	44.4	55.6	45
GR 20	3.4	19.8	61.5	38.5	65
GR 30	5.2	30.1	70.6	29.4	85
Branched PEI					
GR 10	1.7	10.3	44.4	55.6	45
GR 20	3.4	20.1	61.5	38.5	65
GR 30	5.2	30.1	70.6	29.4	85

4.2. Transduction efficiency

The ability of the polymer to facilitate delivery and uptake of adenovirus in a CAR-independent manner was evaluated by forming polymer/Ad complexes that were added to NIH 3T3 cells. As shown in Figures 4.1 and 4.2, polymer/Ad complexes resulted in significantly higher levels of reporter 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 was outperformed slightly by PEG-g-PEG with a GR of 10 (495-fold).

Transduction studies were performed also in the presence of serum to determine the effect on transduction efficiency (Figures 4.1 and 4.2). 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 4.2 shows the reduction in

transduction efficiency in the presence of serum compared to transduction in the absence of serum. At a 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-PEI 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-PEI copolymer with a GR of 10 had only a 44% decrease in gene expression. Both LPEI and BPEI were affected more greatly at higher polymer concentrations. Surprisingly, increasing the grafting ratio 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 grafting ratio actually had a negative impact on the transduction efficiency in the presence of serum. Although higher degree of PEGylation offers higher stability of complexes in a variety of solutions, this stability usually comes with reduced transduction activity, probably due to the hindering effects of PEG on the interactions of the complexes with the cells.

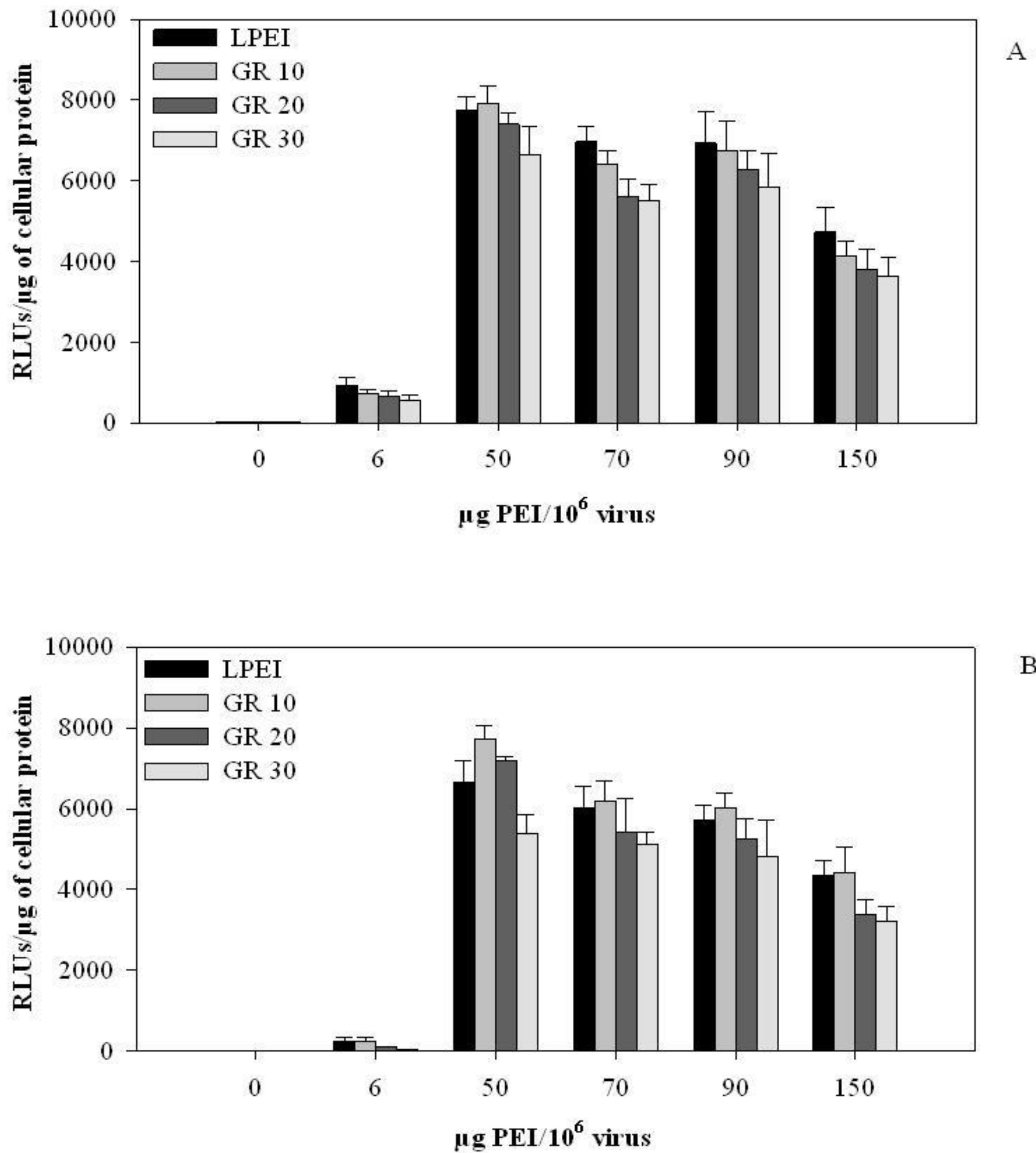


Figure 4.1 Transduction efficiency of LPEI/Ad and PEG-g-LPEI/Ad complexes, (A) in the absence and (B) in the presence of calf serum.

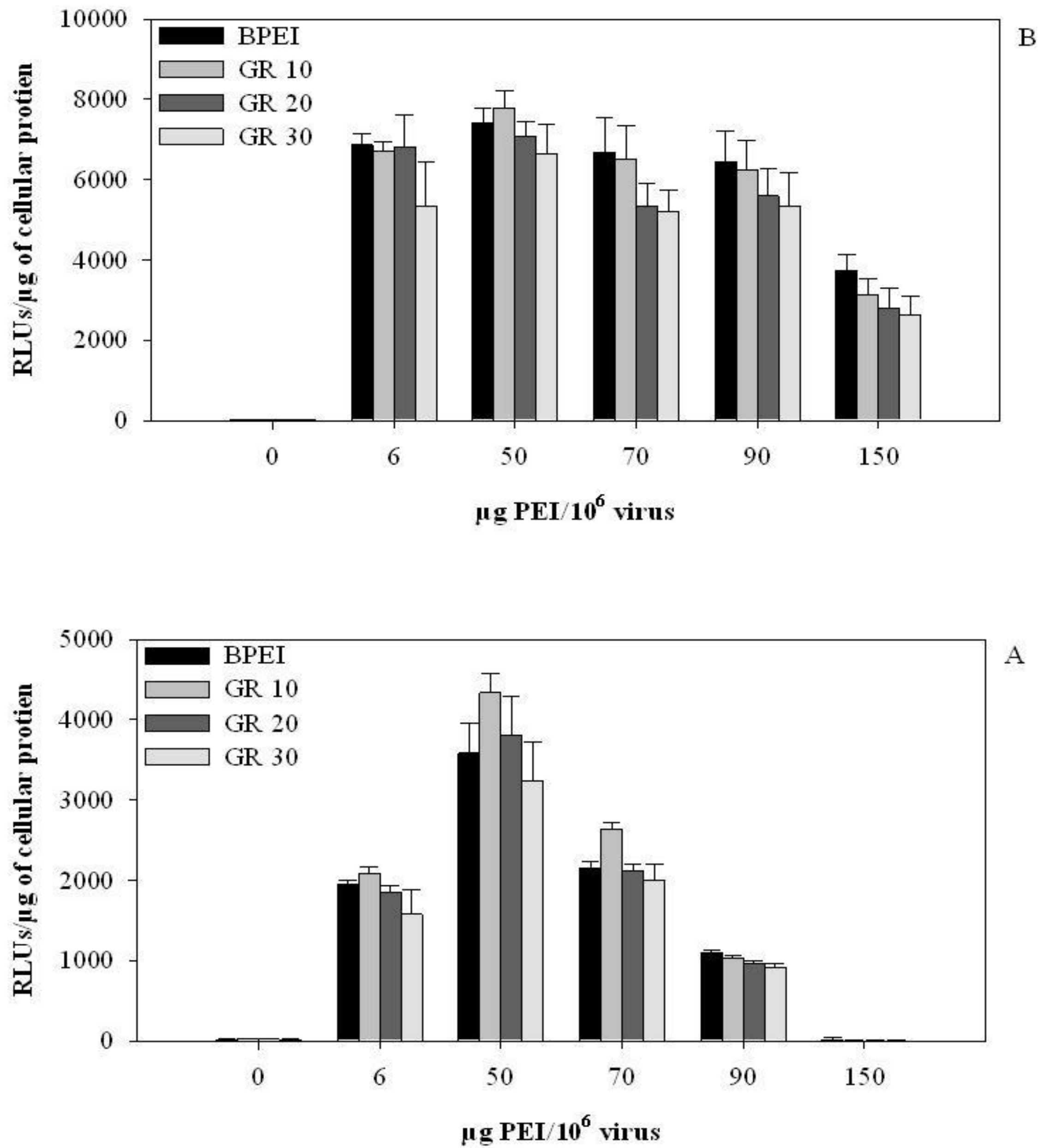


Figure 4.2 Transduction efficiency of BPEI/Ad and PEG-g-BPEI/Ad complexes (A) in the absence and (B) in the presence of calf serum.

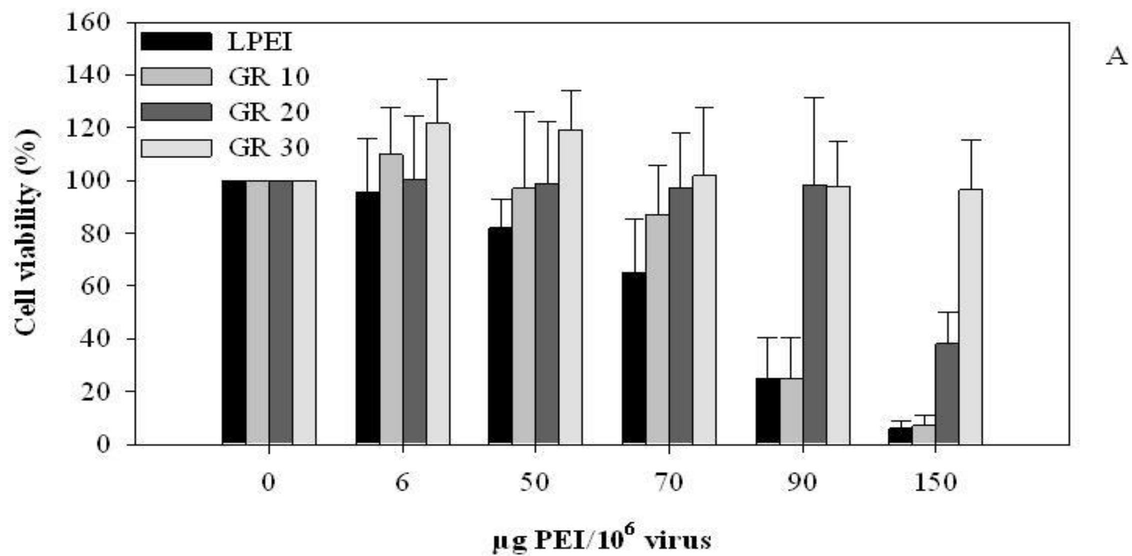
Table 4.2 Reduction in transduction efficiency of the polymer/Ad complexes in the presence and absence of the serum at different polymer concentrations.

Affect of Serum on Transduction Efficiency			
	Polymer Conc. (µg PEI/10^6 virus)		
	50	*70*	*90*
LPEI	-14%	-14%	-18%
GR 10	-2%	-3%	-11%
GR 20	-3%	-4%	-16%
GR 30	-19%	-7%	-18%
BPEI	-52%	-68%	-84%
GR 10	-44%	-59%	-83%
GR 20	-46%	-60%	-83%
GR 30	-51%	-62%	-84%

The effectiveness of the complex was a function of the grafting ratio and the ratio of polymer to virus. Though PEGylation helped overcome interactions with serum proteins, higher GR enhanced interactions. As the GR and concentration increased, the polymers contain higher amounts of PEG, which might also lead to significant interactions with serum proteins. As shown in Figures 4.1 and 4.2, the transduction efficiency is a function of polymer concentration and GR. Therefore, the GR and concentration of the polymer need to be optimized in order to overcome the interactions with serum proteins and thereby achieve the higher transduction efficiency.

4.3. Cytotoxicity

The cytotoxicity of the polymers was studied on NIH 3T3 cells using the same polymer concentration range used for optimizing transduction efficiency. Figure 4.3 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 linear and branched PEI. For both types of polymer, the addition of PEG improved cell viability compared to the unmodified polymer, and greater grafting ratios 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-PEI had cell viabilities of 97, 99, and 119% at grafting ratios of 10, 20, and 30, respectively. Similarly, BPEI had a cell viability of 72%, and PEGylated BPEI had cell viabilities of 93, 93, and 110% at grafting ratios of 10, 20, and 30, respectively. These results demonstrate that the PEGylated PEI is significantly less toxic than the unmodified polymer.



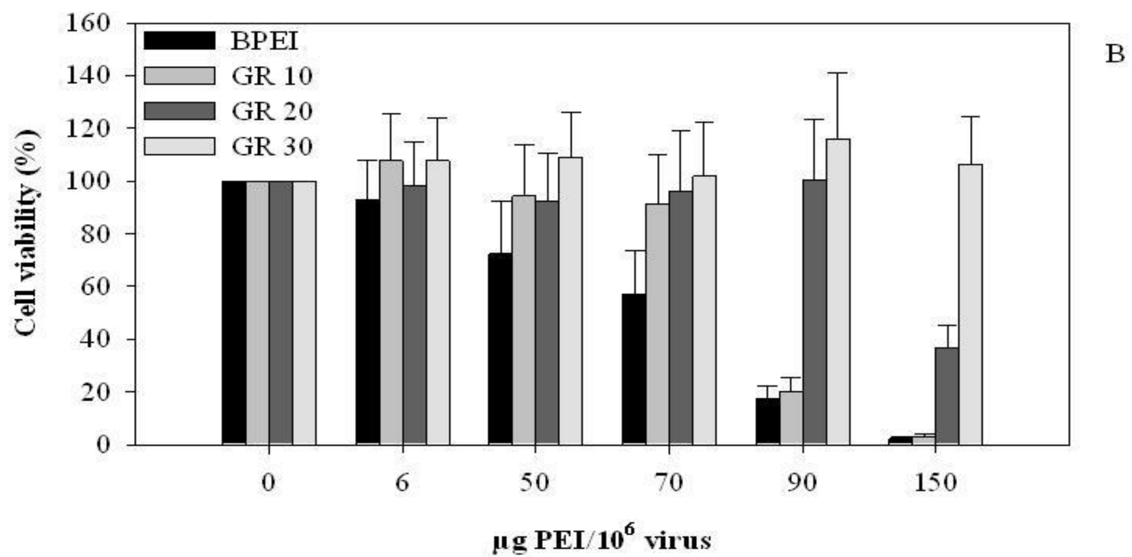
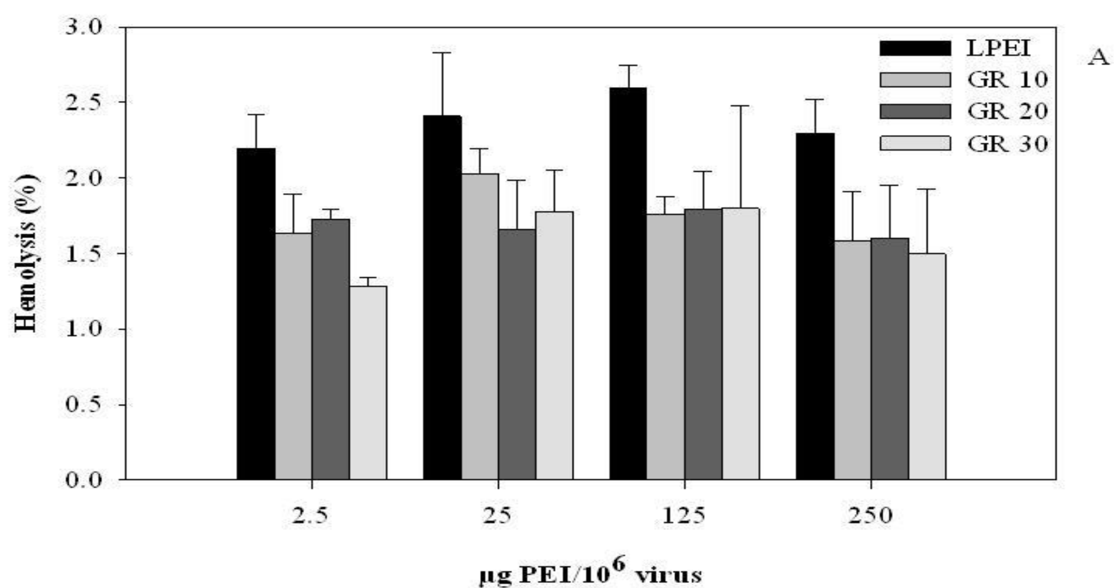


Figure 4.3 Cytotoxicity of (A) LPEI/Ad and PEG-g-LPEI/Ad complexes and (B) BPEI/Ad and PEG-g-BPEI/Ad complexes.

4.4. 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 red blood cells (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 (shown in Figure 4.4). Unmodified linear and branched PEI demonstrated only slightly higher levels of hemolysis with a minor increase with increasing concentration. Based on these results, polymer/Ad vectors composed of PEGylated PEI are expected to perform better *in vivo* since the vector is less likely to result in significant levels of hemolysis.



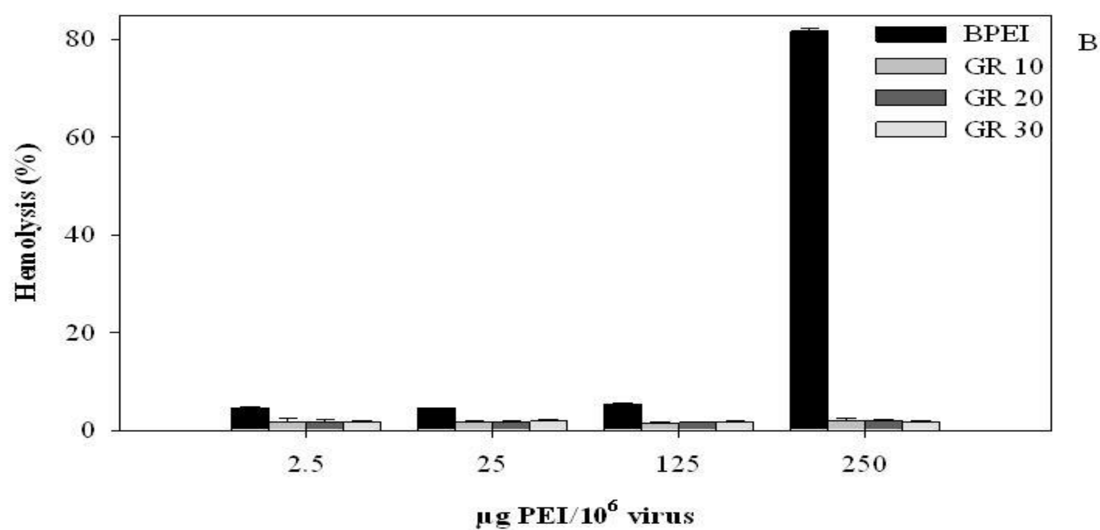


Figure 4.4 Percent hemolysis of (A) LPEI and PEG-g-LPEI polymers and (B) BPEI and PEG-g-BPEI polymers.

4.5. Polymer/Ad complex size

Particle size has been shown to be a major factor in transduction efficiency and cellular trafficking [50, 51]. The efficiency with which particles are internalized is strongly influenced by particle size [50], and if the size of a vector becomes too large it may lead to a loss in transduction efficacy [51]. The effective hydrodynamic diameter of adenovirus was measured as 120 nm, which is within the range often reported for adenovirus [52]. Figure 4.5 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 [53]. Compared to 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 3.4 were performed at the optimum polymer concentration.

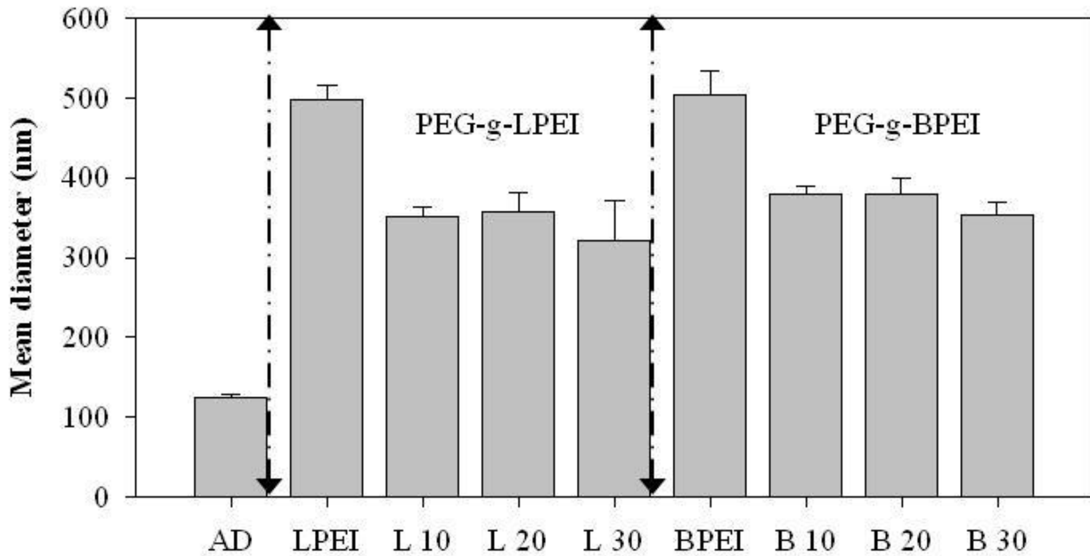


Figure 4.5 Effective diameter of polymer/Ad complexes.

4.6. Polymer/Ad Zeta-potential

Complexes with a net cationic surface charge tend to aggregate in the presence of serum and are often cytotoxic [21]. Therefore, a slightly anionic complex is desired to overcome aggregation, cytotoxicity and undesired interactions with the proteins in the circulatory system [21]. Binding of the cationic polymer to the negatively charged adenovirus (-20 mV) changed the overall surface charge of the complex. As shown in Figure 4.6, the zeta-potential went from being negative to positive when unmodified linear or branched PEI 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 were -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.

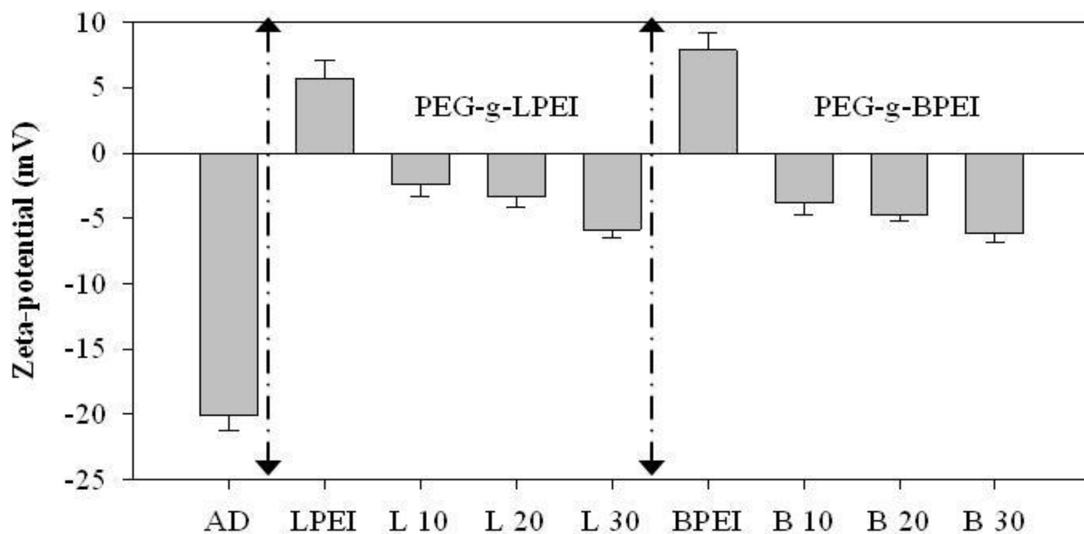


Figure 4.6 Zeta-potential of polymer/Ad complexes.

4.7 Buffer capacity

According to the proton sponge hypothesis, 'proton polymers' including PEI, are assumed to induce endosomal escape due to their high buffer effect between physiological 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 grafting of PEG to PEI has a little effect on the buffering capacity of PEI. As shown in Figure 4.7, the lower GR copolymers showed minor drop in buffer capacity when compared with the 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. It was also observed that the drop in buffer capacity was significant as the grafting ratio increased.

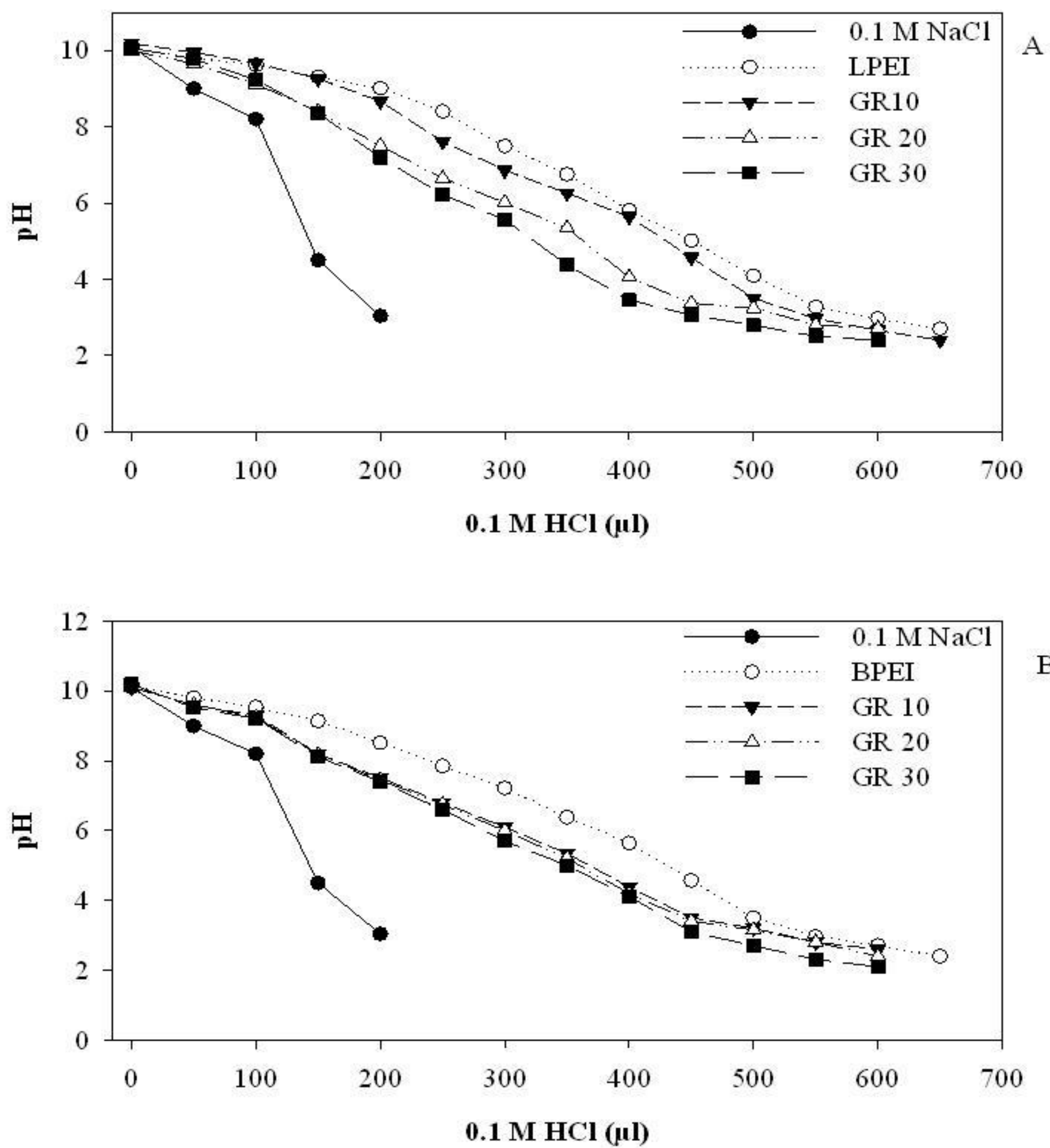


Figure 4.7 Titration curves of LPEI and PEG-g-LPEI (A) and BPEI and PEG-g-BPEI (B).

CHAPTER 5

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 linear or branched PEI 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 in transducing CAR-negative cells. Compared to 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 branched and linear PEI 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 red blood cells. 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 it ability to form complexes that retained their effectiveness in the presence of serum. When PEI was used with Ad, the resulting complex retained only 84% of its transduction efficiency when the

transduction was carried out in the presence of serum. In contrast, the PEG-g-PEI/Ad complex retained 98% of its transduction efficiency under the same conditions. These findings are consistent with previous reports, which showed that PEG-g-PEI improved the transduction efficiency of plasmid DNA complexes compared to PEI alone [42, 54]. Also consistent with previous *in vivo* studies that showed that LPEI performed better than BPEI [55-58], the current study showed that copolymers composed of LPEI performed better than copolymers composed of BPEI.

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 that the PEI/Ad particles in the absence of serum. Increasing the grafting ratio of PEG:PEI reduced the efficiency [42] 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 linear and branched PEI resulted in 18% and 27% cell death, respectively. The reduction in toxicity is not attributed to reduced uptake of polymer/Ad particles since 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 [41], as indicated from the polymer buffering studies. Reducing 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. In addition, the reduction in toxicity in case of copolymers could be due to the fact that the addition of PEG chains which is neutrally charged and non-toxic [59, 60] increases the space and provides steric hindrances which hinders interaction between PEI and cell membrane [46, 61], due to subsequent difficulty in cellular attachment thereby, reduced toxicity.

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 [62]. The linear and branched PEI 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. While 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 linear and branched PEI, 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.

CHAPTER 6

CONCLUSIONS

To summarize, grafted copolymers possess great advantages over unmodified PEI it when comes to gene delivery *in vitro*. First, the linear copolymer with a grafting ratio of 10 produced the highest transduction efficiency compared to any other polymer. Second, complexes formed with PEG-g-PEI showed a significant reduction in interactions with the serum proteins compared to complexes formed with unmodified PEI. Third, the copolymers are less toxic than unmodified branched and linear PEI making it a more suitable material for assisting in the transduction of CAR-negative cells. Fourth, the copolymers showed significant improvement in blood compatibility compared to the unmodified PEI. Copolymers showed less than 2% hemolysis, where as unmodified PEI showed as high as 80% hemolysis at higher concentrations. Therefore, we conclude that PEG chains help in avoiding interactions with RBC. Fifth, the improvement of the surface charge of the adenovirus, after forming complex with copolymer facilitated efficient cellular uptake. Sixth, retention of most of the buffer capacity of the lower GR copolymers helped in overcoming internal barriers of the cell, particularly in endosomal escape. Based on transduction and also from the characterization studies, the polymers

were optimized; and the linear copolymer with a grafting ratio of 10 was the best working polymer at the concentration of 50 µg PEI/10^6 virus.

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. Further, the use of PEG to produce a PEG-grafted-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 produced particles that are near the desired size.

The group copolymers and corresponding analysis performed as part of my study forms the foundation upon which larger, more comprehensive group can be designed and evaluated to better understand how the grafted polymer functions to facilitate viral transduction of target cells.

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APPENDIX A

NMR Calculations:

Molecular weight (MW) of PEI and PEG used is 25,000 kDa and 2,000 kDa.

Functional group of PEI: CH_2CH_2NH (MW=43)

Functional group of PEG: CH_2CH_2O (MW=44)

Number of functional groups in PEI (MW=25,000)

$$\mathrm{N_{PEI}} = \frac{25{,}000g}{mol\ PEI}\frac{mol\ ethyleamine}{44g} = 581\ \frac{ethylene}{PEG} \ldots\ldots\ldots\ldots (1)$$

Number of functional groups in PEG (MW=2,000)

$$N_{PEG} = \frac{2{,}000g}{mol\ PEG}\frac{mol\ ethylene}{44g} = 45 \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots.. (2)$$

Moles of PEI and PEG in PEI-g-PEG can be found from the corresponding values of PEI and PEG integrals from NMR readings. Area that corresponds to PEI after integration is represented as A_{PEI} and that of and PEG is represented as A_{PEG}.

Number of moles of PEI:

$$Mol\ PEI = \frac{A_{PEI}}{N_{PEI}} \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots.\ldots\ldots\ldots\ldots\ldots. (3).$$

Number of moles of PEG:

$$Mol\ PEG = \frac{A_{PEG}}{N_{PEG}} \ldots\ldots\ldots\ldots\ldots\ldots\ldots. (4)$$

Grafting ratio (GR) can be calculated as:

$$GR = \frac{Mol\ PEG}{Mol\ PEI} \ldots\ldots\ldots\ldots\ldots.. (5)$$

Percentage pegylation can be calculated as:

$$\%\ Pegylation = \frac{GR}{PEI\ Chain} \frac{1\ PEI\ Chain}{N_{PEI}} \times 100 \ldots. (6)$$

Percentage mass of PEG

$$\%\ \text{mass of PEG} = \frac{\text{GR} \times 2{,}000}{\text{GR} \times 2{,}000 + 1 \times 25{,}000} \times 100 \ldots\ldots (7)$$

Molecular weight of PEI-g-PEG copolymer

$$MW = GR \times 2{,}000 + 1 \times 25{,}000 \ldots\ldots\ldots\ldots\ldots\ldots\ldots.\ldots\ldots (8)$$

Equivalent concentration of PEI or PEI-g-PEG copolymer can be calculated from:

$$\frac{MW\ of\ PEI}{MW\ of\ copolymer} = \frac{X\ g\ of\ PEI}{Y\ g\ of\ copolymer} \ldots\ldots\ldots\ldots\ldots\ldots (9)$$

MW represents molecular weight. Y and X are the grams of the PEI-g-PEG copolymer, and PEI added. These masses can be calculated from the stock concentration of the polymer and the amount added.

VITA

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Title of Study: MODIFIED POLYETHYLENIMINE USED TO ENHANCE ADENOVIRUS GENE DELIVERY

Pages in Study: 55
Candidate for the Degree of Master of Science

Major Field: Chemical Engineering

Scope and Method of Study: An improved adenoviral-based gene delivery vector was developed by conjugating adenovirus (Ad) with a biocompatible, grafted copolymer of polyethylenimine (PEI) and polyethylene glycol (PEG). Although an adenovirus 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 present study was to determine if adenovirus could be complexed with a PEG-g-PEI copolymer that would enable the virus to transduce cells lacking CAR, the adenovirus receptor, while avoiding 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.

ADVISER'S APPROVAL: Dr. Joshua D. Ramsey