POLYMERIC GENE CARRIER MIXTURES FOR FACILE CARRIER EVALUATION AND IMPROVED GENE DELIVERY VEHICLES

by

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ABSTRACT

Gene therapy offers an alternative therapeutic approach for a variety of diseases and genetic disorders that conventional therapies currently manage. In order to clinically advance this therapeutic alternative, more safe and efficient gene delivery vehicles must be continuously developed. To date, the development of safe and efficient gene delivery reagents for clinical application is hampered by undefined design and formulation requirements. In an attempt to further elucidate these requirements for improved gene delivery reagents, research labs often engineer and study many putative products that possess subtle physiochemical differences that may influence carrier function and biological activity. The synthesis of many putative gene delivery reagents, however, requires multiple optimization cycles for each product, is time-intensive, laborious and costly.

As such, the intent of this dissertation is to implement a method that facilitates evaluation ease of novel, modified gene delivery reagents by avoiding the synthesis of many putative products for the identification of optimal candidates and reagent properties. This method uses mixtures of a promising nonviral gene delivery reagent and its modified counterpart, whereby the relative amount of each species in the formulation mixture is easily controlled, and thus, physiochemical differences and their influence on biological activity can be studied to identify optimal reagent candidates.

The initial part of this dissertation focuses on the chemical modification of a previously published and flourishing non-viral, polycationic gene delivery vehicle, poly(triethylenetetramine/cystamine bisacrylamide) (p(TETA/CBA)) with poly(ethylene glycol) (PEG) to derive poly(triethylenetetramine/cystamine bisacrylamide)-g-poly(ethylene glycol) (p(TETA/CBA)-g-PEG) with physiochemical properties different form p(TETA/CBA) alone. Subsequent studies focused on improving the synthetic ease of p(TETA/CBA)-g-PEG as well as improving its tissue specificity for oncogenic cells and their associated vasculature via modification using a tumor specific peptide. As a result, design and formulation requirements for safe and efficient gene delivery carriers is further clarified and several new and promising gene delivery reagents are born.

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LIST OF ABBREVIATIONS

A549	Human lung carcinoma cells
bPEI	branched poly(ethylene imine)
CBA	N,N'-Cystamine bisacrylamide
c(RGDfC)	cyclic(Arginine-Glycine-Aspartic acid-D-Phenylalanine-Cystine) peptide
CT-26	Mouse colon carcinoma cells
D_2O	Deuterium Oxide
ddH_2O	double-distilled deionized water
DLS	Dynamic Light Scattering
DMEM	Dulbecco's Modified Eagle Medium
DTT	Dithiothreitol
EGM	Endothelial Cell Growth Medium
EtBr	ethidium bromide
FBS	Fetal Bovine Serum
FPLC	Fast-Performance Liquid Chromatography
HIF-1α	Hypoxia Inducible Factor-1α
Luc	Luciferase
NMR	Nuclear Magnetic Resonance
N/P	Nitrogen to Phosphate Ratio
PAA	Poly(amido amine)

pDNA	plasmid DNA
PEA	poly(ester amine)
PEG	poly(ethylene glycol)
PEI	poly(ethylene imine)
PLL	poly(L-lysine)
p(TETA/CBA)	poly(triethylenetetramine/cystamine-bis-acrylamide)
p(TETA/CBA)-g-PEG	poly(triethylenetetramine/cystamine-bis-acrylamide)-grafted-poly(ethylene glycol)
p(TETA/CBA/PEG)	'One-Pot' poly(triethylenetetramine/cystamine bisacrylamide/poly(ethylene glycol)
qRT-PCR	quantitative RT-PCR
RES	Reticuloendothelial System
RNAi	RNA interference
RT-PCR	reverse transcriptase PCR
siRNA	small interfering RNA
SS-PAEI	disulfide reducible poly(amido ethyleneimine)
SVR	mouse pancreatic islet cell
TCEP	tris(2-carboxyethyl)phosphine
TETA	triethylenetetramine
VEGF	vascular endothelial growth factor
w/w	Polymer to nucleic acid weight-to-weight ratio

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CHAPTER 1

INTRODUCTION

1.1 General Introduction

Due to significant research breakthroughs in molecular biology and genetic engineering, nucleic acid-based therapeutics have become plausible candidates for the treatment of genetic diseases that conventional therapies strive to manage. Gene therapy offers the distinct advantage of correcting a genetic defect by delivering nucleic acid cargo in the form of circular DNA (pDNA) or RNA interference (RNAi) sequences to redirect protein translation and establish appropriate cellular function or homeostasis (*1*). Encouraging results have been produced for the treatment of traditional genetic diseases such as cystic fibrosis and muscular dystrophy as well as cardiovascular disease and cancer (*2-5*). Infectious diseases such as Human Immunodeficiency Virus (HIV), have also been impacted by advances in gene therapy (*6*).

While promising gene therapy results have been produced by many research labs, its clinical success is hampered by undefined design and formulation requirements to engineer a safe and efficient gene delivery vehicle. Problems including adverse immunological responses, oncogenesis and limited cargo encapsulation are associated with viral gene carriers. Conversely, if nonviral polymeric gene carriers are designed prudently, they are nonimmunogenic and they can encapsulate relatively large amounts of

therapeutic nucleic acid; however, transcellular cargo delivery of these carriers must be improved for their clinical success (*7-9*).

Because design and formulation requirements are largely undefined for improved nonviral gene delivery reagents, research labs often engineer and study many putative products that possess subtle physiochemical differences that may influence carrier function and biological activity in an attempt to better understand pertinent requirements for improved gene delivery carriers and identify clinically acceptable candidates. The synthesis of many putative reagents, however, requires multiple optimization cycles for each product, is time-intensive, laborious and costly.

The purpose of this study was to improve a promising and flourishing non-viral, polymeric gene carrier for in vitro and in vivo use by modifying the gene carrier with poly(ethylene glycol) (PEG) or PEG-ligand chemical conjugation. Moreover, the studies herein employ a unique method that that promotes evaluation ease of these developed gene carriers by avoiding the synthesis of many putative products in order to identify optimal candidate and reagent properties. Specifically, this method uses mixtures of the existing, flourishing nonviral gene delivery reagent and its modified counterpart(s), whereby the relative amount of each species in the formulation mixture is easily controlled, and thus, physiochemical differences and their influence on biological activity can be empirically studied to identify optimal reagent properties and candidates.

In order to evaluate this unique method, several co-polymeric gene carriers are developed in this work using the disulfide-containing, bioreducible gene carrier, poly(triethylenetetramine/cystamine-bis-acrylamide) (p(TETA/CBA)) as the backbone polycation. p(TETA/CBA) was previously developed as a bioreducible analog of the

efficient, though often toxic, poly(ethylene imine) (PEI) carriers. Researchers have extensively studied various forms of PEI as potential nucleic acid delivery vehicles and its class has become a standard in the nonviral gene delivery field. PEI carriers can condense and protect nucleic acid very easily and provide relatively high cellular transgene expression in vitro and in vivo, which is largely due to its high charge density and buffer capacity (*10-12*). The major problem associated with PEI systems is that because of their high charge density, as the molecular weight of these carriers increases, so does its cellular toxicity and thus are not a viable option for clinical use (*13-16*). The bioreducible PEI analog, p(TETA/CBA), maintains an advantageous high charge density all-the-while avoids cellular toxicity that is imposed by high molecular weight polycations through exploiting the intracellular reductive potential maintained by glutathione and other co-enzymes that trigger its cytosolic degradation (*17*). Despite this advantage, p(TETA/CBA) and other bioreducible gene carriers require further modification to improve their efficacy in vitro and in vivo to advance gene therapy applications. This is especially true for their use as systemic gene delivery vehicles that can be used to combat metastatic cancers.

Modification of bioreducible polycations for systemic application is required to improve their circulation half-life and avoid rapid clearance by the immune system. Insufficient circulation times are due to several factors. One apparent factor is their cationic nature. Often polycation/nucleic acid complexes (polyplexes), if net-positively charged, interact with net-negatively charged serum proteins that lead to particle aggregation, destabilization and often complement system activation. Complement activation facilitates the innate immune response and promotes polyplex sequestration by

the reticuloendothelial system (RES), thereby mitigating polyplex circulation time and putative efficacy (*18, 19*). Research has also shown that significant thiol concentrations exist in the mammalian bloodstream due to the presence of cysteine, homocysteine and glutathione (*20*). These thiols can participate with disulfide-containing bioreducible gene carriers in thiol-disulfide exchange, which leads to premature and uncontrolled carrier degradation and particle instability. In order to avoid the interaction of polycationic carriers with serum proteins, neutrally charged and hydrophilic polymers such as poly(ethylene glycol) (PEG) have been conjugated to polycationic systems to reduce polyplex surface charge and prevent the adsorption of serum proteins to carrier surfaces (*21*). While potentially advantageous, PEG conjugation to polycations has also been shown to adversely affect nucleic acid condensation and carrier function, which is especially true if the polycation has a low molecular weight (*22, 23*). These results taken together reveal the delicate influence of gene carrier chemical properties on their performance and suggest the need to easily alter and study carrier properties following PEG modification in order to optimize carrier function and ensure clinical advancement.

Following this brief introduction chapter, the dissertation herein commences with a literature review that encompasses the use of various nonviral gene delivery systems. Systems discussed will range from initial prototype gene carriers to more advanced systems that have proven to increase payload delivery to solid tumors using targeting moieties, which results in improved therapeutic efficacy and reduced off-target effects. The review will recognize current in vitro and in vivo limitations inherent to nonviral gene delivery systems and how gene carriers can be modified to enhance nucleic acid delivery to cancer-related cells.

Chapter 3 describes the synthesis and characterization of a bioreducible polymer and corresponding copolymer for gene delivery and provides evidence that these two species can be used in conjunction to alter gene carrier properties and study the effects on their bioactivity to optimize and improve gene carrier formulation reagents. The physiochemical characteristics and bioactivity of these products are evaluated using a facile appraisal method of a panel of putative gene delivery reagents and the use of empirical studies to identify improved gene carriers (*24*). Chapter 4 focuses on the synthesis of a copolymer that has the same chemical functionality to that developed in Chapter 3; however, it is generated by a less labor intensive method and has a significantly different molecular weight and degree of modification.

The studies described in Chapter 4 utilize the evaluation method developed in Chapter 3 to derive a panel of putative gene delivery reagents of varying composition to study the characteristics and bioactivity of the reagents, and it provide evidence that the facile evaluation method can be used to identify optimal formulations despite changes in physiochemical properties like molecular weight and degree of modification. This chapter provides evidence to support this claim and suggests that the facile evaluation can be applied to additional gene carriers. The studies maintain in vitro evaluation and extend the use of gene carrier mixtures to in vivo studies where the biodistribution of different carrier formulations is assessed following systemic injection.

Chapter 5 extends the previous studies to engineer a nucleic acid carrier that can enhance payload delivery using a targeted gene carrier that recognizes a cell surface receptor overexpressed on many cancer-related cell types. The study demonstrates that the aforementioned formulation method of mixing multiple delivery reagents can be used

to derive a panel of targeted gene delivery reagents. Targeting of this gene carrier relies on the overexpression of the $\alpha_V\beta_3$ integrin, which is characteristic of many solid tumors and the surrounding vasculature, which can be exploited to improve tissue-specific delivery of therapeutic payload and avoid off-target effects (*22, 23*). This is accomplished by modifying the gene carrier with a peptide moiety that contains the following amino acid sequence: Arginine-Glycine-Aspartic acid (RGD) that can specifically interact with the $\alpha_V\beta_3$ integrin and promote cellular internalization of the gene carrier. The data presented in this chapter demonstrate that targeted gene carrier mixtures can alter carrier properties and be used in vitro to enhance transgene delivery to known $\alpha_V\beta_3$ integrin expressing cells compared to carriers devoid of the targeting peptide. Studies also show cell specificity of the RGD-targeted nucleic acid delivery reagent. The studies provide a foundation for further investigation to determine therapeutic applicability and efficacy in vivo of the RGD-targeted gene delivery reagents.

1.2 Rational for the Study

The full potential of nonviral gene delivery has not been met. PEI systems continue to be the nonviral gene carrier standard because of their ability to facilitate significantly greater transgene delivery and expression compared to other carrier systems. In recent years, however, biodegradable gene carriers have gained tremendous recognition as potentially superior candidates because they are nontoxic and have proven to maintain equal or greater transgene delivery compared to PEI carriers. Despite the ability of biodegradable systems to provide high levels of transgene delivery to a wide range of cell types, their application in vivo is often limited to local administration due to

their degradation and rapid clearance in the blood stream. Modification of these systems with PEG to prevent rapid clearance provides a promising next step towards clinical application. However, if PEG and ligand modification is not thoroughly investigated, optimal carrier performance may not be achieved. Therefore, new methods should be developed that allow PEG and ligand modification of gene carriers that accommodate easy manipulation of the relative amount of PEG or ligand incorporated in gene carrier formulations so that improved gene delivery reagents are more easily developed for in vitro or in vivo use.

1.3 Specific Aims

Provided the aforementioned rationale, the overlying hypothesis is that mixtures of a polycation and PEG-polycation can be used to alter the relative amount of each species in a gene carrier formulation and thus influence carrier properties. Easy manipulation over gene carrier properties and function will allow rapid, facile evaluation and identification of optimal gene carrier reagents when used with nucleic acid. It is also expected that this method can be used for a targeted gene carrier with a PEG spacer to generate an optimal candidate for improved nucleic acid delivery to targetable cell types. The first specific aim was to develop a novel gene carrier comprised of an efficient and nontoxic bioreducible polycation in conjunction with its PEGylated counterpart to improve cellular transgene delivery compared to the polycation itself in serum-conditioned media. By using a feasible and facile approach to tailor the relative amount of PEG to polycation in a gene carrier formulation and derive a panel of putative reagents, the time and costly synthesis of multiple candidate copolymers for use in gene

delivery is avoided. By doing so researchers may study the influence of subtle physiochemical differences on gene carriers properties and bioactivity in order to identify optimal candidates for gene delivery.

The second specific aim is to synthesize a copolymer with the same chemical functionality to that developed in aim one, but with a different molecular weight and degree of PEG conjugation that can be used in conjunction with the bioreducible polycation and substantiate the validity of using mixture formulations for optimization irrespective of synthesis methods and product differences. In vivo biodistribution studies are also employed to evaluate formulation application in a murine adenocarcinoma model.

The third specific aim is to synthesize a targeted copolymer to enhance nucleic acid delivery to oncogenic cell types as well as angiogenic endothelial cells that surround tumor masses. In vitro studies investigate the potential of this targeted copolymer using formulation methods applied in the first two specific aims to verify their use in a targeted gene carrier system and provides a foundation for further studies in vivo. The gene carrier is designed using a nontoxic bioreducible polycation in conjunction with a peptide moiety, cyclic Arginine-Glycine-Aspartic Acid-(D)-Phenylalanine-Cysteine (c(RGDfC)), that that confers specificity toward $\alpha_V\beta_3$ integrins, which are often overexpressed in the tumor environment.

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CHAPTER 2

NONVIRAL GENE DELIVERY SYSTEMS AND CANCER GENE THERAPY: LITERATURE REVIEW

2.1 Introduction

Despite extensive progress in the field of gene therapy, the most prominent obstacles that hinder its clinical success are the development of a safe and efficient delivery system for nucleic acids. Research has produced many different gene carriers that have promising attributes, however carrier-mediated toxicity and/or low transgene expression continue to plague their therapeutic approval. There are two distinct classes of gene carriers: viral and nonviral (*1-4*). Viral gene therapy employs attenuated viruses that are derived using genetic engineering techniques. The most common viruses used are retroviruses, adenoviruses and adeno-associated viruses (*5*). These viral delivery systems often show more effective transgene delivery than nonviral carriers; however, there are significant problems that limit their clinical success (*1*). These problems include oncogenesis that results from chromosomal insertion of genetic payload in the host organism, immunogenicity, limited nucleic acid encapsulation, quality control issues and exorbitantly high production costs (*4, 6, 7*). Therefore, the use of nonviral nucleic acid delivery systems is desirable. Nonviral delivery systems can be designed to avoid immunogenicity, appease quality control mandates, maintain relatively low production

cost, and they are easily modified to possess multifunctional properties that are often advantageous (*8*). Examples of nonviral delivery systems include: physical methods for unprotected (naked) nucleic acid, amphiphilic lipids and polymers. The delivery of nucleic acid using these systems will be examined thoroughly, herein, with emphasis on polymeric systems and how they can be used for cancer gene therapy.

2.2 Nonviral Nucleic Acid Delivery Systems

2.2.1 Delivery of Naked Nucleic Acid

The delivery of unprotected nucleic acid is the most rudimentary method for delivering genetic material to cells. Naked nucleic acid has been delivered to the heart, skeletal muscle, liver, brain, thyroid, skin and to tumors (*9-21*). While transcellular delivery of unprotected nucleic acid has been achieved, its efficiency is limited due to the relatively high molecular weight and negative charge conferred in the phosphodiester backbone of these molecules. Thus, efficient cellular uptake is limited by the charge-charge repulsion between nucleic acid and the negatively charged cell membrane. In order to enhance the cellular delivery of naked nucleic acid, physical methods have been developed. These methods include electroporation, gene gun injection, the use of lasers, hydrodynamic pressure and ultrasound (*22, 23*).

Electroporation relies on a controlled electric field that is applied to cells or tissue in order to enhance cell permeability and uptake of genetic material. This method is useful, however, if the electric fields are not carefully controlled they can impose significant cell mortality and tissue damage (*24*). Ultrasound waves are also used to increase cell permeability and enhance delivery of naked nucleic acid. This method has

advantages because it can use microbubbles loaded with genetic material that when subjected to ultrasonic irradiation release their cargo in a specific location (*25, 26*). Ballistic, or gene gun applications involve shooting genetic payload that coats tungsten or gold nanoparticles into the cytoplasm and the nucleus of cells (*27*). Research presented in the literature has shown that the aforementioned methods can be used to enhance transgene delivery to cells or tissue; however, these methods have limited accessibility to internal organs so as long as minimally invasive applications are maintained. Thus, additional delivery systems are required to circumvent this shortcoming and ensure strong patient compliance.

2.2.2 Cationic Lipids

Minimally invasive delivery methods via systemic administration require the encapsulation and protection of nucleic acid cargo from serum proteins, as nucleic acids are extremely susceptible to nuclease degradation in the extracellular environment. Cationic amphiphiles or lipids can be used for nucleic acid protection and to drive cellular uptake for therapeutic application. Cationic lipids are composed of three basic domains: a positively charged and hydrophilic head group, a hydrophobic chain(s) and a linker group that joins the non-polar and polar regions of the amphiphile. The use of cationic lipids for cellular delivery of nucleic acid was first reported by Felgner and colleagues, who used N-(1-2,3-dioleyloxy)propyl-N,N,N-trimethylammonium chloride (DOTMA) (Figure 2.1) (*28*). Since then, there have been numerous lipids and lipid combinations developed for nucleic acid delivery. Many of them have shown promise in vitro and in vivo, and in fact, some of them have even been used in the clinic (*29-31*).

When cationic lipids are used in conjunction with nucleic acid cargo they form cationic lipid/nucleic acid complexes (lipoplexes) formed spontaneously via electrostatic and hydrophobic interactions. Lipoplexes maintain condensed nucleic acid cores that are devoid of water (*32, 33*). These systems can interact and fuse with the plasmid membrane of cells to facilitate their uptake and are believed to release nucleic acid payload in the cytoplasm by disruption of the endosomal compartment membrane. Membrane disruption occurs via electrostatic interaction between the cationic lipids of the lipoplex and the anionic phospholipids of the endosomal compartment. The dissociation of nucleic acid payload from the lipoplex occurs simultaneously with endosomal escape (*34*).

Research has shown that lipid structure and functionality can greatly influence their amphiphilicty, shape, size and pKa, which in turn profoundly affect their performance as nucleic acid carriers. Thus, these parameters must be rigorously studied (*35, 36*). Unfortunately, many of these lipid-mediated carriers have exhibited toxic side effects in cultured cells and in animals. Moreover, their colloidal stability and immunogenic concerns limit their clinical application, thus requiring the development of additional nucleic acid carriers (*29, 37-40*).

2.2.3 Cationic Polymers

There are numerous polycations that have been used as nucleic acid carriers; both natural and synthetic. Naturally occurring polycations include proteins such as histones and human serum albumin that has been cationized by conjugating hexamethylenediamine to its carboxylic acid groups. Aminopolysaccharides such as

chitosan are also naturally occurring polycations that have been used (*41-44*). Common synthetic polycations include polyamines such as poly(ethylene imine) (PEI), poly-L-lysine, poly(amido amine) (PAMAM) dendrimers and cyclodextrins, among many others (*45-49*).

For quite sometime, poly(ethylene imine)s have been considered the 'gold standard' of nonviral polymeric gene delivery. This class is subdivided into two major types: hyperbranched poly(ethylene imine) (BPEI or bPEI) and linear poly(ethylene imine) (LPEI or lPEI), respectively (Figure 2.2) (*50*). For both classes, the fundamental repeat unit contains a protonable amine that allows the polycation to buffer over a wide pH range. The buffering capacity of these systems is particularly useful within the endosomal pH 5-7 range, as this attribute facilitates endosomal escape and contributes to the delivery efficiency of the carrier. Endosomal escape of the polycationic carrier occurs via 'the proton sponge effect,' and the fundamental hypothesis is that the buffering capacity of a polycation influences the endosomal acidification process, which leads to an exorbitantly high influx of protons and corresponding counterions such as chloride (*45*). The heavy influx of these counter-ions results in osmotic swelling and rupture of the endosome therefore releasing its contents into the cytosol (*51*). It is also believed that these counter-ions destabilize the interaction of polycation with the nucleic acid payload, and thus influence transgene delivery (*52*).

The PEI systems were first used for oligonucleotide delivery in 1995 (*45*). Since then, extensive studies have focused on the biophysical properties of PEI systems, including their branching profiles, molecular weight and polydispersity, all of which greatly influence their gene delivery properties (*53*). In theory, bPEIs maintain a 1:2:1

ratio of primary, secondary and tertiary amines, respectively, that contribute to its preferable buffer capacity and solubility (*54*). However, the high charge density maintained by these amines also greatly increases serum protein interaction and cell membrane destabilization, which leads to reduced transfection efficiency and cellular toxicity (*55*). Moreover, PEI molecular weight and branching have shown to correlate with transfection efficiency and cell toxicity. Attempts to circumvent these negative issues have spawned PEI modification by introducing degradable linkages and neutral, hydrophilic polymers such as poly(ethylene glycol) (PEG) (*56-61*). One very successful modification of a PEI carrier was the incorporation of cholesteryl chloroformate to bPEI 1.8 kDa. This modification proved to increase transfection efficiency of this low molecular weight species significantly above bPEI 25 kDa; however, the polymer product was relatively nontoxic to cells when compared to bPEI 25 kDa. This carrier has been used for in vivo local applications to combat tumor progression as well as attenuate infarct size and cell death following a myocardial infarct. Both applications have proved extremely successful and show clinical promise (*62-64*).

The polycationic backbone of LPEI systems is predominantly comprised of secondary amines and is more water soluble than BPEI (*65*). Nonetheless, LPEIs have shown comparable or greater transfection efficiency compared to their branched counterparts but with considerably less cell toxicity (*66, 67*). Despite these advantages, branched and linear PEIs, alike, still have questionable clinical application due to their cellular toxicity and hampered clearance in vivo. Thus, additional polycationic carriers have been developed and characterized in an attempt to overcome these adversities (*68*).

Poly(L-lysine) (PLL) is a peptidomimetic polymer that contains the ε-amino group

of lysine in each repeat unit, which maintains protonation at physiologic pH and drives its electrostatic interaction with nucleic acid (Figure 2.2). While complexation of PLL with nucleic acid occurs, it has relatively low transfection efficiency compared to the PEIs and is highly variable between cell types. Its limited transfection capacity is primarily attributed to the high pKa of the ε-amines (9.3-9.5), which are almost entirely protonated at physiologic pH and thus possess little buffer capacity (*69*). In order to improve its buffer capacity, modification of PLL with histidine (pka 6.7-7.1) has been performed. Moreover, to also improve its ability to escape the endosome, co-delivery of PLL/nucleic acid with chloroquine, a known endosomolytic agent, has been investigated. Both attempts have been relatively unsuccessful, as these systems have persistent cell toxicity (*47, 70-72*). Additional modifications to improve solubility, mitigate toxicity and improve transfection via PEG conjugation and/or targeting ligands have also been performed (*70, 73-75*). Yet, mandates for clinical success have not been met using PLL gene carriers and the pursuit of improved polycationic nucleic acid carriers continues.

Another large group of polycations that have been developed and rigorously studied for gene therapy application are dendrimers. Dendrimers are highly branched synthetic molecules that possess a functional core from which multiple layers can be radially synthesized in a sequential manner (*76*). Due to their stepwise synthesis, dendrimers are characteristically well defined and maintain a high degree of molecular uniformity and low polydispersity compared to other synthetic polymers (*77*). Provided with their relatively high amine content, poly(amido amine) (PAMAM) dedrimers are commonly used for nucleic acid delivery (Figure 2.3) (*78-80*). Interestingly, these dendrimers can be fractured by heating them in water or butanol, which improves their flexibility and

correlates with transgene delivery efficiency (*81*). Similar to PEIs, however, polycationic dendrimers often exhibit cell toxicity that is influenced by their size, molecular weight and charge density. Moreover, amine terminated dendrimers exhibit a high degree of red blood cell hemolysis that is disadvantageous for in vivo applications (*82, 83*). Despite their advantages, because of their putative toxicity as well as the time consuming nature of their step wise iterative synthesis, their clinical use is avoided.

In general, the molecular weight and branching profile of polycations correlate with transgene expression and cellular toxicity. That is, high molecular weight and branched polycations tend to provide relatively high transgene delivery and expression, whereas, low molecular weight and linear counterparts exhibit less cytotoxicty but lower efficacy (*53, 84*). In lieu of this trend, degradable polycations have been engineered and studied for nucleic acid delivery. As such, degradable polycations can exploit the advantages of high molecular weight or branched systems, all the while degrade into small molecular weight species to limit their cellular accumulation and toxicity (*68, 85-87*).

The earliest degradable cationic polymer used as a nucleic acid delivery reagent was poly(4-hydroxyl-L-proline ester) (PHP) (Figure 2.4). Provided with an ester bond, PHP, similar to other esters, is susceptible to hydrolysis and the polymer product degraded in less than 24 hrs due to ester hydrolysis accentuated by the amine present in the backbone polymer (*88*). While the synthesis of this degradable polymer for nucleic acid delivery was novel, its transfection efficiency is relatively low (*88, 89*). A PLL analogue, poly(α-(4-aminobutyl)L-glycolic acid) (PAGA), which degrades rapidly under physiologic conditions was developed shortly thereafter and proved to be a more efficient

nucleic acid carrier than PHP and PLL (Figure 2.4) (*90*). Other degradable polycationic esters, deemed poly(ester amine)s ((PAE)s) have been synthesized and extensively studied (Figure 2.4). Large libraries of these compounds currently exist. However, despite screening more than 2,000 putative PAE carriers, only a small number of them were able to effectively condense nucleic acid and provide transgene expression comparable to PEIs. Nonetheless, they have proven to be nontoxic irrespective of branching and molecular weight characteristics, thus providing promise for degradable polycations (*85, 91, 92*).

Polyester systems using PEI have also been developed. This was achieved using 1,3-butanediol diacrylate, hexanediol diacrylate or polycaprolactone monomers in conjunction with oligoethylenimine or PEIs (*11, 93, 94*). The gene carrier synthesized using the 1,3-butanediol diacrylate monomer proved to be extremely more efficient (16x greater transgene expression) than bPEI 25kDa with significantly less toxicity. The others, however, were nontoxic but provided relatively low transfection efficiency. Low molecular weight PEI has also been crosslinked with bi-functional poly(ethylene glycol) (PEG) to produce a polyester type PEI-PEG network (*95*). This system proved to be significantly less toxic to cells than bPEI 25kDa; however, transfection paled in comparison due to rapid degradation and reduced charge density. Kim and colleagues also reported Schiff base formation following reaction between gluteraldehyde and PEI to produce an acid-labile PEI counterpart (Figure 2.5). Again, this system showed very little toxicity but similar levels of gene expression compared to its corresponding PEI control (*56*). These hydrolytically unstable PEI systems, as well as the other poly(ester amine)s discussed above, have produced encouraging results in vitro. However, because

of their susceptibility to hydrolytic cleavage, their purification and rapid degradation present problems for potential clinical application as nucleic acid delivery reagents.

In an attempt to overcome the problems associated with hydrolytically degradable nucleic acid carriers, biodegradable polycations that contain reducible disulfide bonds have been synthesized and evaluated. The existence of disulfide bonds in these respective carriers provides hydrolytic stability, all the while providing triggered intracellular degradation of the polycation in order to mitigate putative cellular toxicity. The difference in redox potential between the extracellular and the intracellular space is the driving force for triggered degradation (*96*). Reduced glutathione (GSH) largely participates in the redox cycle of most cells (*97*). In fact, mammalian cells maintain approximately 100x [GSH] compared to [GSSG] inside the cell, depending on the cell line and its oxidative stress (*96, 98*). GSH concentrations are maintained by various enzymes and NADPH via the pentose phosphate pathway (*99, 100*). Several classes of bioreducible polymers capable of exploiting this pathway have been synthesized, including hyperbranched and linear forms (*68, 101-105*). Low molecular weight PEI has also been crosslinked with disulfide-containing monomers to mimic these systems (Figure 2.6) (*50*). The characteristics of these systems such as charge density, rigidity, bascity, hydrophilicity/hydrophobicity influenced by their structure, molecular weight and amino groups profoundly affect their performance as nucleic acid delivery vehicles. Nonetheless, provided with their biodegradable composite, they are relatively non-toxic to cells and many of these gene carriers offer comparable or improved transfection to bPEI 25kDA (*106*). Their hydrolytic stability and synthetic ease make them excellent candidates for clinical application. However, to date, their application is limited to in

vitro use and in vivo local administration. To expand their potential for clinical use, these carriers should be modified for in vivo systemic application in order to avoid their interaction with serum proteins and thus improve their pharmacokinetics. The following section will discuss potential modifications of these systems and polycations alike, which improve carrier use for systemic administration and for cancer gene therapy specifically.

2.2.4 Background and Use of Targeted Polycationic Nucleic Acid Carriers for Cancer Gene Therapy

The success rate for cancer therapy is largely disappointing. In the year 2008, an article in the International Journal of Cancer estimated that more than 12.7 million people had developed a malignant tumor and that approximately 7.6 million others died of the disease that year (*107*). Moreover, cancer is the second leading cause of death in the United States and is responsible for more than half a million deaths per year. The financial implications are multifaceted, as the overall cost of cancer in 2004 for the United States was $189.8 billion: $69.4 billion for direct medical, $16.9 billion for indirect morbidity and $103.5 billion for indirect mortality costs. Even more disconcerting is that as mortality rate of many chronic diseases has decreased significantly in the past 50 years, mortality rates of cancer have remained constant (*108*). Clearly there is a need for more safe and effective anti-cancer therapies.

Systemic administration of drug and nucleic acid carriers is particularly useful for cancer therapy because as malignant tumors grow, oncogenic cells often spread to distant tissues or organs from which they initially arise by migrating through the bloodstream and depositing elsewhere. This process is defined as tumor metastasis. By employing

systemic administration of nucleic acid delivery reagents, whereby these reagents can circulate in the bloodstream, distant and internal organs or tissue(s) including primary tumors and metastatic malignancies that rely on vascularization for their growth and survival can be reached. Oncogenic cells arise from abnormal gene function or gene expression, thus, provided the genetic basis of this disease, gene therapy is particularly useful for overcoming or correcting the responsible genetic defect(s) (*109, 110*). The therapeutic efficacy of nucleic acid based therapies relies, of course, on the ability of therapeutic payload to reach relevant cells or tissue.

Polycationic nucleic acid carriers have been modified to improve their in vivo pharmacokinetics and enhance their delivery to the tumor site following systemic administration(s). Probably the most widely employed modification of these carriers is the covalent coupling of poly(ethylene glycol) (PEG) to them, which can mask their surface charge to avoid undesirable, nonspecific interaction with serum proteins that often leads to opsonization and removal of the delivery vehicle from the blood stream by the reticuloendothelial System (RES) (*111-114*). Relatively high levels of nonspecific deposition in the lung and liver following systemic administration can also be reduced via PEG conjugation of these polycationic carriers by reducing their surface charge and mitigating their adsorption to capillaries and tight junctions (*115-117*). If non-specific deposition and RES uptake of polyplexes are reduced, putative therapeutic efficacy is improved. Moreover, polyplexes that maintain a prolonged circulation time beyond 6 hrs can exploit the Enhanced Permeability and Retention Effect (EPR) (*118*). This phenomenon is commonly observed when macromolecules or nanocarriers are delivered intravenously and with long circulation times tend to deposit to a greater extent at tumor

sites than in normal tissues. The maintained principle behind this effect is the characteristic 'leaky,' fenestration of the vasculature surrounding tumors and their impaired lymphatic drainage. Abundant evidence suggests that this phenomenon is operational in humans (*119*). However, the extent to which the EPR effect can be exploited by drug/nucleic acid delivery reagents depends largely on tumor types and its pathophysiological characteristics that affect tumor vascularization, otherwise known as angiogenesis (*120*). Nonetheless, within the past decade the significance of this vascularization around growing tumors has been widely recognized to have clinical utility (*121, 122*).

While PEG modification of polycations can improve their circulation half-life and exploit the EPR effect for passive targeting of the carriers, PEG modification often decreases transgene delivery and expression compared to un-PEGylated counterparts. The delivery capacity of PEGylated polycations can often be recovered with the incorporation of targeting moieties that possess binding affinity for transmembrane receptors expressed on specific cell types (*123*). Upon receptor binding, the nucleic acid carriers undergo receptor-mediated endocytosis, which has proven to enhance cellular uptake (*124*). Sugar residues, vitamins, peptides, proteins, antibodies and antibody fragments have all been successfully employed as targeting moieties for drug and gene carriers (*124-133*). Of particular interest to this discussion are those molecular entities that recognize transmembrane receptors specifically overexpressed on primary and metastatic cancers or the angiogenic vasculature that surrounds many tumors.

The use of relevant targeting molecules for cancer treatment is particularly important because despite significant progress in surgical, radio and chemotherapies, long

term patient survival is still unmet. Targeting molecules used in conjunction with therapeutic nucleic acid agents provide an alternate means to systemically treat a broad range of tumors and associated angiogenesis. Specific tumor cells have been delineated and targeted individually as well. Prostate cancer cells are one specific example. As such, the transmembrane protein known as Prostate Specific Membrane Antigen (PSMA) is expressed in virtually all prostate cancer cell despite their heterogeneity, and its expression is greatly increased in poorly differentiated, metastatic, and hormone-refractory carcinomas. A humanized monoclonal antibody, deemed J591, was developed and recognizes the extracellular domain of PSMA. This targeting agent has been used for radioimmunotherapy and confirmed as a prostate cancer targeting agent (*134*). J591 has not been used for nucleic acid delivery; however, this targeting agent serves as a platform for other similar agents that can be used for prostate-specific nucleic acid therapy. Further studies are required to elucidate this prospect.

Two of the most widely studied and applied targeting agents for cancer gene therapy are folate (vitamin B9) and Arginine-Glycine-Aspartic Acid (RGD)-containing peptide sequences that possess strong binding affinity for folate receptors and $\alpha_v\beta_3$ integrins, respectively. Both of these receptors are up-regulated on numerous cancer cells and the related tumor vasculature compared to normal, nonpathological tissues (*110, 121, 128, 135-138*).

The folate receptor (FR) has several isoforms. These isoforms exhibit differential binding affinity for folate, though all of them are members of the glycosylphosphatidylinositol (GPI)-linked membrane glycoprotein family. More importantly, once bound by folate, these receptors are able to transport folate-bound

macromolecules and nanoparticles via receptor-mediated endocytosis (*136, 137, 139*). High affinity FR are specifically over-expressed in ovarian, mammary gland, colon, lung, prostate, and leukemic cancers. Enhanced receptor expression correlates with poor prognosis and metastasized cancer cells possess greater amounts of this FR than their primary tumors. Moreover, the only nonpathological tissues that express the FR are placenta, lung, choroids plexus, thyroid and kidney (*139, 140*). With the exception of the kidney, FR expression in these tissues is preserved to the apical side of polarized epithelial cells. Thus, folate-targeting of nanoparticles and nucleic acid carriers is promising for the treatment of a variety of cancers and their related metastasis (*136, 137*). Also noteworthy, folate-targeted therapeutics have proven to be anti-angiogenic, which may provide additive therapeutic affect compared to tumor-targeted eradication alone (*141*).

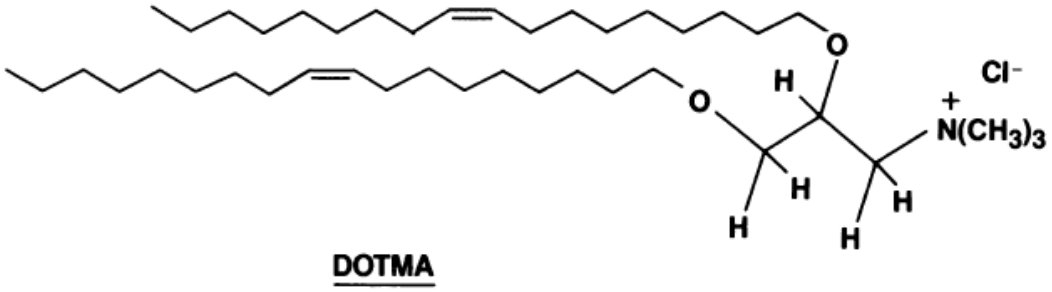
As suggested above, RGD-containing peptides also provide tremendous promise as targeting agents for anti-angiogenic therapy as well as metastasis (*142*). A variety of different RGD peptides have been used to target melanoma, mammary, colon, renal and ovarian carcinomas as well as prostate cancer (*143*). Fundamental for the growth and metastasis of malignant tumors is their ability to perpetrate blood supply through the conquest of preexisting vasculature. This triumph is known as angiogenesis, or is the sprouting of new blood vessels from existing ones, which provides nutrients to the metabolically active and growing tumor (*144, 145*). There is a strong correlation between tumor microvessel density and the clinical stage of cancer, which further correlates with the disease-specific survival rate of patients (*146, 147*).

Extensive research on tumor-associated angiogenesis has shown that its induction, known as the angiogenic switch, is tightly regulated by the expression of endogenous anti-angiogenic and pro-angiogenic molecules (*148-153*). The angiogenic switch occurs in the early stages of tumor growth as a response to hypoxia and decreased cell pH, which increases the expression of hypoxia-inducible factors (HIF) in tumor and stromal cells. Increased HIF production drives the expression of vascular endothelial factor (VEGF) and several other pro-angiogenic factors that promote the angiogenic process by increasing the destabilization, proliferation and migration of endothelial cells (*149, 154, 155*). As mentioned above, tumor-associated angiogenesis leads to 'leaky' and fenestrated blood vessel formation due to the immature and irregular shape of the endothelial cells that comprise the neovasculature (*156*). This vasculature is also characterized by an abnormal increase in $\alpha_v\beta_3$ integrin expression compared to non-pathogenic tissues, which can be exploited for the active targeting of nucleic acid carriers to these tissues using ligands that confer specificity for $\alpha_v\beta_3$ integrins (*130, 131*).

Both tumor cells and the angiogenic vasculature can be targeted using RGD-containing peptide ligands that possess high affinity for the $\alpha_v\beta_3$ integrin receptor. Targeting the angiogenic vasculature using these ligands have several advantages over targeting tumors specifically. First, endothelial targets are common to all solid tumors. Endothelial cells are also immediately accessible to RGD-targeted drug and nucleic acid carriers delivered systemically and are therefore more easily targeted than the tumor cells. These endothelial cells also maintain greater genetic stability than oncogenic cells, which makes them less likely to develop resistance to cancer therapy. Lastly, because one

endothelial cell can support the survival of ~5-50 tumor cells, endothelial cell death can lead to the death of many oncogenic cells (*157, 158*).

Since the initial discovery of RGD-containing peptides by Pierschbacher and Ruoslahti, many different RGD-containing peptides have been employed as tissue-specific targeting agents for oncogenic and angiogenic cells (*159*). For polymeric nucleic acid delivery, probably the most widely used carriers for these targeting purposes are the PEIs. RGD-targeted PEIs used to deliver nucleic acid payload that abrogate oncogenic protein function have provided promising results in vivo demonstrating reduced tumor growth and vascularization compared to untargeted carriers (*130, 131, 160*). RGD-targeted degradable nucleic acid carriers have also been used as promising delivery agents for cancer therapy (*161*). These targeted polycations provide an opportunity to exploit the active and passive targeting of tumors and provide a platform for combination therapy to deliver anti-cancer nucleic acid and chemotherapeutic agents. Moreover, they allow potential increased delivery of therapeutic payload to the tumor site, reduced off-target effects and if applicable, circumvent multi-drug resistance through receptor-mediated uptake at the target site (Figure 2.7). To date, however, no disulfide-containing bioreducible nucleic acid carriers have been used in conjunction with RGD-targeting peptides to evaluate their potential for cancer gene therapy application in vitro or in vivo. Nucleic acid delivery systems in this arena should be developed and studied to elucidate their potential.



DOTMA

Figure 2.1. Structure of the cationic lipid N-(1-2,3-dioleyloxy)propyl-N,N,N-trimethylammonium chloride (DOTMA). A polar head group, hydrophobic tail and ether linkage is shown. Adapted from reference 24.

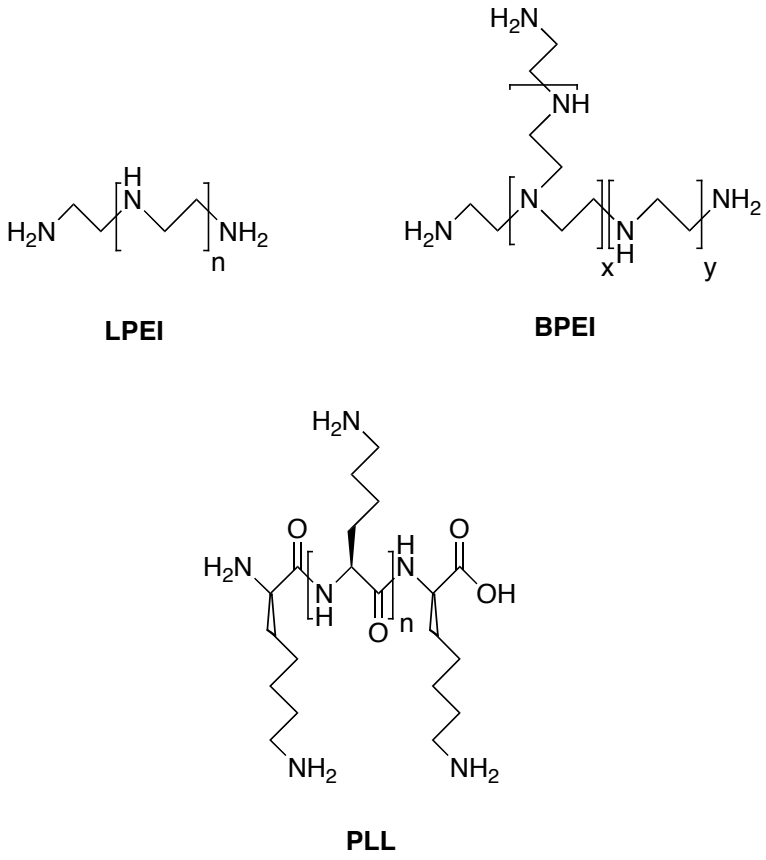


Figure 2.2. Chemical structures of classic, synthetic nondegradable polycations linear poly(ethylene imine) (LPEI); branched poly(ethylene imine) (BPEI); poly-L-lysine (PLL). Adapted from reference 46.

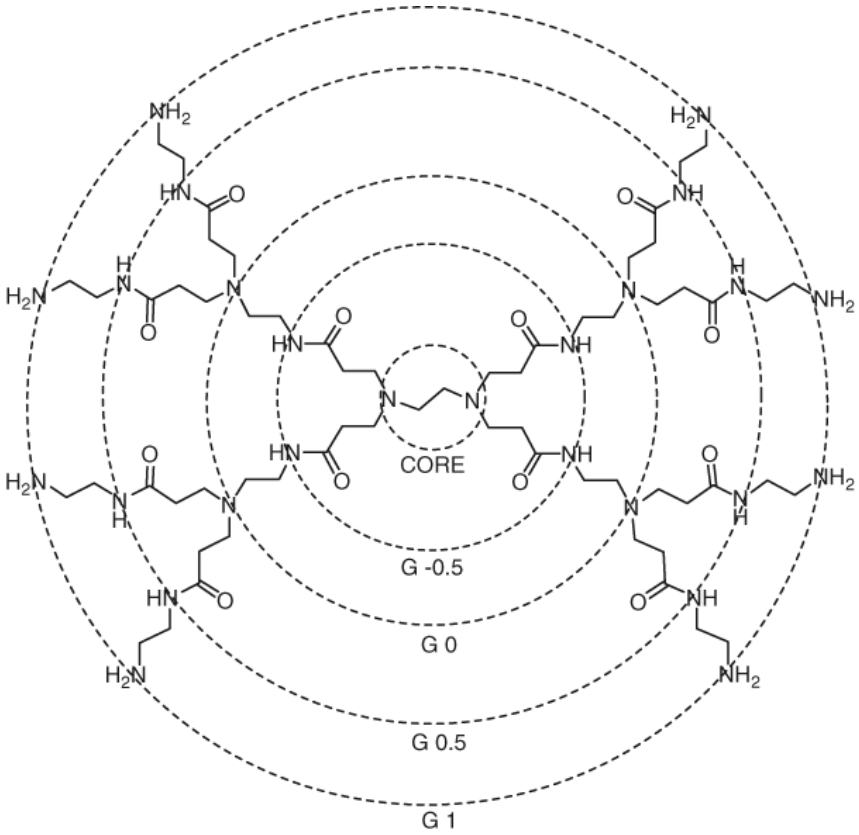
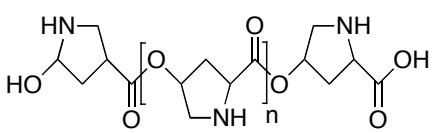
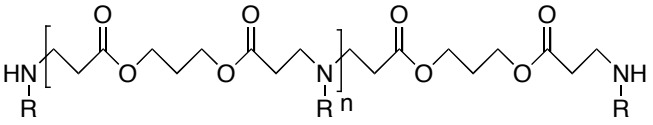


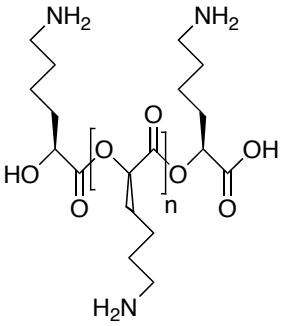
Figure 2.3. Structure of G1 (generation 1) poly(amido amine) (PAMAM) dendrimer. Adapted from reference 80.



PHP



PAE



PAGA

Figure 2.4. Structural examples of degradable nucleic acid carriers; poly(4-hydroxyl-L-proline ester) (PHP), poly(ester amine) (PAE) and poly(α-(4-aminobutyl)L-glycolic acid) (PAGA).

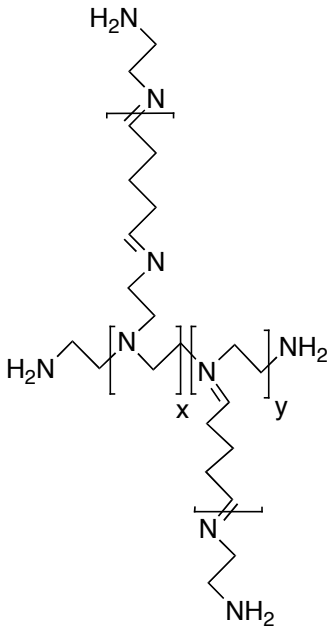


Figure 2.5. Structure of acid-labile PEI. Adapted from reference 51.

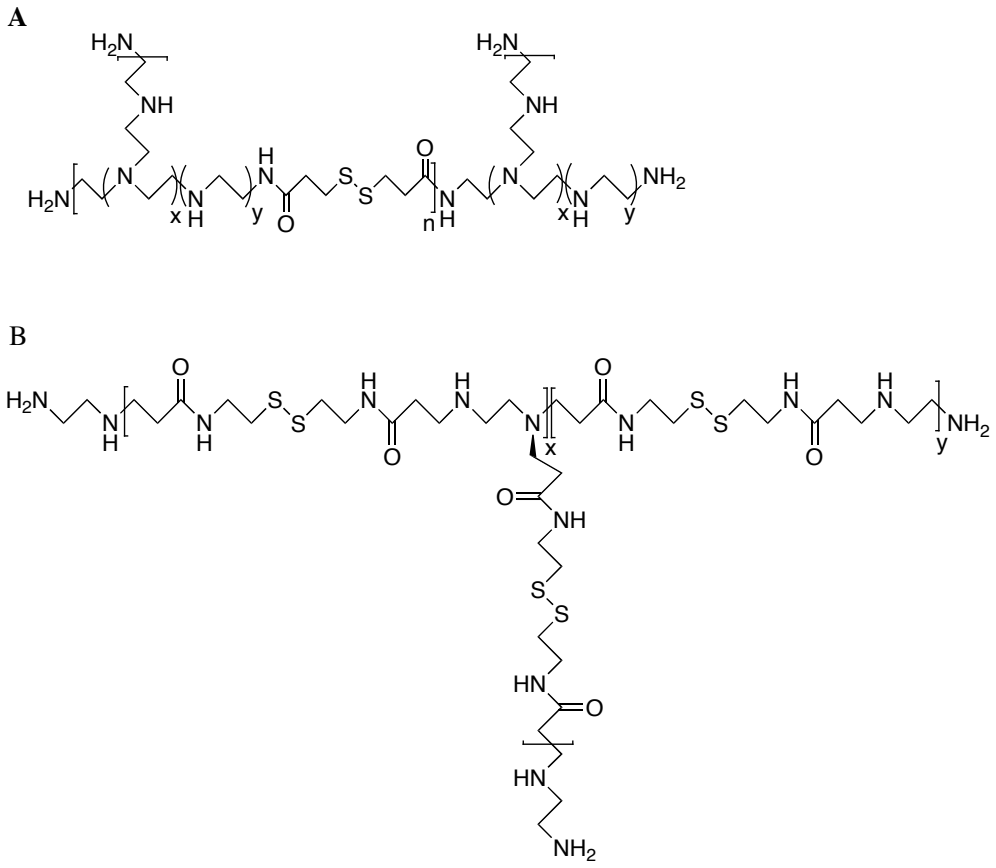


Figure 2.6. General structure of disulfide-containing polycations a) crosslinked low molecular weight poly(ethylene imine); b) poly(ethylenediamine/cystamine-bis-acrylamide) (p(EDA/CBA)). Adapted from reference 46.

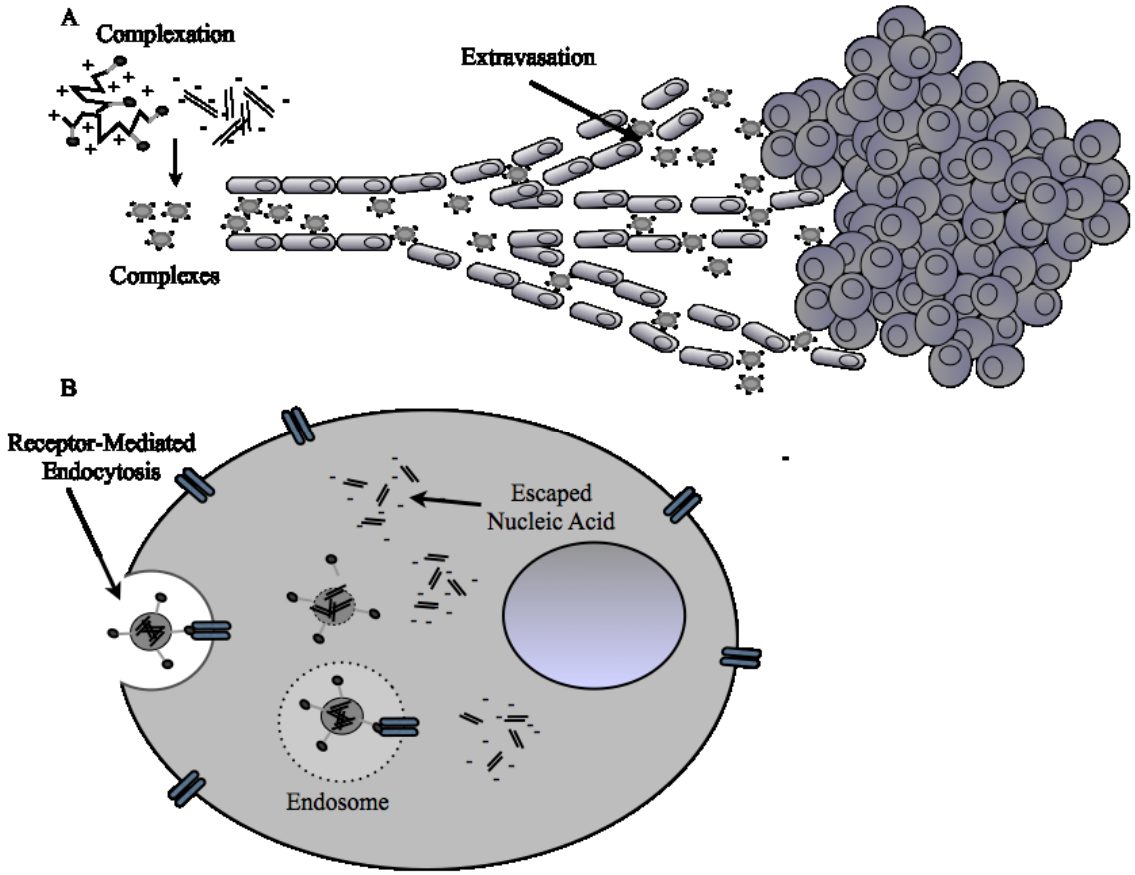


Figure 2.7. General scheme for targeted nucleic acid delivery to tumors. a) Ligand-conjugated polymeric carrier forms electrostatic complex with nucleic acid. When circulating in the bloodstream following systemic injection, complexes extravasate from fenestrated tumor vasculature to access the tumor cells. b) Once the targeted complex binds to the cell surface receptors of tumor and/or vasculature cell, the complex is taken up by receptor-mediated endocytosis. Following uptake, the carrier's buffering capacity facilitates endosomal escape and the complex destabilizes and releases nucleic acid payload intracellularly.

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CHAPTER 3

MIXTURES OF POLY(TRIETHYLENETETRAMINE/CYSTAMINE-BIS-ACRYLAMIDE) AND POLY(TRIETHYLENETETRAMINE/ CYSTAMINE-BIS-ACRYLAMIDE)-g-POLY(ETHYLENE GLYCOL) FOR IMPROVED GENE DELIVERY[1]

3.1 Abstract

Branched disulfide-containing poly(amido ethyleneimines) (SS-PAEIs) are biodegradable polymeric gene carrier analogs of the well-studied, nondegradable and often toxic branched poly(ethylene imines) (bPEIs), but with distinct advantages for cellular transgene delivery. Clinical success of polycationic gene carriers is hampered by obscure design and formulation requirements. This present work reports synthetic and formulation properties for a graft copolymer of poly(ethylene glycol) (PEG) and a branched SS-PAEI, poly(triethylenetetramine/cystamine-bis-acrylamide) (p(TETA/CBA)). Several labs have previously demonstrated the advantages of PEG conjugation to gene carriers, but have also shown that PEG conjugation may perturb plasmid DNA (pDNA) condensation, thereby interfering with nanoparticle formation.

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[1] Brumbach, J. H., Lin, C., Yockman, J., Kim, W. J., Blevins, K. S., Engbersen, J. F., Feijen, J., and Kim, S. W. (2010) Mixtures of poly(triethylenetetramine/cystamine bisacrylamide) and poly(triethylenetetramine/cystamine bisacrylamide)-g-poly(ethylene glycol) for improved gene delivery. *Bioconjug Chem. 21*, 1753-61. (Reprinted with permission).

With this foundation, our studies sought to mix various amounts of p(TETA/CBA) and poly(triethylenetetramine/cystamine-bis-acrylamide)-grafted-poly(ethylene glycol) 2kDa (p(TETA/CBA)-g-PEG2k) together in order to alter the relative amount of PEG in each formulation used for polyplex formation and gene delivery. The influence of different PEG/polycation amounts in the formulations on polymer/nucleic acid nanoparticle (polyplex) size, surface charge, morphology, serum stability and transgene delivery were studied. Polyplex formulations were prepared using p(TETA/CBA)-g-PEG2k, p(TETA/CBA), and mixtures of the two species at 10% p(TETA/CBA)5k-gPEG2k/90% p(TETA/CBA)5k (10% p(TETA/CBA)5k-g-PEG2k) or 50% p(TETA/CBA)5k-gPEG2k/50% p(TETA/CBA)5k (50% p(TETA/CBA)5k-g-PEG2k) at a desired and relevant polymer/pDNA weight-to-weight ratios (w/w) for use. As expected, increasing the amount of PEG in the formulation, adversely affects polyplex formation. However, optimal polymer mixtures could be identified using this facile approach to further clarify design and formulation requirements necessary to understand and optimize carrier stability and biological activity. This work demonstrates the utility of the approach described to identify and potentially overcome typical problems observed when polycations are modified and thus avoid the need to synthesize multiple copolymers to identify optimal gene carrier candidates, which is often timely, laborious and cost prohibitive. This approach may be applied to other polycation-PEG preparations to alter polyplex characteristics for optimal stability and biological activity of newly developed systems.

3.2 Introduction

Gene therapy is a feasible alternative to conventional therapies that simply manage symptoms of diseases and lack an effective treatment. However, gene therapy's clinical success is impaired by uncertain design and formulation requirements for safe and efficient nucleic acid delivery to cells. Recent research advancements have improved carrier safety and efficacy through carrier chemical modification to alter surface charge and/or tissue specificity using poly(ethylene glycol) (PEG) and/or cell-specific targeting ligands (*1*). Polymeric nonviral gene carriers have distinct advantages because if designed prudently they are nonimmunogenic and are easily modified to exhibit multi-functional properties (*2*). Nonviral polycations are also relatively cost-effective, easy to produce industrially and can carry relatively large amounts of therapeutic nucleic acid (*3, 4*).

Many structurally different polymers and copolymers consisting of linear, branched or dendritic architectures have been tested for their efficacy and suitability for in vitro and in vivo gene delivery. Poly(ethylene imine) gene carriers (PEIs) have been most rigorously studied and are a standard for polycationic gene carriers because they easily condense pDNA into nucleic acid/polycation nanoparticles (polyplexes) that protect nucleic acid from serum nuclease degradation as well as facilitate relatively high transgene delivery and expression in many cell types in vitro and in vivo. Unfortunately, PEIs often exhibit cellular toxicity due to intracellular accumulation of non-degradable polycations (*3, 5*). Increased PEI molecular weight and branching, which influence polycation charge density, correlate with increased transgene expression but also with cellular toxicity. Conversely, low molecular weight PEIs show reduced cellular toxicity

that correlates with reduced transgene expression (*6, 7*). As predicted, the design of degradable polycationic gene carriers such as the reducible disulfide-containing poly(amido amine)s (SS-PAAs) and poly(amido ethylenimines) (SS-PAEI), as well as hydrolyzable poly(β-amino ester) families have demonstrated comparable or improved cellular gene delivery and less cell toxicity when compared to PEIs (*8-10*). Reducible SS-PAEIs are synthetic analogs of the PEI family but have the aforementioned advantages of improved biological activity and biocompatibility (*11*). A recent abstract by Martello *et al.* showed that hyperbranched, SS-PAAs can condense plasmid DNA (pDNA) into polyplexes with sizes similar to bPEI 25 kDa, encouraging further functional studies (*12*).

Often cationic polyplexes interact with net negatively charged proteins found in serum, which leads to particle aggregation and reduced efficacy *in vitro* and *in vivo* (*13-15*). In order to overcome this hurdle, poly(ethylene glycol) (PEG) conjugation to polycations has been employed and studies have shown that PEGylation often improves carrier function in the presence of serum. However, previous studies have also clearly shown that increasing targeting ligand and/or PEG conjugation to PEIs, especially to low molecular weight (LMW) PEI ~5kDA, adversely affects polyplex formation and carrier function (*16, 17*).

In order to better design and formulate hyperbranched SS-PAEIs and their corresponding graft PEG copolymers, several SS-PAEI polycationic gene carriers are synthesized and the influence of varying the relative PEG/polycation amounts on polyplex formation, size, surface charge, morphology, serum stability and ultimately biological activity are studied. Polyplex formulations were prepared using pDNA and a known SS-PAEI, poly(triethylenetetramine/cystamine-bis-acrylamide) (p(TETA/CBA)),

its PEGylated counterpart p(TETA/CBA)5k-g-PEG2k, or mixtures of the two species at 10/90% and 50/50% using various polymer to pDNA weight-to-weight ratios (w/w), respectively. Altering the amount of PEG in formulation mixtures was employed as a suitable strategy to easily control and alter gene delivery reagent physiochemical properties in order to identify a suitable gene delivery reagent with improved bioactivity. By utilizing this method, the synthesis of many putative reagents for the same purpose is avoided.

3.3 Materials and Methods

3.3.1 Materials

Triethylenetetramine (TETA), tris(2-carboxyethyl)phosphine (TCEP), N-ethylemaleimide (NEM), hyperbranched polyethylenimine (bPEI, *M*w 25 000 (bPEI25kDa)) and HPLC grade methanol were purchased from Sigma-Aldrich (St. Louis, MO). N,N'-Cystamine-bis-acrylamide (CBA) was purchased from Polysciences, Inc. (Warrington, PA). Ultrafiltration devices and regenerated cellulose membranes (1kDa and 5kDa) were supplied by Millipore Corporation (Billerico, MA). The reporter gene plasmid, pCMVLuc, was designed previously by insertion of luciferase cDNA into a pCI plasmid (Promega, Madison, WI) driven by the pCMV promoter and was purified using Maxiprep (Invitrogen, Carlsbad, CA) protocols. Dulbecco's Modified Eagle's Medium (DMEM), penicillin streptomycin, trypsin-like enzyme (TrypLE Express), and Dubelcco's phosphate buffered saline were purchased from Gibco BRL (Carlsbad, CA). EBM-2 with EGM-2 singlequots was purchased from Lonza. Fetal bovine serum (FBS) was purchased from Hyclone Laboratories (Logan, UT).

3.3.2 p(TETA/CBA) Synthesis

Synthesis of p(TETA/CBA) was performed at 50°C using equimolar amounts of N,N'-cystamine-bis-acrylamide and triethylenetetramine monomers with minor adaptation of the previously described method (*11*). The polymerization reaction was split in half after the pH was adjusted to 7.0 and purified by ultrafiltration using a 1kDa or 5kDa MWCO regenerated cellulose membrane to yield p(TETA/CBA)1k or p(TETA/CBA)5k, respectively. The polycations were subsequently lyophilized (Scheme 1). Composition of the polymer was monitored using ^{1}H NMR (400 MHz, D_2O). p(TETA/CBA) δ 2.61 (COC*H*2CH2NH, 4H), 2.72 (NHCH2C*H*2S-S, 4H), 2.90-3.21 (COCH2C*H*2NHC*H*2C*H*2,16H), 3.41 (NHC*H*2CH2S-S, 4H).

3.3.3 p(TETA/CBA)5k-g-2k Synthesis

Methoxy poly(ethylene glycol) 2 kDa (mPEG2k) was dried using anhydrous toluene and subsequently precipitated in anhydrous ice-cold ether. The white precipitate was collected and dried in vacuo. The mPEG2k was then activated using *p*-nitrophenyl chloroformate in dichloromethane (DCM) as solvent and reacted on ice overnight while stirred. The activated PEG product was collected by precipitation in anhydrous ice-cold ether and dried in vacuo. Following NMR analysis to assess the degree of PEG activation, the amine reactive PEG-carbonate dissolved in anhydrous dimethyl sulfoxide (DMSO) was added dropwise to a solution of p(TETA/CBA)5k dissolved in anhydrous pyridine/DMSO as solvent. The molar feed ratio of PEG-carbonate to p(TETA/CBA)5k was 1.2:1. The reaction was stirred at room temperature and the release of *p*-nitrophenolate was monitored optically at 400 nm using UV-Vis. When the reaction was

complete at approximately 16 hrs. the sample was purified by ultrafiltration (5kDa MWCO) before being lyophilized. The composition of the p(TETA/CBA)-g-PEG2k copolymer conjugate was monitored using ^{1}H NMR (400 MHz, D_20). p(TETA/CBA)5k-g-PEG2k δ 2.5 (COC*H*2CH2NH, 4H), 2.72 (NHCH2C*H*2S-S, 4H), 2.90-3.21 (COCH2C*H*2NHC*H*2C*H*2,16H), 3.41 (NHC*H*2CH2S-S, 4H), 3.5-3.6 (C*H*2CH20, 4H), 2.2.5-2.38 (C*H*3, 3H).

3.3.4 Polymer Characteristics

The absolute molecular weight was determined for p(TETA/CBA)1k, p(TETA/CBA)5k and p(TETA/CBA)5k-PEG2k using AKTA/FPLC (Amersham Pharmacia Biotech Inc.) coupled to a light-scattering detector and using the relevant polymer refractive index increment (dn/dc) for each sample. A SuperdexPeptide column HR 10/30 was used for the separation of p(TETA/CBA)1k (2mg/mL), whereas a Superose 6 10/300 GL column was used for p(TETA/CBA)5k (2mg/mL) and p(TETA/CBA)5k-g-PEG2k (2mg/mL) separation. Poly[N-(2-hydroxypropyl)methacrylamide] (poly(HPMA)) standards were used prior to analysis to ensure that the FPLC columns were clean and functional. Experimental and standard polymer samples were dissolved in degassed and filtered (0.2 μm (Nylon, Alltech)) 0.3 M NaOAc, pH 4.4 with 30% (v/v) acetonitrile eluent buffer. The flow rate was set to 0.4 mL/min.

3.3.5 Determination of Polymer Disulfide Bonds

Disulfide bond content of each polymer was determined using 5,5-dithio-bis-(2-nitrobenzoic acid) (Ellman's reagent) following the manufacturer's protocol with minor adaptation (Pierce). In brief, cysteine hydrochloride monohydrate was used as a standard to generate a standard curve for accurate analysis. Absolute polymer molecular weights were used to estimate the number of disulfide bonds in each polymer sample and to ensure that the average thiolate concentration of the experimental polymers fell within the standard range for accurate analysis of disulfide bond content. Each experimental polymer sample was reduced using 10x immobilized (tris(2-carboxyethyl)phosphine hydrochloride) (TCEP) gel in buffer (0.1 M sodium phosphate, pH 8, 1mM EDTA). Following 30 min. incubation of the polymer samples in the presence of immobilized TCEP reducing agent, the samples were centrifuged at 1000 x g for 2 min and the reduced polymer solutions were collected. Reduced polymers were subsequently added to the Ellman's reagent in the aforementioned buffer and were allowed to incubate at room temperature for 20 min durring constant mixing. The sample absorbance at 412 nm was measured using UV-Vis spectroscopy. The thiol concentration was determined using the standard curve and the absolute molecular weights of the polymers in order to determine the number of disulfide bonds per polymer (n=3).

3.3.6 Polyplex Formation

In all cases polyplex was formed with a known amount of pDNA and a corresponding and desired amount of polymer dissolved in HEPES buffer (20 mM, pH 7.4, 5% glucose). The polymer and pDNA solutions were combined, lightly vortexed

and allowed to equilibrate for 30 min to form electrostatic complexes at room temperature. When the 10% and 50% p(TETA/CBA)-g-PEG2k/p(TETA/CBA)5k polyplexes were formed, a 10% or 50% p(TETA/CBA)-g-PEG2k/p(TETA/CBA) polymer solution was prepared in HEPES buffer at a relevant concentration to obtain a desired w/w ratio with a known amount of pDNA. The respective polymer solutions were then mixed with the known amount of pDNA in HEPES buffer, the solution was lightly vortexed and allowed to equilibrate for 30 min to form electrostatic complexes as described above at room temperature.

3.3.7 Polycation Branching

The relative degree of branching for each polymer sample was determined by the reduction of polymer disulfide bonds using Tris(2-carboxyethyl)phosphine hydrochloride (TCEP) and subsequent free thiol protection using *N*-ethylmaleimide (NEM) prior to sample analysis using MALDI-TOF. MALDI-TOF analysis was performed on the NEM conjugated polymer repeat units using a Voyager-DE STR Biospectrometry Workstation (PerSeptive Biosystems) in positive-ion mode with delayed extraction. Spectra were externally calibrated using a peptide standard mixture spanning a nominal mass range from 325 to 2465 Da. The relative degree of branching (DB) for sample polymers was determined using the following equation:

$$DB = \frac{Bseg}{Bseg + Lseg}$$

where Bseg is the total number of branched repeat unit segments and Lseq is the total

number of linear repeat unit segments.

3.3.8 Acid-Base Titrations

The buffering capacity of each polycation was determined using a previously established method (*11*). In brief, 6 mg polymer was dissolved in 30mL NaCl solution (0.1 M) and initially titrated to pH 10 with 0.1M NaOH. The pH was subsequently lowered with the addition of 0.1 M HCl. Because the absolute molecular weight is not known for these polymers, titration values were determined in μmols of HCl required to lower the pH of the polycation solution from 7.4-5.1. Branched PEI25kDa was used as a reference control.

3.3.9 Light Scattering and ζ-Potential Measurements

The surface charge and polymer/pDNA particle (polyplex) diameters were measured at 25°C using a Zetasizer 2000 instrument (DTS5001 cell) and a dynamic light scattering (DLS) unit on a Malvern 4700 system, respectively. Polyplexes were prepared by adding equal volume polymer solution (200μl) at increasing concentrations in HEPES buffer (20 mM, pH 7.4, 5% glucose) to a desired concentration of 8 μg pDNA in HEPES buffer (200μl). Polyplexes were allowed to equilibrate for 30 min and were subsequently diluted in filtered miliQ water to a final 2 mL volume.

3.3.10 Transmission Electron Microscopy (TEM)

Polyplex was prepared in HEPES buffer (20 mM, pH 7.4, 5% glucose) at 0.05 μg/μl and 5 μl was deposited on TEM copper grid plates to dry. Residual buffer salt was

removed by carefully rinsing each grid with filtered deionized water thrice. The samples were then stained with filtered phosphotungstenic acid (PTA) for 1 min before washing again with filtered deionized water. Images were visualized using a Technai T12 scope (EFM) at 80 kV. Magnification ranging from 20,000 to 200,000x was utilized and the micrograph images were taken at 110,000x. Particle sizes were analyzed using ImageJ software.

3.3.11 Polyplex Stability in 90 % Fresh Rabbit Serum

Polyplex stability and resulting pDNA stability against nuclease activity in serum was evaluated using 500ng free pDNA as a control and 500ng pDNA complexed with polymer mixtures pre-formed in HEPES buffer. Polyplex formation was carried out by combining equal volume solutions of p(DNA) and polymer mixtures using a polymer/pDNA at N/P 50 (24 w/w) and allowed to equilibrate for 30 min. Pre-formed polyplex was then diluted in 90% fresh rabbit serum and incubated at 37°C over time. 25 μl aliquots (125 ng pDNA) were taken at each time point and 10 μl stop buffer (250 mM NaCl, 25 mM EDTA, 2% SDS) was added to each. The samples were frozen at -70°C until further analysis. Once the samples were thawed, they were incubated overnight at 60°C to completely dissociate polycation from the pDNA and 2 μl of 50 mM Dithiothreitol (DTT) was added to each sample and incubated at 37°C for an additional 30 min to ensure complete decomplexation. Lastly, the samples were loaded onto a 2% agarose gel stained with ethidium bromide (EtBr) and subjected to electrophoresis at 96 V for 30 min in TAE (40 mM Tris-acetate, 1 mM EDTA) buffer. The gel image was viewed using GelDoc software (n=2).

3.3.12 Cell Culture

Mouse pancreatic islet endothelial cells (SVR) and colon adenocarcinoma cells (CT-26) (ATCC) were cultured in DMEM containing 10% FBS and 1% penicillin-streptomycin at 37°C in a humidified incubator with an atmosphere containing 5% (v/v) CO_2. Human Umbilical Vein Endothelial Cells (HUVEC) (Invitrogen) were cultured in EBM-2 media with EGM-2 singlequots at 37°C in a humidified incubator with an atmosphere containing 5% (v/v) CO_2.

3.3.13 In Vitro Transgene Expression

Luciferase reporter gene expression in cell culture was performed using each polymer and pCMVLuc plasmid DNA. Cells were plated in 24-well plates containing 0.5mL media. Once the cells were approximately 70% confluent, polyplexes were prepared using 0.5μg pDNA at N/P 50 (24 w/w) in HEPES buffer. Polyplexes were allowed to equilibrate for 30 min. and the cells were transfected in the presence of serum by adding 20 μl polyplex (0.5 μg pDNA) to each well for 4 hrs before replacing with fresh culture media. The cells remained in the incubator for a total of 48 hrs before they were washed with 1ml PBS and treated with cell culture lysis buffer (Promega). Luciferase quantification was performed using a Luciferase assay system (Promega) on a luminometer from Dynex Technologies, Inc. (Chantilly, VA). The amount of protein in the cell lysate was determined using a standard curve of bovine serum albumin (Sigma) and a BCA protein assay kit (Pierce) (n=6).

3.3.14 Cell Viability Assay

Cells were plated in 24-well plates and gene transfections were carried out when the cells were approximately 70% confluent. Polyplexes were prepared as they were for the luciferase reporter gene assay. Respective cell cultures were transfected in the presence of serum with the addition of 20 μl equilibrated polyplex in HEPES buffer solution (0.5 μg pDNA) to each well. Cells were left to incubate for a total of 18 hrs before cell viability using an MTT assay was evaluated (Sigma). Percent cell viability was determined relative to untreated control cells (n=6).

3.3.15 Statistics

The experimental data in this report are assessed as mean ± SD. A one-way ANOVA in conjunction with a Tukey's post-hoc test is used to determine if treatment groups are statistically different from each other. A $p < 0.05$ was considered statistically significant.

3.4 Results

3.4.1 Synthesis and Characterization.

The premise of the studies herein was to develop a functional PEGylated copolymer of a previously developed and promising polycation, p(TETA/CBA). PEGylation of polycationic systems for gene delivery has been shown to improve polycationic carrier function in the presence of serum both *in vitro* and *in vivo* via reducing polyplex surface charge and providing a steric barrier against protein adsorption (*18*). Particles with a surface charge close to neutral tend to aggregate in solution due to their reduced ionic repulsive forces influenced by the isoelectric point, molecular weight

and pH of the solution (*19*). The generation of a PEGylated polycation sufficient to prevent protein adsorption and avoid aggregation often requires the synthesis and evaluation of many putative reagents with various degrees of modification in order to identify optimal candidates, which is often laborious, time consuming and costly. In an attempt to avoid this problem, we sought to synthesize a p(TETA/CBA)5k-PEGylated product with a known degree of modification that could then be mixed with the polycationic counterpart p(TETA/CBA)5k and easily control the amount of PEG included in the gene delivery reagent formulations. It is then easy to control and examine the effects of various PEG content on reagent complexation and carrier functionality to further identify optimal delivery reagents. The synthesis and hypothetical scheme of the p(TETA/CBA) and the p(TETA/CBA)5k-PEGylated products and/or formulation mixtures tested in our studies are shown in Figures 3.1 and 3.3, respectively.

Polymerization of p(TETA/CBA) occurs via Michael-type addition of the amine-containing monomer, TETA, to the N,N'-Cystamine-bis-acrylamide monomer and is shown in Figure 3.1. Four reactive amine groups exist within the unprotected TETA monomer and as a result branched products are produced during polymerization of p(TETA/CBA). In the present study, polymerization reactions were carried out at different temperatures in 100% MeOH and monitored by ^{1}H NMR (Figure 3.2). Synthesis temperature was shown to correlate with the relative degree of branching in each sample (Table 3.1). Purification of polymer products using a relatively high molecular weight cut-off membrane at 5 kDa (5 kDa MWCO) compared to a relatively low molecular weight cut-off membrane at 1 kDa (1 kDa MWCO) proved to eliminate oligomer polycations from the polymer sample as indicated by a 1.6 kDa number average

molecular weight (M_n) increase over the sample purified at 1 kDa MWCO (Table 3.2). This finding is useful for subsequent PEG modification of p(TETA/CBA). Commercial bPEI 25kDa was also analyzed as an external control for comparison using this method. The absolute M_n and weight average molecular weight (M_w) values for bPEI 25kDa determined using AKTA/FPLC and light scattering were lower than the value reported by the commercial provider and thus suggest extrapolations should be made to estimate p(TETA/CBA) molecular weight for subsequent modification.

To obtain the PEG modified p(TETA/CBA)5k, PEG chemical modification of an amine-reactive PEG-phenylcarbonate with p(TETA/CBA)5k was monitored by following the release of *p*-nitrophenolate in anhydrous solvent at 400 nm with a UV-Vis spectrophotometer. The reaction was complete by 16 hrs and the product composition was confirmed using ^{1}H NMR (Figure 3.4). The only side reaction that may occur during PEG conjugation to p(TETA/CBA)5k is the hydrolysis of the amine reactive carbonate present on the activated PEG chain. Analysis and comparison of ^{1}H NMR peak AUC indicated the presence of 0.96 mol PEG to 1 mol p(TETA/CBA)5k (80% conversion) (Table 3.2). These values were determined using the absolute molecular weight values obtained from AKTA/FPLC and light scattering analysis (Table 3.2). The reduced PDI of p(TETA/CBA)-g-PEG2k compared to p(TETA/CBA)5k indicates further removal of un-reacted p(TETA/CBA)5k oligomer polycations during the second purification step used to obtain a pure p(TETA/CBA)-g-PEG2k sample. Nonetheless, analysis of sample buffer capacity indicates that by collecting a higher molecular weight fraction of the produced product (p(TETA/CBA)5k) has similar buffering capacity to the product produced following the purification method initially described which maintains oligomer

polycations of the product produced (p(TETA/CBA)1k). Moreover, PEG modification of p(TETA/CBA)5k does not significantly affects product buffer capacity either, as titration values are not significantly different from each other (Table 3.2). The number of disulfide bonds per molecule of the bioreducible polymer products is also reported for additional characterization of polymer samples (Table 3.2).

3.4.3 Physiochemical Characterization of Polymers

The ability of p(TETA/CBA)5k and p(TETA/CBA)5k-g-PEG2k to form condensed polyplex was investigated by Dynamic Light Scattering (DLS) particle size analysis and polplex surface charge ζ-potential measurements were also taken. Indeed, nanosized particles below or near 100 nm in diameter were formed for both potential gene carriers. As expected, PEG conjugation interfered with polyplex formation at low polymer/pDNA weight-to-weight ratios (w/w) (Figure 3.7). PEG conjugation significantly decreased polyplex surface charge, however, as mentioned above, an increased w/w is required to condense p(DNA) into sub-200nm complexes compared to p(TETA/CBA)5k (Figure 3.7).

Our findings coincide with previous studies that used PEGylated poly(ethylene imine) gene carriers (*17*) and show that PEGylation of p(TETA/CBA) perturbs nucleic acid condensation and polyplex size. To overcome this problem and validate the possibility of premixing polymer/PEG-copolymer solutions in order to control the relative PEG/polycation amounts, as well as identify an ideal formulation that maintains homogenous and stable polyplex with reduced surface charge, polyplexes were prepared using p(TETA/CBA)5k-g-PEG2k, p(TETA/CBA)5k and mixtures of the two molecular

entities at 10/90% and 50/50%, respectively, at a summed polycation/pDNA N/P = 50 (24 w/w). As mentioned previously, mixtures are schematically represented in Figure 3.3 and included to clarity the mixture formulations tested.

In order to test the influence of increasing PEG content on polyplex stability and pDNA protection against serum nuclease activity, polyplexes were formed and following a 30 min equilibration time, were added to fresh rabbit serum to a final serum concentration equal to 90 % at 37°C. Aliquots were taken over time and electrophoresed on an agarose gel to visualize intact pCMVLuc at each time point. Polymer/pDNA formulations were compared to un-complexed pDNA (control) at 0 hours. Figure 3.8 shows that pDNA complexed with polymer formulations is significantly more stable in the presence of serum than uncomplexed, free pDNA over 6 hrs. As the amount of p(TETA/CBA)5k-g-PEG2k in each formulation is increased, lower protective effects are seen, indicating that PEG adversely affects complex stability and pDNA protection. Nonetheless, p(TETA/CBA)5k and 10% p(TETA/CBA)-g-PEG2k sufficiently protect 80% of the pDNA from nuclease degradation over the 6 hrs incubation in 90% serum. Increasing p(TETA/CBA)5k-g-PEG2k to 50 or 100% reduces particle stability and offers less pDNA protection where approximately 60 % and 40 % pDNA is preserved, respectively, at 6 hrs incubation time.

For formulation ease and improved carrier function, stable polyplex was formed using different amounts of PEG. Formulations should display unimodal polyplex size distribution and surface charge with uniform morphology. Polyplex size for each formulation was visualized using TEM (Figure 3.9) and their size and distribution were compared to measurements provided by dynamic light scattering (DLS). The arrows

contained in Figure 3.9 (a-d) show representative polyplexes and the particle size was analyzed using ImageJ software. The values between TEM and DLS are in close agreement with each other. The TEM particle sizes are smaller than those determined using DLS because the polyplexes examined using TEM are in a dehydrated state, whereas polyplex analyzed by DLS are in aqueous buffer and are thus hydrated lending to a larger particle size. Figures 3.9 (a-d) reveal that morphological changes and less compact polyplex occur as the amount of PEG in the formulation increases. p(TETA/CBA)5k-g-PEG2k exhibited aggregation (Figure 3.9 (d)). This aggregation was also seen when analyzed using DLS. The aggregation of these complexes is explained by their reduced surface charge, lending to reduced charge-charge repulsion of polyplex and potential PEG chain entanglement between polyplex and free polymer in solution. Therefore this formulation is excluded from the analysis and not shown. p(TETA/CBA)5k, 10% and 50% PEG formulations generate sub-150nm polyplexes in solution and the amount of PEG inversely correlates with polyplex surface charge, indication the charge-shielding capacity of PEG chains (Figure 3.9 (e)).

3.4.4 Gene Carrier Function and In Vitro Biological Activity of PEG Formulations.

Often times polycationic species interact with and disrupt cell membrane function, and/or they interact with intracellular proteins and nucleic acids, which in turn perturbs intracellular and nuclear processes that leads to cellular toxicity (*20, 21*). Bioreducible polycations such as p(TETA/CBA) in principle mitigate these intracellular interactions as it degrades to small molecular weight species intracellularly and thus limit

intracellular polycation accumulation and subsequent cellular toxicity when transgene delivery occurs using these reagents (*22*). As such, the decreased toxicity of degradable polycations compared to nondegradable counterparts should be maintained irrespective of polycation molecular weight (*22*). In addition to toxicity, serum proteins often reduce polycationic gene carrier performance by destabilizing polyplex, which leads to reticuloendothelial system (RES) uptake, *in vivo,* or exposes the therapeutic gene to serum nucleases where it is degraded and rendered inactive, thus providing another barrier for their successful therapeutic application.

The aforementioned AKTA/FPLC results shown in Table 3.2 indicate that p(TETA/CBA)5k has a slightly higher molecular weight and lower PDI than p(TETA/CBA)1k. In lieu of this result and realizing that polycation molecular weight often influence carrier biological activity, reducing the PDI of p(TETA/CBA) by eliminating destabilizing oligomers and increasing the average molecular weight without perturbing carrier performance is preferred (*17*). Because p(TETA/CBA)5k was used for subsequent PEG modification, it is imperative to ascertain that improved function of bioreducible polycations is not lost with an increase in absolute molecular weight. It is also important to further evaluate the influence of PEG modification on the system's in vitro biological activity. Distinct physiochemical differences of these systems were observed between each other, which may influence their biological activity and putative application as cellular transgene delivery reagents. When tested using several cell lines, the transfection efficiency of p(TETA/CBA)1k and pTETA/CBA)5k populations were significantly better than the 'gold standard' nondegradeable bPEI 25 kDa in the presence of 10% serum. Moreover, p(TETA/CBA)1k and p(TETA/CBA)5k were nontoxic

compared to bPEI 25 kDa (Figure 3.8). There is no significant difference between the biological activity of p(TETA/CBA)1k and p(TETA/CBA)5k, which indicates that increasing the molecular weight of this polycation and eliminating oligomer polycations has no adverse effect on carrier bioactivity and p(TETA/CBA)5k can be used for modification (Figure 3.8). Specifically, p(TETA/CBA)5k is significantly less toxic in primary HUVEC endothelial cells than bPEI 25kDA, as well as providing greater luciferase transgene expression in HUVEC and SVR endothelial cells or the colon adenocarcinoma cell line, CT-26 (Figure 3.8). The toxicity of bPEI 25kDa is likely due to the intracellular accumulation of high molecular weight polycationic species (*3*). The enhanced transgene expression of p(TETA/CBA)5k compared with bPEI 25kDa is also likely explained by its bioreducible nature that lends to its ability to more easily release pDNA intracellularly compared to the nondegradable counterpart bPEI 25kDa (*6, 9*). Three cell lines were used because if trends are seen in multiple cell lines, greater validity is given to the trend, and secondly, endothelial cells (SVR and HUVEC) as well as the adenocarcinoma cells (CT-26) are relevant to in vivo cancer therapy, which is of interest to out lab and is the next step following in vitro characterization. Therefore, we tested the biological activity of our gene carriers using endothelial cells that would be exposed during systemic administration to examine potential activity as well as the adenocarcinoma cell line (CT-26) that is used in our lab for a mouse tumor model when testing therapeutic gene activity in vivo.

To evaluate carrier function of a panel of PEG-modified p(TETA/CBA) gene delivery reagents in an attempt to identify optimal candidates, the biological activity of p(TETA/CBA) and the PEG formulation in vitro was evaluated using colon

adenocarcinoma cells (CT-26) in 10% serum-containing media. Results from this experiment indicate that p(TETA/CBA)5k exhibits significantly better cellular trangene delivery than bPEI 25kDa in the presence of serum-conditioned media and that transfection using p(TETA/CBA)5k is less affected by the presence of serum than for bPEI 25kDa (Figure 3.9). These were collected while evaluating the biological activity of the PEG formulations; however, comparison of p(TETA/CBA)5k with bPEI 25kDa in the presence and absence of serum is shown separately to clearly exemplify the effect of serum on polycation transfection as well as demonstrate the differences of serum effect on transfection between p(TETA/CBA)5k and bPEI 25kDa. Transfection using p(TETA/CBA)5k is less affected by the presence of serum-conditioned media than for bPEI 25kDa, and it indicates potentially less serum adsorption to polyplexes prepared using p(TETA/CBA)5k than those prepared using bPEI 25kDa, as protein adsorption to polyplex often leads to polyplex aggregation and reduced delivery efficacy (*23*). Lower protein adsorption to p(TETA/CBA)5k polyplex compared to bPEI 25kDA polyplex is due to the lower charge density of p(TETA/CBA)5k compared to bPEI 25kDa (*11*). Evaluation of serum effects on gene transfection was performed solely on CT-26 cells because the data presented in Figure 3.8 showed similar trends as in the other two cell lines.

PEGylated p(TETA/CBA)5k formulation biological activity was tested in vitro along with p(TETA/CBA)5k and bPEI 25 kDa in the aforementioned study and results are shown entirely in Figure 3.10. Specifically, two PEGylated formulations of p(TETA/CBA)5k compared with p(TETA/CBA)5k alone and the ‘gold standard’ bPEI 25kDa provide significantly better transgene delivery than p(TETA/CBA)5k and bPEI 25

kDa. The p(TETA/CBA)5k-g-PEG2k alone provided very low transfection to these cells and is explained by their complex aggregation when combined with pDNA. None of the formulations proved toxic to these cells (Figure 3.10b). Moreover, the 10% and 50% PEG formulations are not significantly different from each other when used in the presence and absence of serum-containing media. Combined, these results demonstrate the advantages of p(TETA/CBA)5k over bPEI 25kDa and verify the advantage of PEGylating the p(TETA/CBA) system to generate a polycation with improved in vitro gene delivery capacity utilized in the presence of serum. Most importantly, these results indicate that a panel of PEGylated polycation formulations can be easily derived by premixing various amounts of a PEGylated polycation and polycation to assist the identification of an optimal PEG-modified gene delivery reagent.

3.5 Discussion

Clinical advancements of polycationic gene carriers is hampered by uncertain design and formulation requirements. In the present work, we show that a graft copolymer of poly(ethylene glycol) (PEG) and a branched SS-PAEI can be synthesized and used in formulation mixtures to alter the relative amount of PEG, thereby altering the physiochemical characteristics of the gene carrier in order to easily study the design and formulation requirements to improve gene carrier function. If PEG conjugation to a polycation such as p(TETA/CBA) interferes with polyplex formation and carrier function, as seen here, this work demonstrates the feasibility to identify and potentially rectify this issues by preparing homogenous polyplex formulations using mixtures of p(TETA/CBA) and p(TETA/CBA)5k-g-PEG2k to alter the relative amount of PEG in the

formulations and identify reagents that are functionally viable. More importantly, this approach may be applied to other polycation-PEG preparations to easily alter polyplex characteristics to optimize polyplex stability and biological activity *in vitro*. Further studies need to be performed to understand the feasibility of using this approach for *in vivo* screening and evaluation of gene carriers.

When synthesizing p(TETA/CBA)5k-g-PEG2k, reducing the PDI of p(TETA/CBA) by removing polycationic oligomers with a limited ability to properly condense pDNA is preferred given that PEG conjugation may interfere with polyplex formation as it is. Therefore, in order to reduce the PDI of p(TETA/CBA) following the Michael-addition of TETA with the bisacrylamide group, ultrafiltration was performed using a higher molecular weight cut-off membrane (5 kDa) than was used previously (*11*). As expected, this approach was effective in reducing the polycation's PDI and correlates with a relative increase in molecular weight. Prior research has shown that increasing the molecular weight and branching profile of poly(ethylene imine)s correlates with increased transgene expression, but also cellular toxicity (*24*). Therefore, the present study investigated this putative effect with regards to p(TETA/CBA) and found no significant influence on its biological activity in primary and immortalized cell lines (*6, 7*). These results are explained by the gene carrier's ability to exploit the intracellular redox potential and avoid disruption of intracellular function through relatively high molecular weight polycationic species (*25*).

While p(TETA/CBA) demonstrated significantly better transgene expression than bPEI 25kDa in serum-containing media due to a lower charge density by comparison, p(TETA/CBA)5k delivery capacity was noticeably lower when compared to its activity

in the absence of serum. Therefore, to reduce p(TETA/CBA)/pDNA polyplex interactions with serum proteins and thus improve carrier function in the presence of serum, poly(ethylene glycol) was conjugated to p(TETA/CBA)5k at 1.2/1 PEG-carbonate/p(TETA/CBA)5k ratio and confirmed by ^{1}H NMR following purification. The corresponding relative molecular weight was in agreement with what is expected for the degree of PEG conjugation (0.96/1 moles PEG/p(TETA/CBA)) when analyzed using AKTA/FPLC and light scattering. Conjugating PEG2k to p(TETA/CBA)5k reduced polyplex surface charge, however, adversely affected nucleic acid condensation. This result coincides with prior findings by our lab and others (*16, 26*). Because poly(ethylene glycol) and/or ligand conjugation for cell-specific gene delivery commonly mitigates nucleic acid condensation, synthesis of multiple co-polymeric gene carriers is required to ascertain optimal ratios for maximal carrier performance.

In an attempt to overcome this problem and avoid the need to synthesize multiple carriers for screening, this study investigated the feasibility of altering and optimizing PEG/polycation amounts by formulating mixtures of a polycation and its corresponding PEGylated counterpart. Polyplex stability in serum was evaluated in this study for p(TETA/CBA)5k-g-PEG2k alone, p(TETA/CBA)5k alone, and 10/90 or 50/50 mixtures of p(TETA/CBA)5k-g-PEG2k/p(TETA/CBA)5k, respectively. Polyplex formed using p(TETA/CBA) and 10/90%, sufficiently protects up to 70% of the pDNA from serum nuclease degradation over 6 hrs. Increasing the p(TETA/CBA)5k-g-PEG2k amount to 50 and 100% reduced the relative pDNA protection in serum, which correlates with the capability of each formulation to condense pDNA into nano-sized polyplex determined using DLS and TEM. One potential concern is that if polyplex is formed at a unified 50

N/P (24 w/w) the N/P ratios could be significantly different between p(TETA/CBA) alone and the mixtures. However, NMR results demonstrate that there is approximately 1 PEG chain per TETA/CBA molecule. If a 50 N/P (24 w/w) is used for formulations, there are approximately 1.2% of the amines PEGylated in the 100% p(TETA/CBA)5k-g-PEG2k formulation, ~0.6% for the 50 % formulation, ~0.12% for the 10% formulation and 0% for p(TETA/CBA)5k. This being the case, the difference in N/P ratios between the formulations is insignificant. Moreover, the 10% formulation that demonstrated significantly better transgene expression than p(TETA/CBA)5k in the presence of serum has only 0.12% less amines for use in formulating polyplex.

Luciferase transgene expression and cell viability was investigated in cell culture using the aforementioned formulations to evaluate their bioactivity. As expected, PEG was able to improve gene delivery in serum-containing media compared to p(TETA/CBA) alone. However, this improvement was observed only at specific PEG-formulations: 10 and 50% p(TETA/CBA)-g-PEG2k. These two formulations exhibit a reduced surface charge of approximately 20 mV and 15 mV compared to p(TETA/CBA) at 35 mV, respectively, and their surface charge inversely correlates with PEG concentration. By maintaining a reduced surface charge, protein association with the complexes via electrostatic interaction is mitigated. Moreover, PEG provides a steric barrier for protein adsorption. Taken together, complex aggregation is avoided and more efficient cellular uptake of the complexes is promoted. Despite the reduced surface charge of 100% p(TETA/CBA)5k-g-PEG2k compared to p(TETA/CBA), which can promote transfection in the presence of serum, efficient cellular uptake and transfection is lost due to a high degree of complex aggregation (Figure 3.7). Nonetheless, these results

provide evidence that PEG/polycation ratios can be easily controlled in order to evaluate formulation activity and optimize PEG ratios for improved gene carrier function, which depends on reagent physiochemical properties. In doing so, one can avoid synthesis of multiple bio-reducible co-polymers with different physiochemical characteristics currently employed for gene carrier optimization.

3.6 Conclusion

In the present study, we developed a novel gene carrier comprised of an efficient and non-toxic bioreducible polycation in conjunction with its PEGylated counterpart to improve carrier performance in the presence of serum. In addition we provide a feasible, multifaceted approach to tailor the relative amount of PEG in a polycationic gene carrier reagent by generating controlled mixtures of the polycation and PEGylated polycation for gene delivery in order to elucidate changes in reagent physiochemical properties ideal for gene carrier function. By doing so, the laborious and costly synthesis of multiple candidate copolymers for gene delivery is avoided when designing a gene carrier with preferred physiochemical properties. The methodology described herein may also be employed for other novel gene delivery reagents and can be further used for *in vivo* evaluation of a panel of putative PEGylated polycation delivery reagents.

Table 3.1. Effect of synthesis temperature on polymer branching.

Sample	DB[a]	0 arm	1 arm	2 arm	3 arm
p(TETA/CBA)-4°C	55%	45%	36%	18%	0%
p(TETA/CBA)-30°C	82%	10%	60%	22%	8%
p(TETA/CBA)-50°C	90%	10%	39%	42%	11%

[a]Polymer degree of branching (DB) correlates with polymer synthesis temperature and influences the number of polymer arms per repeat unit. The relative DB was determined using the equation described in section 3.2.7. Polymer branching characteristics were determined by the reduction of polymer disulfide bonds with tris(2-carboxyethyl)phosphine (TCEP) followed by protection of free sulfhydryls with N-ethylemaleimide (NEM) and analyzed by MALDI-TOF

Table 3.2. Polymer and co-polymer characteristics

Sample	M_n (kDa)[a]	M_w (kDa)[a]	PDI (M_w/M_n)	Titration[b] (μmol HCl)	S-S Bonds/ Molecule[c]	Degree[d] Branching	Mol Ratio of PEG[e]
p(TETA/CBA)1k	4.2	8.2	1.95	25.2 ± 3.4	23.2 ± 2.4	0.68	--
p(TETA/CBA)5k	5.8	8.85	1.53	27.6 ± 2.8	27.1 ± 1.8	0.91	--
p(TETA/CBA)5k-g-PEG2k	8.9	10.6	1.19	22.3 ± 3.4	29.1 ± 2.6	--	0.96
bPEI 25 kDa	16.4	21.0	1.28	32 ± 2.9	--	--	--

[a] Number average molecular weight (M_n), weight average molecular weight (M_w), and polydispersity (M_w/M_n) determined using AKTA/FPLC and light scattering using relevant polymer refractive index increments (dn/dc). [b] Polymer fraction buffer capacity titrations determined by the mol of HCl required to shift pH from 7.4 to 5.1 in 0.1M aqueous NaCl. [c] The number of disulfide bonds per molecule was determined by reduction of disulfide bonds with immobilized tris(2-carboxyethyl)phosphine (TCEP) followed by the reaction of free sulfhydryls with 5,5-Dithio-bis-(2-nitrobenzoic acid) and analyzed using UV-Vis spectrometry. [d] Degree branching was determined by reduction of disulfide bonds with tris(2-carboxyethyl)phosphine (TCEP) followed by protection of free sulfhydryls with N-ethylemaleimide (NEM) and analyzed by MALDI-TOF. [e] The molar ratio of PEG to p(TETA/CBA) was determined using ^{1}H NMR and integrating peak Area Under the Curve (AUC).

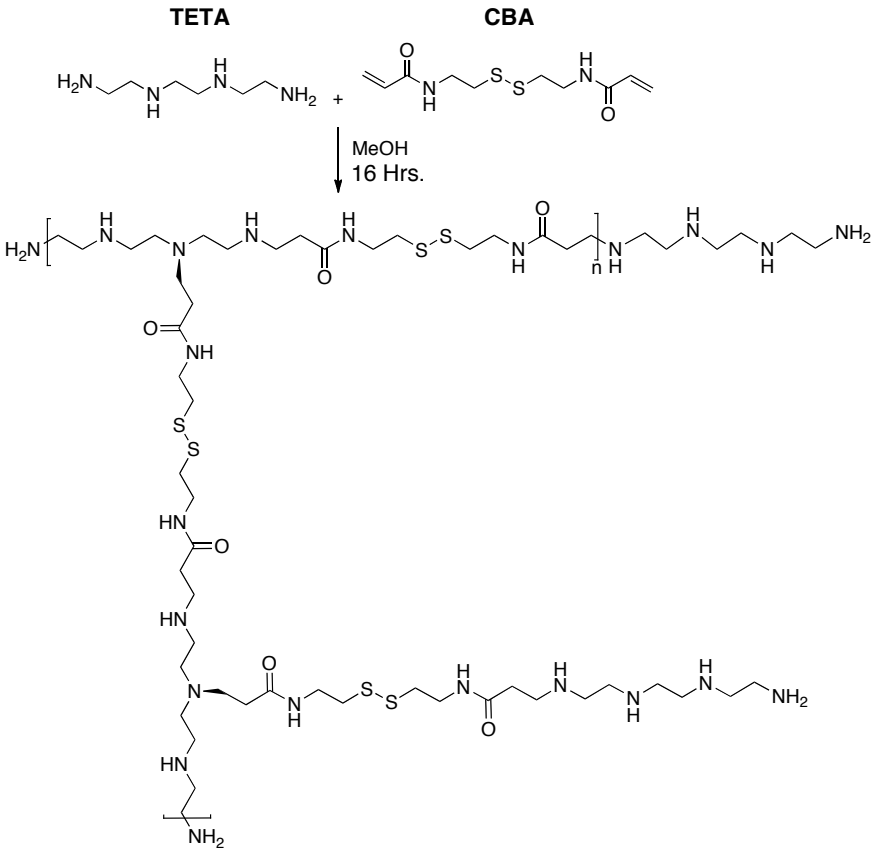


Figure 3.1. Synthetic scheme for poly(triethylenetetramine/cystamine-bis-acrylamide) (p(TETA/CBA)).

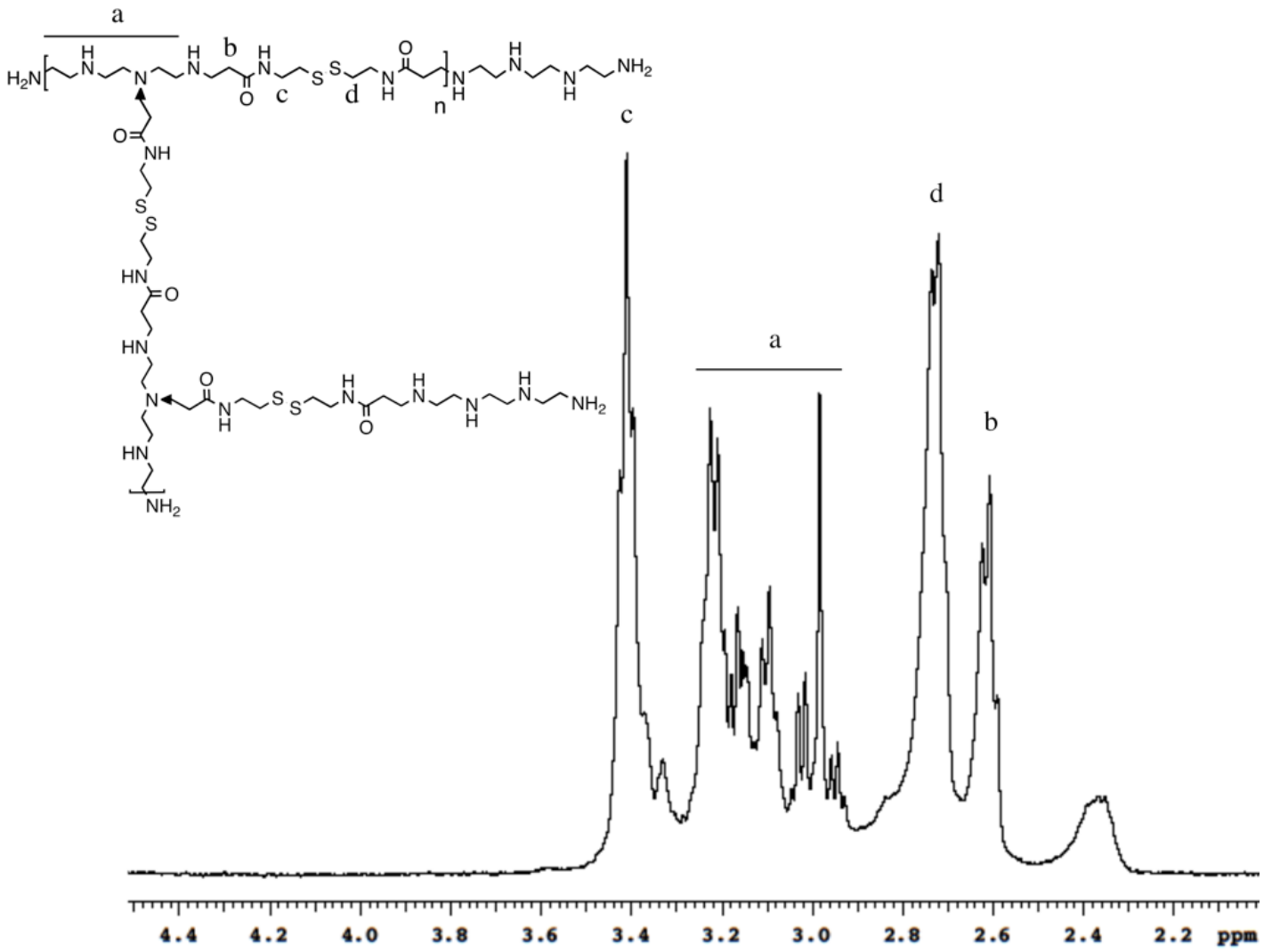
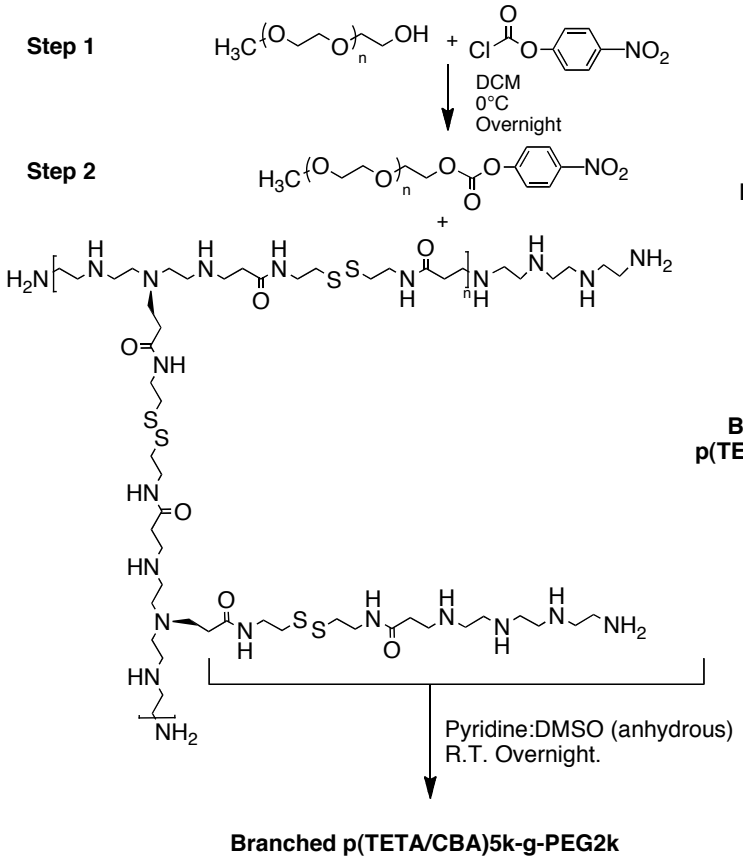


Figure 3.2. ^{1}H NMR of poly(triethylenetetramine/cystamine-bis-acrylamide) (p(TETA/CBA)).



Formulations:

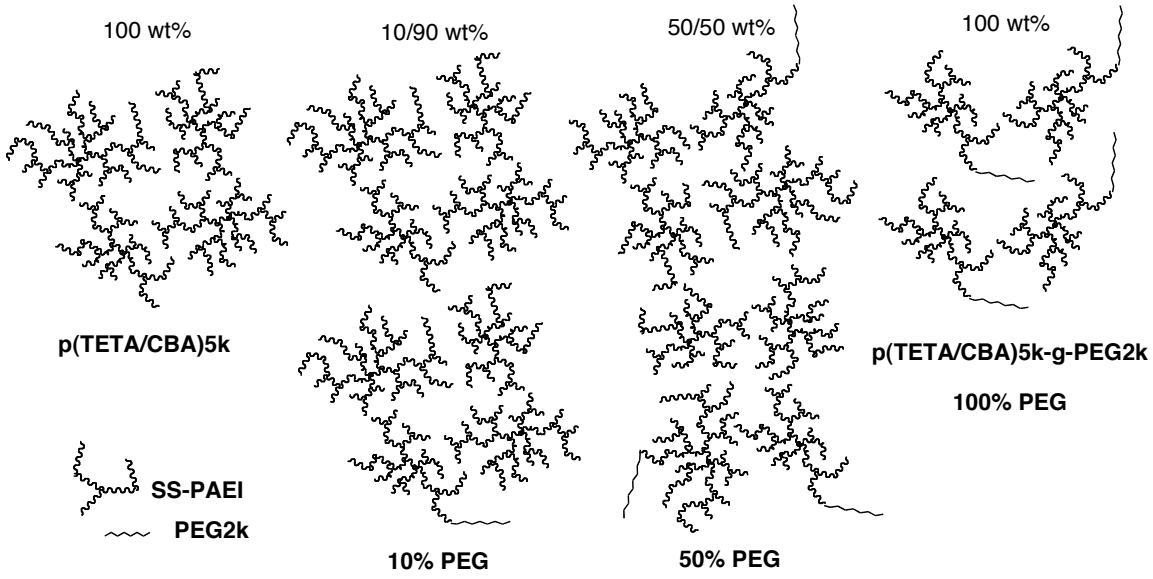


Figure 3.3. Synthesis of p(TETA/CBA)5k-g-PEG2k and corresponding formulation schematics.

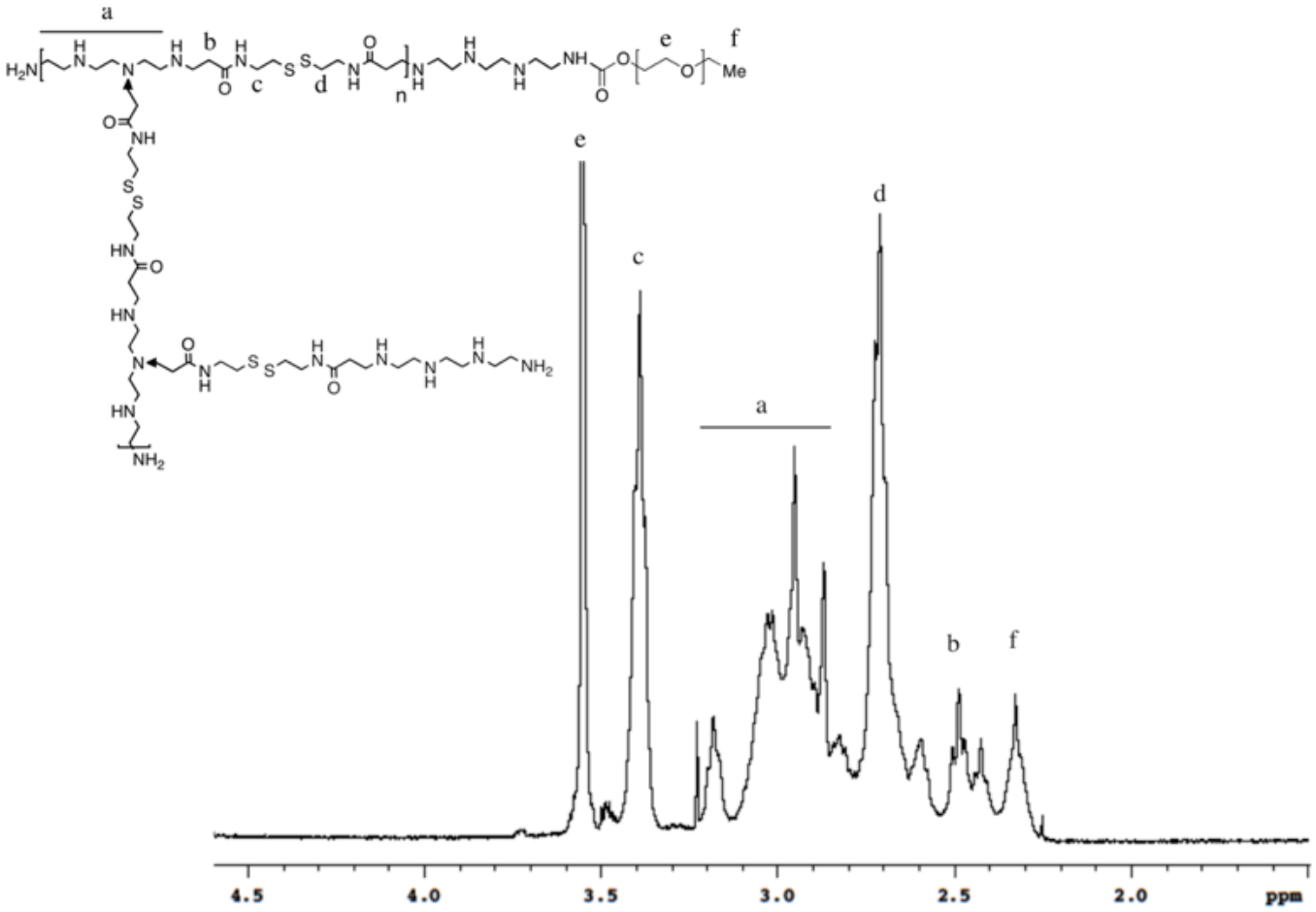


Figure 3.4. ^{1}H NMR of poly(triethylenetetramine/cystamine-bis-acrylamide)5k-g-poly(ethylene glycol)2k (p(TETA/CBA)5k-g-PEG.

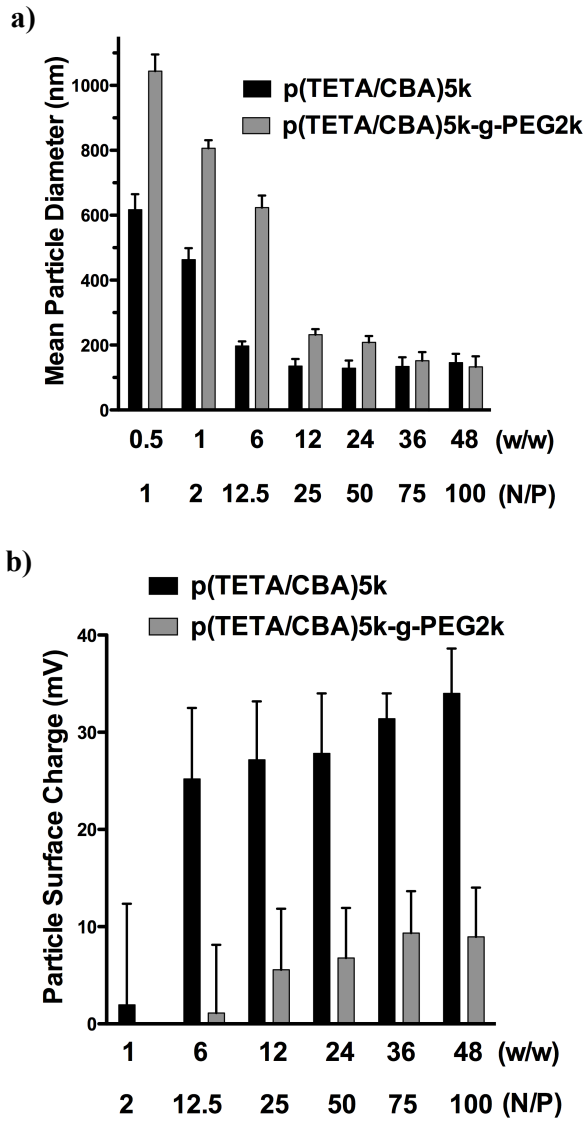


Figure 3.5. Physiochemical characteristics of p(TETA/CBA)5k and p(TETA/CBA)5k-g-PEG2k/pCMVLuc polyplexes. a) Particle size and b) ζ-potential measurements of each respective gene carrier at increasing polymer concentrations using a known amount of pDNA. PEG2k conjugation to p(TETA/CBA) reduces polyplex surface charge, but interferes with pDNA condensation.

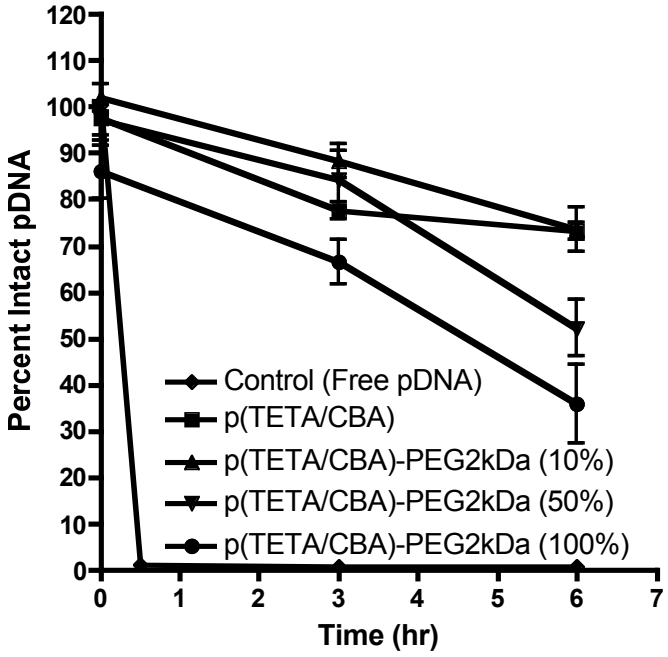


Figure 3.6. Polyplex stability in 90% rabbit serum at 37°C for p(TETA/CBA)5k, poly(TETA/CBA)5k-g-PEG2k, 10/90 (10% PEG) and 50/50 (50% PEG) formulations for p(TETA/CBA)5k-g-PEG2k and p(TETA/CBA)5k, respectively. 500ng pCMVLuc was complexed with each formulation N/P 50 (w/w 24). The relative percent of intact pDNA over time was determined by band pixel intensity using GelDoc software. Increasing the PEG content in the polyplex formulations inversely correlates with pDNA protection (n=2).

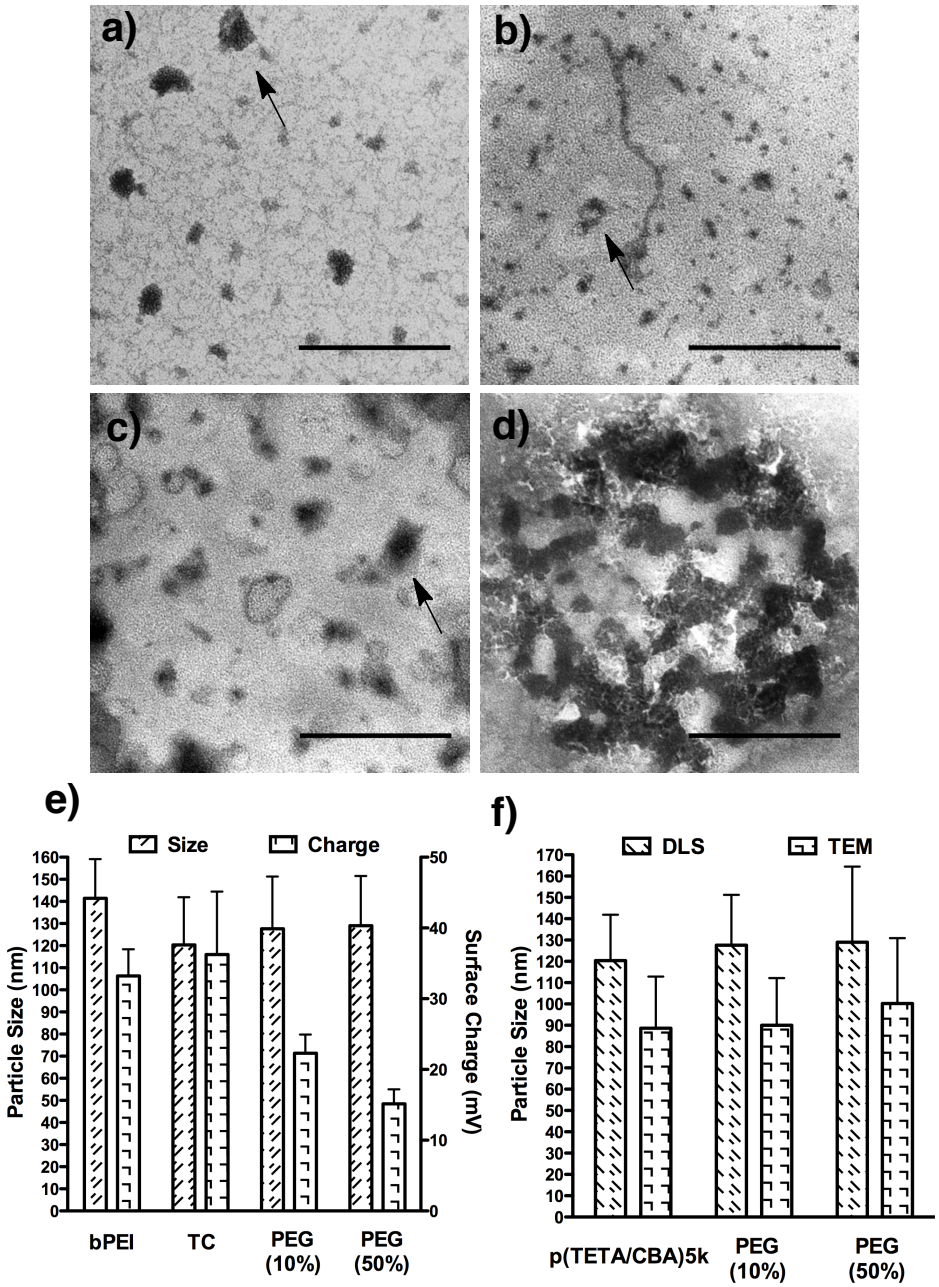
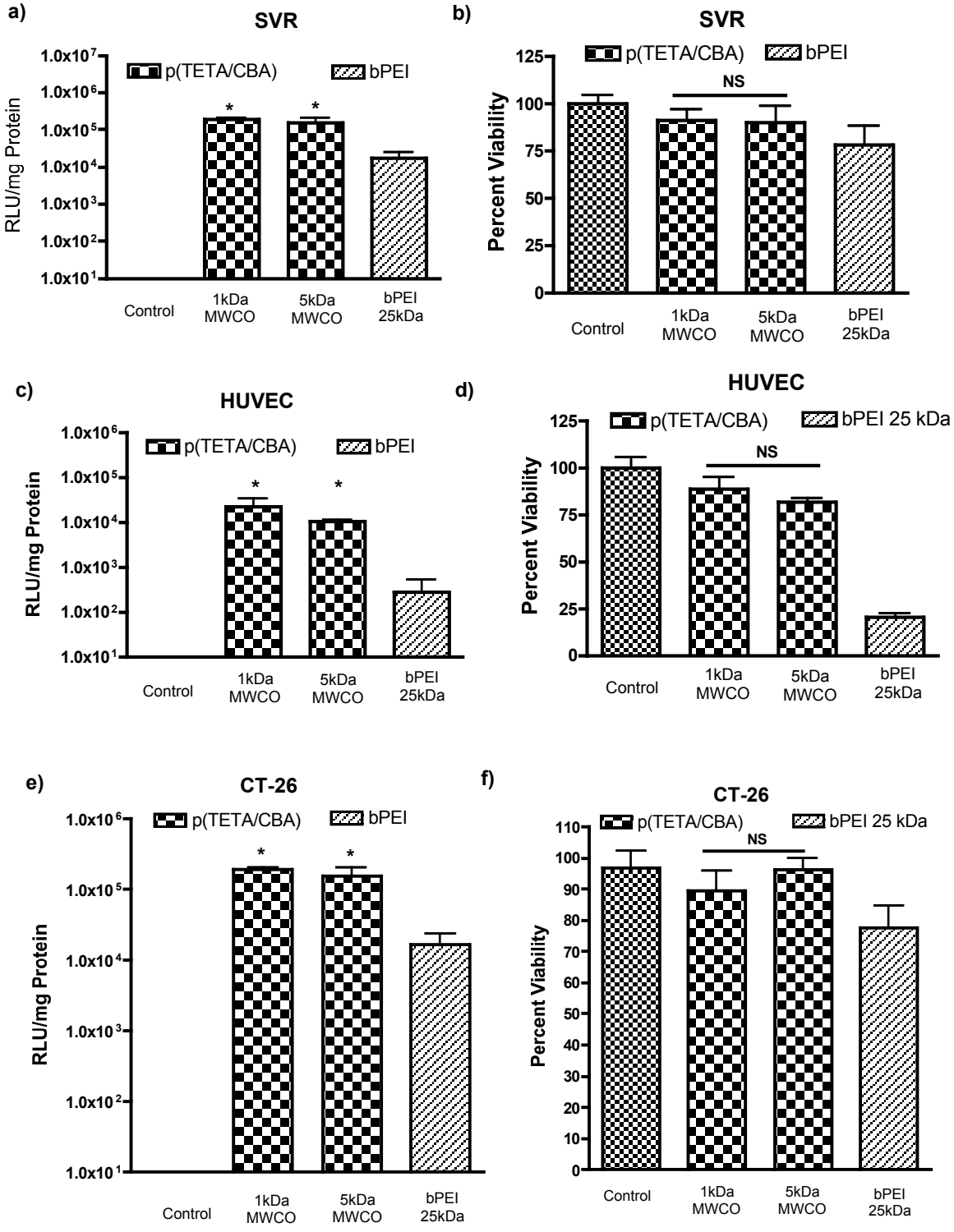


Figure 3.7. Polyplex formulations visualized with TEM (a-d) (scale bar equals 200nm) and studied using DLS (e) at 24 w/w. a) p(TETA/CBA)5k b) 10% PEG c) 50% PEG d) p(TETA/CBA)5k-g-PEG2k. e) Particle size and ζ-potential of polyplex formulations; where p(TETA/CBA)5k is abbreviated as TC. f) Comparison of polyplex size using TEM and DLS.

Figure 3.8. Transfection efficiency (a,c and e) and cell viability (b, d and f) in 10% serum on SVR, HUVEC and CT-26 cells for different p(TETA/CBA) molecular weight analogs compared to the positive control bPEI 25kDa. pCMVLuc was used as the reporter gene. Commercial bPEI 25 kDA and p(TETA/CBA) polyplexes were prepared at N/P 10 (1.3 w/w) and N/P 50 (24 w/w), respectively. Data are represented as mean ± SD, *$p < 0.05$ (n=6).


a)
SVR
RLU/mg Protein
1.0x10^7
1.0x10^6
1.0x10^5
1.0x10^4
1.0x10^3
1.0x10^2
1.0x10^1
p(TETA/CBA)
bPEI
*
*
Control
1kDa MWCO
5kDa MWCO
bPEI 25kDa
b)
SVR
Percent Viability
125
100
75
50
25
0
p(TETA/CBA)
bPEI
NS
Control
1kDa MWCO
5kDa MWCO
bPEI 25kDa
c)
HUVEC
RLU/mg Protein
1.0x10^6
1.0x10^5
1.0x10^4
1.0x10^3
1.0x10^2
1.0x10^1
p(TETA/CBA)
bPEI
*
*
Control
1kDa MWCO
5kDa MWCO
bPEI 25kDa
d)
HUVEC
Percent Viability
125
100
75
50
25
0
p(TETA/CBA)
bPEI 25 kDa
NS
Control
1kDa MWCO
5kDa MWCO
bPEI 25kDa
e)
CT-26
RLU/mg Protein
1.0x10^6
1.0x10^5
1.0x10^4
1.0x10^3
1.0x10^2
1.0x10^1
p(TETA/CBA)
bPEI
*
*
Control
1kDa MWCO
5kDa MWCO
bPEI 25kDa
f)
CT-26
Percent Viability
110
100
90
80
70
60
50
40
30
20
10
0
p(TETA/CBA)
bPEI 25 kDa
NS
Control
1kDa MWCO
5kDa MWCO
bPEI 25kDa

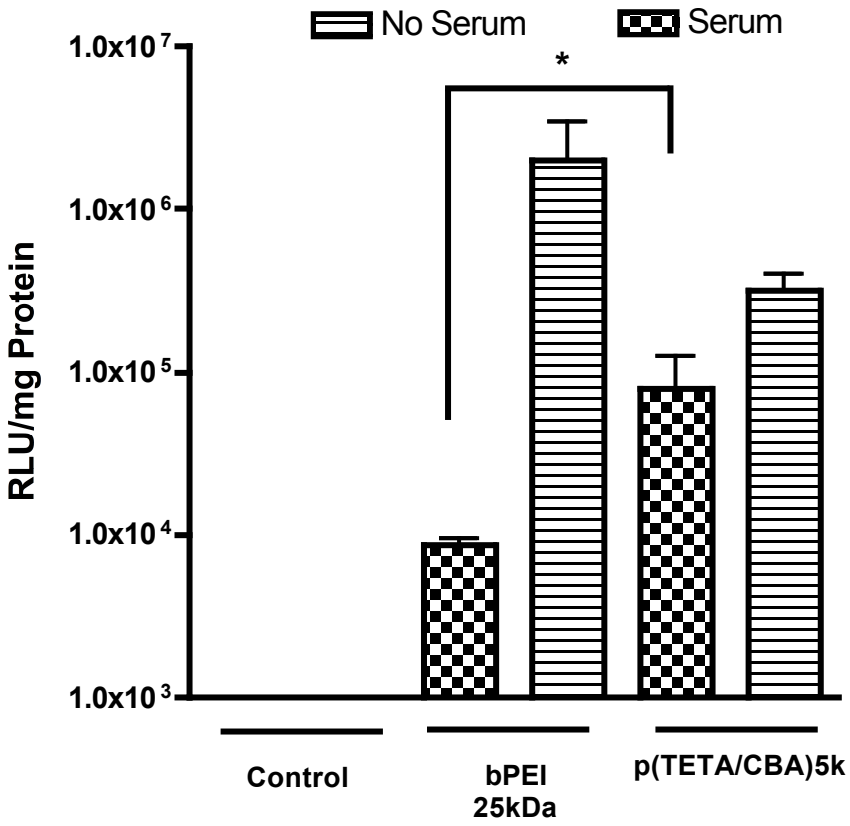
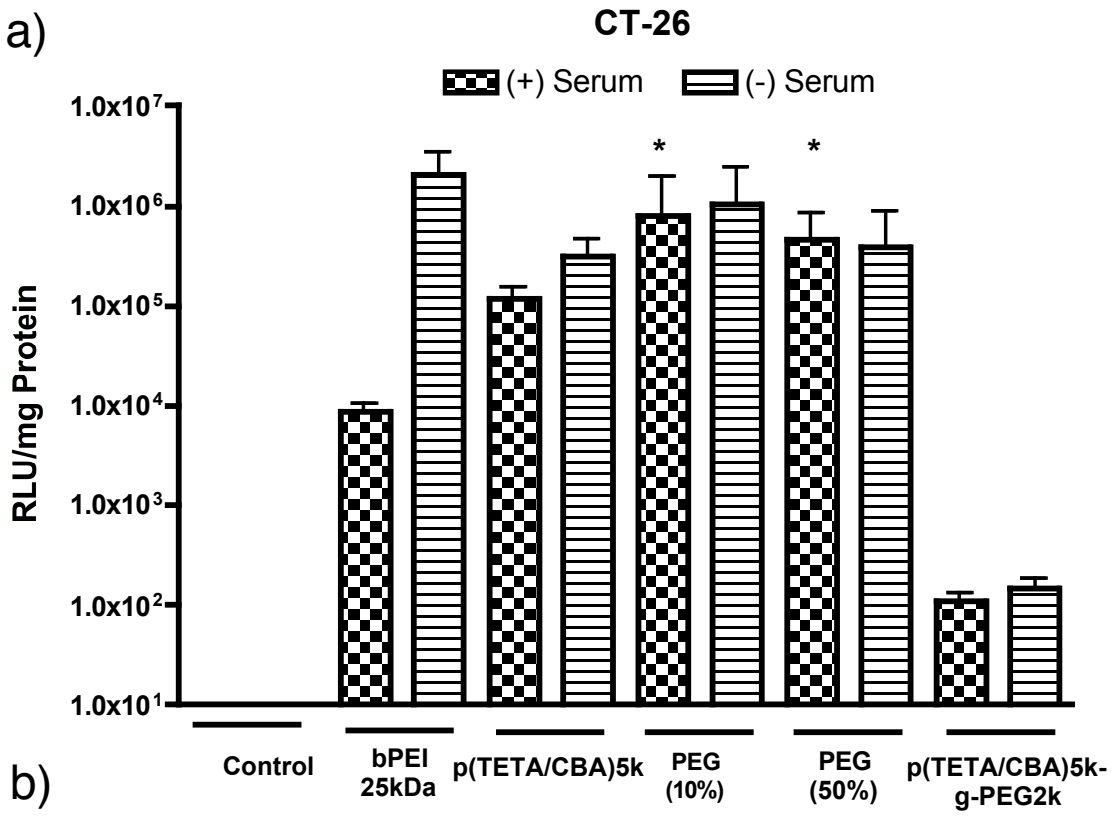
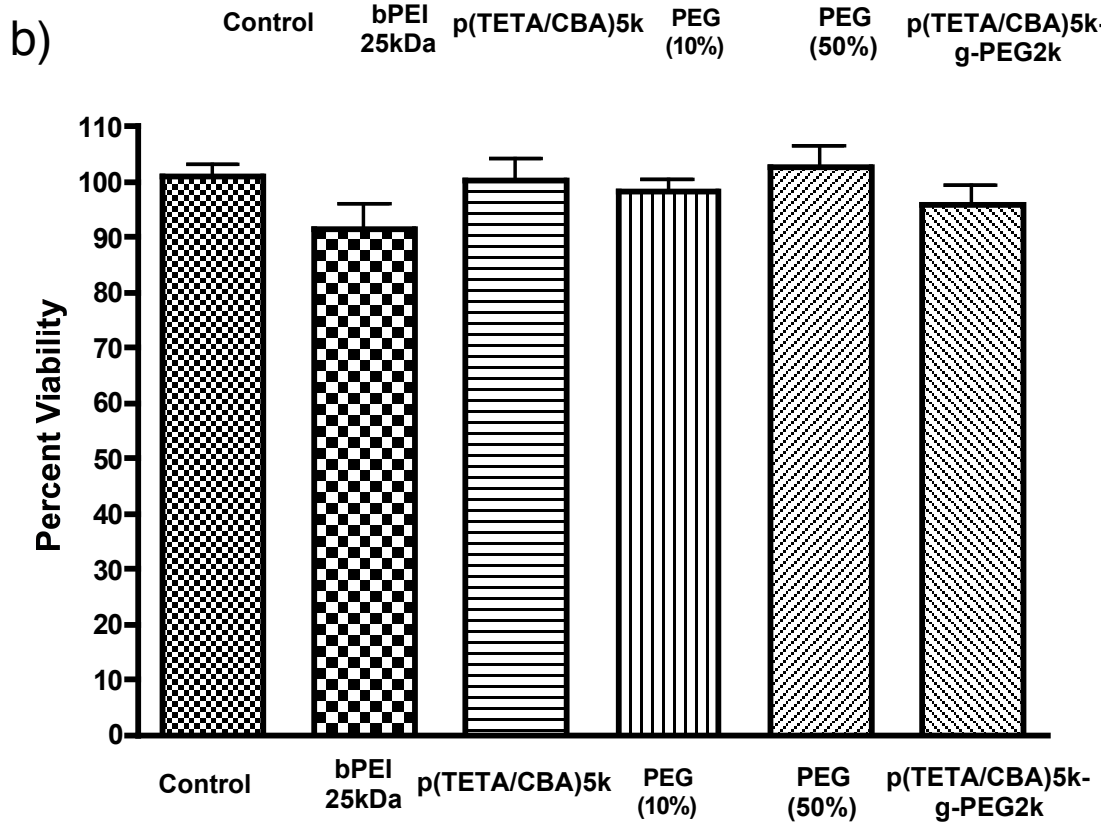


Figure 3.9. Comparison of p(TETA/CBA)5k/pCMVLuc transfection efficiency in the presence and absence of 10% serum in culture media. Transfection efficiency was evaluated by luciferase transgene expression in CT-26 cells. The presence of 10% serum in culture media mitigated transfection efficiencies for bPEI 25kDa N/P 10 (w/w 1.3) and p(TETA/CBA)5k at N/P 50 (w/w 24), however, p(TETA/CBA)5k provides significantly better transfection than bPEI 25kDa in the presence of 10% serum containing media. Data are represented as mean ± SD, *$p < 0.05$ (n=6).

Figure 3.10. a) Transfection efficiency of p(TETA/CBA)5k,10/90, 50/50 and 0/100% p(TETA/CBA)5k/p(TETA/CBA)5k-g-PEG2k polyplex formulations with CMVLuc in the presence and absence of serum. The 10% and 50% PEG formulations provide significantly greater transgene expression than bPEI 25kDa and p(TETA/CBA) in the presence of serum. Moreover, their transfection efficiencies are not significantly affected by the presence of 10% serum in conditioned media. b) Cell viability with analogous polyplex formulations in serum-containing media. Data are represented as mean ± SD, *$p < 0.05$ (n=6).


a)
CT-26
(+) Serum
(-) Serum
RLU/mg Protein
1.0x10^7
1.0x10^6
1.0x10^5
1.0x10^4
1.0x10^3
1.0x10^2
1.0x10^1
*
*
Control
bPEI 25kDa
p(TETA/CBA)5k
PEG (10%)
PEG (50%)
p(TETA/CBA)5k-g-PEG2k


b)
Percent Viability
110
100
90
80
70
60
50
40
30
20
10
0
Control
bPEI 25kDa
p(TETA/CBA)5k
PEG (10%)
PEG (50%)
p(TETA/CBA)5k-g-PEG2k

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CHAPTER 4

FUNCTIONAL PROPERTIES AND BIODISTRIBUTION OF POLY (TRIETHYLENETETRAMINE/CYSTAMINE-BIS-ACRYLAMIDE) AND POLY(TRIETHYLENETETRAMINE/CYSTAMINE-BIS-ACRYLAMIDE)-POLY(ETHYLENE GLYCOL) MIXTURES FORMED WITH NUCLEIC ACID[2]

4.1 Abstract

The clinical success of nonviral gene delivery reagents is hampered by their inefficient cellular transgene delivery, which is largely influenced by carrier properties that are currently undefined and misunderstood. In an attempt to further define and understand the requirements for a safe and efficient nonviral gene delivery reagent, research labs often engineer and evaluate many putative products with subtle physiochemical differences in order to delineate requirements for improved in vitro and in vivo success of their novel systems. The synthesis of many putative reagents is often time-intensive, laborious and costly. In Chapter 3, two physiochemically different polycations were synthesized, poly(triethylenetetramine/cystamine-bis-acrylamide)5k

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(p(TETA/CBA)5k) and its PEGylated counterpart, poly(triethylenetetramine/cystamine-bis-acrylamide)5k-g-poly(ethylene glycol)2k (p(TETA/CBA)5k-g-PEG2k) and they were mixed together at different ratios in order to easily derive a panel of putative gene delivery reagents to study their physiochemical properties and biological activity with a range of PEG concentrations. This approach proved to easily identify improved reagents in vitro, and it avoided the synthesis of many putative candidates for study. The studies herein utilize the aforementioned facile approach to study chemically similar polycationic reagents that are obtained with synthetic ease via one-pot synthesis. Reagent physiochemical properties and in vitro bioactivity are studied prior to investigating their in vivo biodistribution profiles in a murine colon adenocarcinoma model. Findings in these studies indicate that negatively charged complexes formed with the reagents and plasmid DNA (pDNA) exhibit greater passive tumor accumulation than positively charged complexes following their systemic administration. These studies warrant further investigation into the use of negatively charged gene delivery reagents for passive tumor targeting, and they substantiate the use of polycation/PEG-polycation mixtures to easily derive a panel of putative gene delivery reagents for study in order to elucidate design and formulation criteria for successful nonviral gene delivery vehicles for novel systems.

4.2 Introduction

Although polycationic gene carriers have been used in a laboratory setting for over a decade, their clinical application has been stalled because clinical requirements have not been fully achieved. A better understanding of their synthesis, design and

implications of carrier structure and function on biological systems is imperative to their advancement from the lab to the clinic. In addition to ensuring gene carrier safety and efficacy, it is important to design carriers that maintain synthetic ease and avoid laborious production methods that may be inefficient (*1*) . Probably the most important preclinical requirement for gene carrier success is their in vivo safety, followed by efficiency. Unlike viral methods of gene delivery, nonviral gene carriers are relatively safe if designed and/or modified prudently, as they are relatively nonimmunogenic. Another advantage of nonviral gene carriers compared to viral delivery is that they can also be synthesized so that they exhibit multifunctional properties, similar to viral capsids, however, more cost-effectively and with production ease (*2-4*).

Since the dawn of gene delivery, a large number of nonviral polymers and copolymers have been designed, synthesized and tested for their safety and efficacy in vitro and in vivo. Probably the most rigorously studied class of polycationic gene carriers is the poly(ethylene imine)s (PEIs). This is because they were among the first carriers to easily condense nucleic acids into nucleic acid/polycation nanoparticles (polyplexes) that sufficiently protected nucleic acid from serum nuclease degradation and they maintain relatively high transfection efficiency in multiple cell types. In recent years, however, the synthesis and design of biodegradable polycations have been focused on because nondegradable polycations, such as PEI systems, exhibit cellular toxicity at relatively low concentrations. This toxicity results from intracellular accumulation of high-molecular weight species that ultimately interfere with intracellular processes that ultimately lead to cell death (*3, 5*). Interestingly, low molecular weight PEIs maintain relatively low cellular toxicity compared to high molecular-weight PEI and also produce

low levels of gene delivery and subsequent transgene expression (*6-8*). Biodegradable polycations have gained attention because they can be synthesized as high molecular weight products for improved nucleic acid condensation, protection and transfection efficiency, all-the-while exhibit low cellular toxicity because they degrade into small molecular weight species and avoid intracellular accumulation and cell perturbation. Several noteworthy classes of biodegradable polycations include: hydrolysable poly(β-amino ester)s, disulfide containing and reducible poly(amido amine)s (SS-PAAs) and poly(amido ethylenimines) (SS-PAEIs). These classes, in general, have provided similar or improved cellular transgene delivery compared to PEIs; however, because of their degradability, they have proven to be significantly less toxic (*9-12*). Moreover, degradable carriers including cationic methacrylamide-based polymers have been studied in vitro and in vivo using tumor models, and although their molecular weight exceeds the maximum molecular weight to allow efficient glomerular filtration (45 kDA), they maintain efficient renal clearance due to their degradation (*13, 14*).

As mentioned previously, preclinical advancement of polycationic gene carriers is hampered by their safety and efficacy in vivo. Many previous reports have shown that cationic polyplexes interact with negatively charged macromolecules found in serum and the extracellular space. These interactions are therapeutically unfavorable because they often lead to particle aggregation and reduced efficacy (*15, 16*). Poly(ethylene glycol) (PEG) conjugation to various classes of polycations has been employed to overcome this problem and improve carrier performance in vivo. By avoiding this problem, therapeutically active payload can be delivered and the circulation half-life of polyplex is increased, which can be a major advantage to in vivo gene delivery. This is especially

true for cancer therapy where the Enhanced Permeability and Retention (EPR) effect may be exploited by long circulation half-lives of macromolecules (*17, 18*).

Previously published results included in Chapter 3 indicate that mixtures of a polycation and PEG-polycation can be used for gene delivery. Moreover, by altering the relative amount of each species in the mixture, reagent properties could be easily manipulated for study and improved gene delivery candidates could be identified without synthesizing many putative products (*19*). In this present work, chemically similar gene carriers are obtained using a new synthetic method with greater synthetic ease. These products, however, are physiochemically different from those described in Chapter 3, having a higher absolute molecular weight and lower degree of PEG modification. Mixture formulations of these products are developed using the approach outlined in Chapter 3 and the results presented herein provide further evidence that formulation mixtures can be used to derive a panel of gene delivery reagents to study carrier physiochemical properties, bioactivity and their in vivo biodistribution following their systemic administration using a murine colon adenocarcinoma model. Moreover, in vivo studies indicate that negatively charged complexes formed with these reagents and plasmid DNA (pDNA) demonstrate improved passive tumor accumulation compared to positively charged complexes following their systemic administration. These studies warrant additional investigation into the use of negatively charged gene delivery reagents for passive tumor targeting.

4.3 Materials and Methods

4.3.1 Materials

Hyperbranched poly(ethylene imine) (bPEI, *M*w 25 000 (bPEI25kDa)), Triethylenetetramine (TETA) and HPLC grade methanol were purchased from Sigma-Aldrich (St. Louis, MO). N,N'-cystamine-bis-acrylamide (CBA) was purchased from Polysciences, Inc. (Warrington, PA). Ultrafiltration devices along with regenerated cellulose membranes (10 kDa) were purchased through Millipore Corporation (Billerico, MA). The reporter gene plasmid, pCMVLuc, was previously designed by inserting luciferase cDNA into a pCI plasmid (Promega, Madison, WI) that is driven by the pCMV promoter. The resulting plasmid was purified using Maxiprep (Invitrogen, Carlsbad, CA) protocols. Dulbecco's Modified Eagle's Medium (DMEM), penicillin streptomycin, trypsin-like enzyme (TrypLE Express), and Dubelcco's phosphate buffered saline were purchased from Gibco BRL (Carlsbad, CA). Fetal bovine serum (FBS) was purchased from Hyclone Laboratories (Logan, UT).

4.3.2 p(TETA/CBA) Synthesis

Synthesis of p(TETA/CBA) was performed with minor adaptation to the synthesis described in Chapter 3 and that described elsewhere (*10*). The polymerization reaction was run at 30°C for 12 hrs using equimolar amounts of N,N'-cystamine-bis-acrylamide (CBA) and triethylenetetramine (TETA) monomers. At 12 hrs, excess TETA was added to the reaction mixture to terminate polymerization. The reaction was allowed to run for an additional 24 hrs to ensure termination. The pH was subsequently adjusted to 7.0 and the polymer was purified by ultrafiltration using a 10kDa regenerated cellulose

membrane. p(TETA/CBA) was obtained by lyophilization. Composition of the polymer was monitored using ^{1}H NMR (400 MHz, D_2O). p(TETA/CBA) δ 2.61 (COC*H*2CH2NH, 4H), 2.72 (NHCH2C*H*2S-S, 4H), 2.90-3.21 (COCH2C*H*2NHC*H*2C*H*2,16H), 3.41 (NHC*H*2CH2S-S, 4H).

4.3.3 p(TETA/CBA/PEG) Synthesis

The synthesis of p(TETA/CBA/PEG) was performed as a one-pot synthesis, which eliminated the purification step required in Chapter 3 to obtain p(TETA/CBA) prior to its PEG chemical modification. This one-pot synthesis of p(TETA/CBA/PEG) began with the original polymerization reaction conditions described above in Section 4.3.2 for p(TETA/CBA). However, following 10 hrs reaction time methoxy PEG-NHS was added to the reaction mixture and allowed to react for an additional 2 hrs before the addition of excess TETA (100%). The reaction was allowed to proceed for an additional 24 hrs before the pH was lowered to 7.0 and the polymer was purified using ultrafiltration (10 kDa) The composition of p(TETA/CBA/PEG) was also monitored using ^{1}H NMR (400 MHz, D_20). p(TETA/CBA/PEG); δ 2.61 (COC*H*2CH2NH, 4H), 2.72 (NHCH2C*H*2S-S, 4H), 2.90-3.21 (COCH2C*H*2NHC*H*2C*H*2,16H), 3.41 (NHC*H*2CH2S-S, 4H), 3.45-3.7 (CH2CH20, 4H).

4.3.4 p(TETA/CBA) and p(TETA/CBA/PEG) Characterization

Absolute molecular weight analysis for p(TETA/CBA) and p(TETA/CBA/PEG) was performed using AKTA/FPLC (Amersham Pharmacia Biotech Inc.) coupled to a light-scattering detector and using the polymer refractive index increment (dn/dc) for each

sample. A Superose 6 110/300 GL column was used for polymer sample separation. Poly[N-(2-hydroxypropyl)methacrylamide] (poly(HPMA)) standards were injected onto the column prior to experimental sample analysis in order to ensure the column was clean and functional. Experimental and standard polymer samples were dissolved in degassed and filtered (0.2 μm (Nylon, Alltech)) 0.3 M NaOAc, pH 4.4 with 30% (v/v) acetonitrile eluent buffer. The flow rate was set to 0.4 mL/min.

4.3.5 Determination of Polymer Disulfide Bonds

Disulfide bond content of each polymer was determined using 5,5-Dithio-bis-(2-nitrobenzoic acid) (Ellman's reagent) following the manufacturer's protocol with minor adaptation (Pierce). In brief, cysteine hydrochloride was used as a stardard to generate a standard curve monohydrate. Polymer molecular weights were used to estimate the number of disulfide bonds in each polymer and to further normalize the average thiolate concentration so as to fall within the standard range. Each polymer sample was reduced using 10x immobilized (Tris(2-carboxyethyl)phosphine hydrochloride) (TCEP) gel in buffer (0.1 M sodium phosphate, pH 8, 1mM EDTA). Following 30 min incubation to ensure complete polymer reduction, the samples were centrifuged at 1000 x g for 2 min and the reduced polymer solutions were collected. Reduced polymers were subsequently added to the Ellman's reagent in buffer and allowed to incubate at room temperature for 20 min after mixing. The sample absorbance at 412 nm was measured using UV-Vis spectroscopy. The thiol concentration was determined using the standard curve. Polymer average molecular weights were then used to determine the number of disulfide bonds per polymer.

4.3.6 Polyplex Formation

In all cases polyplex was formed with a known amount of pDNA and a corresponding and desired amount of polymer suspended in HEPES buffer (20 mM, pH 7.4, 5% glucose). The two solutions were combined, lightly vortexed and allowed to equilibrate for 30 min at room temperature. When mixtures of the two polymers were used to form polyplex, a known amount of pDNA was brought up in HEPES buffer and a corresponding and desired concentration of p(TETA/CBA) and p(TETA/CBA/PEG) were prepared in buffer. The polymer solutions were mixed prior to polyplex formation at a given polymer concentration and a desired amount of each species before the resulting mixtures were added to the pDNA solution to form complex.

4.3.7 Gel Retardation Assay

Polymer/pDNA complexation was investigated by generating polyplex prepared at increasing polymer-to-pDNA (p(TETA/CBA) or p(TETA/CBA/PEG)) weight-to-weight ratios (w/w) in HEPES buffered solution (20 mM HEPES, pH 7.4, 5% glucose). Polyplex was formed using 500ng pDNA and a corresponding amount of polymer. The polymer/pDNA mixtures were then allowed to incubate at room temperature for 30 min prior to being loaded on a 1% agarose gel that was stained with ethidium bromide (EtBr, 5μg/mL) at 104 V for 30 min in TAE (40 mM Tris-acetate, 1 mM EDTA) buffer. The pDNA was visualized using GelDoc software.

4.3.8 Light Scattering and ζ-Potential Measurements

The surface charge and polymer/pDNA particle (polyplex) diameters were measured at 25°C using a Zetasizer 2000 instrument (DTS5001 cell) and a dynamic light scattering (DLS) unit on a Malvern 4700 system, respectively. Polyplexes were prepared by adding equal volume polymer solution (200μl) at increasing concentrations in HEPES buffer (20 mM, pH 7.4, 5% glucose) to a desired concentration of 8 μg pDNA in HEPES buffer (200μl). Polyplexes were allowed to equilibrate for 30 min and were subsequently diluted in filtered milliQ water to a final 2 mL volume.

4.3.9 Polyplex Stability in Fresh Rabbit Serum

Polyplex stability and resulting pDNA stability against nuclease activity in serum was evaluated using 500ng free pDNA as a control and 500ng pDNA complexed with polymer mixtures pre-formed in HEPES buffer. Polyplex formation was carried out by combining equal volume solutions of pDNA and polymer mixtures using a polymer/pDNA at 12 w/w (N/P 25) and allowed to equilibrate for 30 min. Pre-formed polyplex was then diluted in 90% fresh rabbit serum and incubated at 37°C over time. Twenty-five microliter aliquots (125 ng pDNA) were taken at each time point and 10 μl stop buffer (250 mM NaCl, 25 mM EDTA, 2% SDS) was added to each. The samples were frozen at -70°C until further analysis. Once the samples were thawed, they were incubated overnight at 60°C to completely dissociate polycation from the pDNA and 2 μl of 50 mM DTT were added to each sample and incubated at 37°C for an additional 30 min to ensure complete decomplexation. Lastly, the samples were loaded onto a 2% agarose gel stained with ethidium bromide (EtBr) and subjected to electrophoresis at 96

V for 30 min in TAE (40 mM Tris-acetate, 1 mM EDTA) buffer. The gel image was viewed using GelDoc software (n=2).

4.3.10 Cell Culture and In Vitro Transgene Expression

Mouse colon adenocarcinoma cells (CT-26) (ATCC) were cultured in DMEM containing 10% FBS and 1% penicillin-streptomycin at 37°C in a humidified incubator with an atmosphere containing 5% (v/v) CO_2. Subculturing of cells was performed when cells reached 70-80% confluency. Luciferase reporter gene expression in mouse colon adenocarcinoma cells (CT-26) was performed in vitro using respective polymer and pCMVLuc plasmid DNA mixtures. Cells were plated in 24-well plates containing 0.5mL media. Once the cells were approximately 70% confluent, polyplexes were prepared using 0.5μg pDNA and a respective amount of polymer or polymer mixture in HEPES buffer. Polyplexes were allowed to equilibrate for 30 min and the cells were transfected in the presence of serum by adding 20 μl polyplex (0.5 μg pDNA) to each well for 4 hrs before replacing with fresh culture media. The cells remained in the incubator for a total of 48 hrs before they were washed with 1ml PBS and treated with cell culture lysis buffer (Promega). Luciferase quantification was performed using a Luciferase assay system (Promega) on a luminometer from Dynex Technologies, Inc. (Chantilly, VA). The amount of protein in the cell lysate was determined using a standard curve of bovine serum albumin (Sigma) and a BCA protein assay kit (Pierce) (n=6).

4.3.11 Cell Viability Assay

CT-26 cells were plated in 24-well plates and gene transfections were carried out when the cells were approximately 70% confluent. Polyplexes were prepared as they were for the luciferase reporter gene assay. Respective cell cultures were transfected in the presence of serum with the addition of 20 μl equilibrated polyplex in HEPES buffer solution (0.5 μg pDNA) to each well. Cells were left to incubate for a total of 18 hrs prior to analyzing their viability using an MTT assay (Sigma). Percent cell viability was determined relative to untreated cells (n=6).

4.3.12 Erythrocyte Lysis Assay

Following anticoagulation, fresh rabbit erythrocytes were isolated using a ficoll density gradient using centrifugation on a Sorval 6 swing bucket rotor at 900 x g for 20 min at room temperature. The supernatant was removed and the Red Blood Cell (RBC) pack was resuspended in 10ml of 0.5% NaCl and mixed thoroughly by pipetting. The solution was then spun at 600 x g in order to remove any remaining serum proteins. The erythrocytes were then diluted 100x in 20mM HEPES buffer containing 5% glucose. An equal number of cells were plated in each well using a 24-well cell culture dish. Polyplexes were formed as described above at increasing polymer concentrations using 500ng pDNA and added to the culture dish. The study was done in triplicate with representative images taken following 4 hrs incubation in the presence of polyplex.

4.3.13 In vivo Polymer Biodistribution Study

Mouse colon adenocarcinoma cells (CT-26) suspended in 100μl sterile PBS were subcutaneously injected (1 x 10^6 cells) into the right flank of 6-week old female Balb/c mice. Primary tumors were allowed to form over 2 weeks or until they reached an average tumor volume near 80 mm^3. Outliers were discarded to maintain this average. A total volume of 200μl polyplex solution was injected intravenously into each mouse via the tail vein after polyplex was formed. Polyplex was prepared using the p2CMVIL-12 (Figure 4.1) plasmid and 25, 50 75 and 100% p(TETA/CBA/PEG) polymer formulations at 0.5 w/w and 3 w/w in HEPES buffer (20 mM HEPES, pH 7.4, 20% glucose). The weight-to-weight (w/w) ratios were chosen based on particle size and surface charge. Mice were sacrificed at 40 hrs post-injection by isoflurane overdose and tissues (heart, lung, liver, spleen, kidneys, and tumors) were excised. Excised tissues were immediately flash-frozen with liquid nitrogen (N_2) and placed in 2ml shatter-proof tubes with zirconia/silica beads (Bio-Spec Products, Bartlesville, OK) that had been preweighed. The samples were weighed in the tubes and the previously recorded weight was subtracted to get the weight of the tissue. One milliliter of protease inhibitor solution (complete Ultra Tablets (Roche)) was added to each tube and the samples were homogenized on a mini-bead beater. Samples were subsequently stored at -80°C until used for DNA isolation. DNA was isolated using a Wizard SV 96 Genomic DNA Purification Kit (Promega) in accordance with the manufacturer's directions. The obtained DNA was measured by UV spectrophotometry using a Nanodrop ND-1000. 260/280 values lower than 1.7 were discarded and re-processed. qPCR was performed using FastStart Universal master mix and a StepOne Real-Time Thermocycler (Applied

Biosystems). Taqman primers specific for the p2CMVIL-12 plasmid were used. A serial dilution of total DNA was set-up using an untreated control sample to generate a standard curve with spiked controls each time qPCR was run. Concentrations ranged from 500 to 10 ng in order to determine optimal concentrations with the spiked controls. Each concentration was spiked with 1 x 10^5 copies of p2CMVIL-12. The primer sequences are as follows: AmpF-CGTGTCGCCCTTATTCCCTTTT; AmpR-AAACGCTGGTGAAAGTAAAAGATGC. The total copy number for each sample was determined and normalized to mg tissue.

4.3.14 Statistics

The experimental data in this report are assessed as mean ± SD. A one-way ANOVA in conjunction with a Tukey's post-hoc test is used to determine if treatment groups are statistically different from each other. A $p < 0.05$ was considered statistically significant (n=6). For serum stability studies, a posttest for linear trend was performed in addition to a two-way ANOVA with a Bonferroni post-hoc test. Data are represented as mean ± SD, $p < 0.05$ (n=3).

4.4 Results

4.4.1 p(TETA/CBA) and p(TETA/CBA/PEG) Synthesis and Characterization

The objective of the studies herein was to develop a co-polymeric gene carrier with the same chemical functionalities as that developed in Chapter 3 but with a higher molecular weight and different degree of modification in order to justify the use of gene

carrier mixture formulations for carrier optimization beyond the synthetic products produced previously in Chapter 3. This newly synthesized co-polymeric gene carrier, deemed p(TETA/CBA/PEG), was produced using a one-pot synthetic approach. Evidence herein demonstrates that p(TETA/CBA/PEG) can be used alone for cellular transgene delivery of pDNA and in conjunction with p(TETA/CBA) to derive a panel of putative gene delivery reagents with various physiochemical properties that influence carrier bioactivity. By doing so, efficacy studies are more easily performed to understand the design and formulation requirements that govern ideal nonviral gene carrier properties and their bioactivity in vitro and in vivo. We have previously shown that a graft copolymer of PEG and p(TETA/CBA)5k (p(TETA/CBA)5k-g-PEG2k) can be used in this manner for similar studies in vitro (*19*).

^{1}H NMR analysis was used to confirm the synthesis of p(TETA/CBA/PEG) and identify the degree of PEG modification for p(TETA/CBA/PEG) (Figure 4.2). ^{1}H NMR confirmed that a 10 wt % feed ratio of PEG during synthesis resulted in a final conjugation of ~4 wt % PEG (0.68/1 moles PEG/p(TETA/CBA)) following purification (Table 4.1). PEG conjugation to the p(TETA/CBA) backbone is competed by hydrolysis of the PEG-NHS-ester and/or reaction with un-polymerized TETA monomer that is eliminated during subsequent polymer purification, which explains why there is only 40% conversion. Absolute molecular weight analysis using AKTA/FPLC indicates that similar molecular weight polymers were obtained following synthesis and purification, with polydispersity indices (PDIs) of 1.54 and 1.27 for p(TETA/CBA) and p(TETA/CBA/PEG)), respectively (Table 4.1). Chemical conjugation of the amine-reactive PEG-NHS-ester with unpolymerized TETA monomers will reduce the incidence

of TETA monomer addition to late-stage growing oligomer chains. As such, p(TETA/CBA) oligomers generated during the final stages of polymerization more likely exist in the p(TETA/CBA) sample than p(TETA/CBA/PEG) and explain the lower PDI and higher M_n of p(TETA/CBA/PEG) compared to p(TETA/CBA). The number of disulfide bonds per molecule are similar between the two samples and were determined using Ellman's reagent and calculated based on the molecular weight of each sample (Table 4.1). Moreover, the degree of branching for p(TETA/CBA) is in agreement with our previously published result and those described in Chapter 3, which demonstrate that synthesis temperature influences the degree of branching for the obtained products (Table 4.1) (*19*).

Plasmid DNA (pDNA) complexation using p(TETA/CBA) and p(TETA/CBA/PEG) were investigated. Results from this study indicate that both gene carriers form interactions with pDNA as polymer concentrations increase. The p(TETA/CBA) product demonstrates partial pDNA complexation at 0.1 w/w (p(TETA/CBA)/pDNA) and complete complexation as the polymer concentrations are increased to achieve 4 w/w (Figure 4.3). Moreover, complexation with pDNA using p(TETA/CBA/PEG) begins around 0.2 w/w and complete pDNA encapsulation is achieved at 6:1 w/w (Figure 4.3). The increased amount of p(TETA/CBA/PEG) (6 w/w) required to fully encapsulate the pDNA compared to p(TETA/CBA) (4 w/w) suggest that incorporation of PEG to p(TETA/CBA) mitigates complexation as seen in Chapter 3 and in other studies (*20, 21*). The steric effects of the PEG chains reduce polycation association with pDNA.

Given the aforementioned influence of PEG on polyplex formation using

p(TETA/CBA/PEG) alone, mean particle diameter and surface charge were investigated using mixtures of p(TETA/CBA) and p(TETA/CBA/PEG) at different ratios and as individual species. Both gene carriers and the mixture formulations (25, 50 and 75% p(TETA/CBA/PEG)/p(TETA/CBA)) formed polyplex at or below 100nm when a polymer/pDNA w/w ratio equal to 3 and greater are used. A dramatic increase in particle size is seen at 1 w/w regardless of the formulation mixture tested, which indicates that complete pDNA/polymer complexation does not occur at or below this ratio and aggregation of the complexes occurs (Figure 4.4). Aggregation is overcome as the polymer amount is increased to 3 w/w via charge-charge dispacement of the aggregated complexes and excess polycation. When particle surface charge was evaluated for each formulation, positive polyplex was formed at 3 w/w (Figure 4.4), which further indicates that complete pDNA encapsulation occurs at this ratio.

The ability of each polymer formulation to condense and protect pDNA from serum nuclease degradation was evaluated. Following polyplex formation using each polymer formulation at 12 w/w, the samples were added to fresh rabbit serum (90% serum) and allowed to incubate at 37°C for 6 hrs. All formulations significantly enhance nucleic acid protection from serum nuclease degradation when compared to free pDNA at 1, 2, 4 and 6 hrs (Figure 4.5). A posttest for linear trend indicated significant changes in the percent of intact pDNA remaining over time for all treatments. Moreover, increasing amounts of p(TETA/CBA/PEG) correspond with lower protective effects in serum, as 75% and 100% p(TETA/CBAPEG) treatments provide approximately 25 and 20% less intact p(DNA) compared to p(TETA/CBA) at 6 hrs incubation in 90% serum, respectively. Increasing the amount p(TETA/CBA/PEG) in reagent formulations

mitigates the protective affects of the polycation system because PEG sterically mitigates complexation with pDNA, which leads to less payload protection. Despite their reduced capacity to protect pDNA from serum nuclease degradation, 75 and 100% p(TETA/CBA/PEG) protect approximately 65% pDNA over 6 hrs (Figure 4.5). These data indicate the advantage of formulating pDNA with polycations for improved pDNA protection against serum nuclease degradation, and they demonstrate the influence of PEG content on formulation stability, which may be tailored to influence formulation stability and functional payload delivery in vitro and in vivo were serum proteins are present.

4.4.2 p(TETA/CBA) and p(TETA/CBA/PEG) In Vitro Bioactivity

The bioactivity of each formulation was assessed in the mouse colon adenocarcinoma cell line CT-26. To do so, luciferase transgene expression and cell viability were evaluated using each polymer mixture formulation (0, 25, 50 and 75% p(TETA/CBA/PEG)/p(TETA/CBA)). When p(TETA/CBA) was used alone for transfection, it provided similar transgene delivery to control bPEI 25kDA used at 0.5 w/w (Figure 4.6) . The transfection efficiency for bPEI 25kDA when performed at 1 w/w was not included because this condition exhibited significant cell toxicity and accurate transgene expression could not be determined for this treatment (Figure 4.6). The p(TETA/CBA) and p(TETA/CBA/PEG) formulations presented no toxicity to the cells when used at 12 w/w, exemplifying its biocompatibility in this cell line, as these concentrations are 12x greater than that used for bPEI 25kDa, which imposed approximately 80% cell death. Moreover, the p(TETA/CBA/PEG) formulations showed

comparable, or greater, transgene delivery and expression compared to the relevant bPEI 25kDa control (Figure 4.6). Most importantly, the 75% p(TETA/CBA/PEG) reagent provided significantly better transfection than the other formulations when compared to the bPEI 25kDa control. The other formulations tested do not provide significantly better transfection compared to bPEI 25kDa (Figure 4.6).

In order to study the effect of p(TETA/CBA) and p(TETA/CBA/PEG) on red blood cell (RBC) lysis, polyplexes were prepared at various polymer/pDNA w/w ratios and administered to each well, respectively. Cells were visualized by light microscopy following 4 hrs incubation with polyplex. Increasing concentrations of p(TETA/CBA), but not p(TETA/CBA/PEG), correlated with RBC aggregation and lysis (Figure 4.7). Most noticeably, nearly 85% of the erythrocytes had been lysed by p(TETA/CBA) treatments around 6 w/w over the 4 hrs incubation period, whereas, p(TETA/CBA/PEG) treatments at the same concentration had no noticeable lysis and/or increase in cell aggregation (Figure 4.7). It is useful to also evaluate the affect of formulation mixtures on RBC lysis. Formulations were not tested; however, these results indicate the advantage that p(TETA/CBA/PEG) has on maintaining RBC integrity.

4.4.3 p(TETA/CBA) and p(TETA/CBA/PEG) In Vivo Biodistribution

The biodistribution of 25, 50, 75 and 100% p(TETA/CBA/PEG)/p(TETA/CBA) formulations was studied using a murine adenocarcinoma model (CT-26), following systemic administration via mouse tail vein. The p(TETA/CBA) gene delivery reagent alone was not evaluated because previous studies have demonstrated the importance of PEG shielding in polyplex administration performed systemically and the RBC lysis

assay indicated adverse effect on RBC viability and aggregation (*2, 22*). In lieu of this understanding and finding for the RBC assay, tumor bearing mice were injected with polyplex formulations at 0.5 and 3 w/w ratios using the panel of 25, 50, 75 and 100% p(TETA/CBA/PEG)/p(TETA/CBA) formulations.

Organ accumulation values at 48 hrs postinjection were determined using quantitative PCR (qPCR) and are represented as the Percent Initial Dose (%ID) for lung, liver, spleen kidney, heart and tumor (Table 4.2). These data indicate that positively charged complexes formed at 3 w/w had markedly high liver accumulation (9.35 ± 7.19 - 14.78 ± 5.14 %ID), compared to the other organs that ranged between 0.02 ± 0.00 and 4.69 ± 5.99 %ID (Table 4.2). No significant trend, however, was observed between the p(TETA/CBA/PEG) formulations. Conversely, the negatively charged complexes had similar payload deposition between organs with the exception of heart that possessed nearly no pDNA accumulation. One-hundred and 75% p(TETA/CBA/PEG) formulation used at 3 w/w exhibited lower liver accumulation but relatively higher spleen accumulation compared to the 50 and 25% p(TETA/CBA/PEG) formulations, which may be due to their relative size and surface charge. No significant trend was observed between negatively-charged p(TETA/CBA/PEG) formulations.

The %ID values for the negatively charged complexes were markedly lower than those values obtained for the positively charged complexes, which indicates decreased encapsulation and/or protection of pDNA at 0.5 w/w compared to 3 w/w during systemic circulation. Interestingly, however, negatively charged complexes exhibited greater passive tumor accumulation compared to the positively charged complexes and the data is represented graphically in Figure 4.8. Moreover, despite the reduced pDNA protection

of the negatively charged complexes compared to positively charged complexes in vivo, the %ID values at the tumor site were similar between the two (Table 4.2 and Figure 4.8). By achieving similar payload delivery to the tumor site using complexes formed using 6x less polycation, potential dose-limiting toxicities from the polymer reagents can be avoided during systemic administration of nucleic acids using modified polycation formulations. As such, dosing regimens can be increased to drive delivery to the tumor environment.

4.5 Discussion

In the present work, a new synthetic strategy is employed in order to obtain a PEGylated SSPAEI (p(TETA/CBA/PEG)) that can be used in conjunction with the polycationic backbone polymer, p(TETA/CBA), and by itself for nucleic acid delivery. By formulating mixtures of the two species, the relative amount of each polymer can be easily altered to affect gene carrier characteristics that influence carrier efficacy, bioactivity and biodistribution. Using this facile approach, various gene carrier characteristics are studied more easily and identification of optimal candidates is expedited.

Pegylation of p(TETA/CBA) proved to reduce the ability of the gene carrier to condense and form complex with pDNA (Figure 4.3). This finding is in agreement with previous reports, which showed that complexation is influenced by the molar amount and the chain length of PEG introduced to a polycation carrier that further influences complex size and stability (*23*). DLS studies herein, in conjunction with findings provided in Chapter 3 corroborate this influence of PEG, as complex size and stability tend to

correlate with the amount of PEG in each formulation mixture (*19*). Particles around 100-120 nm in diameter were formed for all formulations when complexed at 3 w/w and above. Interestingly, similar unimodal particle sizes (90-140 nm) were seen at 0.5 w/w ratios with a marked increase in size at 1 w/w. This indicates that complete pDNA condensation does not occur below 3 w/w. The large particles found at 1 w/w appear to be partially complexed, not having enough polymer to fully condense the pDNA and large complex sizes indicate probable interparticle interaction and aggregation. Charge-charge displacement of these aggregates occurs as the polycation concentration is increased to reach 3 w/w. Moreover, the surface charge of polyplexes below 3 w/w are close to neutral and in some cases negatively charged, which further suggests the incomplete protection of pDNA by the polycation formulations.

In vitro bioactivity was evaluated for p(TETA/CBA) and the respective p(TETA/CBA/PEG) formulations, which include 25, 50, 75 and 100% by weight of the latter species. Significant cell toxicity is seen using bPEI 25kDa at 1 w/w, while the bioreducible polymer formulations p(TETA/CBA) and p(TETA/CBA/PEG) imposed no toxicity at 12 w/w. The amount of polycation used in the p(TETA/CBA) and p(TETA/CBA/PEG) formulations is significantly greater than that used for bPEI controls, thus exemplifying the advantage of using a degradable gene carrier system with respect to biocompatibility and cell toxicity. Mixture formulations of p(TETA/CBA/PEG)/p(TETA/CBA) are able to deliver viable transgene to CT-26 cells as well or better than the bPEI 25kDa control at 0.5 w/w. Cells treated with 75% p(TETA/CBA/PEG)/pDNA showed a statistically significant increase in transgene expression compared to the bPEI 25kDa control. This formulation exhibited good

complexation characteristics with pDNA and due to the amount of PEG incorporated in this formulation likely maintains the steric repulsion of serum proteins present in the transfection media and avoids their adverse affect on transfection. All the while this reagent's positive surface charge assisted in cell association of complexes and their subsequent endocytosis (*24*). The 100% p(TETA/CBA/PEG) formulation may have provided less transgene expression than the 75% p(TETA/CBA/PEG) formulation despite sufficient PEG present to sterically reduce serum protein adsorption because this formulation showed a marked decrease in surface charge seen at this weight-to-weight ratio (12 w/w) (Figure 4.4) This formulation also exhibited reduced stability and pDNA protection in serum during the initial hours of incubation. Increasing amounts of p(TETA/CBA/PEG) in polyplex formulations corresponded with lower protective effects in serum over 6 hrs, and strong effects in the first 2 hrs, which may be explained by reduced nucleic acid condensation (Figure 4.5) (*23*).

The ability of p(TETA/CBA) and p(TETA/CBA/PEG) to induce RBC lysis were investigated prior to evaluating the biodistribution profiles of the polymer formulations. Noticeably, increasing concentrations of p(TETA/CBA), and not p(TETA/CBA/PEG), induced erythrocyte lysis and aggregation. The difference in the behavior of these two treatment groups may be explained by their respective ability to disrupt RBC membrane stability, where cell membrane intercalation of polycationic chains is sterically reduced in the p(TETA/CBA/PEG) formulation due to the presence of PEG chains. In addition, oxidant strength has been shown to influence RBC viability (*25*). Because intracellular enzymes that facilitate the degradation of disulfide-containing systems function to protect the cell from oxidative stress, it is plausible that this difference of RBC lysis between

p(TETA/CBA) and p(TETA/CBA/PEG) is due to a slower degradation profile of p(TETA/CBA/PEG) compared to p(TETA/CBA). A slower degradation rate for p(TETA/CBA/PEG) can occur due to the presence of PEG that sterically reduces enzymatic degradation of the gene delivery reagent. If degradation is slowed due to the putative steric effects of PEG, the cell can manage oxidative stress more effectively, as the enzymes are more free to maintain their redox homeostasis and thus maintain RBC viability. Further studies must be performed to determine this potential.

A biodistribution study was performed using a murine colon adenocarcinoma model in order to examine the potential differences in organ distribution of pDNA following systemic administration using each gene carrier formulation (25, 50, 75 and 100% p(TETA/CBA/PEG)) in vivo. Injection of positively and negatively charged complexes was also investigated in order to study potential differences of their biodistribution profiles. Each aforementioned gene carrier formulation was administered using 0.5 or 3 w/w, which derive negatively or positively charged complexes, respectively (Figure 4.4). Results from this study indicate that all p(TETA/CBA/PEG) formulations administered at 0.5 w/w exhibit significantly lower %ID values in each organ compared to the formulated complexes derived using 3 w/w. The reduced %ID values determined in each organ following systemic delivery of the negatively charged complexes is explained by the insufficient protection of pDNA payload from serum nuclease degradation during circulation, as DLS results indicated that pDNA was not fully encapsulated by the formulations at 0.5 w/w. Interestingly however, the negatively charged complexes showed greater passive tumor accumulation than positively charged complexes. Moreover, those complexes formed at 0.5 w/w that possessed the greatest

negative surface charge, 75% p(TETA/CBA/PEG) (-20 ± 2.5 mV) and 25% p(TETA/CBA/PEG) (-9.8 ± 3.5 mV), exhibited greater tumor accumulation than 100% TCP (1.1 ± 0.9 mV) and 50% p(TETA/CBA/PEG) (-4.3 ± 3.8 mV) at 0.5 w/w, which is irrespective of PEG content. A similar result was found in a previous study and may be explained by the ability of negatively charged complexes to avoid interaction with glycosaminoglycans (GAGs) present at relatively high levels in the tumor environment. By avoiding interactions with GAGs, these complexes can penetrate tumors more effectively than complexes with a high positive surface charge that often adsorb to GAGs, which leads to their destabilization and tumor delivery (*26, 27*). Taken together, these results suggest potential advantages of negatively charged gene and drug delivery reagents for passive tumor targeting and warrant further investigation using polycations or other polymeric delivery reagents.

The biodistribution profile of positively charged complexes demonstrated a high degree of liver accumulation compared to the other major organs that were evaluated. This finding is similar to other previously published results using PEGylated polycations as gene delivery reagents (*28*). Moreover, positively charged complexes formed using 100% and 75% p(TETA/CBA/PEG) formulations demonstrated reduced liver deposition compared to 50% and 25% p(TETA/CBA/PEG) formulations, with an inverse relationship toward their accumulation in the spleen. A correlation between complex physiochemical characteristics and this difference in organ deposition does not exist. However 25% p(TETA/CBA/PEG) complexes formed at 3 w/w did provide the lowest complex size and surface charge compared to the other formulations, which correlated with relatively high liver deposition and significantly low spleen accumulation. This

distribution profile suggests potentially low interaction of these complexes with serum proteins and thus the evasion of the reticuloendothelial system (RES). Moreover, the relatively small size of these complexes may facilitate their extravasation through liver endothelial fenestrae, as extravasation of nanoparticles is size dependant (*29*). While this explanation may explain the observed organ distribution profile, more rigorous studies are required to assert this putative correlation, as the aforementioned differences are subtle and the correlation is not statistically definitive. Nonetheless, these studies demonstrate that mixtures of a synthesized polycation and its PEGylated counterpart can be formulated together in order to easily alter and control the relative amount of each species in a gene delivery reagent formulation and delineate changes in gene carrier properties and influence on bioactivity and biodistribution. This facile evaluation method is particularly useful for novel, PEG-modified delivery reagents that possess a high degree of PEG modification that needs to be easily manipulated or controlled to identify optimal reagents that are delicately balanced by the advantages PEG chemical modification offers all-the-while avoiding putative adverse effects often seen when chemical modification is not optimized.

4.6 Conclusion

The clinical application of polycationic gene carriers is impeded by un-defined design and formulation requirements that optimize safety and efficacy in vitro and in vivo. The work herein demonstrates that a copolymer comprised of polyethylene glycol (PEG) and a branched SS-PAEI, p(TETA/CBA/PEG) can be easily synthesized and used alone or in combination with the polycation, p(TETA/CBA), in order to easily formulate

putative polymeric gene delivery reagents mixtures that possess different physiochemical properties and function in order to identify optimal properties or delivery reagents. By using mixtures of a polycation and modified polycation, the synthesis of many putative gene delivery reagents is avoided, which is often required to study reagent characteristics on biological activity in order to identify products that are functionally viable for in vitro and in vivo use. Most importantly, this approach should be applied to other novel polycation-PEG preparations for this purpose and that described in Chapter 3. Lastly, biodistribution results suggest the potential advantage of negatively charged, nano-sized delivery reagents for passive tumor targeting compared to positively charged reagents and as such warrant further investigation.

Table 4.1. Polymer Characteristics

Sample	M_n (kDa)[a]	M_w (kDa)[a]	PDI (M_w/M_n)	Degree[b] Branching (DB)	S-S Bonds/ Molecule[c]	Moles PEG/p(TETA/CBA)[d]
p(TETA/CBA)	21.7	33.4	1.54	0.79	82.3 ± 3.2	-
p(TETA/CBA/PEG)	24.5	31	1.27	-	70.4 ± 2.7	0.68

[a]Absolute number average molecular weight (M_n), weight average molecular weight (M_w), and polydispersity (M_w/M_n) were determined using AKTA/FPLC. [b]Degree branching (DB) was determined by reduction of disulfide bonds with tris(2-carboxyethyl)phosphine (TCEP) followed by the protection of free sulfhydryls with N-ethylemaleimide (NEM) and analyzed by MALDI-TOF. DB was calculated using the equation presented in Chapter 3, Section 3.3.7. [c] The number of disulfide bonds per molecule was determined by reduction of disulfide bonds with immobilized TCEP followed by the reaction of free sylfhydryls with 5,5-Dithio-bis-(2-nitrobenzoic acid) and analyzed using UV-Vis spectrometry. [d]NMR was used to determine the molar ratio of PEG to p(TETA/CBA) backbone polycation by integrating p(TETA/CBA) and p(TETA/CBA/PEG) peak area under the curve (AUC).

Table 4.2. Biodistribution percent initial dose (%ID) of p(TETA/CBA/PEG) formulations

Formulation	w/w	Lung	Liver	Spleen	Kidney	Heart	Tumor
25% TCP	3	2.03 ± 0.94	14.78 ± 5.14	0.72 ± 0.78	2.02 ± 0.88	0.33 ± 0.67	0.61 ± 0.44
50% TCP	3	2.69 ± 1.30	14.67 ± 10.08	1.18 ± 0.39	0.27 ± 0.21	0.01 ± 0.00	0.32 ± 0.11
75% TCP	3	1.01 ± 2.23	9.35 ± 7.19	4.69 ± 5.99	0.46 ± 0.66	0.02 ± 0.00	0.35 ± 0.28
100% TCP	3	4.26 ± 3.28	10.59 ± 9.38	3.35 ± 1.69	0.88 ± 0.75	0.54 ± 0.99	1.37 ± 1.09
25% TCP	0.5	0.17 ± 0.10	0.22 ± 0.12	0.35 ± 0.07	0.16 ± 0.08	0.01 ± 0.02	0.52 ± 0.03
50% TCP	0.5	0.03 ± 0.01	0.14 ± 0.03	0.16 ± 0.07	0.01 ± 0.01	0.00 ± 0.00	0.30 ± 0.08
75% TCP	0.5	0.23 ± 0.03	0.22 ± 0.13	0.35 ± 0.06	0.08 ± 0.09	0.01 ± 0.02	0.52 ± 0.05
100% TCP	0.5	0.18 ± 0.11	0.63 ± 0.27	0.34 ± 0.15	0.14 ± 0.11	0.02 ± 0.00	0.24 ± 0.07

*Values are shown as percent of injected dose (%ID) 48 hrs. following systemic injection and represent the mean ± standard deviation (mean ± SD) (n=5)) . The p(TETA/CBA/PEG) formulations are abbreviated as TCP in this table.

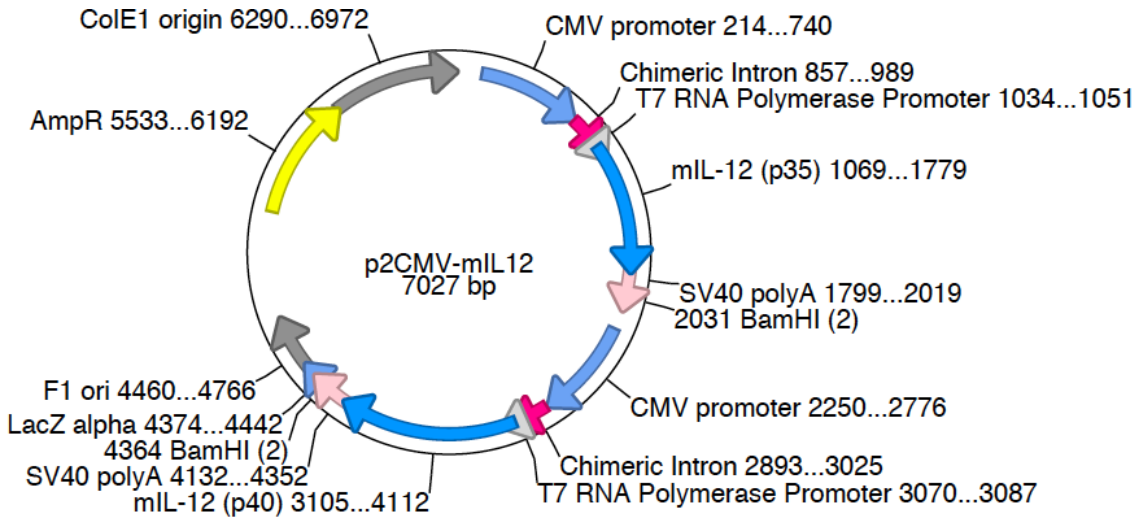


Figure 4.1. p2CMV-mIL12 plasmid map

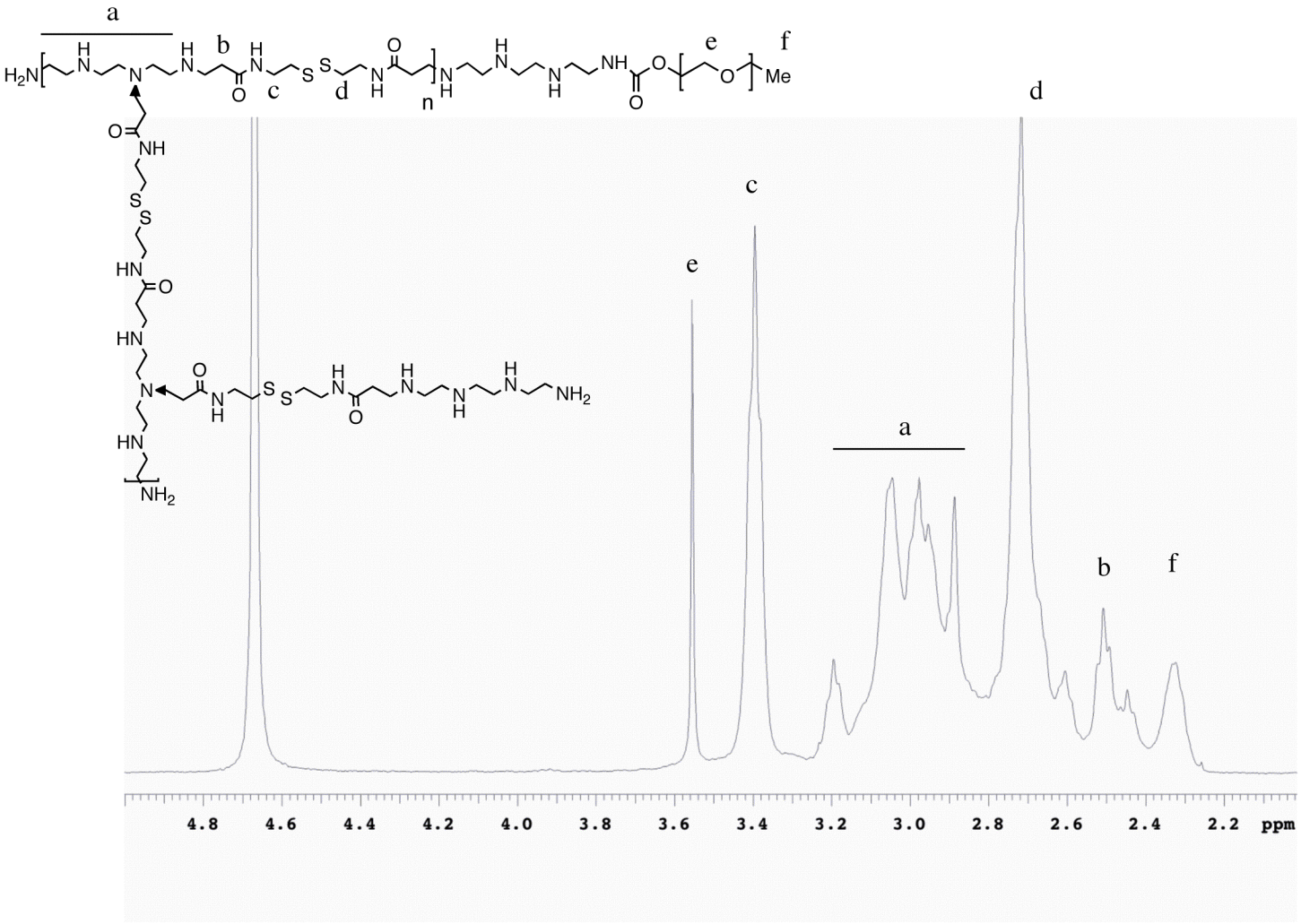


Figure 4.2. ^{1}H NMR Spectrum of p(TETA/CBA/PEG)

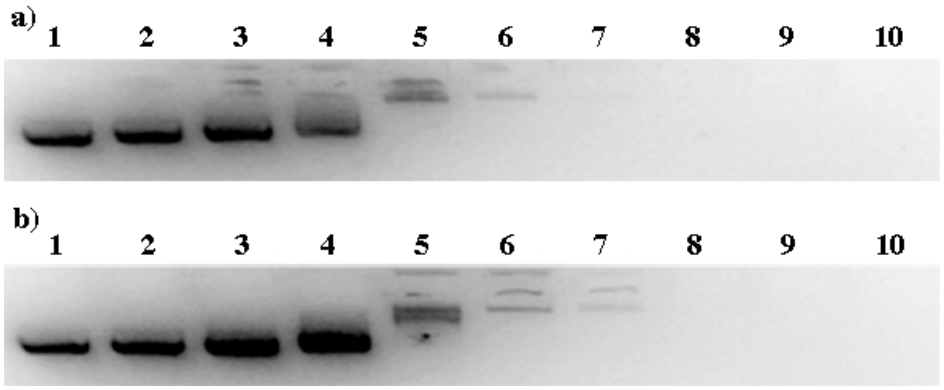
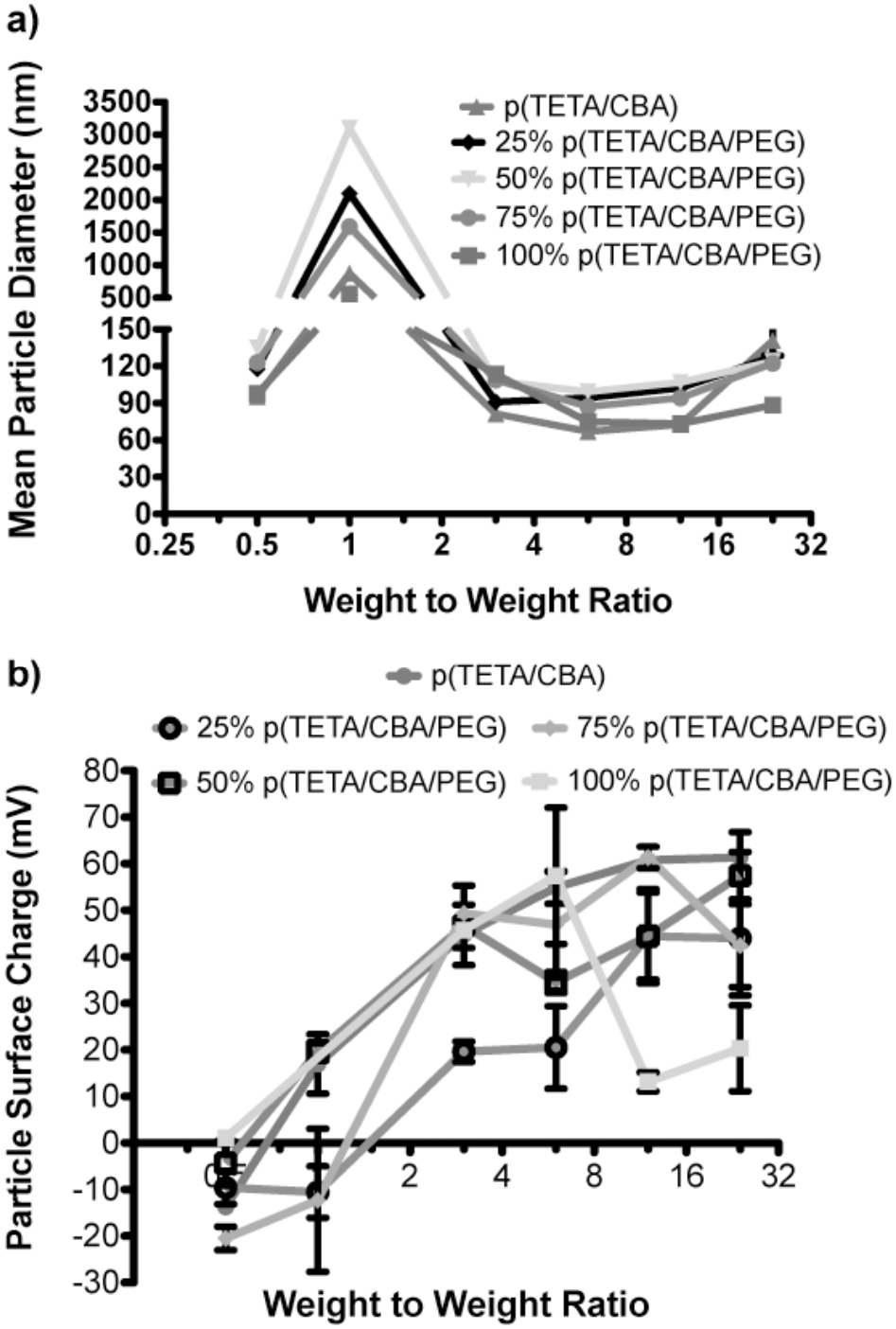


Figure 4.3. Polymer/pDNA complexation was assessed using a gel retardation assay. An equal volume solution of a) p(TETA/CBA) or b) mixture of p(TETA/CBA)/p(TETA/CBA/PEG) (75% p(TETA/CBA/PEG) was added to 500ng pDNA in HEPES buffered saline and allowed incubate for 30 min before being loaded on a 1% agarose gel, stained with EtBR, and then run for 30 min in TAE buffer. Total polymer amounts in solution used for both experiments (a and b) where 1) 0.0μg (0 w/w), 2) 0.025μg (0.05 w/w), 3) 0.05μg (0.1 w/w), 4) 0.1μg (0.2 w/w), 5) 0.5μg (1 w/w), 6) 1μg (2 w/w), 7) 2μg (4 w/w), 8) 3μg (6 w/w), 9) 6μg (12 w/w), 10) 8μg (16 w/w). Gel images were obtained using GelDoc Software.

Figure 4.4. Light scattering and ζ-potential measurements were performed to determine polymer/pDNA (polyplex) particle size (a) (diameter) and surface charge (b) at increasing polymer concentrations, indicated by polymer/pDNA weight-to-weight ratios on the graphs.


a)
Mean Particle Diameter (nm)
3500
3000
2500
2000
1500
1000
500
150
120
90
60
30
0
0.25
0.5
1
2
4
8
16
32
Weight to Weight Ratio
p(TETA/CBA)
25% p(TETA/CBA/PEG)
50% p(TETA/CBA/PEG)
75% p(TETA/CBA/PEG)
100% p(TETA/CBA/PEG)
b)
Particle Surface Charge (mV)
80
70
60
50
40
30
20
10
0
-10
-20
-30
0
2
4
8
16
32
Weight to Weight Ratio
p(TETA/CBA)
25% p(TETA/CBA/PEG)
75% p(TETA/CBA/PEG)
50% p(TETA/CBA/PEG)
100% p(TETA/CBA/PEG)

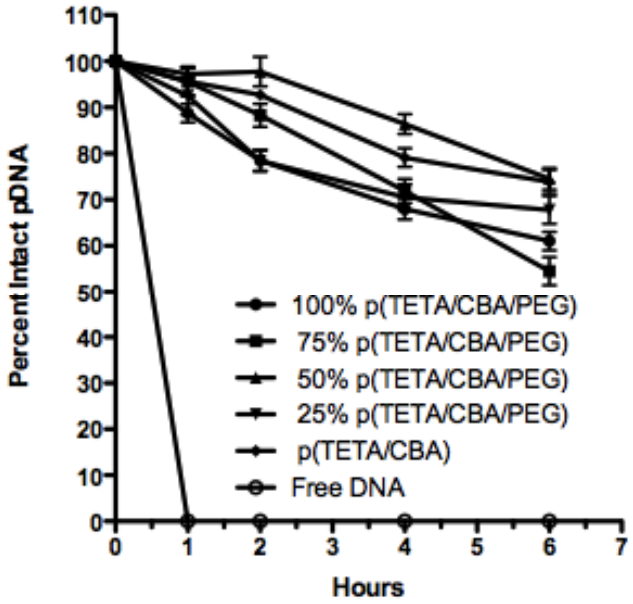


Figure 4.5. Polymer protection of pDNA from serum nuclease degradation was assessed over time. Free pDNA or polymer/pDNA mixtures at increasing polymer concentrations incubated in fresh rabbit serum. Samples were taken at 0, 1, 2, 4 and 6 hrs and evaluated for intact pDNA using gel electrophoresis (1% agarose). Percent intact pDNA was normalized to the amount of intact free (unprotected) pDNA at 0 hrs. using GelDoc software. All treatments offered significantly greater amounts of intact p(DNA) when compared to untreated control (Free p(DNA)) at 1, 2, 4 and 6 hrs. A posttest for linear trend proved significant for each treatment group and the percent intact p(DNA) at 6 hrs. was significantly different between all treatment groups with the exception of 50% p(TETA/CBA/PEG) and p(TETA/CBA). Data are represented as mean ± SD, $p < 0.05$ (n=3).

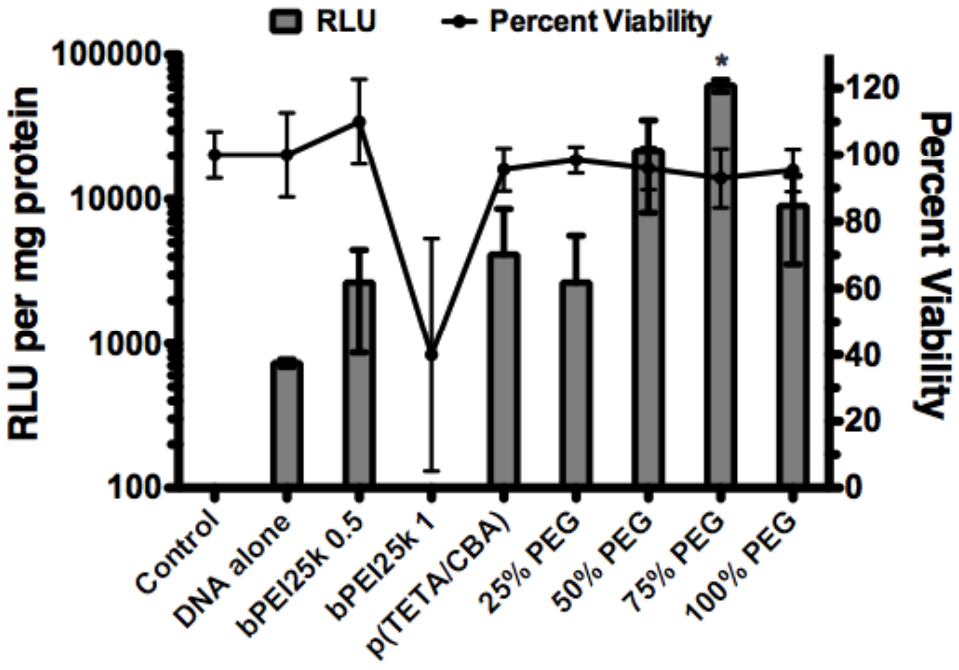


Figure 4.6. Transfection efficiency and cell viability for each polymer formulation were assessed in CT-26 cells. The formulations are denoted at the bottom of the graph. The wt% p(TETA/CBA/PEG) formulations are abbreviated as % PEG. bPEI was used at 0.5/1 and 1/1 w/w ratio for transfections. The other formulations were used at 12 w/w. The RLU/mg protein was not included for bPEI 1/1 due to the significant toxicity observed. The transfection data for 75% p(TETA/CBA/PEG) proved to be significantly better than p(TETA/CBA) and all other data sets were not significantly different from one another. Data are represented as mean ± SD, *$p < 0.05$ (n=6).

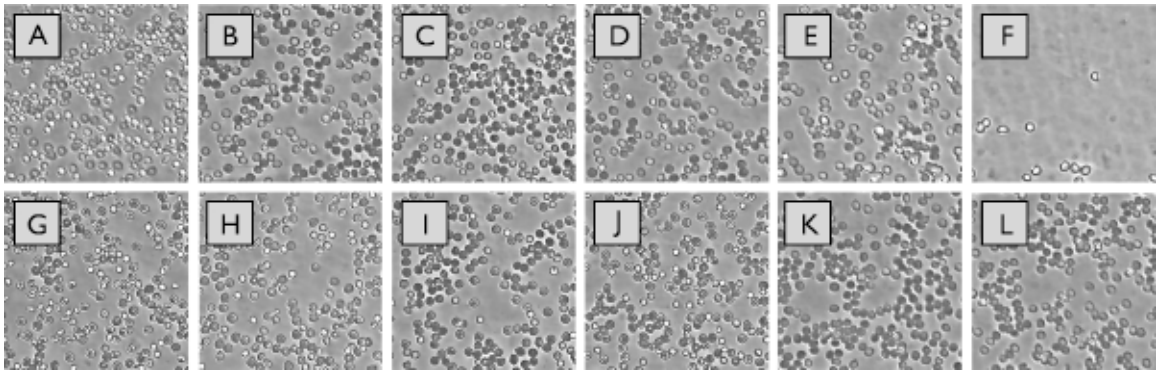


Figure 4.7. The erythrocyte lysis assay demonstrates that Red Blood Cell (RBC) lysis and aggregation occur at increasing concentrations of p(TETA/CBA) (A-F); whereas high concentrations of p(TETA/CBA/PEG) do not correlate with RBC lysis. Polymer concentrations are as follows: A and G, 0.277μg; B and H, 0.415μg; C and I, 0.83μg; D and J, 1.66 μg; E and K, 2.5μg; F and L, 3.3μg.

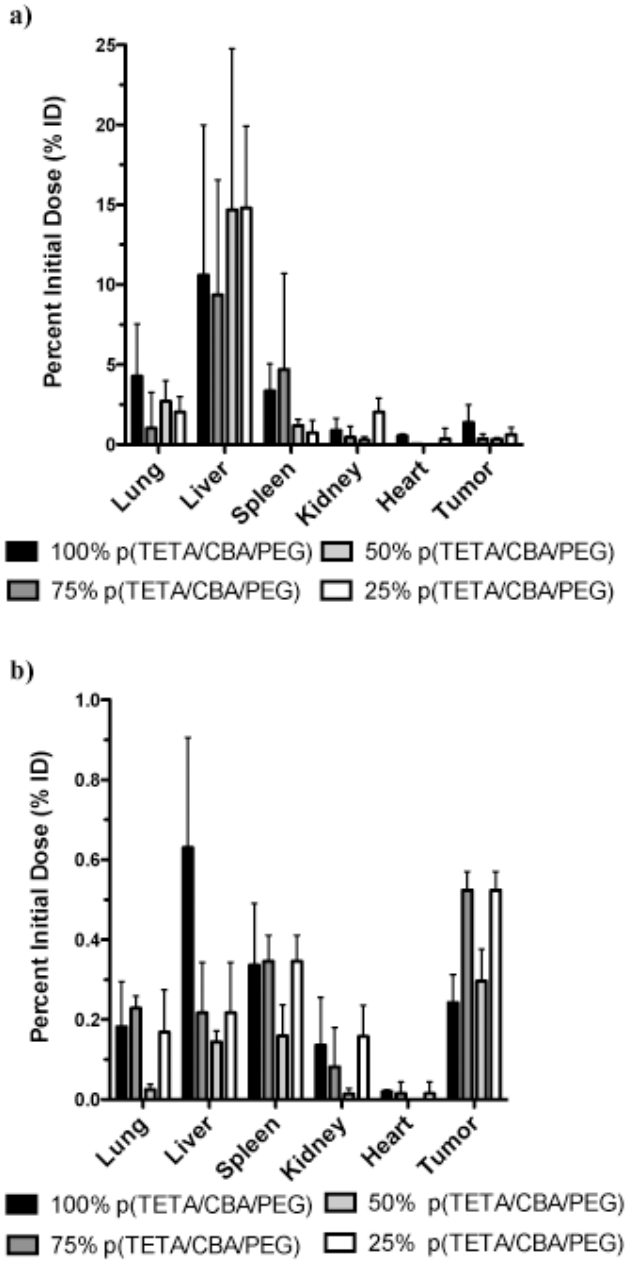


Figure 4.8. Biodistribution of pDNA following systemic injection using 100, 75, 50 and 25% p(TETA/CBA/PEG) formulations as delivery agents in a murine colon adenocarcinoma model. Data are represented as mean ± standard deviation (n = 5) of Percent Initial Dose (%ID). a) Positively charged complexes formed at 3 w/w. b) Negatively charged complexes formed at 0.5 w/w.

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CHAPTER 5

MIXTURE OF POLY(TRIETHYLENETETRAMINE/CYSTAMINE-BIS-ACRYLAMIDE) AND POLY(TRIETHYLENETETRAMINE/ CYSTAMINE-BIS-ACRYLAMIDE)-g-POLY(ETHYLENE GLYCOL)-c(RGDfC) TO ENHANCE siRNA DELIVERY TO CANCER-RELATED CELLS

5.1 Abstract

The cell-specific delivery of therapeutic nucleic acids using targeted gene carriers is vital for the clinical success of gene therapy. This is especially advantageous for cancer gene therapy regimens, as these therapeutics often abrogate cell proliferation and survival, which is detrimental to noncancerous tissues and may lead to adverse side effects. In the previous chapters, empirical evidence has shown that polycations can be used in conjunction with their PEGylated counterparts to improve the biological activity of gene carrier systems. The studies presented herein provide evidence that peptide-targeted gene carriers can also be used in conjunction with their nontargeted counterpart to improve gene carrier bioactivity and cell-specific delivery of nucleic acids in vitro.

5.2 Introduction

The delivery of nucleic acid payload constitutes one of the most critical steps for successful gene therapy (*1, 2*). Despite extensive research in the field, a major limitation for the clinical success of nonviral gene delivery reagents is their low transfection performance. Nonetheless, further research perseveres to modulate structural sizes, shapes and functional surface properties to improve their delivery. This research has proven to be quite slow and often costly, as multiple optimization steps and synthetic products are required to gradually improve the performance of gene delivery systems. In the previous two chapters, a novel approach through combining a polycation gene carrier with its modified/PEGylated counterpart has proven effective for easily altering gene carrier characteristics to identify optimal candidates for pDNA delivery. Using this approach, the synthesis of multiple candidates during the optimization process is avoided. The work outlined in this chapter covers the synthesis of a peptide-targeted polycation system to improve the delivery of therapeutic siRNA to targetable, cancer-related cells. Following synthesis, this putative siRNA delivery reagent will be evaluated, in conjunction with its nontargeted PEG-polycation and polycation counterpart for its ability to condense siRNA into nano-sized polyplex and enhance the delivery of therapeutic siRNA to specific cells that are pertinent to cancer and its therapy.

Since its discovery, RNA interference (RNAi) has showed increasing promise as a new approach toward the inhibition of gene expression in vitro and in vivo. As such, it presents large potential for the treatment of human diseases (*3-5*). The inhibition of gene expression via RNAi occurs with the sequence-specific binding of siRNA to the target messenger RNA (mRNA) and subsequent mRNA degradation, which halts the translation

of the encoded protein. Specifically, once inside the cell, duplexed siRNA associates with a multiprotein complex known as RNA induced silencing complex (RISC) and upon its incorporation, the siRNA sense strand is cleaved while the antisense strand remains incorporated and directs the RISC complex to the target mRNA for cleavage (*6*). Once the mRNA is cleaved, the antisense strand loaded RISC can cleave additional target mRNAs, thus making RISC-mediated regulation of gene expression a powerful catalytic event (*7*).

The ability of siRNA to down-regulate the production of specific mRNA sequences is particularly useful for the treatment of diseases that arise from the over-expression of specific genes, which is often the case for cancer. A major genetic player for cancer progression and metastasis is hypoxia-inducible factor 1 (HIF-1), which regulates the transcription of multiple genes involved in cancer cell survival, cell differentiation, vascularization of surrounding tissues and metastasis. HIF-1α over-expression, specifically, is a prognostic factor in many cancers and is associated with increased patient mortality. As a result, HIF-1α inhibitors have been developed as novel anticancer agents and have shown clinical promise (*8, 9*). RNAi of HIF-1α over-expression provides a promising therapeutic approach as well. However, in order to be therapeutically viable, delivery of RNAi sequences to the appropriate cells is mandatory. As with other nucleic acid based therapeutics, RNAi sequences have a difficult time crossing the cell membrane, as they have relatively high molecular weight (~13 kDa) and strong anionic charge imposed by the phosphodiester backbone. Naked siRNA is also highly susceptible to nuclease degradation, which renders it inactive. Therefore, the development of safe and efficient delivery vehicles for siRNA therapeutics is imperative

to circumvent these obstancles. As with pDNA, non-viral polycations offer great potential as siRNA delivery reagents (*10*).

Previous reports have demonstrated the enhanced delivery and tissue specificity of nucleic acid payload to cancer-related cell types that overexpress the cell-surface integrin receptor, $\alpha_V\beta_3$, which is a known marker of angiogenic vascular tissue and cancer cells although it is also expressed at relatively low levels on other cell types such as macrophages (*11, 12*). Enhanced gene delivery to these tissues was accomplished by incorporation of the $\alpha_V\beta_3$ integrin-binding peptide motif, RGD on the polycationic gene carrier branched polyethylenimine (bPEI) with a hydrophilic poly(ethylene glycol) (PEG) spacer (*13, 14*).

Based on these previous studies, a peptide-targeted polycation system was developed to improve the delivery of therapeutic siRNA against HIF-1α to $\alpha_V\beta_3$ expressing cells. This peptide targeted gene carrier was developed using a known $\alpha_V\beta_3$ integrin-binding RGD peptide with high affinity, c(RGDfC), in conjunction with a PEG spacer chemically conjugated to the biocompatible polycation used in Chapter 3, p(TETA/CBA) (*15, 16*). Mixtures of this targeted polycation, p(TETA/CBA)-g-PEG-c(RGDfC) and p(TETA/CBA) are evaluated to study their ability to complex with the therapeutic HIF-1α siRNA cargo as well as there biological activity in vitro. In vitro biological activity is investigated using a lung carcinoma cell line (A549), fibrosarcoma cell line (HT-1080) and an endothelial cell line (HUVEC) that are known to exhibit high levels of $\alpha_V\beta_3$ integrin and the therapeutic target, HIF-1α (*17-21*). To evaluate the tissue specificity of the gene carrier mixtures, an epithelial cell line (HEK293T) devoid of $\alpha_V\beta_3$ expression was used as a negative control (*22*). The results herein provide a promising

foundation for the use of polymeric mixtures to study targeted polymeric gene carrier systems and their use in siRNA delivery.

5.3 Materials and Methods

5.3.1 Materials

Triethylenetetramine (TETA), 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (EDC), *N*-hydroxysulfosuccinimide (sulfo-NHS) and HPLC grade methanol were purchased from Sigma-Aldrich (St. Louis, MO). N,N'-Cystamine-bis-acrylamide (CBA) was purchased from Polysciences, Inc. (Warrington, PA). Ultrafiltration devices and regenerated cellulose membranes (10 kDa) were supplied by Millipore Corporation (Billerico, MA). HO-PEG3500-COOH and MAL-PEG3500-NHS were purchased from JenKem USA (Allen, TX). The c(RGDfC) peptide was purchased from Peptides International, Inc. (Louisville, KY). siRNA sequences were purchased from Integrated DNA Technologies (IDT) (San Diego, CA). Primers specific for HIF-1α and GAPDH mRNA were obtained from the University of Utah Protein/Nucleic Acid Core Facility (Sat Lake City, UT). RNAeasy Purification columns were purchased from Qiagen (Valencia, CA). SuperScript III First-Strand synthesis system for RT-PCR was supplied by Invitrogen, Corp. (Carlsbad, CA) and the GoTaq Green Master Mix for PCR was obtained through Promega (Madison, WI). Dulbecco's Modified Eagle's Medium (DMEM), penicillin streptomycin, trypsin-like enzyme (TrypLE Express), and Dubelcco's phosphate buffered saline were purchased from Gibco BRL (Carlsbad, CA). EBM-2 with EGM-2 singlequots was purchased from Lonza. Fetal bovine serum (FBS) was purchased from Hyclone Laboratories (Logan, UT).

5.3.2 p(TETA/CBA) Synthesis

Synthesis of p(TETA/CBA) was performed with minor adaptation of the previously described method. The polymerization reaction was run at 30°C for 12 hrs, at which time excess TETA was added to the reaction mixture and the reaction was allowed to run for an additional 24 hrs to ensure that the reaction had been terminated. The pH was subsequently adjusted to 7.0 and the polymer was purified by ultrafiltration using a 10kDa regenerated cellulose membrane. p(TETA/CBA) was obtained by lyophilization. Composition of the polymer was monitored using ^{1}H NMR (400 MHz, D_2O). p(TETA/CBA) δ 2.61 (COC*H*2CH2NH, 4H), 2.72 (NHCH2C*H*2S-S, 4H), 2.90-3.21 (COCH2C*H*2NHC*H*2C*H*2,16H), 3.41 (NHC*H*2CH2S-S, 4H).

5.3.3 p(TETA/CBA)-g-PEG Synthesis

The carboxylic acid of HO-PEG-COOH was activated via NHS/EDC coupling using 4x molar excess of NHS and EDC to HO-PEG-COOH for 1 hr in PBS buffer (pH 7.4). The active HO-PEG-NHS ester was subsequently added dropwise to a stirred solution of p(TETA/CBA) dissolved in PBS buffer (pH 7.4). The reaction proceeded at room temperature for 4 hrs. When the reaction was complete, the sample was purified by ultrafiltration (10kDa MWCO) before being lyophilized. The composition of p(TETA/CBA)-g-PEG3500 copolymer conjugate was analyzed using ^{1}H NMR (400 MHz, D_20). p(TETA/CBA)-g-PEG3500 δ 2.61 (COC*H*2CH2NH, 4H), 2.72 (NHCH2C*H*2S-S, 4H), 2.90-3.21 (COCH2C*H*2NHC*H*2C*H*2,16H), 3.41 (NHC*H*2CH2S-S, 4H), 3.45-3.7 (CH2CH20, 4H).

5.3.4 p(TETA/CBA)-g-PEG-c(RGDfC) Synthesis

The synthesis of p(TETA/CBA)-g-PEG-c(RGDfC) began by dissolving a known amount of p(TETA/CBA) in PBS buffer (pH 7.4), While the p(TETA/CBA) solution stirred at room temperature, 1.3x molar excess of MAL-PEG3500-NHS solution was added dropwise to p(TETA/CBA). The reaction proceeded for 30 min, at which time 2x molar excess c(RGDfC) dissolved in 3% acetic acid was added to the reaction mixture. The pH of the reaction mixture was quickly adjusted to pH 7 using a NaOH. The reaction proceeded for 2 additional hours to allow the free sulfhydryl on the free cysteine contained in c(RGDfC) to react with the maleimide moiety at the distal end of the PEG chains. The reaction mixture was subsequently purified by ultrafiltration (10kDA) in order to remove un-reacted peptide and PEG before being lyophilized. The composition of p(TETA/CBA)-g-PEG-c(RGDfC) copolymer was analyzed using ^{1}H NMR to determine degrees of chemical conjugation to p(TETA/CBA). ^{1}H NMR (400 MHz, D_20). p(TETA/CBA)-g-PEG-c(RGDfC) δ 2.61 (COC*H*2CH2NH, 4H), 2.72 (NHCH2C*H*2S-S, 4H), 2.90-3.21 (COCH2C*H*2NHC*H*2C*H*2,16H), 3.41 (NHC*H*2CH2S-S, 4H), 3.45-3.7 (CH2CH20, 4H), 7.1-7.3 (CH, 5H).

5.3.5 Polymer Characteristics

Absolute molecular weight analysis for p(TETA/CBA), and p(TETA/CBA)-g-PEG and p(TETA/CBA)-g-PEG-c(RGDfC) was performed using AKTA/FPLC (Amersham Pharmacia Biotech Inc.) coupled to a light-scattering detector and using the polymer refractive index increment (dn/dc) for each sample. A Superose 6 110/300 GL column was used for polymer sample separation. Poly[N-(2-

hydroxypropyl)methacrylamide] (poly(HPMA)) standards were injected onto the column prior to experimental sample analysis in order to ensure the column was clean and functional. Experimental and standard polymer samples were dissolved in degassed and filtered (0.2 μm (Nylon, Alltech)) 0.3 M NaOAc, pH 4.4 with 30% (v/v) acetonitrile eluent buffer. The flow rate was set to 0.4 mL/min.

5.3.6 Determination of Polymer Disulfide Bonds

Disulfide bond content of each polymer was determined using 5,5-Dithio-bis-(2-nitrobenzoic acid) (Ellman's reagent) following the manufacturer's protocol with minor adaptation (Pierce). In Brief, cysteine hydrochloride was used as a stardard to generate a standard curve monohydrate. Polymer molecular weights were used to estimate the number of disulfide bonds in each polymer and to further normalize the average thiolate concentration so as to fall within the standard range. Each polymer sample was reduced using 10x immobilized (Tris(2-carboxyethyl)phosphine hydrochloride) (TCEP) gel in buffer (0.1 M sodium phosphate, pH 8, 1mM EDTA). Following 30 min incubation to ensure complete polymer reduction, the samples were centrifuged at 1000 x g for 2 min and the reduced polymer solutions were collected. Reduced polymers were subsequently added to the Ellman's reagent in buffer and allowed to incubate at room temperature for 20 min after mixing. The sample absorbance at 412 nm was measured using UV-Vis spectroscopy. The thiol concentration was determined using the standard curve. Polymer average molecular weights were then used to determine the number of disulfide bonds per polymer.

5.3.7 Polyplex Formation

In all cases polyplex was formed with a known amount of siRNA and a corresponding and desired amount of polymer each suspended in HEPES buffer (20 mM, pH 7.4, 5% glucose). The two solutions were combined, lightly vortexed and allowed to equilibrate for 30 min. When mixtures of two different polymers were used to form polyplex, a known amount of siRNA was brought up in solution and a corresponding and desired concentration of p(TETA/CBA) and p(TETA/CBA)-g-PEG or p(TETA/CBA)-g-PEG-c(RGDfC) were prepared by mixing the appropriate species at specified ratios before being added to the siRNA solution, lightly vortexed and allowed to equilibrate.

5.3.8 Light Scattering Measurements

The particle size of polymer/siRNA particles (polyplex) was measured at 25°C on a dynamic light scattering (DLS) unit, BI-9000AT with a Digital Autocorrelator. Polyplexes were prepared by adding equal volume polymer or polymer mixture solutions (200μl) at increasing concentrations in filtered HEPES buffer (20 mM, pH 7.4, 5% glucose) to a desired amount of siRNA (40μg), also suspended in filtered HEPES buffer (200μl). Polyplexes were allowed to equilibrate for 30 min and were subsequently diluted in filtered milliQ water to a final 2 mL volume.

5.3.9 Gel Retardation Assay

Polymer/siRNA complexation was investigated by forming polyplex prepared at increasing polymer-to-siRNA weight-to-weight ratios (w/w) in HEPES buffered solution (20 mM HEPES, pH 7.4, 5% glucose). Polyplexes were formed using 200ng siRNA and

corresponding amount of polymer or polymer mixture. The polymer/siRNA solutions were mixed, lightly vortexed and were then allowed to incubate at room temperature for 30 min. Following equilibration, the solutions were loaded on a 1% agarose gel that was stained with ethidium bromide (EtBr, 5μg/mL) at 104 V for 30 min in TAE (40 mM Tris-acetate, 1 mM EDTA) buffer. The siRNA was visualized using GelDoc software.

5.3.10 Cell Culture

Human lung carcimoma cells (A549), human fibrosarcoma cells (HT-1080) and human embryonic kidney cells (HEK293T) were all cultured in DMEM containing 10% FBS and 1% penicillin-streptomycin at 37°C in a humidified incubator with an atmosphere containing 5% (v/v) CO_2. Human Umbilical Vein Endothelial Cells (HUVEC) (Invitrogen) were cultured in EBM-2 media with EGM-2 singlequots at 37°C in a humidified incubator with an atmosphere containing 5% (v/v) CO_2.

5.3.11 In Vitro Transfections

The delivery of siRNA sequences against HIF-1α mRNA in cell culture was performed using polyplex formed by the addition of p(TETA/CBA), 75% p(TETA/CBA)-g-PEG/25% p(TETA/CBA) and/or 75% p(TETA/CBA)-g-PEG-c(RGDfC)/25% p(TETA/CBA) to 0.115μg siRNA in HEPES buffer at polymer-to-siRNA 6 w/w and equilibration for 30 min. Cells were previously plated in 24-well plates containing 0.5mL media. When HUVEC cells were plated, the media was supplemented with 200ng/mL $VEGF_{165}$. Once the cells were approximately 70% confluent, the polyplexes prepared, as described above, were added to each respective well (20nM

siRNA) containing fresh culture media with 10% FBS. The cells remained in the incubator for a total of 48 hrs before being collected and analyzed for HIF-1α mRNA expression. A c(RGDfC) competition assay was performed using each cell line by pre-incubating the cells with a 20-fold molar excess of free peptide (1.5μM) 30 min. prior to transfection as previously described (*23, 24*). The siRNA sequences specific for HIF-1α mRNA were: 5'-TAGUUGGGUCUGUAUAGGUG-3'and 5'-TCACCUAUACAGACCCAACU-3'. The scrambled siRNA sequences were: 5'-TUUUAAGGGCGCAAUGCGCA-3' and 5'-TTGCGCATTGCGCCCTTAAA-3'.

5.3.12 Cell Viability Assay

Cells were plated in 24-well plates and gene transfections were carried out when the cells were approximately 70% confluent. Polyplexes were prepared as they were for transfection experiments. The respective cell cultures were transfected in the presence of serum with the addition of 20 μl equilibrated polyplex in HEPES buffer solution (0.115μg siRNA) to each well. Cells were left to incubate for a total of 18 hrs before analyzing cell viability using an MTT assay (Sigma) following the manufacturers directions. Percent cell viability was determined relative to untreated cells (n=6).

5.3.13 RT-PCR

In order to semiquantitatively evaluate HIF-1α mRNA expression and its potential knockdown following cell transfection, 48 hrs following treatment the cells were rinsed with 1ml PBS and collected using trypsin digestion. Total RNA was collected using Qiagen RNeasy Mini Spin Columns by following the manufacture's

directions and the amount of RNA collected was assessed using a Nanodrop ND-1000 UV spectrophotometer (Absorbance 240nm). A total of 200ng of RNA was reverse transcribed using SuperScript III First-Strand synthesis system (Invitrogen). Resulting cDNA was further amplified (2μl) using GoTaq Green Master Mix for PCR (Promega). The sequence of the specific nucleotide primers HIF-1α (176 bp) were forward: 5'-TGGTTCTCACAGATGATGGTGAC-3' reverse: 5'- GCTTCGCTGTGTGTTTTGTTC-3', GAPDH (216 bp) forward: 5'-GACCCCTTCATTGACCTCAACTAC-3' reverse: 5'-AGATGATGACCCTTTTGGCTCC-3'. The PCR reaction consisted of 95°C for 2 min, 28 cycles at 95°C for 30 sec then 57°C for 30 sec and 72°C for 30 sec, which was followed by an extension of 5 min at 72°C before the PCR block dropped to 4°C to maintain the PCR product. PCR products were separated by electrophoresis in a 2% agarose gel stained with ethidium bromide (EtBr, 5μg/mL) and run at 110 V for 30 min. Products were visualized using GelDoc software.

5.3.14 Statistics

The experimental data for cell viability are assessed as mean ± SD (n = 6). For RT-PCR analysis and mRNA knockdown, the data are represented as mean ± SEM derived from two separate experiments where n = 3. A one-way ANOVA is used in conjunction with a Tukey's post-hoc test to determine if treatment groups are statistically different from each other. A $p < 0.05$ was considered statistically significant.

5.4 Results

5.4.1 p(TETA/CBA)-g-PEG and p(TETA/CBA)-g-PEG-c(RGDfC) Synthesis and Characterization

The nucleic acid carrier p(TETA/CBA)-g-PEG-c(RGDfC) was synthesized to enhance the delivery of siRNA to cancer-related cell types compared to non-cancerous cells, and to provide evidence that polymeric gene carrier mixtures can be used for targeted nucleic acid delivery systems in addition to the PEGylated systems covered in Chapters 3 and 4. The use of mixtures for gene carrier formulation can assist in carrier development without the need of synthesizing many different putative products, which can be time and labor intensive as well as costly. The synthesis scheme for p(TETA/CBA)-g-PEG-c(RGDfC) and the structure of the c(RGDfC) peptide used are shown in Figure 5.1. ^{1}H NMR was used to confirm the synthesis of p(TETA/CBA)-g-PEG-c(RGDfC) (Figure 5.2) and p(TETA/CBA)-g-PEG (Figure 5.3) as well as estimate the amount of PEG-c(RGDfC) or PEG incorporated in the respective gene carrier products by integrating peak AUC. Peak integration indicates approximately 0.88 and 0.93 moles PEG incorporated in p(TETA/CBA)-g-PEG-c(RGDfC) and p(TETA/CBA)-g-PEG, respectively. ^{1}H peak integration also indicates 97% of the PEG chain chemically conjugated to p(TETA/CBA) had c(RGDfC) peptide also present (Table 5.1). Molecular weight analysis using FPLC indicates similar molecular weights and polydispersity indices (PDIs) below 1.8 for all three polymers used. Molecular weight and PDI differences between the three polymer products were observed. Specifically, p(TETA/CBA) had a relatively low absolute molecular weight and high PDI. Because p(TETA/CBA) was only purified once, while the modified p(TETA/CBA) products were

obtained following two purification steps, oligomer polycations may not have been completely eliminated from the sample. Moreover, p(TETA/CBA)-g-PEG-c(RGDfC) had the a relatively high molecular weight and PDI. Because c(RGDfC) was conjugated to this product via its free sulfhydryl group, potential disulfide exchange with the p(TETA/CBA) backbone may occur as a side reaction, which will lead to uncontrolled crosslinking of the polycation resulting in higher molecular weight products and an increase in PDI. The number of disulfide bonds per molecule is also presented for completeness (Table 5.1).

The complexation of siRNA with p(TETA/CBA), 25%p(TETA/CBA)/75%p(TETA/CBA)-g-PEG, 25%p(TETA/CBA)/75%p(TETA/CBA)-g-PEG-c(RGDfC) and p(TETA/CBA)-g-PEG-c(RGDfC) alone was tested by gel retardation. Results from this study demonstrate strong interaction of p(TETA/CBA) with siRNA as the amount of polymer is increased, as siRNA migration through the gel is abrogated at 1 w/w and above indicating complete complexation of the two entities. Interaction between siRNA and p(TETA/CBA)-g-PEG-c(RGDfC) appears to occur at 3 w/w, as indicated by a shift in siRNA band migration, however, complete entrapment of siRNA does not occur at 12 w/w or below. Combining p(TETA/CBA) with p(TETA/CBA)-g-PEG-c(RGDfC) at 25%/75%, respectively, improves siRNA complexation compared to p(TETA/CBA)-g-PEG-c(RGDfC) alone and fully encapsulated the siRNA at 3 w/w ratio and above. Complete siRNA encapsulation is also seen at 3 w/w using 25%/75% p(TETA/CBA) and p(TETA/CBA)-g-PEG, respectively (Figure 5.4). As shown in previous chapters, increased polymer concentrations of the modified polycations are required to encapsulate nucleic acid, which indicates that PEG

and/or PEG-ligand modification of a gene carrier can mitigate its ability to interact with the payload of interest. This effect has been seen in studies where PEG and targeting ligand conjugation affect nucleic acid condensation (*14, 24*).

To further investigate the influence of PEG-ligand modification on nucleic acid complexation, mean particle diameter of polyplex formed between siRNA and the aforementioned gene carrier formulations was assessed using Dynamic Light Scattering (DLS). The p(TETA/CBA), 25%p(TETA/CBA)/75%p(TETA/CBA)-g-PEG and 25%p(TETA/CBA)/75%p(TETA/CBA)-g-PEG-c(RGDfC) formulations form particles near 200nm at 6 w/w. The p(TETA/CBA)-g-PEG-c(RGDfC) copolymer generates slightly larger particle than these formulations when combined with siRNA at 6w/w. No further siRNA condensation is accomplished when p(TETA/CBA)-g-PEG-c(RGDfC) is used at 12 w/w (Figure 5.5). These complexation results corroborate those from the gel retardation study. The mean particle sizes using the p(TETA/CBA) formulation at 6 w/w began to show smaller particle sizes biased by excess, uncomplexed polymer in solution. This effect was seen using 25%p(TETA/CBA)/75%p(TETA/CBA)-g-PEG and 25%p(TETA/CBA)/75%p(TETA/CBA)-g-PEG-c(RGDfC) formulations at 12 w/w. This, however, did not occur when p(TETA/CBA)-g-PEG-c(RGDfC) was used alone, which indicates that complete siRNA condensation does not occur at or below 12 w/w and that PEG- c(RGDfC) modification may interfere with complexation.

5.4.2 p(TETA/CBA)-g-PEG and p(TETA/CBA)-g-PEG-c(RGDfC) In Vitro Bioactivity

The bioactivity of p(TETA/CBA), 25%p(TETA/CBA)/75%p(TETA/CBA)-g-PEG and 25%p(TETA/CBA)/75%p(TETA/CBA)-g-PEG-c(RGDfC) formulations was assessed in vitro. These specific formulations were chosen based on the aforementioned results obtained from the complexation studies, which indicated that these gene carrier formulations could completely complex with siRNA cargo at 6 w/w. Each formulation was complexed with HIF-1α siRNA for a final concentration equal to 20 nM siRNA per well. Formulation transfection efficiency was evaluated by assessing the relative knockdown of HIF-1α mRNA relative to untreated control in a lung carcinoma cell line (A549), a fibrosarcoma cell line (HT-1080) and an endothelial cell line (HUVEC) all known to express relatively high levels of $\alpha_v\beta_3$ integrins on their cell surface compared to normal, noncancerous tissues (*17-21*). Transfections were also performed in Human Embryonic Kidney cells (HEK293T), which serves as a negative control, as the basal levels of αvβ3 integrin expression are extremely low (*22*). For accurate analysis HIF-1α expression levels were normalized by the internal control, GAPDH.

Significantly enhanced HIF-1α mRNA knockdown occurred in the $\alpha_v\beta_3$ expressing cell lines when p(TETA/CBA)-g-PEG-c(RGDfC) was used in formulation with P(TETA/CBA) to deliver HIF-1α siRNA compared to the untargeted formulations, p(TETA/CBA) and p(TETA/CBA)-g-PEG (Figure 5.6). This result indicates that incorporation of the targeting peptide c(RGDfC) on the p(TETA/CBA)-g-PEG backbone is sufficient to enhance nucleic acid delivery to these cell lines. When these cells are pre-incubated with 20-fold excess soluble c(RGDfC) peptide prior to transfection with the

formulation including p(TETA/CBA)-g-PEG-c(RGDfC), HIF-1α siRNA transcellular delivery is abrogated (Figure 5.6). This result suggests that the c(RGDfC) peptide is necessary for enhanced nucleic acid delivery to these cell types. Conversely, when HEK293T cells are treated with the same formulations, no significant HIF-1α mRNA knockdown is seen, which further indicates that the enhancement in siRNA delivery using p(TETA/CBA)-g-PEG-c(RGDfC) is enhanced by high levels of $\alpha_v\beta_3$ integrin expression an specific cell lines (Figure 5.6). In all cell lines, the delivery of scrambled HIF-1α siRNA using the p(TETA/CBA)-g-PEG-c(RGDfC) formulation resulted in no significant knockdown in HIF-1α mRNA (Figure 5.6). This result indicates that the HIF-1α siRNA used for the transfection experiments is sequence specific for HIF-1α mRNA. Moreover, the cell viability of each cell line was assessed following treatment with each gene carrier formulation. No significant toxicity was observed, indicating that the nucleic acid formulations tested are biocompatible and preincubation with free c(RGDfC) peptide imposes no cellular toxicity (Figure 5.6).

5.5 Discussion

The co-polymer, p(TETA/CBA-g-PEG-c(RGDfC), was synthesized to develop a nucleic acid carrier that can enhance payload delivery to cancer-related cell types. The studies described in this chapter provide evidence that peptide-targeted gene carriers can be used in conjunction with their nontargeted counterpart to formulate a gene carrier to improve cell-specific delivery of nucleic acid compared to carriers devoid of the targeting peptide in vitro. Cell specific targeting of cancer-related cell types was accomplished using a peptide moiety, c(RGDfC) that has known affinity for $\alpha_v\beta_3$ integrin, which is

often highly expressed in the tumor environment compared to normal tissues (*14, 23*). The high expression levels of these integrins on related cell types can be exploited using peptide moieties that confer specificity for these integrins to drive cellular uptake of nucleic acid payload.

Complexation studies using gel electrophoresis and Dynamic Light Scattering (DLS) revealed that p(TETA/BA), 25%p(TETA/CBA)/75%p(TETA/CBA)-g-PEG and 25%p(TETA/CBA)/75%p(TETA/CBA)-g-PEG-c(RGDfC) could efficiently form polyplex with siRNA at 6 w/w. Conversely, p(TETA/CBA)-g-PEG-c(RGDfC) alone was not able to completely complex siRNA at 6 w/w. These results indicate that the covalent attachment of PEG-c(RGDfC) to the p(TETA/CBA) backbone interferes with siRNA condensation. This effect could be overcome using a gene carrier mixture comprised of 25%p(TETA/CBA)/75%p(TETA/CBA)-g-PEG-c(RGDfC). In order to confirm that this mixture formulation maintained p(TETA/CBA)-g-PEG-c(RGDfC) in the polyplexes formed, and thus improve cell-specific delivery to cells containing high levels of $\alpha_v\beta_3$ integrin expression, cell transfection experiments were performed.

Cell transfection experiments using the 25%p(TETA/CBA)/75%p(TETA/CBA)-g-PEG-c(RGDfC) formulation demonstrated enhanced and cell-specific delivery of HIF-1α siRNA to $\alpha_v\beta_3$ integrin expressing cell lines, indicating that this formulation maintained the p(TETA/CBA)-g-PEG-c(RGDfC) species in polyplexes formed. This finding is particulary important for this study, which aims to extend the use of mixture formulations to targeted nucleic acid delivery systems. A previous report, and that which is outlined in Chapter 3, indicates that polycation and PEG-polycation mixtures can be used to alter gene carrier properties and improve their bioactivity. If both species are not

incorporated into formed polyplex, altering the relative amount of each species between the formulations to monitor effective changes on bioactivity or biocompatibility is moot. It is possible that because of steric implications or charge masking effects, that the PEGylated or targeted species will exhibit a reduced capacity to complex with nucleic acid. If this is the case, when high N/P or w/w ratios are used for polyplex formation and nucleic acid delivery, free uncomplexed polymer can interact with serum proteins to prevent serum interaction with polyplex, which may otherwise abrogate transcellular delivery, and will result in high transgene delivery that is nonspecific. Because this study reveals that enhanced delivery occurs using a mixture with a targeted gene carrier, compared to nontargeted formulations, the enhanced nucleic acid delivery observed is not a product of nonspecific masking of serum proteins by free polymer, but rather receptor mediated enhancement due to the incorporation of the targeted species in the polyplexes formed.

The putative cell toxicity of each formulation was examined in the cell lines used to evaluate transcellular delivery of HIF-1α siRNA. The formulations used were non-toxic to all cell lines. Previous reports have indicated that free, soluble peptide that contain the RGD motif used in our studies, can adversely affect the cell viability of human endothelial cells (HUVEC) and human leukemia cells (HL-60) (*25*). If cell viability is compromised, transfection results have limited validity. In fact, our studies indicate no cell toxicity of soluble c(RGDfC) in any of the cell lines tested, including cells pretreated with free c(RGDfC) peptide to perform the competition assay. The discrepancy between the previous studies and ours is likely explained by the amount of free peptide used in the respective experiments. Chemotaxis and cell viability of

HUVEC cells was compromised when peptide concentrations reached approximately 380 μM, whereas the free peptide concentration used for the competition assay in this study was substantially less (1.5 μM) (*25*). Taken together, the studies described in this chapter provide evidence for the use of polymeric mixtures to study peptide-targeted gene carrier systems to improve gene carrier performance and biological activity.

5.6 Conclusion

In conclusion, the empirical studies herein demonstrate that polymeric mixtures comprised of a peptide-targeted gene carrier and a corresponding polycationic backbone can be used to improve gene carrier properties and achieve enhanced siRNA delivery to targeted cell types. These findings are important for more rapid and cost-effective development of improved nucleic acid carriers, as this approach may be applied to other peptide-targeted preparations to easily alter carrier characteristics and identify optimal formulations for laboratory and clinical application.

Table 5.1. Polymer Characteristics

Sample	M_n (kDa)[a]	M_w (kDa)[a]	PDI (M_w/M_n)	DB[b]	S-S Bonds/ Molecule[c]	Moles PEG[d]	Moles c(RGDfC)[d]
p(TETA/CBA)	5.5	9.83	1.78	0.74	28.9 ± 4.2	--	--
p(TETA/CBA)-g-PEG	15.5	20.47	1.32	--	52 ± 2.6	0.93	--
p(TETA/CBA)-g-PEG-c(RGDfC)	15.7	29.06	1.8	--	73 ± 3.3	0.88	0.85

[a]Number average molecular weight (M_n), weight average molecular weight (M_w), and polydispersity (M_w/M_n) determined using FPLC. [b] Degree branching (DB) was determined by reduction of disulfide bonds with Tris(2-carboxyethyl)phosphine hydrochloride (TCEP) followed by the protection of free sulfhydryls with *N*-ethylmaleimide (NEM) and analyzed by MALDI-TOF. Calculation was performed using the equation presented in Chapter 3 Section 3.3.7. [c] The number of disulfide bonds per molecule was determined by reduction of disulfide bonds with immobilized TCEP followed by the reaction of free sulfhydryls with 5,5-dithio-bis-(2-nitrobenzoic acid) and analyzed using UV-Vis spectrometry. [d] NMR was used to determine the amount of PEG and c(RGDfC) in the samples and the values are expressed as the molar ratio of PEG or c(RGDfC) per mole p(TETA/CBA). The molar ratio was determined by integrating peak AUC and using the absolute molecular weight shown above.

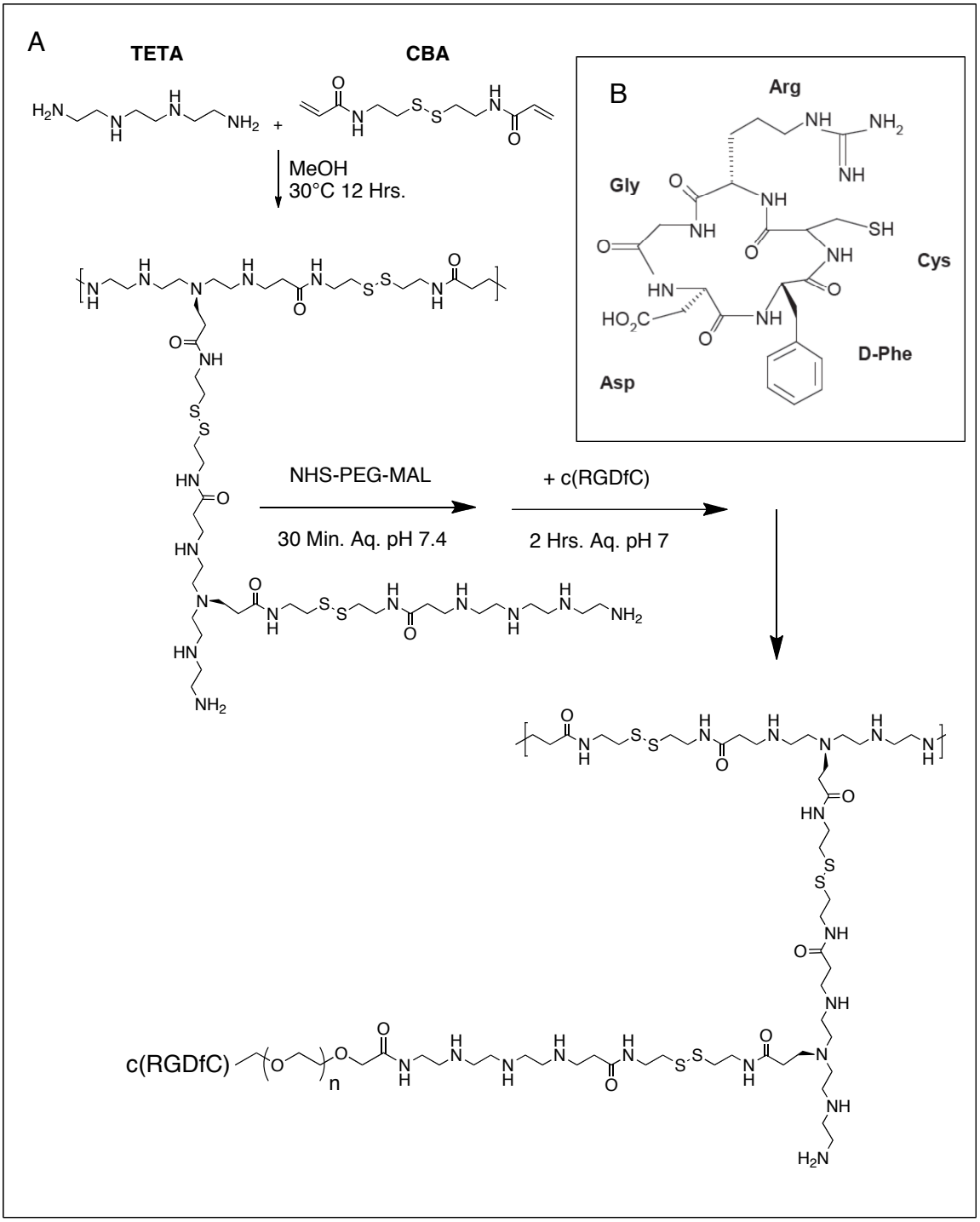

Figure 5.1. p(TETA/CBA)-g-PEG-c(RGDfC) a) synthesis scheme and b) structure of c(RGDfC) peptide used in the synthesis.

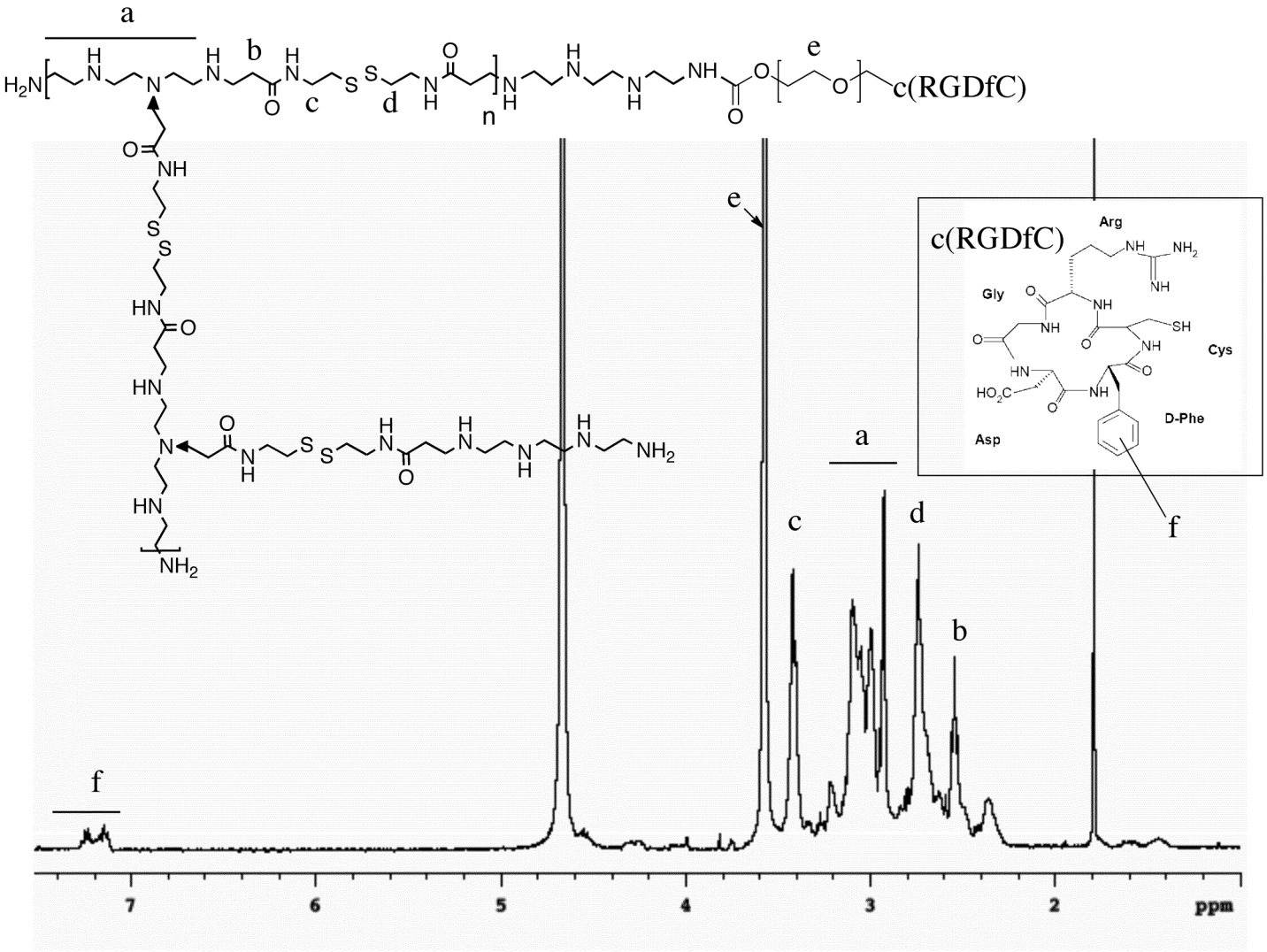

Figure 5.2. ^{1}H NMR Spectrum of p(TETA/CBA)-g-PEG-c(RGDfC). The chemical structure of c(RGDfC) is shown separately.

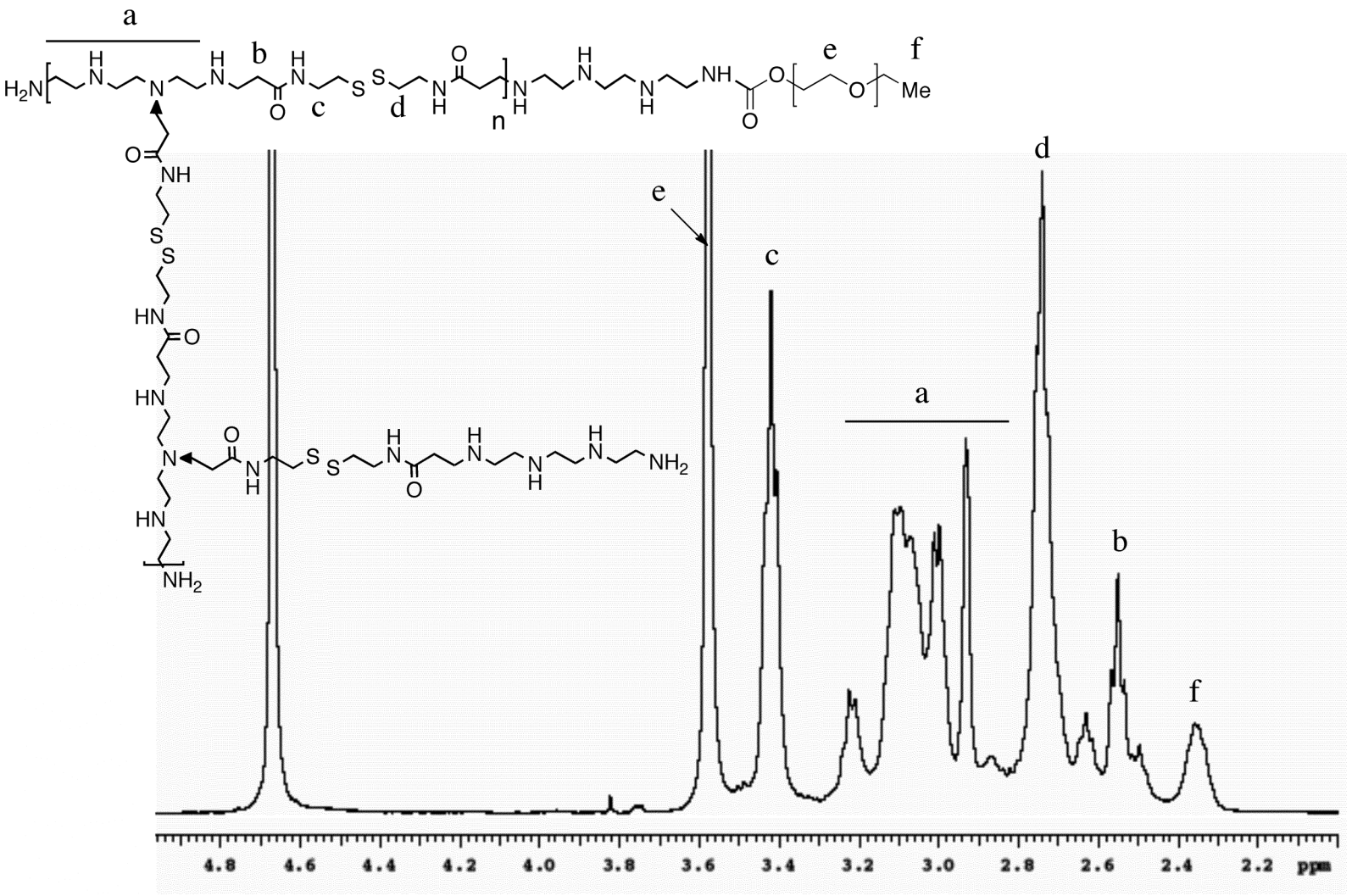

Figure 5.3. ^{1}H NMR Spectrum of p(TETA/CBA)-g-PEG.

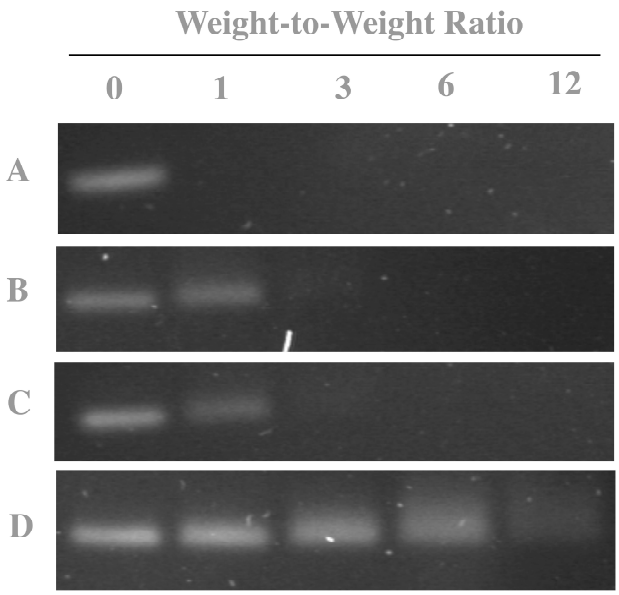

Figure 5.4. Polmer/siRNA complexation assessed using a gel retardation assay. Equal volume solutions of a) p(TETA/CBA), b) 25% p(TETA/CBA)/75%p(TETA/CBA)-g-PEG, C) p(TETA/CBA)/75%p(TETA/CBA)-g-PEG-c(RGDfC) or D) 100% p(TETA/CBA)-g-PEG-c(RGDfC) and 200 ng siRNA in HEPES buffered saline were combined at 0, 1, 3, 6, and 12 polymer to siRNA w/w ratios, respectively. Following 30 min equilibration, each sample was loaded on a 2% agarose gel, stained with EtBR, and then run for 30 min in TAE buffer. Gel images were obtained using GelDoc Software. Combining 25% p(TETA/CBA) with p(TETA/CBA)-g-PEG or p(TETA/CBA)-g-PEG-c(RGDfC) rescues complexation at low polymer/pDNA weight-to-weight ratios.

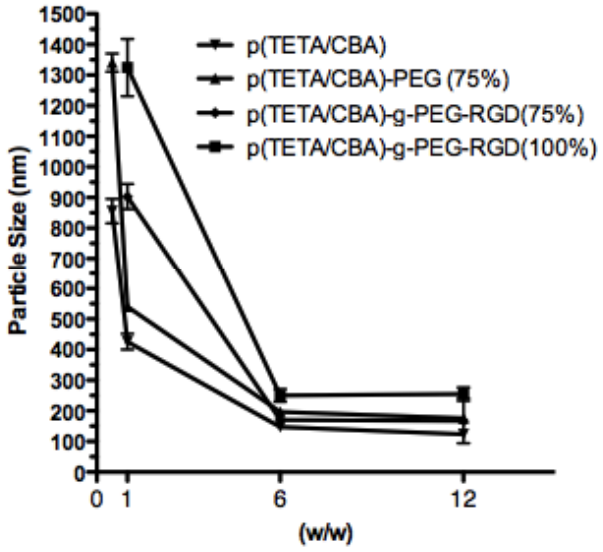

Figure 5.5. Intensity-based particle sizes of gene carrier formulations with siRNA using Dynamic Light Scattering (DLS). Formulation mixtures of 25%p(TETA/CBA)/75%p(TETA/CBA)-g-PEG-c(RGDfC) show improved siRNA condensation compared to p(TETA/CBA)-g-PEG-c(RGDfC) alone.

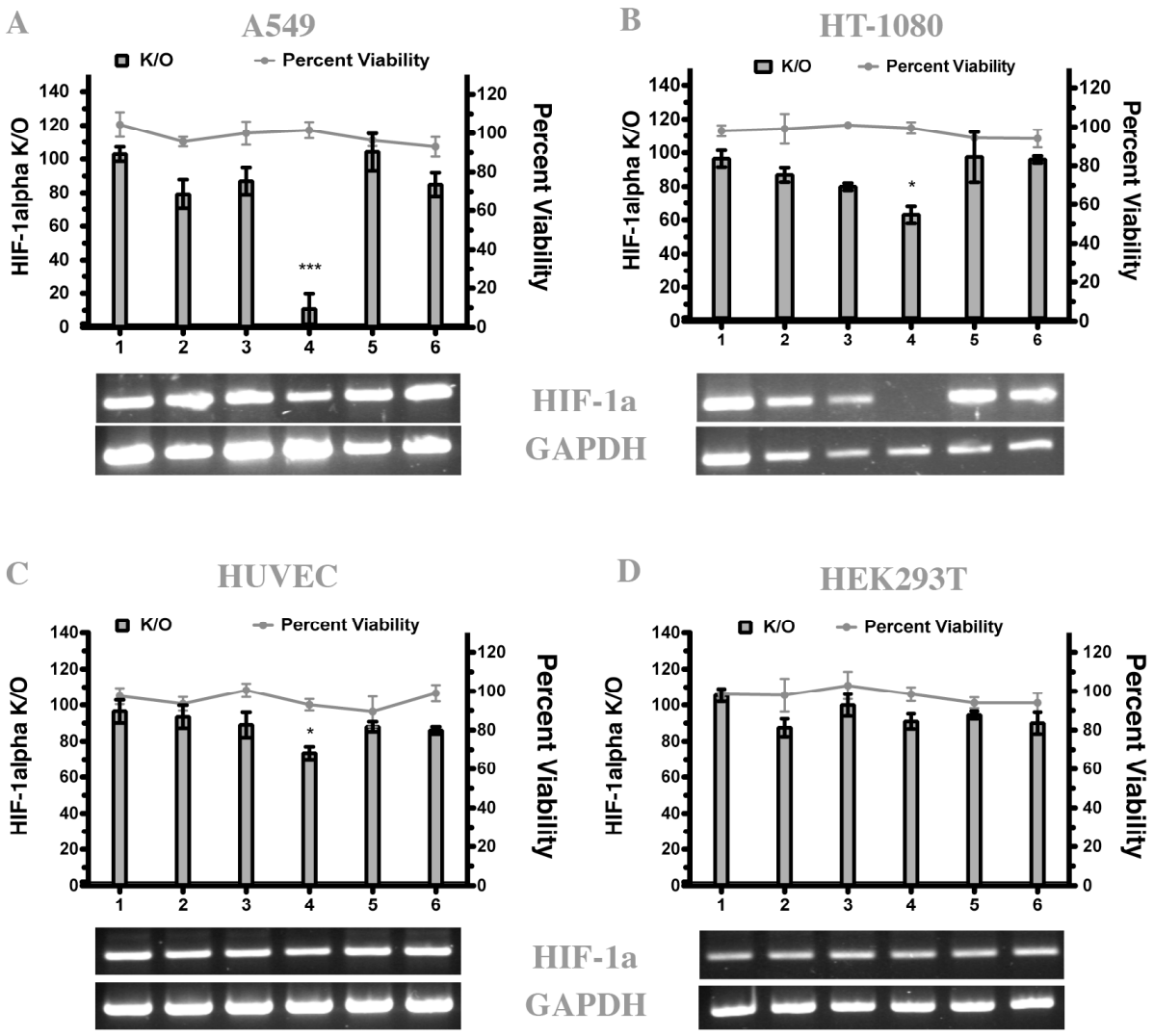

Figure 5.6. Transfection efficiency and cell viability in a) A549 cells b) HT-1080 cells c) HUVEC cells d) HEK293T cells. In all graphs, 1) Untreated control 2) p(TETA/CBA) 3) p(TETA/CBA)-g-PEG 4) p(TETA/CBA)-g-PEG-c(RGDfC) 5) p(TETA/CBA)-g-PEG-c(RGDfC)/c(RGDfC) competition 6) p(TETA/CBA)-g-PEG-c(RGDfC)/scRNA. HIF-1α and GAPDH RT-PCR products are shown below each graph. Each formulation was combined with 20nM siRNA at 6 w/w. Transfection data are represented as mean ± SEM, *$p < 0.05$ or ***$p < 0.0001$ using a One-way ANOVA and Tukey's post-hoc test (n=2). Cell viability data are represented as mean ± SD (n=6).

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CHAPTER 6

SYNOPSIS OF RESULTS, CONCLUSIONS AND FUTURE PROSPECTS

6.1 Synopsis of Results and Conclusions

Gene therapy offers a promising therapeutic alternative to conventional therapies that are currently marketed. Its clinical success, however, is hampered by undefined design and formulation requirements to engineer a safe and efficient nucleic acid delivery vehicle. Previous studies have demonstrated that the use of disulfide-containing polycations, such as bioreducible poly(amido amine)s (SS-PAAs) and poly(amido ethyleneimine)s (SS-PAEIs), provide increased transgene expression and reduced cell toxicity compared to the 'gold' standard, poly(ethylene imine) systems (1, 2). While these degradable systems maintain promising attributes for safe and efficient gene delivery vehicles, as with all other nonviral polycationic gene carriers, they must be further modified to improve their biocompatibility and maintain efficient application in vivo. The chemical conjugation of a neutrally charged and hydrophilic polymer, poly(ethylene glycol) (PEG), to nonviral polycations has proven effective in promoting biocompatibility in vitro and in vivo, and it can also accommodate the incorporation of a targeting moiety to improve cell-specific activity (3). While PEG and/or PEG-ligand modification can be advantageous, it has also been shown to adversely affect nucleic acid

condensation and carrier performance depending on the gene carrier being used and the degree to which the carrier is modified (4, 5). Resultantly, multiple synthetic products and optimization steps are required to gradually identify or improve the performance of gene therapy reagents (6). In lieu of these findings, several copolymers were synthesized using the SSPAEI backbone, poly(triethylenetetramine/cystamine-bis-acrylamide) (p(TETA/CBA)), chemically grafted with PEG or PEG-peptide to improve the bioactivity and-or cell specificity of the SS-PAEI in the presence of serum. Moreover, a novel method was employed to study the influence of PEG modification on the SSPAEI, which allowed easy manipulation over the relative amount of PEG-to-SSPAEI polycation incorporated in a gene carrier reagent to easily study and optimize formulations and avoid the synthesis of multiple potential candidates for optimization. Each of the synthesized products was characterized by ^{1}H NMR, acid-base titration linking an inverse correlation with the degree of PEG modification and buffer capacity, and FPLC for molecular weight analysis.

The ability of each synthetic product to form electrostatic interaction with nucleic acid (pDNA or siRNA) was evaluated at increasing polymer-to-nucleic acid concentrations by gel electrophoresis and/or dynamic light scattering (DLS) measurements. Chemical modification of the polycations with PEG or PEG-ligand proved to mitigate the gene carrier's ability to electrostatically interact with nucleic acid payload. Incorporation of the polycation with the co-polymer formulation proved to overcome this effect and drive complexation of the polycation formulation with nucleic acid. When various gene carrier mixtures were used to test their ability to protect pDNA from serum nuclease degradation, the formulations significantly enhanced the amount of

viable pDNA available after 6 hrs when compared to unformulated, free pDNA. The results also indicated an inverse correlation between the amount of PEG maintained in a formulation and the protection of pDNA from serum nuclease degradation.

The bioactivity of the polymer formulations complexed with p(DNA) demonstrated that PEG modification of p(TETA/CBA) could improve cellular transgene delivery and expression in the presence of serum compared to the unmodified polycation and bPEI, however, the amount of PEG incorporated in the formulation had to be tailored to achieve an optimum, which is similar to previous reports (7). Tailoring the amount of PEG in the gene carrier formulations was made easy by using the aforementioned method, where the polycation and PEG-polycation species are mixed at different ratios. This facile method can be used for other gene delivery reagents in order to facilitate the optimization process and avoid the synthesis of many putative candidates.

The biodistribution of several mixture formulations was evaluated at a polymer-to-pDNA weight-to-weight ratio that resulted in net-negatively charged polyplex and a weight-to-weight ratio that resulted in a net-positive charge. Polyplex size of the formulations injected was similar irrespective of charge. The relative amount of PEG present in each formulation provided no significant trend on biodistribution, however, differences between formulations were observed. More noteworthy was that the formulations that maintained a net-negative charge exhibited significantly greater tumor accumulation compared to other major organs. Conversely, the formulations that maintained a net-positive charge on polyplexes did not show any enhanced tumor accumulation compared to other organs, but they did deposit more payload in the respective tissue than the negatively charged polyplexes.

Similar to the PEG-copolymers synthesized and evaluated in Chapters 3 and 4, the targeted siRNA delivery reagent, p(TETA/CBA)-g-PEG-c(RGDfC), showed a reduced ability to electrostatically interact with siRNA when compared to p(TETA/CBA) alone. Moreover, incorporation of p(TETA/CBA) with p(TETA/CBA)-g-PEG-c(RGDfC) in solution, helped drive electrostatic interaction with siRNA to encapsulate the siRNA and maintain nanosized particles (<200nm) that are sufficient for cellular uptake. More importantly, in vitro cell transfection experiments using a 25%p(TETA/CBA)/75%p(TETA/CBA)-g-PEG-c(RGDfC) formulation mixture proved to enhance the delivery of HIF-1α siRNA specifically to $\alpha_V\beta_3$ integrin expressing cell lines and not to cells devoid of this integrin receptor. All formulations were non-toxic to the cell lines tested. Enhanced transfection was abrogated in $\alpha_V\beta_3$ integrin expressing cell lines when a competition assay was performed using free, soluble c(RGDfC) peptide, indicating that enhanced delivery to these respective cells is specific and driven by the $\alpha_V\beta_3$ integrin-binding peptide, c(RGDfC). This cell specific, enhanced delivery unequivocally indicates the inclusion of the p(TETA/CBA)-g-PEG-c(RGDfC) species in the polyplexes formed and substantiates the use of mixture formulations to study targeted gene delivery reagents. For this practice to be commonly employed in order to expedite the product development of gene delivery reagents, further studies are inevitable.

6.2 Future Prospects

The studies outlined in this dissertation demonstrate the improved bioactivity and tissue-specificity of PEG or PEG-ligand modified p(TETA/CBA), respectively. In both cases, combining the polycation, p(TETA/CBA) with its modified counterpart proved to

influence carrier properties and bioactivity. The use of gene carrier mixtures provides a promising approach to easily study the effects of modification on a nucleic acid delivery reagent and identify an optimal candidate. Once the relative abundance of each species in an optimal delivery reagent is known, further research studies should retroactively engineer a copolymer system that is a direct counterpart to the optimal formulation and test the two gene carrier reagents against each other. In principle, the two reagents should exhibit similar biocompatibility and bioactivity. Additional studies are required to affirm this principle and provide stronger evidence for using mixture formulations to expedite the development of improved nucleic acid delivery vehicles.

While PEG modification of p(TETA/CBA) and the use of p(TETA/CBA)/p(TETA/CBA)-g-PEG mixtures has proven useful to improve the bioactivity of p(TETA/CBA), previous reports have indicated that PEG modification of a polycation can interfere with its buffering capacity and thus the intracellular release of nucleic acid from the endosome (*8*). The reports herein indicate a modest reduction in buffering capacity of the PEG modified polycations, however, no significant adverse effect was seen on bioactivity. The buffering capacity of the mixture formulations was likely rescued by the presence of unmodified p(TETA/CBA). Additional studies characterizing the number of 1°, 2° and 3° amino groups in the gene delivery reagents and respective formulations would be useful, as the composition of amino groups in a polycationic system critically influences buffering capacity and complexation properties with nucleic acid. The amino content can be determined via Ninhydrin and 2,4,6-trinitrobenzenesulfonic acid (TNBS) assays. Moreover, to further improve the bioactivity of PEG-modified polycations, PEG conjugation that results in a physiologically triggered

release of the PEG chain should be evaluated. Possible triggers may include changes in pH, enzyme concentrations or redox potential. Acid-labile and disulfide-containing conjugates, or linkers susceptible to acid protease cleavage via cathepsins are all plausible for physiological release of PEG (*1, 9, 10*). In addition, because the PEG content of formed polyplexes is an important parameter influencing their function, future studies that utilize mixture formulations as described throughout this dissertation should elucidate PEG and/or PEG-ligand content present in polyplexes formed. Evidence in this dissertation demonstrated correlating trends between the amount of PEG included in formulation mixtures and reagent physiochemical and functional properties, however, a more thourough understanding of precise PEG/PEG-ligand incorporation to polyplex is important to further undrstand and advance gene delivery reagents.

6.2.1 c(RGDfC) Binding Activity with αvβ3 Integrins In Vitro

Additional studies should be performed to further assert c(RGDfC) activity in vitro, and evaluate the putative effect of polymer formulations on peptide binding affinity with the $\alpha_V\beta_3$ integrin. The binding activity of free c(RGDfC) and c(RGDfC)-copolymer mixtures with the $\alpha_V\beta_3$ integrin receptor on respective cell lines can be assessed using a competitive binding assay with ^{125}I-echistatin, a known $\alpha_V\beta_3$ integrin agonist (*11, 12*). This is performed by washing $\alpha_V\beta_3$ integrin expressing cells with sterile PBS and subsequently re-suspending them in binding buffer (20mM Tris, pH 7.4, 150mM NaCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 0.1%bovine serum alumina (BSA)) before seeding them in 96-well Multiscreen HV filter plates (0.45 μm; Millipore, Billerica, MA). At $5x10^4$ cells/well. The cells are then co-incubated with ^{125}I-echistatin (0.05 nM)

and increasing concentrations of free peptide or equivalent concentrations of c(RGDfC)-copolymer mixtures (0-100μM) at 4°C for 2 hrs in a final 200μl volume of buffer. Following co-incubation, the plates are subsequently filtered using a Multiscreen manifold with vacuum (Millipore) and rinsed several times with cold binding buffer. In this case, ^{125}I-echistatin can be harvested from the filters and the cell-bound ^{125}I-echistatin can be quantified using an automatic gamma-counter to determine binding activity. A nonlinear regression analysis can be used to determine IC_{50} values indicative of peptide binding affinity. It is expected that the binding affinity of free and polymer-bound c(RGDfC) is similar for the 25%p(TETA/CBA)/75%p(TETA/CBA)-g-PEG-c(RGDfC) mixture given the in vitro cell culture results that indicate strong $\alpha_V\beta_3$ integrin targeting of this formulation.

6.2.2 In Vivo Biodistribution Study of p(TETA/CBA)-g-PEG-c(RGDfC) for siRNA Delivery to Primary Tumors

The synthesis and use of p(TETA/CBA)-g-PEG-c(RGDfC) produced promising in vitro results for the delivery of therapeutic HIF-1α siRNA to multiple cancer cell lines and angiogenic endothelial cells that have high levels of $\alpha_V\beta_3$ integrin expression. To continue this positive data and fully understand the therapeutic potential of this gene therapy combination, in vivo biodistribution and efficacy studies must be performed in an animal cancer model. To perform the in vivo biodistribution study, an IVIS 200 Series Living image device (Xenogen) can be used, which would allow gene carrier dosing and biodistribution to be studied concurrently prior to the efficacy studies. For this application, CT-26-Luc/GFP expressing colon carcinoma cells should be injected into

female BALB/c mice and allowed to grow until the average tumor volume is approximately 100mm^3. Respective formulations should be complexed with cy3-labeled siRNA against luciferase or cy3-labeled scrambled siRNA as a negative control. Three appropriate but different w/w ratios should be used for polyplex formation. Buffer (20mM HEPES with 20% glucose) and naked siRNA controls should also be used. The mice should be injected with 200μl of complexes totaling 5mg/kg of siRNA. To monitor luciferase expression and the siRNA biodistribution (cy3 emission) mice must be injected intraveinously with 150 mg/kg of d-Luciferin (Xenogen Corporation, Alameda, CA) approximately 10 min before imaging. For imaging, the mice must be anaesthetised with isofluorane (2.5%). Mice should be imaged at approximately 6, 12, 24, 36 and 48 hrs postinjection using Living Image software with XFO-6 fluorescence option and a bandpass filter program (Xenogen Corporation, Alameda, CA).

6.2.3 In vivo Efficacy Study of p(TETA/CBA)-g-PEG-c(RGDfC) for HIF-1α siRNA Delivery to Primary Tumors

Respective gene carrier formulations should be complexed with 50ug of HIF-1α siRNA at the preferred w/w ratios identified in the biodistribution study. Buffer and naked siRNA controls should also be used. Repeated injections should be performed intravenously every three days once the average tumor volumes reach approximately 100mm^3. Tumor volume should be measured each time animals are injected in order to derive a tumor growth/inhibition curve. Animals should be sacrificed when the buffer control animals are sacrificed due to excessive tumor size or complications. Tumors and other organs (lung, liver, kidney, spleen, blood, heart, stomach, ovary) should be excised

immediately and flash frozen in liquid nitrogen. The capillary density of the tumors should be evaluated and HIF-1α protein expression should be determined using an ELISA kit.

In addition, the potential immune response of the injected formulations should be studied. To do so, blood samples should be taken at 6 and 12 hrs postinjection from the treated mice and evaluated for cytokine production using an appropriate ELISA kit. Relevant cytokines to be evaluated are: IFN-α, IFN-β, IFN-γ, IL-2 and IL-6. Buffer-only animals should be used for comparison and mice treated with polyinosinic:polycytidylic acid poly(I):(C) should serve as positive controls.

Further work with these gene delivery reagents may include the use of conventional chemotherapy in conjunction with therapeutic nucleic acid such as HIF-1α siRNA. Chemical modification of the polycation with chemotherapeutic agents is plausible if physiological release of the agent is achieved in order to maintain the therapeutic activity. Examples for triggered physiological release are mentioned at the beginning of section 6.2. Using a combined therapy with the targeted carrier, p(TETA/CBA)-g-PEG-c(RGDfC), may lend a synergistic anticancer effect and can reduce the off-target cytotoxicity of the chemotherapeutic agent. If nonspecific cytotoxicity is reduced and synergistic anti-tumor effects are achieved, promising clinical application is near.

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APPENDIX

POLY(TRIETHYLENETETRAMINE/CYSTAMINE-BIS-ACRYLAMIDE) AND POLY(TRIETHYLENETETRAMINE/CYSTAMINE-BIS-ACRYLAMIDE)-POL(ETHYLENE GLYCOL) MIXTURES FOR THERAPEUTIC SIRNA DELIVERY IN VIVO

A.1. Introduction

The systemic delivery of pDNA with 75% p(TETA/CBA/PEG)/25% p(TETA/CBA) at 0.5 w/w ratio denoted here as 75% p(TETA/CBA/PEG), and the 100% p(TETA/CBA/PEG) at 3 w/w denoted herein as 100% p(TETA/CBA/PEG) formulations provided the greatest tumor accumulation of pDNA among the tested formulations (Section 4.4.3). Most notably, the 75% p(TETA/CBA/PEG) formulation exhibited passive tumor accumulation and provided nearly 10-fold higher copy number/mg tissue in the tumor than the other major organs. On the other hand, 100% p(TETA/CBA/PEG) showed comparable pDNA accumulation in the major organs when compared to the tumor, however showed approximately 5-fold higher copy number/mg tissue than the 75% p(TETA/CBA/PEG) formulation. These nucleic acid carrier formulations are comprised of a polycationic unit and a hydrophilic, neutrally charged poly(ethylene

glycol) (PEG) chain. Often the covalent incorporation of PEG to polycationic carriers can mask their surface charge and provide a steric barrier to avoid undesireable, non-specific interaction with serum proteins that can lead to opsonization and removal of the delivery vehicle from the blood stream by the reticuloendothelial system (RES) (*1-4*). If RES uptake of polyplexes is avoided, passive tumor accumulation and putative therapeutic efficacy may be improved because delivery carrier circulation times are lengthened. Improved passive tumor accumulation of macromolecules and nucleic acid carriers is a product of the Enhanced Permeability and Retention Effect (EPR), which is commonly observed following systemic administration. However, the extent to which the EPR effect can be exploited by drug/nucleic acid delivery reagents depends on carrier characteristics, largely on individual tumor types that possess different pathophysiological characteristics, which affect tumor vascularization, or angiogenesis (*5*). Delivery vehicles with a negative surface charge and small particle sizes have been shown to preferentially accumulate in tumors compared to positively charged carriers with a large size (*6*).

As mentioned above, 75% p(TETA/CBA/PEG) complexed with pDNA showed passive tumor accumulation and it possesses a net-negative surface charge. Conversely, 100% p(TETA/CBA/PEG) possesses a net-positive surface charge and demonstrated no preference in tumor accumulation compared to nondiseased organs. Each formulation produced similar complex size when used with pDNA. Provided with these results, we sought to study the ability of p(TETA/CBA/PEG) formulations to complex with siRNA and evaluate their potential efficacy in vivo for the delivery of therapeutically useful siRNA against a gene target known to promote tumor growth and vascularization. This

gene target is Hypoxia Inducible Factor-1α (HIF-1α) and many inhibitors of this protein have been used clinically to mitigate cancer progression (*7, 8*). Complexation studies of the polymer formulations with siRNA provided similar particle sizes and surface charge to the results obtained using them in conjunction with pDNA. Furthermore, when used in vivo for the delivery of HIF-1α siRNA to tumor bearing mice, they provide promising results when tumor growth inhibition and median animal survival is assessed.

A.2 Materials and Methods

A.2.1 Materials

Triethylenetetramine (TETA), 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (EDC), *N*-hydroxysulfosuccinimide (sulfo-NHS) and HPLC grade methanol were purchased from Sigma-Aldrich (St. Louis, MO). N,N'-Cystamine-bis-acrylamide (CBA) was purchased from Polysciences, Inc. (Warrington, PA). Ultrafiltration devices and regenerated cellulose membranes (10 kDa) were supplied by Millipore Corporation (Billerico, MA). The siRNA sequences were purchased from Integrated DNA Technologies (IDT) (San Diego, CA).

A.2.2 p(TETA/CBA) Synthesis

Synthesis of p(TETA/CBA) was performed as described in section 4.3.2. at 30°C for 12 hrs, at which time excess TETA was added to the reaction mixture and the reaction was allowed to run for an additional 24 hrs to ensure that the reaction had been terminated. The pH was subsequently adjusted to 7.0 and the polymer was purified by ultrafiltration using a 10kDa regenerated cellulose membrane. p(TETA/CBA) was

obtained by lyophilization. Composition of the polymer was monitored using ^{1}H NMR (400 MHz, D_2O). p(TETA/CBA) δ 2.61 (COC*H*2CH2NH, 4H), 2.72 (NHCH2C*H*2S-S, 4H), 2.90-3.21 (COCH2C*H*2NHC*H*2C*H*2,16H), 3.41 (NHC*H*2CH2S-S, 4H).

A.2.3 p(TETA/CBA/PEG) Synthesis

The synthesis of p(TETA/CBA/PEG) is described as a one-pot synthesis in section 4.3.3 that began with the original polymerization reaction conditions described for p(TETA/CBA) at 30°C. However, following 10 hrs reaction time, amine-reactive methoxy PEG-NHS (mPEG-NHS) was added to the reaction mixture and allowed to react for an additional 2 hrs before the addition of excess TETA (100%). The reaction was allowed to proceed for an additional 24 hrs before the pH was lowered to 7.0 and the polymer was purified using ultrafiltration (10 kDa) The composition of p(TETA/CBA/PEG) was monitored using ^{1}H NMR (400 MHz, D_20). p(TETA/CBA/PEG); δ 2.61 (COC*H*2CH2NH, 4H), 2.72 (NHCH2C*H*2S-S, 4H), 2.90-3.21 (COCH2C*H*2NHC*H*2C*H*2,16H), 3.41 (NHC*H*2CH2S-S, 4H), 3.45-3.7 (CH2CH20, 4H).

A.2.4 Polymer Characteristics

Absolute molecular weight analysis for p(TETA/CBA) and p(TETA/CBA/PEG) was performed using AKTA/FPLC (Amersham Pharmacia Biotech Inc.) coupled to a light-scattering detector and using the polymer refractive index increment (dn/dc) for each sample. A Superose 6 110/300 GL column was used for polymer sample separation. Poly[N-(2-hydroxypropyl)methacrylamide] (poly(HPMA)) standards were injected onto

the column prior to experimental sample analysis in order to ensure the column was clean and functional. Experimental and standard polymer samples were dissolved in degassed and filtered (0.2 μm (Nylon, Alltech)) 0.3 M NaOAc, pH 4.4 with 30% (v/v) acetonitrile eluent buffer. The flow rate was set to 0.4 mL/min.

A.2.5 Polyplex Formation

In all cases polyplex was formed with a known amount of siRNA and a corresponding and desired amount of polymer suspended in HEPES buffer (20 mM, pH 7.4, 20% glucose). The two solutions were combined, lightly vortexed and allowed to equilibrate for 30 min. When mixtures of two different polymers were used to form polyplex, a known amount of siRNA was brought up in solution and a corresponding and desired concentration of p(TETA/CBA) and p(TETA/CBA/PEG) were prepared by mixing the appropriate species at specified ratios before being added to the siRNA solution and lightly vortexed. The resulting complexes were and allowed to equilibrate for 30 min. at room temperature.

A.2.6 Light Scattering and ζ-Potential Measurements

The surface charge and polymer/pDNA particle (polyplex) diameters were measured at 25°C using a Zetasizer 2000 instrument (DTS5001 cell) and a dynamic light scattering (DLS) unit on a Malvern 4700 system, respectively. Polyplexes were prepared by adding equal volume polymer solution (200µl) at increasing concentrations in HEPES buffer (20 mM, pH 7.4, 5% glucose) to a desired concentration of 40µg siRNA in HEPES buffer (200µl). Polyplexes were allowed to equilibrate for 30 min and were subsequently

diluted in filtered milliQ water to a final 2 mL volume.

A.2.7 In Vivo Tumor Regression and Survival

Human non-small cell lung carcinoma cells (A549) suspended in 100µl sterile PBS were subcutaneously injected (1 x 10^6 cells) into the right flank of 6-week old, BALB/c nude female mice. Tumors were allowed to form over 2 weeks or until they reached an average tumor volume around 80 mm^3. Outliers were discarded to maintain this average. Once sufficient tumors formed, animals were randomly assigned to groups of 5 and were injected systemically with sterile HEPES buffer (20 mM, pH 7.4, 20% glucose), or the following polyplex formulations also prepared in sterile HEPES buffer: 75% p(TETA/CBA/PEG)/25% p(TETA/CBA) complexed with HIF-1α siRNA at 0.5 w/w (75% p(TETA/CBA/PEG)/siHIF), 100% p(TETA/CBA/PEG) complexed with HIF-1alpha siRNA at 3 w/w (100% p(TETA/CBA/PEG)/siHIF), 75% p(TETA/CBA/PEG)/25% p(TETA/CBA) complexed with scrambled siRNA at 0.5 w/w (75% p(TETA/CBA/PEG)/scRNA), 100% p(TETA/CBA/PEG) complexed with scrambled siRNA at 3 w/w (100% p(TETA/CBA/PEG)/scRNA). Treatments were administered every three days for each group. Mice treated with formulated siRNA received a total siRNA dose of 55ug, which is approximately 2.5mg/kg. Tumor volume was measured on each injection day prior to treatment using digital calipers. The siRNA seqyuences used are as follows: The siRNA sequences specific for HIF-1α mRNA: 5'-TAGUUGGGUCUGUAUAGGUG-3'and 5'-TCACCUAUACAGACCCAACU-3'. The scrambled siRNA sequences: 5'-TUUUAAGGGCGCAAUGCGCA-3' and 5'-TTGCGCATTGCGCCCTTAAA-3'. Animal survival was also assessed during these

studies to generate a survival curve for analysis. Due to Institutional Animal Care and Use Committee (IACUC) standards, the animals were sacrificed when tumor ulcerations reached a standard surface area equal to $1mm^2$ or when the individual tumor volume exceeded $1500mm^3$. No animal death associated with treatment was observed. Animals were checked everyday for survival and the onset of tumor ulceration.

A.2.8 Statistical Analysis

The statistical analysis for tumor growth inhibition was performed by comparing the AUEC for each treatment, which is reported as mean ± SD (n = 4-5), using a one-way ANOVA in conjunction with a Tukey's post-hoc test. A $p < 0.05$ was considered statistically significant. The animal survival curve was analyzed using a built-in survival analysis using GraphPad Prism 5 Software that ran a log-rank (Mantel-Cox) test and a log-rank test for trend reporting Chi square values (n = 4-5).

A.3 Results

A.3.1 Physiochemical Characterization

Because complexation of polycationic gene carriers with pDNA may be different than it is with siRNA, we tested the ability of the aforementioned and relevant p(TETA/CBA/PEG) formulation to complex with siRNA. These formulations were 75% p(TETA/CBA/PEG), and the 100% p(TETA/CBA/PEG) at polymer/siRNA w/w ratios equal to 0.5 and 3, respectively. For simplicity, these formulations are referred to as 75% p(TETA/CBA/PEG) and 100% p(TETA/CBA/PEG), that is 75% p(TETA/CBA/PEG) is always complexed with siRNA at 0.5 w/w and 100% p(TETA/CBA/PEG) at 3 w/w. The

intensity-based mean particle size for 75% p(TETA/CBA/PEG) and 100% p(TETA/CBA/PEG) were similar to those observed using pDNA. Particle sizes were maintained at approximately 100nm in diameter with little standard deviation (Figure A.1). The surface charge of the complexes formed between the formulations and siRNA were also similar to those observed using pDNA (Figure A.1). Based on the similarity of pDNA and siRNA complex physiochemical characteristics alone, we believe these two gene carrier formulations complexed with siRNA will possess similar in vivo biodistribution characteristics to those seen with pDNA. A biodistribution study using the relevant formulations and siRNA should be performed to unequivocally assert this putative similarity. Nonetheless, the therapeutic efficacy of siRNA against HIF-1α (siHIF) using these carrier formulations was studied.

A.3.2 In Vivo Tumor Regression and Survival

Human lung carcinoma cells were injected into female BALB/c nude mice and allowed to grow sufficient tumors over approximately 2 weeks. Once sufficient tumors formed, therapeutically active siHIF or scrambled siRNA (scRNA), serving as a negative control, was complexed with 75% p(TETA/CBA/PEG) and 100% p(TETA/CBA/PEG) and administered to the mice two times per week. Buffer only injections also served as a control. Following 18-20 days of treatment, The 75% p(TETA/CBA/PEG)/siHIF treated group had average tumor volumes that were 55% smaller than 75% p(TETA/CBA/PEG)/scRNA and also smaller than the buffer only group (Figure A.2). This result indicates that the carrier-mediated delivery of siHIF is therapeutic and tumor delivery of the siRNA payload occurs using the 75% p(TETA/CBA/PEG) formulation.

The 100% p(TETA/CBA/PEG)/siHIF treatment group exhibited nearly 30% reduction in tumor volume compared to 100% p(TETA/CBA/PEG)/scRNA also suggesting carrier activity and target specific siHIF therapeutic activity (Figure A.2). These results suggest a promising trend toward efficacy for 75% p(TETA/CBA/PEG)/siHIF and 100% p(TETA/CBA/PEG)/siHIF, however no statistical significance between treatment groups was found. A power analysis performed using StatMate 3.0 software (Graphpad Inc.) indicates that nine animals per treatment group are required to achieve significant tumor growth inhibition with a 95% confidence limit and assuming a standard deviation in tumor volume equal to $100mm^3$. Thus, a repeated study using more animals has potential to provide significance.

Animal survival curves were generated from the tumor regression studies. As described above, animals were sacrificed for tumor ulceration or when the tumor volume exceeded 1500 mm^3. Interestingly, tumor ulceration was not size dependent, thus large and small tumors were susceptible to tumor ulceration. Also noteworthy, animals treated with 75% p(TETA/CBA/PEG)/siHIF and 100% p(TETA/CBA/PEG)/siHIF appeared to exhibit more tumor ulceration than the other groups (data not shown). This character may be due to tumor necrosis imposed by effective delivery and treatment with siHIF, however, this is not confirmed and additional studies are required to ascertain this postulation. Nonetheless, 75% p(TETA/CBA/PEG)/siHIF and 100% p(TETA/CBA/PEG)/siHIF treated animals did demonstrate a significant survival trend compared to the 75% p(TETA/CBA/PEG)/scRNA and 100% p(TETA/CBA/PEG)/scRNA negative control groups (Figure A.2). This significance was determined using the logrank test for trend with a Chi square equal to 6.649 and a p value

less than 0.0099. Median survival times for 75% p(TETA/CBA/PEG)/scRNA and 100% p(TETA/CBA/PEG)/scRNA negative control groups ranged between 17 and 21 days, respectively. Conversely, the 75% p(TETA/CBA/PEG)/siHIF group had a median survival equal to 52 days (Figure A.2). As with the tumor growth inhibition studies, these results are promising with respect for 75% p(TETA/CBA/PEG)/siHIF and 100% p(TETA/CBA/PEG)/siHIF therapeutic efficacy and appears to be target specific for HIF-1α, as scRNA demonstrated no significant trend. However, a repeated study using more animals per treatment group is required to see putative significance in survival rate for these formulations.

A.4 Discussion

The results presented here coincide strongly with those obtained using the 75% p(TETA/CBA/PEG) and 100% p(TETA/CBA/PEG) formulations for pDNA complexation and in vivo biodistribution. Based on physiochemical characteristics and the biodistribution patterns alone, it is likely that the significant survival and tumor growth inhibition trend observed for 75% p(TETA/CBA/PEG) may be due to an increased ability to penetrate the tumor environment due to its negative charge. This reason may be 2-fold. First, by having a negative surface charge, the carrier is less likely to interact with serum proteins that are often positively charged and thus less likely to be removed from circulation by the RES system, which allows for passive tumor accumulation. Secondly, the tumor environment is often overcome with negatively-charged cell-surface glycosaminoglycans (GAGs), which have been shown to limit particle diffusion of positively charged carriers into the tumor and/or may cause complex

destabilization which will result in reduced efficacy (*9-12*). Multiple nanoparticle delivery systems that exhibit small size and a negative charge have demonstrated increased tumor accumulation compared to other organs and to highly positively charged carriers (*6*). Carriers with a low positive surface charge and small particle size are also preferred because they have limited interaction with serum proteins and cell surfaces. The significant trend in animal survival using 75% p(TETA/CBA/PEG)/siHIF also suggests increased efficacy by tumor penetration of these nucleic acid delivery reagent compared to the positively charged formulation, 100% p(TETA/CBA/PEG).

A.5 Conclusion

The results from the biodistribution study presented in Chapter 4.4.3 showed that when 75% p(TETA/CBA/PEG) and the 100% p(TETA/CBA/PEG) were used to deliver pDNA systemically, they showed the greatest degree of tumor accumulation compared to the other formulations. Specifically, the 75% p(TETA/CBA/PEG) formulation showed passive tumor accumulation and provided nearly 10-fold higher copy number/mg tissue in the tumor than the other major organs. The 100% p(TETA/CBA/PEG) formulation showed comparable pDNA accumulation in the major organs when compared to the tumor, however showed approximately 5-fold higher copy number/mg tissue than the 75% p(TETA/CBA/PEG) formulation. These formulations were tested for their ability to interact and form complex with therapeutically relevant siRNA specific for HIF-1alpha, a target gene known to promote tumor growth and vascularization. Complexation studies of the polymer formulations with siRNA provided similar particle sizes and surface charge to the results obtained using them to complex with pDNA. Furthermore, when

used in vivo for the delivery of siHIF to tumor bearing mice, the treatments provided promising results with respect to tumor growth inhibition and median survival times, which appear to be target specific for HIF-1alpha. These results provide a promising foundation for further studies to unequivocally assert the therapeutic efficacy of these treatments.

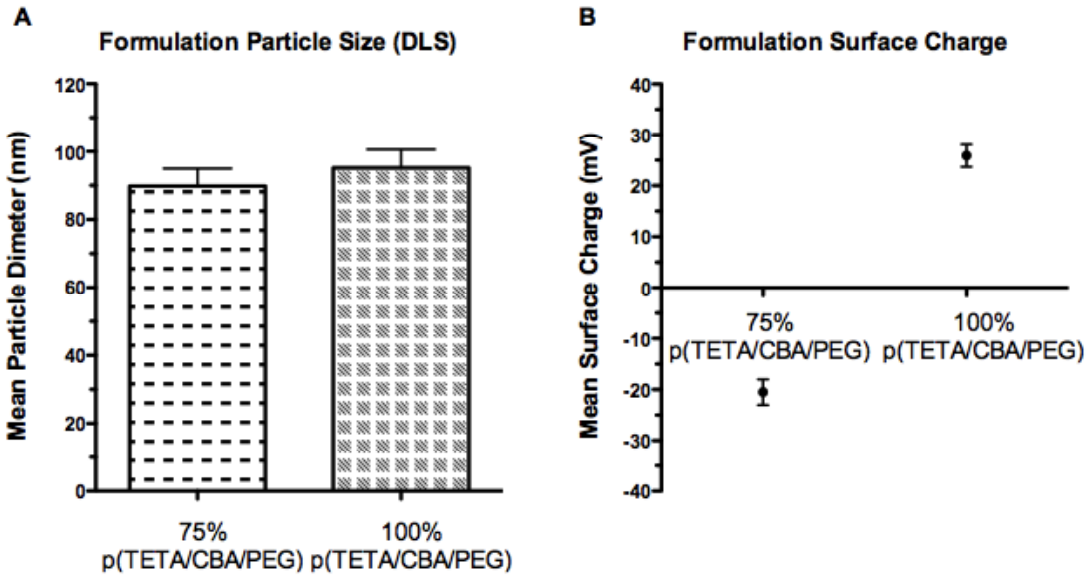

Figure A.1. a) dynamic light scattering (DLS) was performed to evaluate complexation of 75% p(TETA/CBA/PEG) and 100% p(TETA/CBA/PEG) with siRNA. b) ζ-Potential measurements to determine mean surface charge of 75% p(TETA/CBA/PEG) and 100% p(TETA/CBA/PEG) complexes with siRNA.

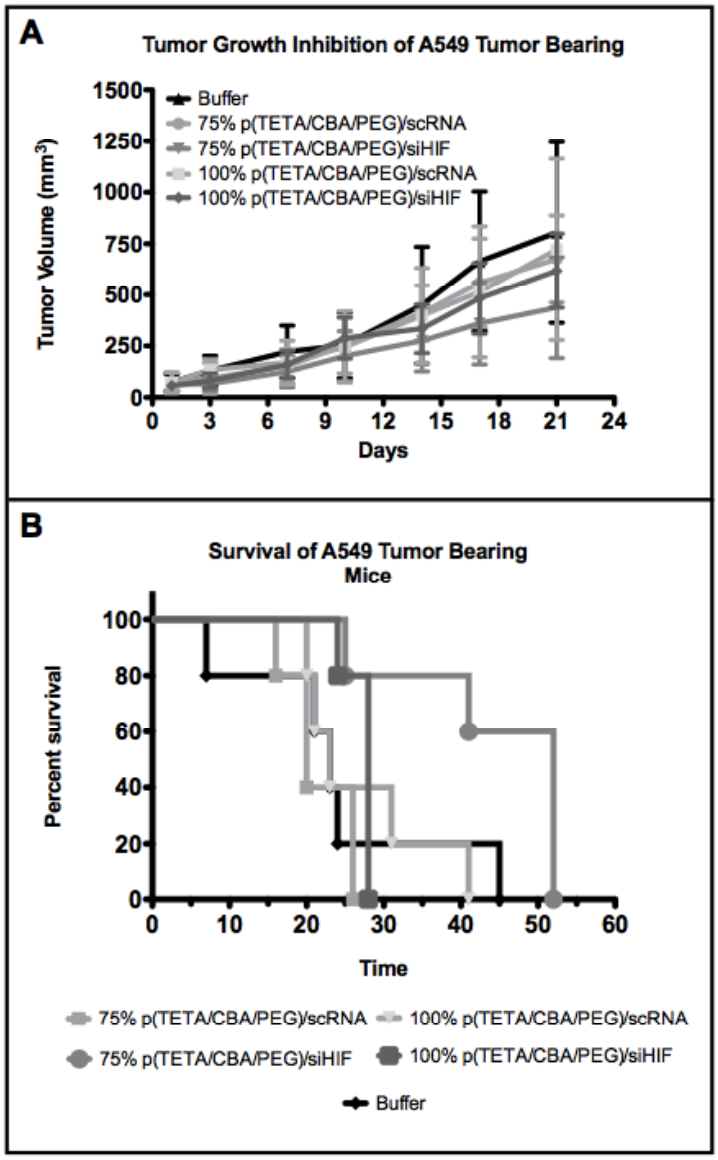

Figure A.2. Efficacy studies using 75% p(TETA/CBA/PEG) and 100% p(TETA/CBA/PEG) for siHIF-α and scRNA negative controls. a) Tumor volume over time. Treatments were given every 3 days following tumor volume measurement each time. b) Survival curve generated from the same experiment. Respective treatments are listed in each graph.

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