A Focused Poly(Aminoether) Library for Transgene Delivery to Cancer Cells

by

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ABSTRACT

Cancer diseases are among the leading cause of death in the United States. Advanced cancer diseases are characterized by genetic defects resulting in uncontrollable cell growth. Currently, chemotherapeutics are one of the mainstream treatments administered to cancer patients but are less effective if administered in the later stages of metastasis, and can result in unwanted side effects and broad toxicities. Therefore, current efforts have explored gene therapy as an alternative strategy to correct the genetic defects associated with cancer diseases, by administering genes which encode for proteins that result in cell death. While the use of viral vectors shows high level expression of the delivered transgene, the potential for insertion mutagenesis and activation of immune responses raise concern in clinical applications. Non-viral vectors, including cationic lipids and polymers, have been explored as potentially safer alternatives to viral delivery systems. These systems are advantageous for transgene delivery due to ease of synthesis, scale up, versatility, and in some cases due to their biodegradability and biocompatibility. However, low efficacies for transgene expression and high cytotoxicities limit the practical use of these polymers.

In this work, a small library of twenty-one cationic polymers was synthesized following a ring opening polymerization of diglycidyl ethers (epoxides) by polyamines. The polymers were screened in parallel and transfection efficacies of individual polymers were compared to those of polyethylenimine (PEI), a current standard for polymer-mediated transgene delivery. Seven lead polymers that demonstrated higher transgene expression

efficacies than PEI in pancreatic and prostate cancer cells lines were identified from the screening. A second related effort involved the generation of polymer-antibody conjugates in order to facilitate targeting of delivered plasmid DNA selectively to cancer cells. Future work with the novel lead polymers and polymer-antibody conjugates developed in this research will involve an investigation into the delivery of transgenes encoding for apoptosis-inducing proteins both in vitro and in vivo.

ACKNOWLEDGMENTS

There are a number of people that I would like to acknowledge for helping me with this work. First I would like to thank and acknowledge Dr. Kaushal Rege for giving me the opportunity to join his lab and work with his group. I also would like to thank Dr. Rege for all of his support and guidance throughout this past year. I also would like to acknowledge Dr. Michael Sierks and Philip Schulz for allowing me to access the ÄTKA FPLC system that was used numerous times throughout my research. In addition to these professors, I also would like to acknowledge Dr. Thrimoorthy Potta for his time, effort, advice, and friendship throughout all of this work. Dr. Potta has been a loyal and helping colleague and has directly and indirectly contributed to this work. I also would like to acknowledge Fred Peña for his continued support with mechanical issues in the lab. Next, my group members; graduate and undergraduate students, have also been very helpful and influential throughout my graduate studies. I would like to acknowledge and thank them for providing me with the support and willingness to always help out a fellow member in need of assistance. Finally, I would like to acknowledge my parents and brothers for their unconditional love up to this point and into the future as I continue to pursue new endeavors.

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Chapter 1

INTRODUCTION

1.1 Background

Cancer is one of the leading causes of death in the United States and around the world (Yu, Tai, Xue, Lee, & Lee, 2010) among men and women younger than 85 years of age (Jemal, 2011). Among the different types of cancer, prostate cancer is one of the leading causes of death among men in the United States with an estimated mortality rate of 20,093 men in 2007 (Jemal, 2011). In addition to prostate cancer, one other form of cancer that has a survival rate of less than 5% is pancreatic cancer (Simeone, Cascarelli, & Logsdon, 1997). Even with current treatments, the estimated death rates are still projected to increase by 11% and 6% at the end of 2011 for prostate and pancreatic cancer respectively (Siegel, Ward, Brawley, & Jemal, 2011). Due to these alarming statistics, there exist a great need for development of new treatments and therapies aimed at inducing cancer cell death or suppressing the development of tumors. The use of anti cancer agents (chemotherapeutics), given systematically is the currently one commonly used method of treatment used in the medical profession (Yu, et al., 2010). However, these treatments are most effective if a tumor is found in a defined localized state in the early stages of metastasis (Merdan, Kopecek, & Kissel, 2002). Although chemotherapeutics can be effective to some extent, lack of cancer cell specificity leaves a toxic effect not only on cancer cells but also healthy cells. This toxicity induces unwanted side effects in patients and requires

determination of the correct dosing to achieve a balance between effectiveness and toxicity. This presents a great challenge to physicians because patient responses to these chemotherapeutic agents vary making under dosing and over dosing very problematic (Saha, Vasanthakumar, Bende, & Snehalatha, 2010).

Cancers diseases have been characterized to result from the presence of defective genes that induce uncontrollable cell growth. Recently, alternative strategies aimed at correcting these defects have been a highly researched topic. A promising method that has been shown to be effective *in vitro*, involves the use of viruses or nanoparticles to deliver either genes (transgene delivery), to correct the defective sequences or inhibit their expression, or anti cancer drugs (drug delivery). Some advantages to the use of nanoparticles are the ability to control the sizes allowing for easier penetration of tumors, and also protecting the genetic material from being degraded by DNAses and harmful pH conditions present in tumors. This versatility warrants the continued investigation of these systems.

There have been two modes of transgene delivery that are traditionally investigated throughout the literature. One method involves the use of viruses (i.e adenoviruses) to deliver genetic materials. Another method is through the use of polymeric systems (i.e Polyethylenimine, poly amido-amines, etc). Each of these methods offers certain advantages and disadvantages; however, both have demonstrated some success for transgene delivery *in vitro* and *in vivo*.

1.2 Viral Vectors

1.2.1 Adenoviral delivery. Use of viruses in gene delivery has been a well studied topic throughout the literature. One of the most commonly used vehicles is adenoviruses. The use of adenoviruses for delivery of a wide range of genes has been shown to be successful in various cancer cells lines both *in vitro* (Kasman, Barua, Lu, Rege, & Voelkel-Johnson, 2009; Park, et al., 2010; Simeone, et al., 1997; Varga, et al., 2005) and *in vivo* (Jounaidi, Chen, Veal, & Waxman, 2006; Jounaidi & Waxman, 2004). Simeone et al. found a maximum transfection of 80 % using an adenovirus with the lacZ adenoviral marker gene (adlacZ) in BXPC-3 pancreatic cancer cell line. In addition to the lacZ gene, the retinoblastoma gene, which is important in cell cycle regulation, was also delivered using the same adenovirus. This resulted in inhibition of cell proliferation up to 60 % in BXPC-3, Mia-PaCa-2, and PANC-1 cells (Simeone, et al., 1997). Other methods have also been developed using adenoviruses with cationic polymers and lipids to induce a synergistic effect that enhanced transfection efficacies (Fasbender, et al., 1997; Han, et al., 2010; Kasman, et al., 2009; MeunierDurmort, Grimal, Sachs, Demeneix, & Forest, 1997), thus demonstrating their relevance to cancer therapeutics.

1.3 Non-Viral Vectors

While the use of viral vectors shows high efficiencies and promising results, there are some limitations that come with their use. These limitations include high immunogenicity triggered by the mammalian immune systems leading to elimination of the viral vectors and insertional mutagenesis (Merdan, et al., 2002). For these reasons, non-viral vectors have been explored and developed as safer alternatives to viral vectors. Most vectors being explored currently are polymeric systems which have been shown to be effective. These systems are advantageous due to the ease of synthesis, scale up, and versatility (Elsabahy, Nazarali, & Foldvari, 2011). These systems can also be designed to be biocompatible, biodegradable, and can be functionalized with targeting ligands or other enhancers to improve functionality and transfection in cancer cells. Throughout the literature, cationic lipids and polymers are a widely investigated kind of system. These positively charged molecules possess the ability to bind to the negatively charged DNA backbone forming a spherical polymer/DNA complex (polyplex), which can interact with the negatively charged cell membrane and other surface proteins as illustrated schematically in figure 1. This interaction induces uptake of the polyplex into cell via endocytosis resulting in the encapsulation of the polyplex in an endosome. According to the proton sponge theory (Akinc, Thomas, Klibanov, & Langer, 2005; Berthold, Shiraishi, & Nielsen, 2010; Boussif, et al., 1995), due to the acidic nature of the cytoplasm in a cancer cell and the high buffering capacity of the polymer, protons are imported

into the endosome, resulting in osmotic swelling of the endosomal vesicles. Once the osmotic pressure reaches its maximum capacity, endosomal rupture occurs releasing the DNA into the cytoplasm where it is speculated to enter the nucleus to be processed in a similar manner to the cell's native DNA.

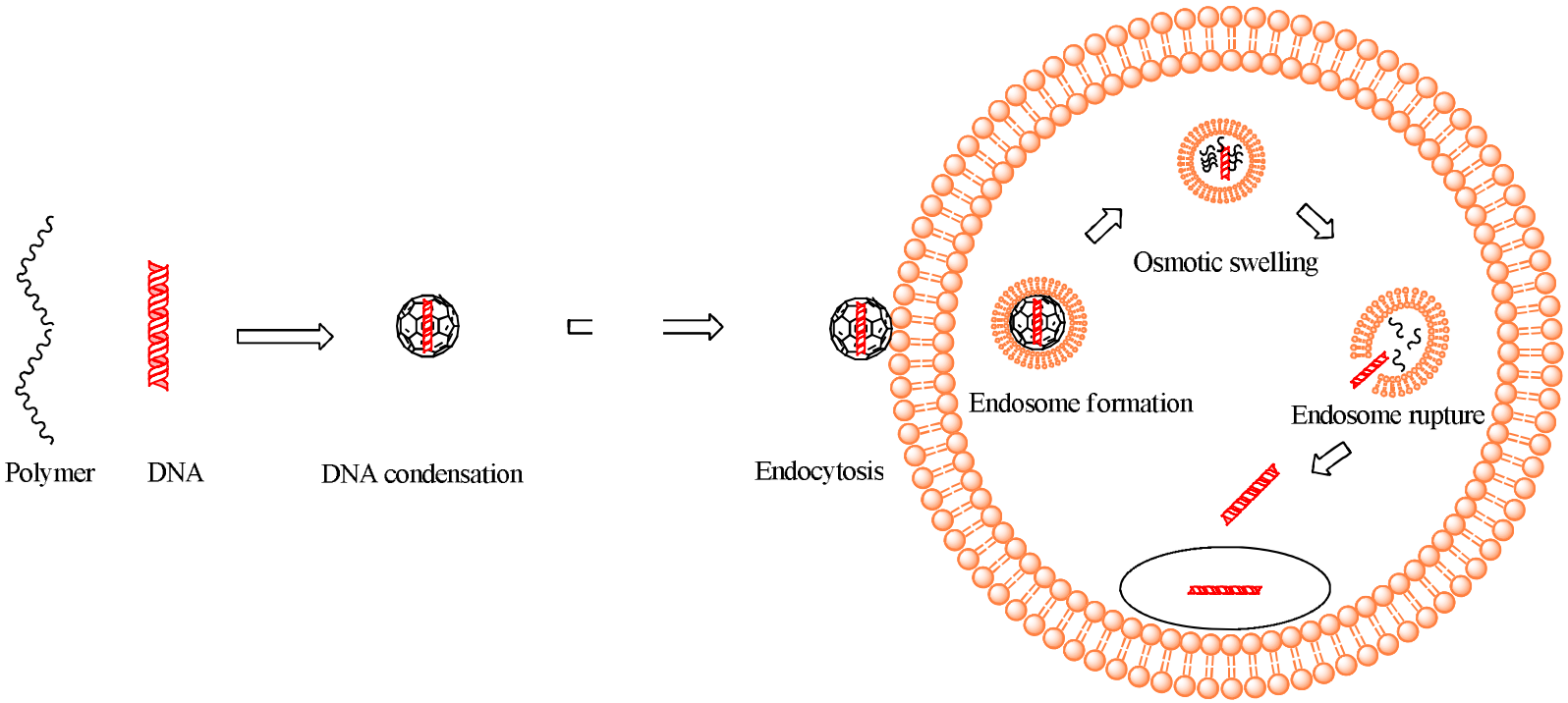

Figure 1 Schematic demonstrating the process of transgene delivery as described in the text.

1.3.1 Polyethylenimine (PEI). PEI is currently the standard for polymer mediated transgene delivery and has been studied exhaustively throughout the literature. Many have proven continued success with transgene delivery in many types of cells using PEI/DNA both *in vitro* (Boussif, et al., 1995; Cherng, Hung, & Kao, 2011; Gabrielson & Pack, 2009; Kang, Kang, & Bae, 2011; M. Lee, 2007; Mishra, Kang, & Bae, 2011; Y. H. Wang, et al., 2011) and *in vivo* (Chumakova, et al., 2008; Coll, et al., 1999). This polymer has also been characterized (von Harpe, Petersen, Li, & Kissel, 2000) extensively for its intrinsic properties such as buffering capacity which is believed to promote endosomal rupturing releasing the genetic material into the cytoplasm. This is the defining characteristic that is believed to be the contributing factor to the proton sponge effect (Akinc, et al., 2005; Berthold, et al., 2010; Boussif, et al., 1995; Y. H. Wang, et al., 2011). This key feature is also believed to be one of the reasons that PEI has such superior transfection efficacies. In addition to the buffering capacity, the size of the polyplexes formed using PEI allows for ease of entry into cells by endocytosis. It has been proven that the endocytotic range of less than 200 nm is required for efficient transgene delivery (Akinc, Lynn, Anderson, & Langer, 2003). PEI has been found to satisfy this requirement forming polyplex sizes that range from 100-130 nm (Merdan, et al., 2002; Y. H. Wang, et al., 2011). Although this polymer seems ideal for use as a non-viral therapeutic agent, there are some limitations to its use. One noteable finding throughout the literature is that transfection efficiency with PEI is heavily dependent on the molecular weight of

the polymer, such that higher molecular weight yields higher transgene expression (Godbey, Wu, & Mikos, 1999). However, it has also been determined that cytotoxicity increases with increasing molecular weight thus limiting use of this polymer beyond these limits (Brunot, et al., 2007). Due to this, many have attempted to modify PEI with other moieties such as polyethylene glycol (PEG), which was shown to lower cytotoxicity and in some cases enhance gene delivery with particles sizes forming within the endocytotic range (Chen, et al., 2011; Peng, et al., 2008; Zhang, et al., 2010; Zhuang, et al., 2011). In addition many have used PEI to coat the surfaces of adenoviruses to reduce specific interactions, resulting in enhanced viral gene delivery (Han, et al., 2010; MeunierDurmort, et al., 1997; Varga, et al., 2005). From these examples, it demonstrates that PEI serves as an ideal polymer for transgene delivery.

1.3.2 Addition of targeting ligands for enhanced transgene delivery. It is evident that PEI possesses great potential as a non-viral vector for transgene delivery. In the previous section, PEI was shown to be used and characterized successfully *in vitro*. One major limitation to its use *in vivo* is the lack of targeting ability towards specific types of cells. In theory, addition of these targeting abilities would enhance transgene delivery towards targeted cells by reducing non-specific interactions. This would also result in lower cytotoxicity towards non targeted cells. Overcoming this problem would make polymeric transgene delivery with PEI a more effective treatment *in vitro* as well as *in vivo*. It is known that many cancers have overexpressed receptors on the cell surfaces that

could be used as potential targets for these types of treatments. For example, prostate cancer cell overexpress prostate-specific membrane antigen (PSMA) (Moffatt, Papasakelariou, Wiehle, & Cristiano, 2006) and breast cancer cells overexpress the HER2 antigen (Acharya, Dilnawaz, & Sahoo, 2009; Germershaus, Neu, Behe, & Kissel, 2008). Researchers have been able to take advantage of this fact by introducing targeting ligands to the polyplexes, such as antibodies that have specificity towards these antigens. Many have demonstrated this concept successfully by conjugating antibodies to PEI and performed targeted transgene delivery to prostate cancer cells (He, et al., 2010; Lu, Jackson, Gleave, & Burt, 2008; Moffatt & Cristiano, 2006; Moffatt, et al., 2006; Patri, et al., 2004), breast cancer cells (Acharya, et al., 2009; Germershaus, et al., 2008), ovarian carcinoma cells (Merdan, et al., 2003), and lymphocytes (Buschle, et al., 1995) with positive results. With the introduction of the antibodies to the polyplexes, it is expected that an increase in the particle size occurs. This was determined not to be true with particles that formed within the endocytotic range between 100-230 nm with low zeta potentials ranging from ±2 mV - ±6 mV using antibodies and other targeting ligands (Germershaus, et al., 2008; Ghiamkazemi, Amanzadeh, Dinarvand, Rafiee-Tehrani, & Amini, 2010; Merdan, et al., 2003). Most importantly, all groups that have used this concept have all shown higher transgene expressions with lower cytotoxicity. With these positive results already being shown *in vivo* (Fujita, et al., 2007), there still exists room for improvement.

This could be facilitated with the use of different targeting ligands in combination with different polymers. For the time being, this field remains open.

1.3.3 Poly amido-amines. With toxicity presenting a major limitation to the use of untargeted PEI in clinical trials, there exists a need to explore other polymers that enhance transfection at higher levels. One other class of polymers that has been studied more recently and have shown to be comparable to PEI are poly amido-amines. These polymers are synthesized using a Michael type addition of various primary or secondary amines to bisacrylamides and have shown, in some cases, to have higher transfection efficacies than PEI and form polyplexes in the endocytotic range. This class of polymers also has good water solubility and biodegradability showing their applicability in transgene and drug delivery (Emilitri, Ranucci, & Ferruti, 2005; Guo, et al., 2011) and advantage over the non-biodegradable PEI. In addition to these characteristics, it has been shown that these polymers have lower cytotoxicity than PEI (R. B. Wang, et al., 2010), which is attributed to its biodegradability (Ferruti, Marchisio, & Duncan, 2002). Throughout the literature, modifications have also been performed on these polymers such as addition of disulfide linkages to enhance transgene delivery (Lin, et al., 2007) and incorporation of hydrophobic moieties (Piest & Engbersen, 2010). These modifications have been shown to induce lower surface charge density, (Lin & Engbersen, 2008; Lin, et al., 2007; Piest & Engbersen, 2010) while still maintaining a buffering capacity higher than PEI (Lin, et al., 2007). Along with the biodegradability of the polymer, low surface charge density is another

contributing factor to the lower cytotoxicity resulting from less interaction between polymer and the negatively charged cellular membrane. Based on these results it is encouraging to see that there are potentially other polymers that have to ability delivery genes better than PEI with or without modifications.

1.3.3 Amine-epoxide polymers. Amine-epoxide polymers can serve as another potential for transgene delivery as they have been extensively investigated and characterized in the literature for their intensive properties and reaction kinetics (Ghaemy, Barghamadi, & Behmadi, 2004; Klee, Hagele, & Przybylski, 2003; Rosu, Mititelu, & Cascaval, 2004; Theis & Ritter, 2010). Synthesis of these polymers involves a ring opening polymerization (ROP) of diglycidyl ether (epoxide) by polyamines (Guo, et al., 2011; Theis & Ritter, 2010) as shown in figure 1.

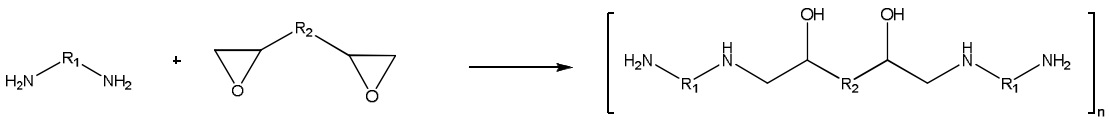

Figure 2: General scheme for a ring opening polymerization between an amine and epoxide .

For simplicity, figure 2 depicts a linear polymerization although it is possible to branching can occur in this type of reaction (Barua, et al., 2009). Although these polymers are relatively easy to synthesize, there have been no applications shown in the literature. To my knowledge, these polymers have not been thoroughly investigated for transgene delivery. Recently, it was found that one amine-epoxide polymer, named 1,4 C-1,4 Bis, was shown to have higher transgene expression than PEI at certain polymer:DNA weight ratios (Barua & Rege, 2010). These

results warranted more investigation into these types of polymers for evaluation as potential alternative strategy to viral vectors and forms the basis for investigation in this thesis.

1.4 Conclusion

Although this is not an exhaustive list of polymers studied, it illustrates the potential for their use in transgene delivery. Polymers provide the advantage of ease of synthesis, ability to be functionalized, and in some cases are biodegradable. In addition, they also possess the ability to carry larger genes than viral vectors making them far more practical for gene therapy. Although toxicity still remains as one of the limiting factors to the use of polymers, great strides have been made to reduce this effect through the incorporation of PEG and other functional groups. Even with the advantages to the use of polymers over viral vectors outweighing the disadvantages, more research is still needed to exploit the full potential of these as gene therapy agents.

Chapter 2

POLYMER SYNTHESIS AND EVALUATION OF TRANSFECTION EFFICACY

2.1 Introduction

As stated in the previous chapter, polymeric gene delivery systems have become a heavily investigated non-viral alternative to viral vectors. Currently, one polymer that is commercially available, known as PEI has become the standard for polymeric transgene delivery (Merdan, et al., 2002). This is due to the excess positive charges on the polymer backbone resulting in effective condensation of DNA into nanoparticles allowing for sufficient uptake by cells via endocytosis. While this polymer has been shown to be used successfully *in vitro*, cytotoxicity issues arise from its use. More recently, it was discovered that an amine-epoxide polymers enhanced transgene delivery at levels higher than PEI at certain polymer:DNA weight ratios. It was found that the optimum polymer:DNA ratio that gave the highest transgene expression using the polymer name 1,4C-1,4Bis was 10:1 (w/w) (Barua & Rege, 2010). Based on this result, it was encouraging to see that there is a possibility for polymers synthesized using these types of monomers, to produce better transfection efficacies than PEI. This provided the motivation behind the work presented in this chapter.

In this work, synthesis of a library of 26 polymers using the same reaction as described in section 1.3.3 occurred. From this library, only 21 polymers were successfully synthesized and those polymers were carried forward in transfection

studies comparing their respective transfection efficacies to that of PEI at a polymer:DNA weight ratio of 10:1. The cell lines used in the experiment were the human pancreatic cancer cell line, Mia-PaCa-2 cells and the human prostate cancer cell lines, PC-3 cells and PC-3 PSMA cells. From the screening, lead polymers were identified as those having greater transfection efficacies than that of PEI.

2.2 Materials and Methods

2.2.1 Materials. The amines (as shown in figure 3); 2,2 dimethyl-1,3-propanediamine (1), N-(2-aminoethyl)-1,3-propanediamine (2), 3,3'-Diamino-N-methyldipropylamine (3), Tris-(2-aminoethyl)amine (4), Diethylenetriamine (5), Pentaethylenehexamine (6), Ethylenediamine (7), Triethylenetetramine (8), 2,2'-(ethylenedioxy)bis(ethylamine) (9), 1,5-Diamino-2-methylpentane (10), 1,3 Diaminopropane (11), N,N-Dimethylethylenediamine (12), and 1,3 Diaminopentane (13) were obtained from Sigma-Aldrich (St. Louis, MO) and used as received without any further modification. The ethers (as shown in figure 4); Neopentyl glycol diglycidyl ether (NPGDE) and 1,4-cyclohexanedimethanol diglycidyl ether (1,4C) were also both obtained from Sigma-Aldrich and used as received without any further modification. The control polymer, branched polyethyleneimine (MW ~ 25,000) was also obtained from Sigma-Aldrich. Ninhydrin reagent was purchased from Sigma-Aldrich. Luciferase and BCA protein assay kits were purchased from Promega Corporation (Madison, WI) and

Thermo Fisher Scientific Inc. (Rockford, IL) respectively. The pGL3 control vector was also purchased from Promega Corporation.

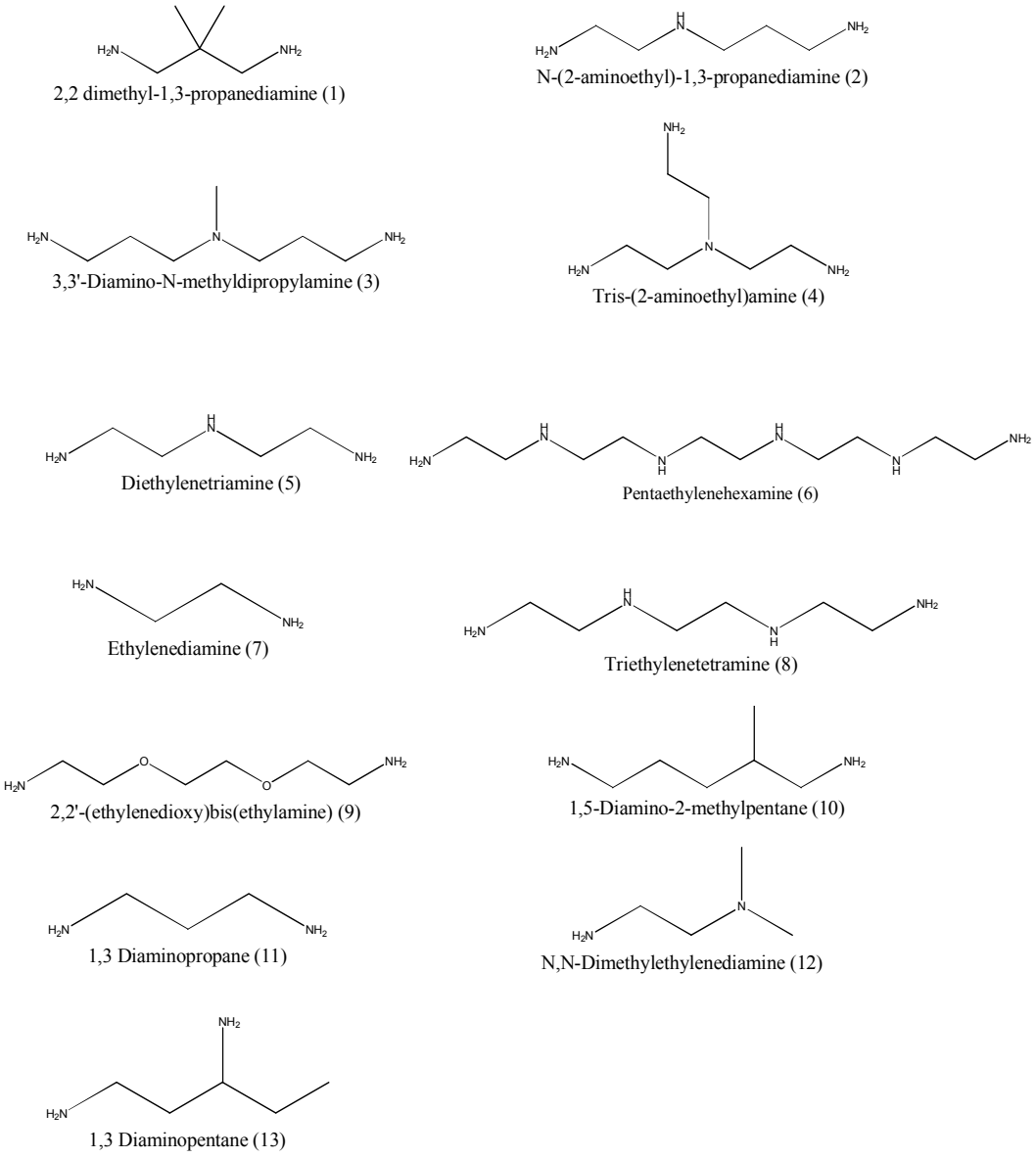

Figure 3: Amine monomers used in the synthesis of the NPGDE and 1,4C libraries. The polymers were numbered according to the amines as labeled above.

Figure 4: Epoxides monomers used in the synthesis of the NPGDE and 1,4C libraries. The libraries were named according to the epoxide used.

2.2.2 Synthesis of the polymers (parallel polymer synthesis). Synthesis of the polymers was carried out as a parallel synthesis approach as described previously (Barua, et al., 2009). Two libraries of 26 polymers was synthesized using the two ethers, 1,4C and NPGDE, and the amines listed above (1-13) in a ring opening polymerization (ROP) (Guo, et al., 2011) resulting in 13 polymers for each library. Figure 3 shows the structures of all of the amines and figure 4 shows all of the structures of the epoxides used in the synthesis of these libraries. The libraries formed using these monomers will be referred to henceforth as the NPGDE library and 1,4C library labeled as such according to the epoxides used to form these polymers. Additionally, each polymer was numbered according to the numbering system given to the amines in figure 3. The epoxide (1,4C or NPGDE) and the amine were mixed in a 1:1 molar ratio in a 4 mL scintillation vial (Wheaton) and incubated at room temperature (22°C - 25°C) for 16 hours. After 16 hours, the resulting polymer was removed from the vial and dissolved in 1X PBS (10 mM Na_2HPO_4, 140 mM NaCl, 27 mM KCl, pH 7.4) to form a polymeric solution with a concentration of 10 mg/mL. The pH of the polymer solution was adjusted to 7.4 using 6N hydrochloric acid to compensate for the alkalinity of the solution (Barua, et al., 2009). The degree of polymerization was determined by

quantifying the amine content using the ninhydrin assay (Gabrielson & Pack, 2009). Briefly, a standard curve was made using glycine with varying amine concentrations (0 μM - 250 μM, total volume of 200 μL for each standard). A 1 mL sample of 5 mg/ml of the resulting polymer was diluted 50X to a total volume of 200 μL. 100 μL of ninhydrin reagent was added to each of the standards and samples and the absorbance was read using a plate reader (Bio-Tek Synergy 2) at a wavelength of 570 nm to quantify the amine concentration of the polymer. Only the polymers that were soluble in solution after the 16 hour reaction were carried further and used in transfection studies.

2.2.3 Purification of lead polymers. Purification of the lead polymer solutions, as identified from the screening, occurred using a 1000 Da molecular weight cutoff (MWCO) membrane. Dialysis occurred in nanopure water for 48 hours and replacement with fresh water occurred every 12 hours. After 48 hours of purification, the polymer solutions were lyophilized, reconstituted in 1X PBS, and filtered with a 0.22 μm syringe filter before use in transfection experiments.

2.2.4 Cells and cell culturing. PC-3 cells (prostate cancer cell line) were obtained from the American Type Culture Collection (Manassas, VA), PC-3 PSMA cells (prostate cancer cell line) were obtained from Dr. Michael Sadelain from the Memorial Sloan-Kettering Cancer center (New York, NY), and Mia-PaCa-2 cells (Pancreatic cancer cell line) were obtained from Dr. Haiyong Han from The Translational Genomics Research Institute (Phoenix, AZ). All of these cells were cultured in serum containing media (RPMI 1640, 10% FBS, 1%

penicillin, 1% streptomycin) and were grown in an incubator under humidified air containing 5% CO_2 at 37°C.

2.2.5 DNA purification. DH5α E. Coli cells transformed with the pGL3 control vector, which encodes for the luciferase protein, under control of the SV40 promoter was cultured in 150 mL of terrific broth (MP Biomedicals, Solon, OH) containing 100 mg/mL Ampicillin, overnight in an incubator (200 RPM, 37°C) for 14 hours. The cultures were centrifuged at 6000 RCF at 4°C for 15 minutes and were purified according to the QIAGEN plasmid purification maxi kit protocol (QIAGEN Inc., Valencia, CA). DNA concentration and purity was determined by measuring optical density (OD_{260} and OD_{280}) using the NanoDrop spectrophotometer (ND-1000, NanoDrop Technologies). Only DNA within the acceptable absorbance range (1.8-2, indicates purity) of the 260 nm: 280 nm ratio were used in the transfection experiments. The plasmid was stored at -20 °C until use.

2.2.6 Transfection experiments. Transfection of PC-3, PC-3 PSMA, and Mia-PaCa-2 cells using DNA/polymer complexes (polyplexes) consisting of pGL3 plasmid DNA and the synthesized polymers was performed. Prior to the transfection, cells were seeded at a density of 50,000 cells/well in 500 µl of serum containing medium in a 24-well plate and allowed 24 hours for attachement. Luciferase plasmid, pGL3, was diluted to a concentration of 50 ng/µL using Tris EDTA buffer (Thermo Fisher Scientific, Rockford, IL). Polymer solutions were prepared and diluted with 1X PBS to a concentration of 500 ng/µL and filter

sterilized by using a 0.22 μm filter. Complexes for transfection were prepared by the dropwise addition of 2000 ng (4 μL) of each polymer on top of 200 ng (4 μL) of pGL3 plasmid DNA to form a polymer:DNA weight ratio 10:1. Complexes were incubated for 20 minutes at room temperature prior to use. Cells were then treated with polyplexes in serum free media (RPMI RPMI 1640, 1% penicillin, 1% streptomycin) for 6 hours in an incubator under humidified air containing 5% CO_2 at 37°C. Subsequently, the medium was replaced with fresh serum containing medium and allowed to incubate for an additional 48 hours. Luciferase protein expression, expressed in terms of relative luminescence units or RLU, was determined using the luciferase assay kit according to the manufacturer's protocol 48 hours after transfection. The luminescence was quantified using the plate reader. The protein content in each well was determined using the BCA Protein Assay Kit. Transgene expression in all cell lines was calculated and normalized with the protein content and expressed as RLU per milligram (mg) protein (RLU/mg protein).

2.2.7 Dose response using the lead polymers. Dose response experiments were carried out using the same method as in the previous section. From the studies in the previous section, lead polymers were identified as those with higher transfection with PEI across all cell lines from each library (NPGDE and 1,4C). These leads were purified according to the method in section 2.2.3 and were used in subsequent treatments of the cells at different polymer:DNA weight ratios. The ratios used in these studies were 1:1, 5:1, 10:1, 20:1, 25:1. and 50:1.

2.2.8 Statistical analyses. All experiments were carried out at least in triplicate unless mentioned otherwise. The significance between the control and each experimental test condition was determined using the two tailed student's t-test with $p < 0.05$ being considered significant.

2.3 Results and Discussion

2.3.1 Polymer synthesis. Out of the 26 polymers that were synthesized, only 11 of the polymers from the NPGDE library (1-11) and 10 polymers from the 1,4C library (1-3, 5-6, and 9-13) were soluble in 1X PBS making a total of 21 successful polymerizations. The polymers that were insoluble after the 16 hour reaction can be explained by the possibility of extensive cross linking. This was confirmed physically by the hardness of the resulting polymer that inhibited removal from the reaction vial. Ideally, these polymers should be re-synthesized with the molar ratios of each monomer adjusted however, with the focus being on rapid generation and identification of potential therapeutic polymers, this was not employed. All of the successful polymer amine concentrations were quantified using the ninhydrin assay (Gabrielson & Pack, 2009). All polymer solutions were adjusted to a pH of 7.4 and store in a stock solution at a concentration of 10 mg/mL. Dilution of the polymer solutions to a concentration of 500 ng/μL was performed before use in transfection experiments.

2.3.2 Transfection in Mia-PaCa-2, PC-3, and PC-3 PSMA cell lines.

All three cell lines were transfected with polyplexes consisting of the NPGDE library and pGL3 plasmid DNA. Two of the cells lines, Mia-PaCa-2 and PC-3 cells were transfected using the 1,4C library and pGL3 plasmid DNA. PC-3-PSMA cells were not used for the evaluation of the 1,4 C library due to comtamination issues that surfaced during the screening process. All transfection experiments for the purpose of this screening were performed at a 10:1 polymer:DNA weight ratio as explained previously.

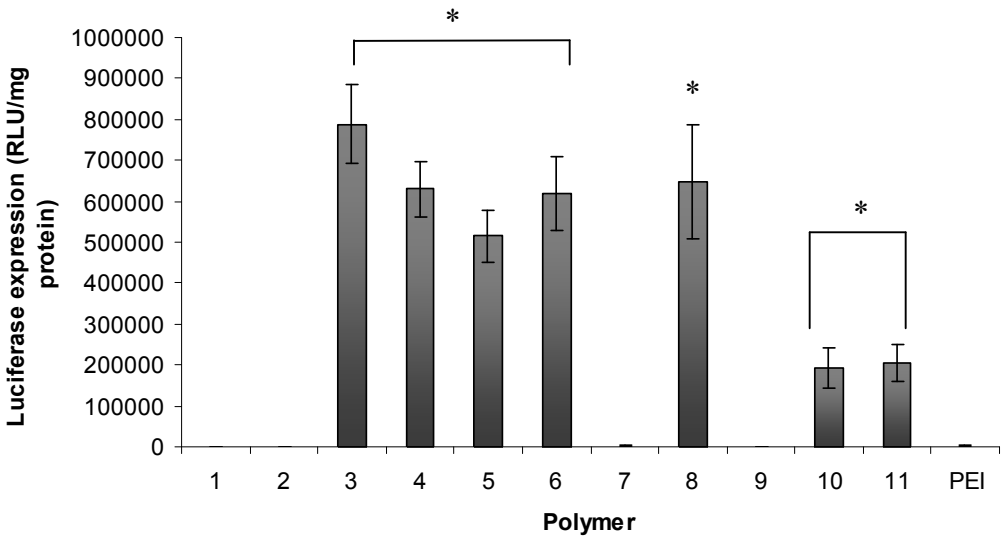

Figure 5: Luciferase gene expression (RLU/mg protein) 48 hours after transfection using the NPGDE based polymers in Mia-PaCa-2 cells. Only polymers that were soluble in 1X PBS after synthesis were used. All Transfections were performed at a polymer:DNA weight ratio 10:1 (n = 3, * = p < 0.05). P-values were obtained by comparing data from each polymer to PEI. All negative luminescence values were not considered significant due to lack of physical meaning, and were not included in the calculations.

As shown in figure 5, polymers 3, 4, 5, 6, 8, 10, and 11 from the NPGDE library showed higher transfection than PEI with statistical significance ($p < 0.05$). It was calculated that these polymers had between approximately 170 - 700 fold

higher luciferase expression in Mia-PaCa-2 pancreatic cancer cells. Through visual inspection it was observed that PEI, used at this weight ratio (10:1), exhibited high toxicity towards the Mia-PaCa-2 cells, which is presumed to be the reason for poor transfection. Polymers 1, 2, 7, and 9 were also screened at this condition and showed very poor transfection efficacies. It is worthy to note that toxicity was not visually observed with these polymers and thus cannot be the explanation for the poor transfection.

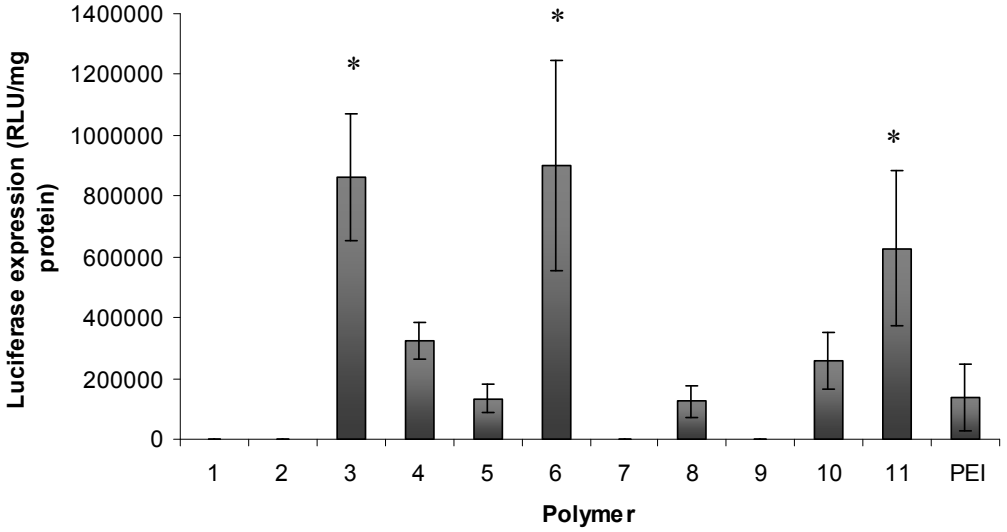

Figure 6: Luciferase gene expression (RLU/mg protein) 48 hours after transfection using the NPGDE based polymers in PC-3 cells. Only polymers that were soluble in 1X PBS after synthesis were used. All transfections were performed at a polymer:DNA weight ratio 10:1 (n = 3, * = p < 0.05). P-values were obtained by comparing data from each polymer to PEI. All negative luminescence values were not considered significant due to lack of physical meaning, and were not included in the calculations.

A similar trend was observed when using the same NPGDE library with PC-3 cells with the exception of polymers 5 and 8, which showed slightly less luciferase expression as compared to PEI as shown in figure 6. Polymers 3, 6, and 11 showed statistically higher transfection efficacy than PEI. These polymers

exhibited a 4 - 6 fold higher luciferase expression than PEI. In addition, polymers 4 and 10 showed a 2 fold higher luciferase expression than PEI. While polymers 4 and 10 were not statistically significant, the error bars are within reasonable limits and thus it is presumed that these results are reliable. Moreover, it was observed that PEI was not toxic at this weight ratio in the PC-3 cells, which correlates to what was previously shown by others (Barua & Rege, 2010).

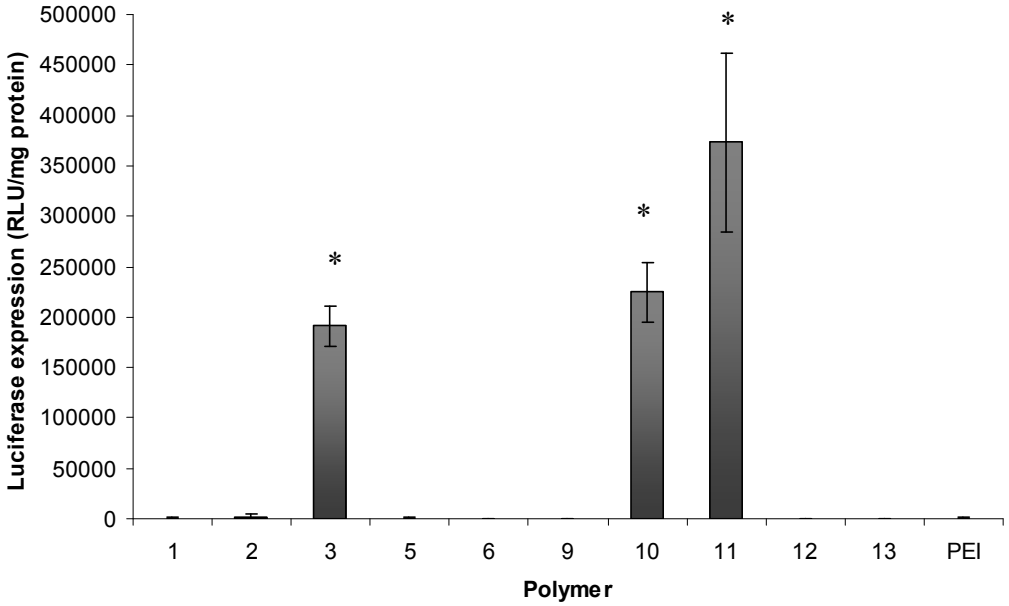

Figure 7: Luciferase gene expression (RLU/mg protein) 48 hours after transfection using the 1,4C based polymers in Mia-PaCa-2 cells. Only polymers that were soluble in 1X PBS after synthesis were used. Transfections were performed at a polymer:DNA weight ratio 10:1 (n = 3, * = p < 0.05). P-values were obtained by comparing data from each polymer to PEI. All negative luminescence values were not considered significant due to lack of physical meaning, and were not included in the calculations.

Screening of the 1,4 C library in Mia-PaCa-2 cells only showed three polymers that had better transfection efficacies than PEI as demonstrated in figure 7. Polymers 3, 10, and 11 showed statistically higher transfection efficacies of about 1700 – 3000 fold over PEI. Interestingly, polymer 6 from this library did

not show significant transfection efficacy as was demonstrated with polymer 6 from NPGDE library even though the amine used to synthesize both was the same. Comparing the result for PEI to that shown in figure 5, there exists a correlation of toxicity when using PEI to transfect Mia-PaCa-2 cells thus confirming the results in both cases are true. This can also accounts for the large fold transfection efficacies demonstrated by polymer 3, 10, and 11 from this library.

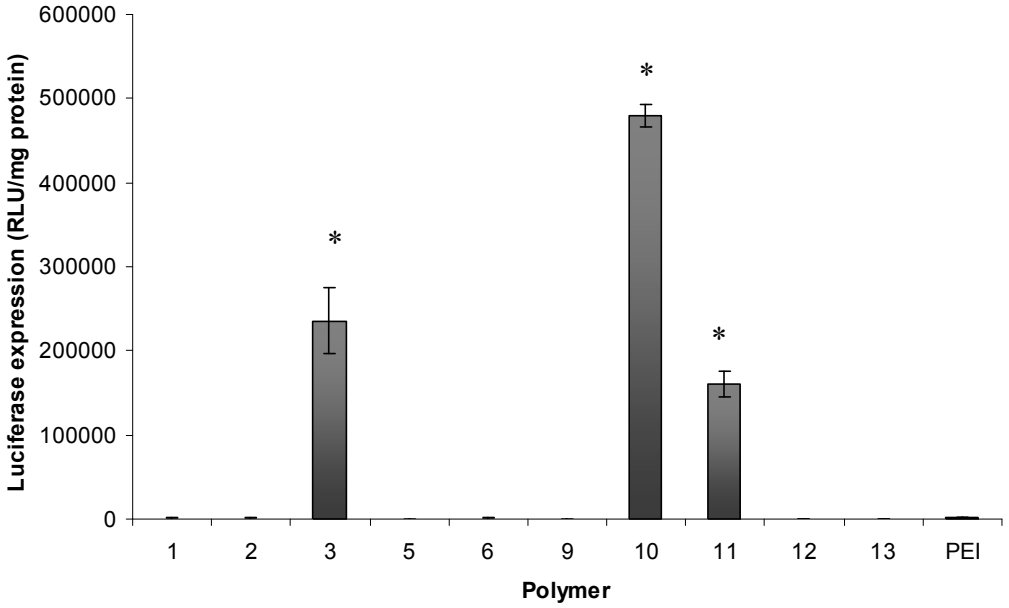

Figure 8: Luciferase gene expression (RLU/mg protein) 48 hours after transfection using the 1,4C based polymers in PC-3 cells. Only polymers that were soluble in 1X PBS after synthesis were used. Transfections were performed at a polymer:DNA weight ratio 10:1 (n = 3 for all polymers except 3 (n = 2) and 10 (n = 2) , * = $p < 0.05$). P-values were obtained by comparing data from each polymer to PEI. All negative luminescence values were not considered significant due to lack of physical meaning, and were not included in the calculations.

From figure 8, a similar trend was observed in PC-3 cells using the 1,4 C library. Polymers 3, 10, and 11 again showed statistically higher transfection efficacies than PEI. These polymers showed between 80 - 360 fold higher

transfection efficacies than PEI. It was also observed again that polymer 6 did not show significant transfection efficacies.

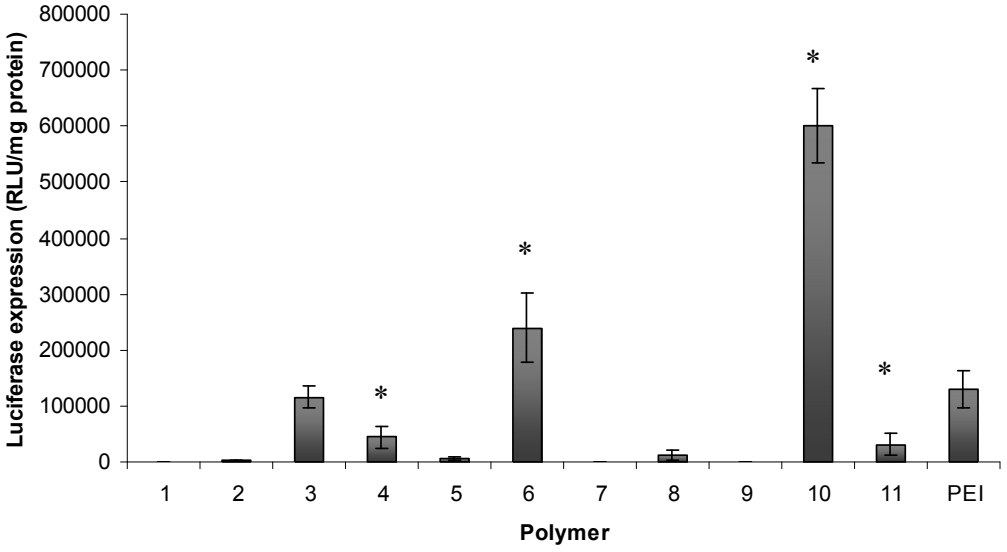

Figure 9: Luciferase gene expression (RLU/mg protein) 48 hours after transfection using the NPGDE based polymers in PC-3 PSMA cells. Only polymers that were soluble in 1X PBS after synthesis were used. Transfections were performed at a polymer:DNA weight ratio 10:1 (n = 3, * = p < 0.05). P-values were obtained by comparing data from each polymer to PEI. All negative luminescence values were not considered significant due to lack of physical meaning, and were not included in the calculations.

As mentioned previously due to the discovery of contamination with PC-3 PSMA cells, the 1,4C library was not screened using these cells. Prior to the contamination, the NPGDE library was screened and again showed similar trends in that the same polymers were able to transfect these cells as was shown in the other two cell lines. It was found however, that only polymers 6 and 10 showed statistically higher transfection efficacies than PEI showing only between a 2 – 5

fold increase. Polymers 3, 4, and 11 showed slightly less transfection efficacy than PEI.

From the results shown in figures 5 – 9, lead candidate polymers can be identified as those that consistently showed higher transfection efficacies than PEI across multiple cell lines. From the NPGDE library the leads were NPGDE - 3, NPGDE - 6, NPGDE - 10, and NPGDE – 11. While NPGDE - 4, NPGDE – 5, and NPGDE – 8 showed higher transfection efficacies in some cell lines, they were not consistent enough to be considered lead polymers. From the 1,4C library three leads were identified and they are 1,4C-3, 1,4C-10, and 1,4C-11. From these results it is apparent that the polymers made using amines 3,3'-Diamino-N-methyldipropylamine (3), 1,5-Diamino-2-methylpentane (10), and 1,3 Diaminopropane (11) as monomers perform well for transgene delivery. Based on the structures of these three amines it is concluded that methylene spacing between amine groups plays a crucial role in transfection. It was determined previously that DNA binding efficiency is related methylene spacing in that three or four methylene spacing in between amine groups showed better DNA binding than two methylene spacing (Rege, et al., 2005; van Dam, Korolev, & Nordenskiold, 2002). Based on the results obtained from these experiments, this result holds true because all three lead amines (3, 10, and 11) have between three to five methylene spacing. Additionally, they also showed higher transfection than PEI in most cases. This is further confirmed based on the structures of amines 4, 5, 6, and 8, which all have two methylene spacing, resulting in lower transfection

efficacies when compared to the lead amines in most cases. Interestingly, Pentaethylenehexamine (6) transfected well when synthesized using NPGDE but did not transfect well when synthesized with 1,4C. This may be due to some cytotoxicity effects although that was not investigated in these experiments. From these results it is valid to conclude that between three and five methylene spacing plays a crucial role in transfection. This is explained by increased DNA binding as others have shown, which can possibly lead to more effective condensation of the DNA allowing easier uptake via endocytosis.

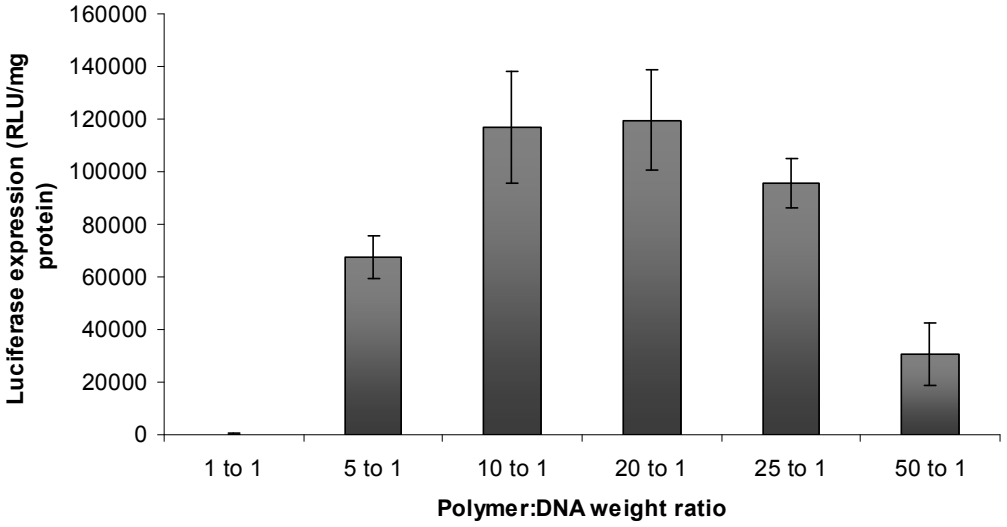

Figure 10: Luciferase gene expression (RLU/mg protein) 48 hours after transfection using the purified NPGDE based polymer number 3 in Mia-PaCa-2 cells. Transfections were performed at various polymer:DNA weight ratios (n = 3) All negative luminescence values were not considered significant due to lack of physical meaning, and were not included in the calculations.

2.3.3 Dose responses using lead polymers identified from the NPGDE library.

After completion of the screening, the next logical step is to proceed to testing the lead polymers at different polymer:DNA weight ratios which henceforth will be referred to as dose responses. This needs to be determined to see if these polymers are truly comparable to PEI. Currently, it was discovered in the literature that PEI is most effective at a polymer:DNA weight ratio of 1:1 (Barua & Rege, 2010; Cherng, et al., 2011). Screening of the first lead from the NPGDE library (polymer 3) in Mia-PaCa-2 cells showed a typical bell shaped curve response (Lin, et al., 2007) with the maximum transfection occurring when using polymer:DNA weight ratio of 20:1. It is worthy to note that this polymer was purified using the method as described in section 2.2.3 resulting in lower

amine content as demonstrated with the ninhydrin assay. This lowering in amine content could be a potential explanation for the difference in transfection efficacies between the 10:1 from the "unpurified" polymer 3 (ref. figures 5 and 10) and the purified polymer 3 both of which are from the same library and same stock.

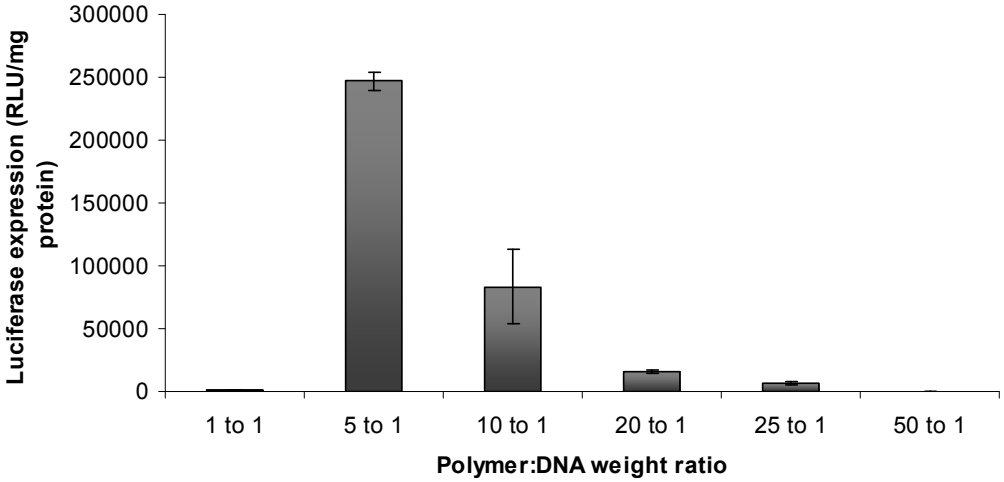

Figure 11: Luciferase gene expression (RLU/mg protein) 48 hours after transfection using the purified NPGDE based polymer number 6 in Mia-PaCa-2 cells. Transfections were performed at various polymer:DNA weight ratios (n = 3) All negative luminescence values were not considered significant due to lack of physical meaning, and were not included in the calculations.

Another lead indentified from the screening of the NPGDE polymers was polymer 6. This polymer was carried into the dose response similarly to polymer 3 from the NPGDE library in Mia-PaCa-2 cells. Results from the dose response of the purified NPGDE polymer 6 shows that the optimum ratio occurs at 5:1 (w/w). One consistent result that is found from the dose responses from both of these polymers is that there exists an optimum ratio at which transfection is the highest.

It is also observed that after this optimum ratio, transfection efficacy decreases to significantly lower levels. While this was not tested in these experiments, it is hypothesized that this may be due to cytotoxic effects. It is known that cationic polymers have a tendency to interact with the negatively charged cellular membrane thus the possibility of damaging the cells exists (Choksakulnimitr, Masuda, Tokuda, Takakura, & Hashida, 1995; Lv, Zhang, Wang, Cui, & Yan, 2006; Moghimi, et al., 2005). This is primarily due to the excess of positive charges on the polyplex surface as demonstrated by zeta potential measurements (Kang, et al., 2011; Lin, et al., 2007). While increasing the polymer:DNA ratio may result in more effective condensation of the DNA, there also exists issues with higher toxicity that has been demonstrated exhaustively with the use of PEI. This seems to be the case with the use of these polymers as demonstrated in figures 10 and 11. Therefore, it is concluded that there is a need to achieve a balance between these two factors in order to have an ideal vector for transgene delivery.

2.4 Conclusion

In this chapter, synthesis of a new library of polymers was performed using the reaction between an amine and an epoxide in a ring opening polymerization (ROP) using the monomers NPGDE and 1,4C with various amines. Evaluation of these polymers for transgene expression using the pGL3 control vector, identified four lead polymers from the NPGDE library and three lead polymers from the 1,4 C library that showed up to a 3000 fold higher transfection efficacy than PEI at 10:1 polymer to DNA weight ratio. Based on the structures of these monomers, it is evident that methylene spacing between amines not only play a role in DNA binding (Rege, et al., 2005; van Dam, et al., 2002) as others have shown but also have a significant effect on transfection efficacy. Out of these leads, two were purified and used in dose responsing experiments where it was found there exists optimum weight ratios at which transfection is the highest. With the data presented here it is concluded that polymers synthesized using these monomers hold great potential as gene therapeutic agents.

Chapter 3

INTRODUCTION OF AN ANTIBODY AS A TARGETING LIGAND FOR IMPROVED TRANSGENE EXPRESSION

3.1 Introduction

Conjugation chemistry has been a well explored topic throughout the literature. Use of the heterobifunctional linker molecule, N-succinimidyl 3-(2-pyridyldithio) propionate (SPDP) has been explored greatly in the literature (Carlsson, Drevin, & Axen, 1978) mostly due to its diversity and simplicity. There are two reactive sites of the SPDP molecule that many researchers take advantage of. One end of the molecule contains a highly amine-reactive N-hydroxysuccinimide (NHS) ester which allows for easy conjugation to amine containing molecules such as proteins, antibodies, and polymers similar to PEI. The other is a 2-pyridylthio group that is highly reactive with sulfhydryls making it easy to bind the linker molecules to each other. This becomes important when conjugating antibodies to polymer molecules. This molecule has been used extensively in the literature for the conjugation of antibodies to PEI. Performing this type of conjugation has been shown to enhance transgene delivery towards cancer cells that contain overexpressed antigens on their surfaces (Germershaus, Merdan, Bakowsky, Behe, & Kissel, 2006; Germershaus, et al., 2008; Kircheis, et al., 1997; J. Lee, et al., 2010; Merdan, et al., 2003; Moffatt, et al., 2006; Slutter, et al., 2010).

Previously, a library of 80 polymers were synthesized in our lab and screened for their respective transgene expressions. One lead candidate, known as 1,4C-1,4Bis, was identified as having improved transgene expression over PEI at certain polymer/DNA weight ratios (Barua, et al., 2009). Since then, this polymer has become the golden standard of amine-epoxide polymers. However, it is hypothesized that enhanced transgene delivery will result with a targeting ligand conjugated to the polymer. The goal of this study was to successfully synthesize the polymer-antibody conjugates. Ideally, evaluation would be needed to conclude if addition of a targeting ligand to the polyplex would induce an improved transgene expression in PC-3 PSMA prostate cancer cells. Unfortunately, the evaluation experiments were not able to take place due to unforeseen contamination of the PC-3 PSMA cells.

3.2 Materials and Methods

3.2.1 Materials. 1,4-cyclohexanedimethanol diglycidyl ether (1,4C) and 1,4-bis(3-aminopropyl) piperazine (1,4Bis) were both obtained from Sigma Aldrich and used as received without any further modification. The monoclonal antibody, J591, was obtained from Dr. Neil Bander from Cornell University Medical College (New York, NY). The linker molecule, N-succinimidyl 3-(2-pyridyldithio) propionate (SPDP) and Ellman's reagent were obtained from Thermo Fisher Scientific. Ninhydrin reagent was also obtained from Sigma-Aldrich. BCA protein assay kit and Dithiothreitol (DTT) were purchased Thermo Fisher Scientific Inc. (Rockford, IL).

3.2.2 Synthesis of the polymer 1,4C-1,4Bis. Synthesis of this polymer was carried out using a synonymous method as described in section 2.2.2 and also as described previously (Barua, et al., 2009). 1,4C (269.5 μL) and 1,4Bis (238.2 μL) were mixed in a 1:1 molar ratio in a 4 mL scintillation vial and incubated at room temperature for 16 hours. After 16 hours, the resulting polymer was removed from the vial and dissolved in 1X PBS (10 mM Na_2HPO_4, 140 mM NaCl, 27 mM KCl, pH 7.4) to form a polymeric solution with a concentration of 10 mg/mL. The pH of the polymer solution was adjusted to 7.4 using 6N hydrochloric acid to compensate for the alkalinity of the solution (Barua, et al., 2009). The degree of polymerization was quantified by quantifying the amine content using the ninhydrin assay (Gabrielson & Pack, 2009) in the exact manner as described in chapter 2.

3.2.3 Purification of 1,4C-1,4Bis. Purification of the polymer 1,4C-1,4Bis occurred in the same exact manner as described previously in section 2.2.3. 1,4C-1,4Bis polymer solution was purified using a 1000 Da molecular weight cutoff (MWCO) membrane. Dialysis occurred in nanopure water for 48 hours and replacement with fresh water occurred every 12 hours. After 48 hours of purification, the polymer solution was lyophilized and reconstituted in 1X PBS.

3.2.4 Molecular weight determination of the 1,4C-1,4Bis. Gel permeation chromatography (GPC) was used to determine the molecular weight of the polymer, 1,4C-1,4bis, against Poly (2-vinylpyridine) standards (American polymer standards Corporation Mentor, OH). The standard curve was based on

five standards (3000, 7000, 12000, 35000, and 70000 Da). This was performed using one ultrahydrogel column, (ultrahydrogel 250, Waters Corporation, Milford, MA). The mobile phase used was 0.1 M Triflouroacetic acid and 40% Acetonitrile at a flow rate of 0.5 mL/min and a column temperature of 35°C. The pump used to deliver the polymers to the columns was a waters 1515 isocratic HPLC pump and the refractive index was measured with a waters 2414 refractive index detector.

3.2.5 Synthesis of the polymer-antibody conjugates. Synthesis of the conjugate was carried out as shown schematically in figure 13. 1,4C-1,4Bis (0.83 μmol) was dissolved in 1 mL of 1X PBS and reacted with 1.05 μmol (10 mM in DMSO) of N-succinimidyl 3-(2-pyridyldithio) propionate (SPDP) at room temperature for 1 hour under vigorous stirring (Reaction A from figure 7). Purification of the product (1,4C-1,4Bis-SPDP) was performed using gel chromatography on a PD-10 desalting columns equilibrated with 1 X PBS to remove the excess SPDP. Since dilution of the SPDP activated 1,4C-1,4Bis occurs, concentration was performed by lyophilization at -48°C and 44 x 10^{-3} mbar (Labconco, Kansas city, MO) for 24 hours. Prior to use, the conjugate was reconstituted in Nanopure water. Reconstituted SPDP activated 1,4C-1,4Bis was reduced for 30 minutes at room temperature using excess dithiothreitol (DTT) to produce 1,4C-1,4Bis-SH. The excess DTT was removed using a PD-10 desalting column. This sample was again subsequently freeze dried at -48°C and 44 x 10^{-3} mbar for 24 hours. Prior to analysis, the 1,4C-1,4Bis-SH was reconstituted again

in Nanopure water. The presence of the –SH group on the polymer was confirmed through the use of the Ellman's reagent according to the manufacturer's protocol. The degree of substitution of SPDP to the polymer was determined using the pyridine 2-thione assay according to the manufacturer's protocol.

SPDP was conjugated to J591 using a similar method as described above (Reaction B from figure 7). J591 (0.013 μmol) in 1 mL of 1X PBS was reacted with 0.13 μmol of SPDP (10 mM in DMSO) at room temperature for 1 hour under vigorous stirring. Purification of the product was performed using gel filtration on a PD-10 desalting columns equilibrated with 1 X PBS to remove the excess SPDP. Concentration of the J591 antibody was obtained from the BCA assay according to the manufacturer's protocol. With dilution of the SPDP activated J591 occuring from the gel chromatography, concentration was required and performed using Slide-A-Lyzer dialysis cassettes (10 MWCO) with the Slide-A-Lyzer concentrating solution as described by the manufacturer's protocol. Concentrated SPDP activated J591 was added to the 1,4C-1,4Bis-SH and allowed to react for 18 hours at 4°C to produce the final antibody-polymer conjugate (Reaction C in figure 7). Purification of the conjugate was performed using two different methods with an ÄTKA FPLC system equipped with a UV detector (GE Healthcare, Piscataway, NJ). First, strong cation exchange chromatography was performed with a BioRad Macroprep High S column (40 mm x 5.6 mm, 1 mL). Gradient elution was performed using 22-100% buffer A; Buffer A, 3M NaCl and 20 mM HEPES pH 7.4, Buffer B, 20 mM HEPES pH 7.4. Second, weak cation

exchange chromatography was performed with a GE Healthcare Hi Trap CM FF column (1.6 cm x 2.5cm, 5 mL). Gradient elution was also performed using 22-100% buffer A; Buffer A, 3M NaCl and 20 mM HEPES pH 7.4, Buffer B, 20 mM HEPES pH 7.4.

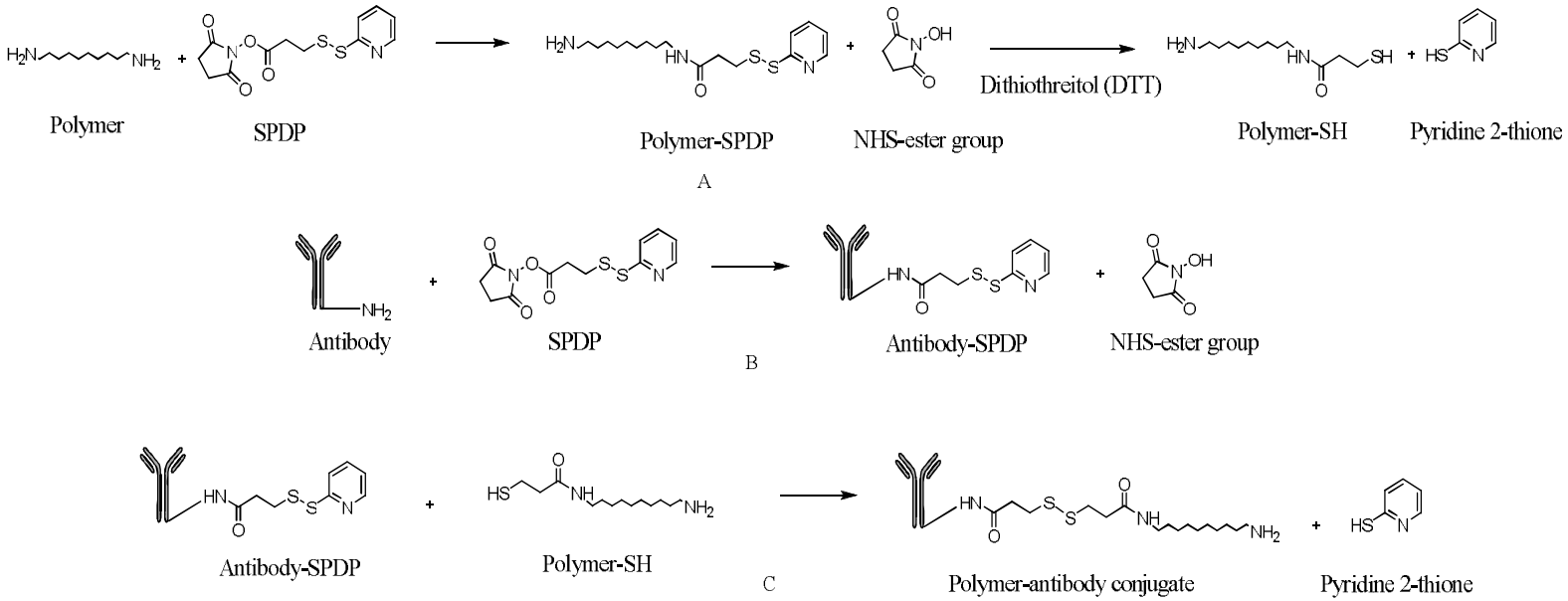

Figure 12: General schematic outlining the conjugation of the antibody (J591) to the polymer 1,4C-1,4Bis. A) Reaction between the amine-epoxide polymer and the linker molecule (SPDP). In the second step of the reaction DTT is added to the conjugated polymer reducing the disulfide bond which releases the pyridine-2-thione group. B) Reaction between the J591 antibody and SPDP. C) Final conjugation between the SPDP modified antibody and the –SH functionalized epoxide amine polymer resulting in release of the pyridine-2-thione group.

3.3 Results and Discussion

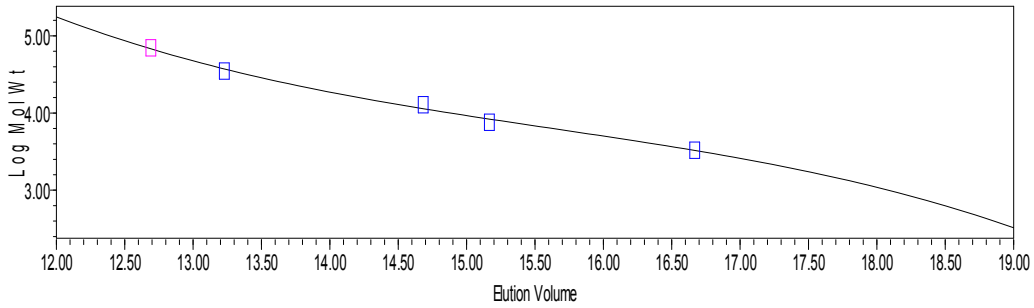

Figure 13: Calibration curve obtained from the Poly (2-vinylpyridine) standards

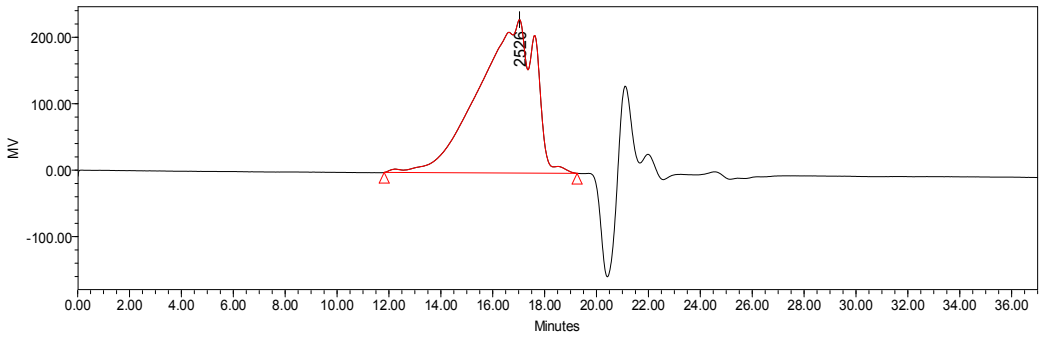

Figure 14: Chromatogram for 1,4C-1,4Bis

3.3.1 Molecular weight determination. GPC is a very common method that is used to characterized polymer molecular weights based on elution times (Georgiou, Vamvakaki, Patrickios, Yamasaki, & Phylactou, 2004; Peetz, Moustafa, & Kennedy, 2003a, 2003b). Based on the calibration curve obtained from the Poly (2-vinylpyridine) standards in figure 13 and the elution time of the 1,4C-1,4Bis polymer (~16 minutes) as shown in figure 14, it was calculated using the machine software that the molecular weight of this polymer, synthesized by the method as described in sections 3.2.2. and 3.2.3, is 6000 Da.

3.3.2 Conjugation of the antibody (J591) to the polymer (1,4C-1,4Bis).

Conjugation of J591 to the SPDP molecule (reaction B in figure 12) can be verified through the release of the Pyridine-2-Thione group, which has a detectable absorbance at 343 nm. The release of this group is induced upon addition of excess DTT according to the manufacturer's protocol and as demonstrated in the literature (Germershaus, et al., 2008; Merdan, et al., 2003). This same assay can also be used to verify the conjugation of 1,4C-1,4Bis to SPDP (reaction A in figure 12).

Table 1:

Absorbance values confirming linkage of SPDP to J591

A_{343} prior to addition of DTT (a.u.)	A_{343} after addition of DTT (a.u.)
0.011	0.128

Based on the absorbance values from table 1 it is shown that there was a definite increase in absorbance after mixing the SPDP to J591, showing a difference of 0.117 absorbance units (a.u) between the readings before and after addition of DTT. Using the calculation provided by the manufacturer yields a degree of substitution of approximately 2 moles of SPDP per mole of J591.

Table 2:

Absorbance values confirming linkage of SPDP to 1,4C-1,4Bis

A_{343} prior to addition of DTT (a.u.)	A_{343} after addition of DTT (a.u.)
0.011	0.743

Based on the absorbance values from table 2 it is again observed that an increase in absorbance, showing a difference of 0.732 a.u. between the readings before and after addition of DTT. The degree of substitution was not able to be conclusively determined due to lack of knowledge of the polymer concentration after use of the PD-10 desalting column.

Table 3:

Absorbance values confirming linkage of J591 to 1,4C-1,4Bis

A_{343} prior to addition of 1,4C-1,4Bis (a.u.)	A_{343} after addition of 1,4C-1,4Bis (a.u.)
0.011	0.132

Finally upon addition of the –SH functionalized 1,4C-1,4Bis to the J591-SPDP, an absorbance change of 0.120 was observed. From this result it can be qualitatively concluded that conjugation of the J591 to 1,4C-1,4Bis did occur. This is further confirmed by comparing absorbance readings after the addition of DTT (A_{343} after addition of DTT) in both tables 1 and 3. From these absorbance values it is clear that the two pyridine-2-thione groups present from the

conjugation of J591 to SPDP were released. Without the presence of reducing agents, the only logical explanation for is that the 1,4C-1,4Bis-SH successfully conjugated to form the final product, J591 conjugated to 1,4C-1,4Bis.

3.3.3 Purification of the polymer-antibody conjugate. Purification of the polymer-antibody conjugate using cation exchange chromatography is a necessary step in the synthesis. This ensures the removal of the unconjugated antibodies which could potentially interfere with targeted transgene delivery by competitive inhibition, in which free antibody could bind to the overexpressed antigens instead of the polymer conjugated antibody. This lessens the chance to transfect the cells via targeted delivery In order to detect the antibody a UV detector set at 280 nm was used.

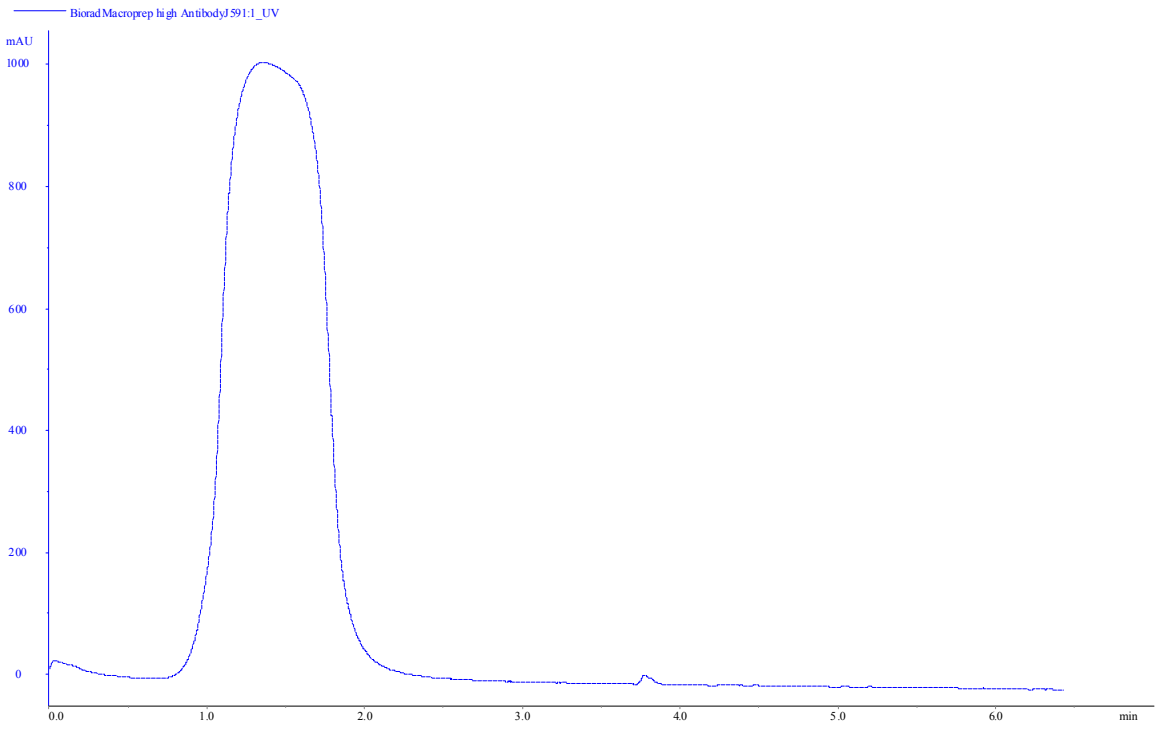

Figure 15: Chromatogram of free unconjugated J591 in the BioRad Macroprep high S column (Strong cation exchange)

The first attempt at purification was done using strong cation exchange chromatography. This was performed with BioRad high S column which is the column that has been used quite extensively in the literature (Germershaus, et al., 2006; Germershaus, et al., 2008; Kircheis, et al., 1997). Figure 15 shows the result from a control experiment where a 2 milligrams (mg) of the antibody (2 mg/mL, 1mL) was injected into the system to determine if the antibody exhibits any binding to the column using a gradient elution of 22% - 100% buffer A; Buffer A, 3M NaCl and 20 mM HEPES pH 7.4, Buffer B, 20 mM HEPES pH 7.4. This was a necessary experiment because in theory, the polymer antibody conjugate should elute at a different retention time (RT) than the antibody due to the interaction of the polymer (1,4C-1,4Bis) with the column. It was confirmed that the antibody did not bind to the column as is evident by single large peak that appears at a RT of about two minutes, corresponding to a 0% buffer A. This result was found to be consistent with the literature results (Germershaus, et al., 2006; Merdan, et al., 2003). This was further confirmed by the BCA assay which verified that mass was conserved (~2 mg elution from the column).

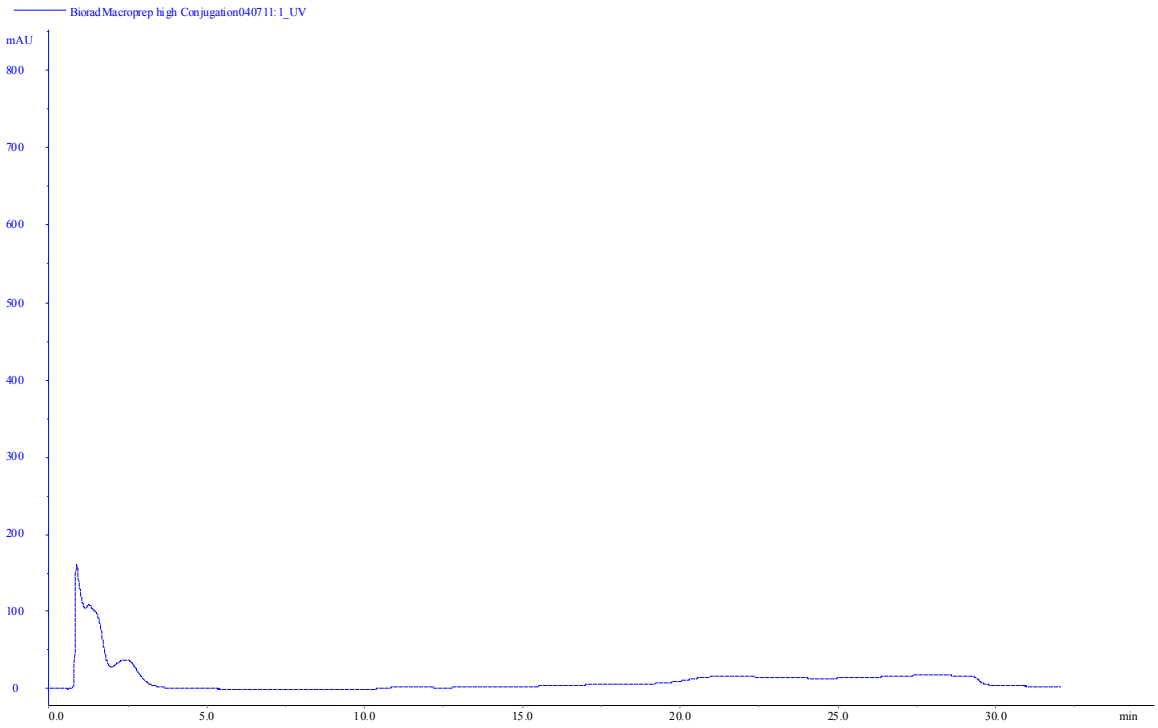

Figure 16: Chromatogram of the polymer-antibody conjugate in the BioRad Macroprep high S column (Strong cation exchange)

With the result from the control experiment confirmed, the polymer - antibody conjugate was subsequently injected into the system. Consistent with the previous result, the free antibody eluted from the column at a RT of ~ two minutes which corresponds to 0 % buffer A. Performing BCA assay on this fraction confirms negligible amounts of antibody are present indicating that most of the antibody has been conjugated to the antibody. Interestingly, there was not second peak that is representative of the polymer-antibody conjugate. It has been shown in the literature that the polymer- antibody conjugate should elute towards the end of the gradient where a high concentration of buffer A is applied to the column (Germershaus, et al., 2006; Merdan, et al., 2003). This was not the case

with this polymer-antibody conjugate as shown in figure 16 due to the lack of a second peak. This is a strong indication of an unknown interaction between the polymer-antibody conjugate and the column functional ligand (SO_3^{2-}), possibly hydrogen bonding, that is not allowing elution of the polymer – antibody conjugate from the column.

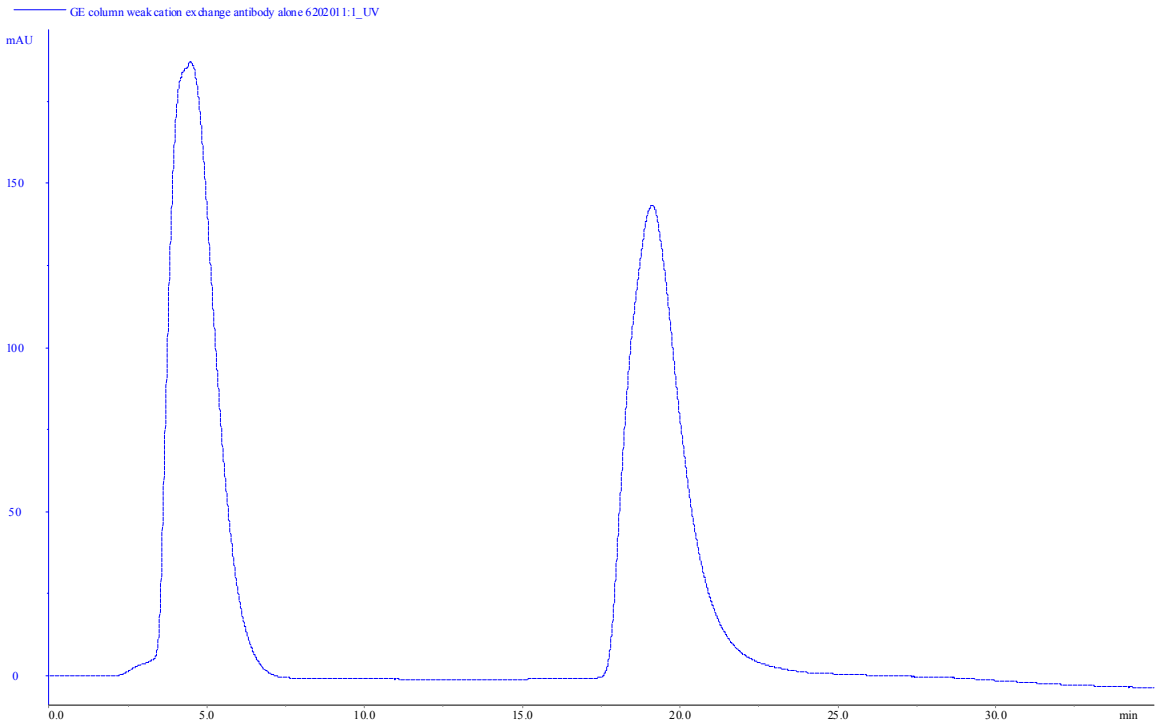

Figure 17: Chromatogram of free unconjugated J591 in the GE Healthcare Hi Trap CM FF column (weak cation exchange column)

Due to the previous result, it hypothesized that using a weaker binding column (GE Healthcare Hi Trap CM FF column) with a different functional ligand (-COOH) would allow for elution of the polymer-antibody conjugate. Before this could be performed the same control experiment was performed and the results are presented in figure 17. There exist two separate and distinct

antibody peaks present, indicating that the antibody binds to the column. The first peak, corresponding to 0% buffer A, shows the antibody elutes at a RT of five mintues. The second peak, also representative of an antibody, corresponds to ~11% - 13% buffer A with an RT of approximately twenty minutes. BCA assay confirms that the sum of both fractions yields approximately 2 mg, thus mass is conserved.

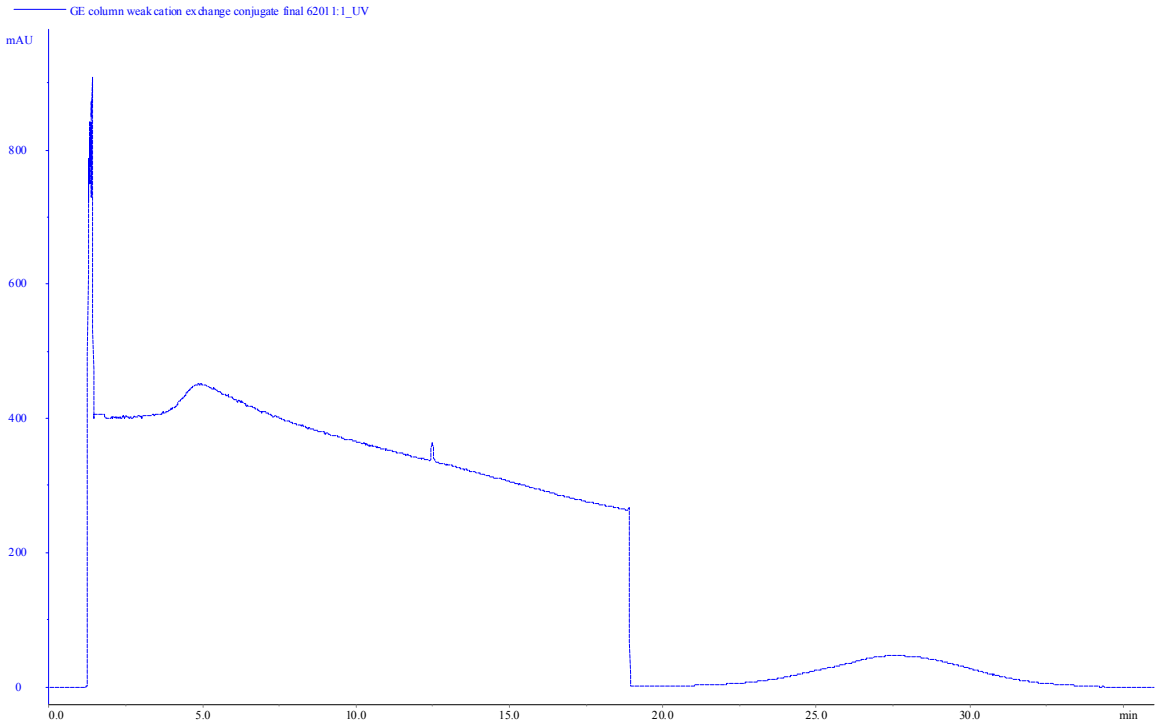

Figure 18: Chromatogram of the polymer-antibody conjugate in the GE Healthcare Hi Trap CM FF column (weak cation exchange column)

Finally, the polymer - antibody conjugate was injected into the system and the same gradient elution protocol was used. The results in figure 18 show consistency with the control experiment in that there is a small peak present at a

RT of five minutes. There is a second peak that exists at a RT of approximately twenty five minutes corresponding to 42% buffer A. This is not indicative of free antibody as demonstrated in figure 15 (RT = 20 mintues) but could possibly be the polymer - antibody conjugate. Further characterizations are needed in order to prove that this is true.

3.4 Conclusion

In this chapter, synthesis of the polymer-antibody conjugate using the polymer 1,4C-1,4Bis and the PSMA antibody J591, was performed using the heterobifunctional linker molecule, SPDP. The results show a strong indication that the polymer-antibody conjugate did form however, further characterizations are needed to determine the polymer:antibody ratio of the conjugate. In addition to the synthesis, purification of the polymer-antibody conjugate was attempted using both a strong cation exchange column (BioRad High S) and a weak cation exchange column (GE Healthcare CM FF) in order to separate the excess antibody. It was determined that there exists strong interactions between the polymer and the functional ligands thus inhibiting elution of the polymer-antibody conjugate from the BioRad high S column. Performing the separation using the GE Healthcare CM FF column yielded a second peak with a different elution time than was shown in the control experiment, indicating the possibility of the elution of the conjugate. With the data presented in this chapter, it is concluded that polymer-antibody conjugate was successfully synthesized with purifications still needed.

Chapter 4

FUTURE WORK

4.1 Chapter 2 Future Work

4.1.1 Purification of the polymer solutions. While this process has already begun, the rest of the lead polymers, as identified in chapter 2, need to be purified via dialysis using a 1000 Da MWCO membrane against water. This will ensure that all excess amines, which are soluble in water, are removed from the solution. The excess amines present a potential problem in that they can also bind to the DNA and occupy binding sites where the polymer usually binds. This could result in less condensation of the DNA which could inhibit endocytotic uptake and in addition also contribute to higher cytotoxicity. With all of the excess monomer removed from the polymer solution, it is speculated that only the polymer will bind to the DNA. This could lead to more efficient condensation of the DNA and allow higher transfection efficacy due to smaller particle sizes. Currently, PEI/DNA forms spherical particles with sizes that range from ~100 nm -150 nm (Merdan, et al., 2002; Y. H. Wang, et al., 2011). It is hypothesized that with the removal of the monomer, sizes to similar to that of PEI can be formed thus yielding more efficient DNA delivery.

4.1.2 Molecular weight determination. Molecular weight of the purified lead polymers should be measured using the same methods as described in section 3.2.4. Molecular weight determination is important because it has been shown that lower molecular weight polymers exhibit lower toxicity (Merdan, et al., 2002).

Unfortunately, low molecular weight polymers also have a lower transfection efficacy. Although this does not seem to be the ideal case, it is possible to functionalize the polymers with side groups that lower toxicity while enhancing transfection. It is encouraging that the molecular weight of 1,4C-1,4Bis which was synthesized using the same method as NPGDE and the 1,4C libraries, had a low molecular weight (6000 Da) after purification. This demonstrates the possibility that the polymers in the NPGDE and 1,4C libraries will have similar molecular weights.

4.1.3 Structural studies. Structural determination of the polymers is a necessary characterization. This allows for more valid conclusions on polymeric properties that enhance transgene delivery. Structural studies should be performed using ^{1}H NMR and FT-IR. Both of these techniques will allow for sufficient characterization of the polymers and will allow a valid conclusion on the structures of all of the polymers.

4.1.4 Particle size and surface charge determination. Particle size and zeta (ζ)-potential of the polyplexes should be determined by dynamic light scattering (DLS). This should be performed with the purified polymers to determine if they are comparable to PEI.

4.1.5 Dose response with lead polymers. While the results shown in chapter 2 seem to be very promising use of a polymer to condense DNA at a 10:1 polymer:DNA weight ratio is not ideal. It has been shown that use of PEI at a 1:1 polymer:DNA weight ratio has the highest transfection efficacy (Barua & Rege, 2010; Cherng, et al., 2011) proving yet again why this polymer is the standard for polymeric transgene delivery. In order to determine whether the lead polymers found in this study are truly more efficient than PEI, transfection studies will need to be performed at varying polymer:DNA weight ratios, which is work that is currently being performed. The weight ratios that are planned to be tested are 1:1, 5:1, 10:1, 20:1, 25:1, and 50:1. Only after these experiments are carried out can a valid conclusion be made as to whether these polymers are more efficient at delivering DNA to the three cell lines mentioned above. Currently, the optimum polymer:DNA ratio that has been shown to be the most effective when using PEI to condense reporter genes such as pGL3 and E-GFP is a 1:1 (w/w) (Barua & Rege, 2010; Cherng, et al., 2011), which has also been confirmed in our lab to be true

4.1.6 Cytotoxicity studies. In addition to evaluating the transgene delivery of the polyplexes, toxicity studies also need to be performed. Cytotoxicity of the polyplexes should be evaluated using the methyl thiazolyl tetrazolium (MTT) assay to validate the claims made in chapter 2.

4.2 Chapter 3 Future Work

4.2.1 Purification of the polymer-antibody conjugates. As shown previously in chapter 3, purification of the polymer-antibody conjugates has been a great challenge. This step of the process is necessary because there exists the possibility that unconjugated antibody is present in the solution along with the polymer-antibody conjugate. This presents a problem in that these free antibodies can occupy the PSMA binding sites over-expressed on the surface of PC-3 PSMA cells, thus lowering the potential of gene delivery using the antibody-polymer conjugate. Throughout the literature, it has been found that separation of the unconjugated antibody from the polymer-antibody conjugate has been fairly simple (Ref. Figures 15 and 17). However, due to some unknown interactions the polymer-antibody conjugate has not been shown to elute from the columns in this case. This is proven by the one peak that is present on all of the chromatograms using the strong cation exchange column, without the presence of the second peak that is representative of the antibody-polymer conjugate as demonstrated by others (Germershaus, et al., 2006; Germershaus, et al., 2008; Kircheis, et al., 1997). One hypothesis for these unknown interactions is the possibility of hydrogen bonding between the functional ligand of the columns and the polymers. In addition separation using the weak cation exchange column has not shown to be greatly successful as well possibly for the same reasons. Changing of pH of the elution buffers may alleviate some of these binding effects and allow for easier elution of the polymers. Changing of the composition of the buffers may also aid

in elution. For example, using $CaCl_2$ instead of NaCl could help better displace the polymer. However, use of $CaCl_2$ should be monitored very carefully due to the possibility of precipitation onto the column which could result in loss of the polymer-antibody conjugates and loss of column functionality.

4.2.2 Cell transfection studies with PC-3 PSMA cells. Following completion of the polymer-antibody conjugates synthesis and purification, transfection studies are needed to if enhanced transgene delivery occurs in PC-3 PSMA cells. In theory, transgene delivery should be enhanced, to some extent, due to addition of the targeting ligand as previously shown in the literature (Buschle, et al., 1995; Germershaus, et al., 2006; Germershaus, et al., 2008; Kircheis, et al., 1997; J. Lee, et al., 2010; Merdan, et al., 2003; Moffatt, et al., 2006). These results are encouraging and should be confirmed using the conjugate formed using 1,4C-1,4Bis and compared against the conjugate formed using PEI. The experiment should be performed using the same polymer:DNA weight ratios as previously described in section 4.1.5 and in addition should be compared against the untargeted polyplexes.

4.2.3 Cytotoxicity studies. Using the same methods as described in section 4.1.6, the cytotoxicity of the complexes formed from the polymer - antibody conjugates and DNA will be evaluated. These studies are important to determine what ratios are toxic to cells and to see whether these results correlate with published literature values.

4.2.4 Structural studies. Structural determination of the complexes formed from the antibody-polymer/DNA is a necessary characterization. This will ensure that the conjugation of the antibody to the polymer was successful and will give insight as to where binding of the SPDP linker molecule binds on the antibody. This becomes an important factor because if the SPDP molecule interferes with the antigen binding sites, lower transfections could potentially result due to inefficient binding of the antibody. Structural studies will be performed using ^{1}H NMR and FT-IR. Both of these techniques will allow for sufficient characterization and will allow a valid conclusion on the structures of all of the complexes.

4.2.5 Particle size determination. Determination of the size and zeta potential will need to be evaluated on the antibody-polymer/DNA complex using the same methods as described in section 4.1.4.

4.2.6 Click chemistry. Although conjugation using the linker molecule SPDP was shown to be effective, the efficiency of the reaction remains in question. Other alternative conjugation methods exist that claim to be better than heterobifunctional linkers. One method that has been used in the literature is commonly known as click chemistry. Antibodies are typically conjugated to nanoparticles through a copper-catalyzed terminal alkyne-azide cycloaddition (CuAAC) (Elias, Cheng, & Tsourkas, 2010; Thorek, Elias, & Tsourkas, 2009). This same concept could be employed with the polymers synthesized in this thesis and efficiencies between the use of SPDP and CuAAC should be compared.

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