Synthesis and Chemical Modification of Degradable Polymers to Enhance Gene Delivery

by

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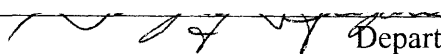DOCTOR OF PHILOSOPHY IN CHEMICAL ENGINEERING

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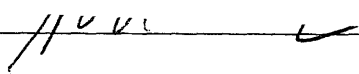MASSACHUSETTS INSTITUTE OF TECHNOLOGY

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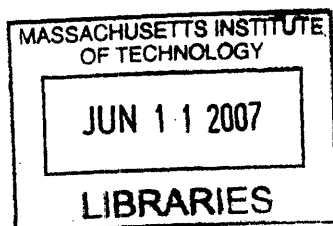Department of Chemical Engineering
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Abstract

Poly(β-amino ester)s are a class of cationic, degradable polymers that have shown significant promise as gene delivery agents, more effective than the state-of-the-art, commercially available non-viral systems. The main objective of this thesis is to synthesize new poly(β-amino ester)s and modify existing ones to further improve their gene delivery properties for clinical applications. This has been accomplished by developing both side- and end-chain chemistries for poly(β-amino ester)s.

A series of novel poly(β-amino ester)s were prepared using a new amine monomer 2-(2-pyridyldithio)-ethylamine. The polymer side chains display fast and selective reactivity towards thiol ligands, as demonstrated using mercaptoethylamine (MEA) and RGDC, a ligand that binds with high affinity to certain integrin receptors on angiogenic endothelial cells. The MEA derivatives in particular, were able to self-assemble with plasmid DNA to form nano-complexes that can partially disassemble in response to intracellular glutathione concentrations. These polymers also displayed low cellular toxicity and were able to mediate transfection at high levels in human hepatocellular carcinoma cells. It is envisioned that the PDA poly(β-amino ester)s can serve as cationic, degradable platforms to attach targeting ligands, viral peptides and other molecules to a single chain to improve gene delivery.

A two-step end-modification strategy is also presented to optimize the functionality at the polymer end points. Conditions were developed so that many structurally diverse end groups could be explored, without the need for polymer purification. Using a highly efficient poly(β-amino ester), C32, optimization of the terminal amine group improved *in vitro* gene transfection by 30% and reduced the polymer:DNA ratio 5-fold. Differences of single carbons and functional groups at the polymer ends were shown to affect many polymer-DNA properties, including the binding affinity, complex size and surface charge, levels of endocytosis, cytotoxicity and transfection. Intraperitoneal gene delivery in mice using several end-modified C32 polymers proved an order-of-magnitude more effective than unmodified C32, as measured in whole body scans and harvested organs.

The end- and side-chain modification strategies presented here have led to the discovery of improved poly(β-amino ester)s for gene delivery and may aid in their future development into clinically useful delivery systems.

Thesis Advisor: Robert Langer
Institute Professor

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Chapter 1 – Introduction

1.1 Overview

Advances in functional genomics and proteomics have provided a detailed understanding of normal cell physiology and the alterations that exist in many pathological states. Such knowledge at the molecular level of the genes and proteins that are abnormal or malfunctioning has naturally led to the discovery of new targets for therapeutic intervention. Traditionally, this information is used to design or identify small molecule drugs that that work more effectively and with higher specificity to treat an illness. Recent progress in molecular biology has also allowed protein drugs and monoclonal antibodies in particular, to be synthesized, isolated and used as alternative therapeutics to small molecules. With more than 200 monoclonal antibodies in over 350 clinical trials worldwide,[1] these macromolecules are gaining widespread use mainly in the treatment of cancer and arthritis due to their high specificity towards virtually any target. While these approaches have led to the routine treatment of many diseases and prolonged patient survival, there still remains the need to effectively cure cancer, neurodegenerative disorders, infectious diseases, and various cardiovascular diseases.

Gene therapy is a new and promising alternative to small molecule and protein drugs that could theoretically treat and even cure any disease. The central goal of gene-based therapies is to introduce a new gene(s) into a target cell population to replace a defective gene or allow for the production of new RNA or protein macromolecules that have a therapeutic effect. One of the biggest advantages of gene therapy is the potential for long-term or permanent protein expression after one dosing schedule. As a result, patients may no longer need to take drugs or, for the case of protein therapeutics, receive injections on a regular basis. Instead, the therapeutic protein can be manufactured and used or secreted by cells in the patient directly. This also drastically reduces the cost of treating an illness, which is particularly important when considering expensive protein therapeutics such as monoclonal antibodies which may otherwise be very effective. In addition, gene therapy easily allows for the production of multiple proteins that may work in a cooperative or synergistic manner to treat a disease. Since often times these proteins need to function inside the cell, there is no concern with intracellular delivery of the desired protein, provided that the DNA is effectively transduced into the target cells.

Of the many diseases that are amenable to gene therapy, the most obvious application is for the treatment of inherited monogenic diseases, in which one faulty gene and its encoded protein give rise to the pathology.[2] These include cystic fibrosis, severe combined immunodeficiency syndrome (SCID), haemophilia, and orthinine transcarbamylase (OTC) deficiency to name a few. Another obvious application of gene therapy is for the treatment of polygenic diseases such as cancer. Over two-thirds of the gene therapy clinical trials underway in 2006 are dedicated to cancer treatment as essentially all forms of cancer are amenable to nucleic acid therapy.[3] Clinical trials for many other diseases are also underway including infectious diseases (e.g., HIV/AIDS, tetanus), vascular diseases (e.g., peripheral vascular disease, coronary heart disease), and others (e.g., Alzheimer's, Parkinson's, rheumatoid arthritis, glaucoma, inflammatory bowel disease).

The two components for successful gene therapy are an appropriate gene construct to permit expression of the desired protein and a delivery system to safely and efficiently transport the DNA to the target cell nuclei. For many viral delivery systems, the therapeutic gene can be grafted into the vial genome at sites that do not interfere with virus assembly, production and infection. Non-viral systems require the use of DNA plasmids to carry the desired gene.[4] These are circular chromosomes that have an origin-of-replication to allow for bacterial amplification, viral promoter and enhancer sequences (e.g., cytomegalovirus – CMV, simian virus 40 – SV40) to provided maximal protein expression, and several other important features (restriction sites, antibiotic resistance genes, polyA signal, etc.). Plasmid optimization is gaining interest and can lead to increased or prolonged levels of protein expression, which are important limitations of gene therapy today. Furthermore, incorporation of tissue-specific promoters can allow for selective gene expression in target tissues such as the breast, pancreas, lung, liver, muscle, ovary, and prostate. This specificity is particularly important for treating cancers arising in these tissues since the end result is death or growth inhibition that may cause damage or adverse effects to normal, healthy cells.

The optimized gene construct usually needs to be combined with an effective delivery system. The exception to this rule is for the transfection of muscle, skin and liver, where naked DNA can be processed and used to produce protein at relatively low levels.[5] Due to the low, transient expression and lack of transfection in most tissues of interest, the DNA is usually coupled to a carrier system to facilitate its delivery. In general, delivery systems can be loosely classified as either viral or non-viral, with many sub-divisions existing in each category. Viruses have become the preferred delivery vehicles due to their high efficiency and the ability to genetically refine their structure and activity. In this case, a top-down approach is taken to modify an already existing system to improve its safety and selectivity. Although viruses are very efficient, none have been discovered or synthetically altered to be used safely for routine clinical treatments. As a result, much effort has been initiated in the past ten years to develop non-viral alternatives that can mediate gene delivery. These include cationic lipids and polymers that either electrostatically bind DNA or physically entrap it for delivery. Although they suffer from low transfection efficiencies, especially in non-dividing cells, synthetic delivery systems

can be engineered from the bottom-up to potentially control the toxicity, biodistribution, and cellular effects of the treatment.

Despite the difficulty in designing and identifying an appropriate gene delivery system, clinical trials have been underway for over 15 years to test the feasibility of gene therapy with existing technologies. Although a few major setbacks have been encountered along the way, there have been a few significant trials that provide proof-of-concept experiments that continue to drive the field today.

1.2   A Brief History of Trials

1.2.1 In The Beginning

The first clinical gene therapy trial occurred in 1990 and involved the treatment of two patients suffering from severe combined immunodeficiency (SCID) syndrome arising from adenosine deaminase (ADA) deficiency.[6,7] This devasting illness occurs from the lack of a functional ADA enzyme in B and T lymphocytes, which results in the intracellular accumulation of adenosine to toxic levels that impede lymphocyte replication and function. Consequently, SCID patients cannot amount an effective immune response against infections and can die at a young age if not diagnosed early. The current standard of care is still either bone marrow transplants or periodic infusion of pegylated ADA (PEG-ADA). Bone marrow transplants can be effective but require a suitable match and virus-free donor cells. Supplementation with PEG-ADA substantially improves the T cell count and decreases the occurrence of infections but does not restore normal immune function, largely because it only provides an extracellular source of the enzyme. At the time of this trial and even today, gene therapy is regarded as the most promising cure and treatment option for SCID patients.

Gene therapy can be potentially used to treat and cure SCID-ADA by inserting a functional ADA gene into the lymphocytes. In the first clinical trial, an ex vivo viral-based approach was used to incorporate the ADA gene into the patient's T cells. This involved isolation of the T cells from the patient's blood, proliferation in culture, transfection with modified murine leukemia retrovirus carrying the ADA gene, and insertion of the tranduced cells back into the patient. Both patients responded well to the treatment, with the number of T cells reaching normal levels for several years during and after the treatment. In addition, delayed-type hypersensitivity skin tests to environmental and vaccine antigens were positive in many cases for both patients, indicating that their immune systems are responsive to foreign pathogens. Increased levels of circulating antibodies were detected to indicate that the humoral arm of the immune system was also functioning. For one patient, circulating ADA levels increased to roughly half of the concentrations found in heterozygous carriers.

The results of this first trial are largely considered successful in light of the substantial improvement in immune system function and response. Both patients have benefited from an improved quality of life and increased ADA levels, although weekly PEG-ADA injections are still necessary. Importantly, this study showed that a replacement gene can

be stably expressed in a cell over a long period of time. However, while the results are encouraging, several problems were noticed from this trial which included low gene transfection of the T cells, transient T cell numbers in circulation, and variable immune responses. Despite these issues and the inability to unequivocally cure SCID, this trial demonstrated for the first time that gene therapy may be used to effectively treat and potentially cure genetic diseases with further advances in vector development.

1.2.2 The Shortcomings: Trials on Trial

The initial gene therapy trial provided a surge of motivation for the continued development and testing of gene-based therapeutics. Over the years, refinements in vector function and the identification of new targets for genetic intervention have been utilized in numerous studies to provide the necessary proof-of-concept experiments in animal studies. However, little progress was seen in clinical gene therapy throughout the 1990's. In fact, several SCID trials after the first failed to demonstrate any clinical benefit to the patients. No approved therapies emerged during this decade and many trials were deemed unsuccessful due to low transfection efficiencies *in vivo* and immune-mediated responses towards the viral delivery systems and infected (i.e., "treated") cells. Despite the discovery of new delivery systems and therapeutic approaches, clinical trials have met with limited success and, in some cases, resulted in tragic outcomes.

Perhaps the largest setback to gene therapy development came in 1999 with the death of a patient enrolled in a trial to correct OTC deficiency.[6] The OTC protein is an enzyme in the liver necessary for the normal functioning of the urea cycle and the elimination of nitrogenous waste. In the absence of OTC, ammonia builds up to toxic levels that can damage the central nervous system, resulting in convulsions, coma, and death if not treated. Currently, a cocktail containing sodium benzoate and phenylacetate is intravenously administered to treat patients in large medical facilities that have laboratories for close monitoring. In light of this treatment regime and the existence of a single genetic defect, OTC deficiency is an obvious choice for gene replacement therapy. In the 1999 OTC study, patients were infused in the right hepatic artery with an adenovirus carrying the OTC gene to correct the hepatic defect. In the last patient treated in the one year trial, a high dose of viral particles was administered that caused transfection and damage of liver cells. In addition to a toxic build up of ammonia, an innate immune response to the viral infection caused the onset of disseminated intravascular coagulation (DIC). Eventually, release of interleukins from an adaptive immune response triggered inappropriate blood coagulation throughout the circulatory system. The system-wide formation of blood clots led to excessive tissue swelling and multiple organ failure that resulted in the patient's death within a few days after the virus infusion. This single instance underscores the inherent danger of viral-based gene delivery due to the potential immune responses towards the infected tissues. In addition, an NIH investigation a few months after the trial pointed out four significant flaws which included (1) a failure to adhere to the stopping rules after toxic side effects were observed in earlier patients, (2) a failure to adhere to the principle of informed consent by not notifying trial participants of animal toxicity information and adverse effects seen in earlier cohorts in the trial, (3) failure to keep adequate records of vector lineage and titer

as some patients were unknowingly injected with 10-fold higher virus than was approved, and (4) changing the protocol without approval since some patients were treated that had ammonia levels higher than the trial limits. Taken together, the profound anti-viral immune response and poor trial procedures brought a significant halt to clinical gene therapy and the much needed redesign and close monitoring of future trials.

Another major setback came to gene therapy in 2002 during a trial for SCID conducted in France.[8] In this trial, bone marrow stems cells from each patient were harvested, transfected with a modified retrovirus carrying the correct γ_c gene of an interleukin receptor that is faulty in SCID-X1, and transplantation back into the patient. Subsequent differentiation of the gene-corrected stem cells will give rise to B and T lymphocytes with normal replication and functioning. Interestingly, this trial was first regarded as the most successful application of gene therapy and a "miracle" trial, as most patients treated showed a dramatic improvement in their immune response characterized by increased numbers of B, T, and NK cells. For every patient, the overall number and functioning of the lymphocytes was similar to age-matched controls. Unfortunately, three of the ten patients have developed T cell leukemia, caused by retroviral genetic insertion within or near the *LMO2* proto-oncogene.[9] One of the three patients with leukemia passed away in October 2004, slowing down the progression of gene therapy until safer delivery systems can be developed.

1.2.3 The Current Landscape

Following the beginning of clinical gene therapy in 1990, a steady rise in the number of approved trials has been seen during the 1990s (Figure 1.1).[3] The number has dropped off somewhat in the early part of the new millennium, largely due to the tragic results of the OTC deficiency trial described above. Currently in 2006, only 28 trials are approved or ongoing for correcting a wide range of genetic abnormalities. This number is expected to grow in the remainder of the year, however, and only reflects the first part of 2006.
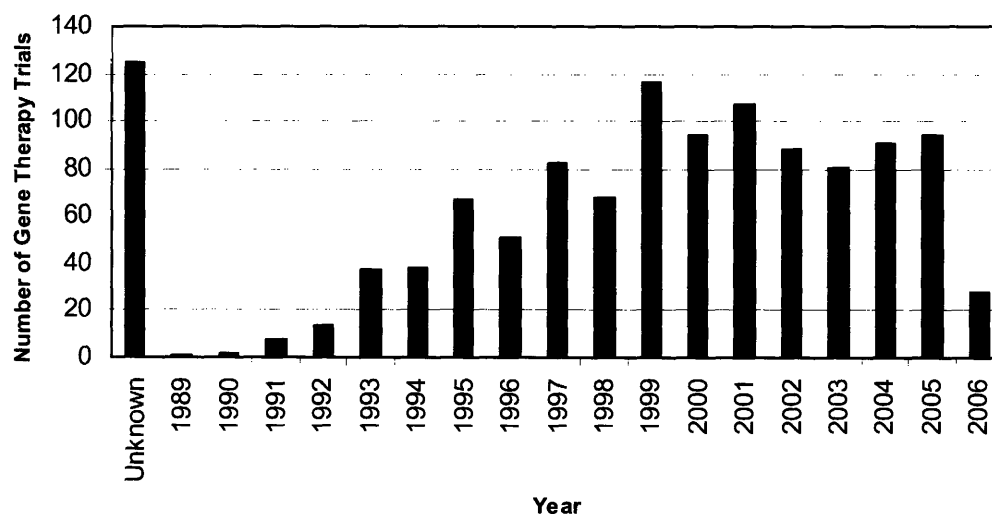
Figure 1.1 Number of gene therapy clinical trials by year between 1989 – 2006.

The most common disease targets in clinical trials are shown in Figure 1.2(A), the majority of which are only in a Phase I or I/II setting (82%).[3] Cancer applications are the most common indication, with over two-thirds of clinical trials focused on slowing or eradicating abnormal growths. The high prevalence and mortality associated with cancer, the lack of suitable medications in late stages, and a thorough genetic roadmap for most forms of cancer that allows for a clear, potentially beneficial, genetic intervention, are the main reasons for its large emphasis in gene therapy trials. A number of cancer gene therapy approaches are the subject of active clinical investigation including immunotherapy, inactivation of oncogenes, replacement of tumor suppressor genes (e.g., p53), antisense and ribozyme supplementation, and expression of suicide genes. After cancer, vascular diseases represent the next largest focus in gene therapy trials with 8.9% of trials in this area. Most are focused on therapeutic angiogenesis for myocardial ischemia (due to coronary artery disease) and lower limb ischemia brought on by peripheral artery disease. In many cases, genes encoding growth factors such as fibroblast growth factor (FGF) or vascular endothelial growth factor (VEGF) are delivered into patient tissues to spur new blood vessel formation. In similar number to vascular diseases, various monogeneic diseases account for 8.6% of ongoing trials, mostly because of the intuitive and obvious application of gene therapy to correct the single genetic defect in these conditions. Cystic fibrosis is by far the most commonly explored monogeneic disease, with SCID and various others (e.g., hemophilia, muscular dystrophy, OTC deficiency) accounting for far less. Infectious diseases such as HIV/AIDS, tetanus, CMV infection, and adenovirus infection are the subject of a smaller percentage of clinical trials (6.5%). Finally, many other disease targets not listed here are currently been investigated using gene therapy and demonstrate the potential widespread application of nucleic acid therapeutics in medicine.

(A)

- Cancer (67%)
- Vascular diseases (8.9%)
- Monogeneic diseases (8.6%)
- Infectious diseases (6.5%)
- Other diseases (3.4%)
- Gene marking (4.2%)
- Healthy volunteers (1.6%)

(B)

- Adenovirus (26%)
- Retrovirus (24%)
- Naked DNA (17%)
- Liposomes (8.3%)
- Poxvirus (6.9%)
- Vaccinia virus (6.5%)
- Adeno-assoc. virus (3.4%)
- Herpes simplex virus (3.4%)
- Others (3.7%)
- Unknown (3.1%)
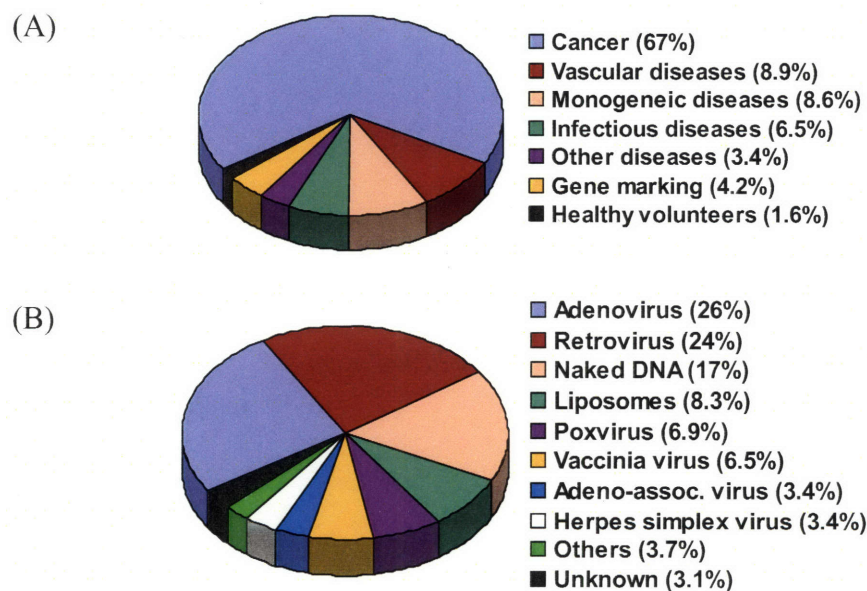
Figure 1.2 (A) Disease targets and (B) delivery systems used in current gene therapy clinical trials

The current vectors used in clinical trials to deliver new or corrective genetic material into target cells are shown in Figure 1.2(B).[3] Despite the safety risks and adverse events realized with modified viruses, these natural carriers are still the most widely used systems for gene delivery in clinical trials. Adenoviruses and retroviruses are the most common vectors, accounting for half of the actively used systems. Although inefficient and very transient, naked DNA applications continue to be an active area of clinical research (17%). Lipofection represents a small but significant delivery strategy (8.3%). Surprisingly, despite the use of poly-L-lysine for over 20 years as a gene transfection agent and even 11 years now after the introduction of PEI, no clinical trials have been initiated using polymeric delivery systems. This lack of use is due to the inherent toxicity of these and other polycations and their low delivery efficiencies *in vivo*. In the coming years, as more functionality is engineered into cationic polymers, it is expected that their effectivenss and safety profile will be improved to permit their use and exploration in gene therapy clinical trials.

Although gene therapy trials have met with limited success over the years, slow progress is evident and encouraging for the future of this field. In October 2003, China became the first country to approve gene therapy for head and neck cancers.[10] The product, Gendicine, uses an adenovirus to deliver a p53 tumor suppressor gene to inhibit tumor growth. A similar product from Shanghai Sunway Biotech, H101, was also approved in November 2005. In the United States, no gene therapy products have been launched but recent clinical trials suggest that they may be developed soon. A recent study in August 2006 reports that genetically modified lymphocytes can be used to achieve "full clinical regression" of rapidly progressive metastatic melanoma.[11] Even though this effect occurred in only two of the 17 patients in the trial, this study highlights the possibilities and promise of gene therapy that can be expected by developing more effective delivery systems.

1.3 Gene Delivery Systems

1.3.1 Basic Considerations for Effective Delivery

In order for gene therapy to be successful, many obstacles must be overcome to reach long-term gene expression at therapeutic levels. Perhaps the most important requirement is the development and use of safe delivery systems. In this context, a safe system would be one that delivers genetic material to the target cells without any unintended, adverse effects such as insertional mutagenesis. In addition, the vector must not elicit an immune response towards itself and the cells it ultimately transfects. The delivery system must also possess favorable pharmacokinetic and pharmacodynamic profiles that give rise to sustained protein expresion. In the first respect, the vector must demonstrate stability in extracellular compartments and the ability to locate target cells. These characteristics are not essential if an *ex vivo* approach is used to harvest the cells, transfect and then re-insert them into a patient. Pharmacodynamic effects pertain to the action of the delivery system on cells and include events associated with uptake, intracellular transport and transcription. These issues are common to all gene therapy approaches.

In addition to a favorable safety profile, an important requirement for all effective delivery systems is the ability to interact with the therapeutic gene. For modified viruses, the desired gene can be grafted into the viral genome at pre-determined sites that still permit viral production and infection. For non-viral systems, DNA must be encapsulated, electrostatically bound, or in some way associated with an appropriate carrier. Typically, a cationic material is used that electrostatically binds the negative charged DNA. The physicochemical properties of the resulting complex is determined by the relative cation/anion amounts, formation technique (mixing speed, time, temperature, concentration, etc.) and various material properties (hydrophobicity, charge density, molecular weight, steric effects, etc.). By adjusting these properties, nanoscopic complexes can be formed with nucleic acids that have favorable characteristics for delivery, depending on the application. Both the size and surface charge are particularly important properties which affect the transport and function of the system at all levels.

For a gene delivery system to be effective *in vivo*, a few basic requirements exist that are common to all vectors. Many additional constraints apply depending on the route of administration as well but are not individually addressed here. Again, it should be pointed out that these requirements do not pertain to *ex vivo* applications.

(1) **Stability in the extracellular environment.**[12] In transit to the target cells, a gene delivery system must protect the DNA and retain its physicochemical properties for effective transfection. Nucleases in the circulation and various tissues can rapidly degrade plasmids if they are not sufficiently protected by the delivery system. The half-life for plasmid degradation in whole blood is around 10 minutes, with substantially faster breakdown occurring after intravenous injection in mice.[13] Resistance to nucleases can be achieved with liposomal formulations and cationic polymers that can encapsulate or shield DNA.[14,15] In addition to nucleases, the carrier must protect DNA from uptake and elimination by the reticuloendothelial system (RES).[16] The RES consists of phagocytic immune cells in the lymph nodes and spleen, along with Kupffer cells of the liver than act to non-specifically engulf, digest and purge the body of foreign matter. Avoidance of the RES has been shown for some liposomal and polymer formulations, either by changing the composition or shielding with inert materials such as polyethyleneglycol (PEG).[17,18] Finally, the delivery system must retain its physicochemical properties in the extracellular spaces. The ionic strength alone in these compartments is capable of inducing aggregation of non-viral gene delivery systems by screening and weakening electrostatic interactions between particles. In addition, negatively charged proteins and glycosaminoglycans, on cell surfaces and in the circulation, can also facilitate aggregation of non-viral assemblies and effectively compete for DNA binding. The use of hydrophilic, non-ionic polymers such as PEG has been shown to substantially improve the colloid stability of both lipid and polymer-based systems.[18,19]

(2) **Transport to the target cell population.** The considerations associated with DNA delivery to the desired site and cells depends on the target location and route

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of administration. In the simplest case, DNA can be delivered by direct inject into the target tissue. Although various systemic barriers are not confronted, the DNA must still traverse the extracellular matrix and avoid the immune system surveillance. For most other situations in which the DNA cannot reach its intended site by direct application, it must be coupled to a carrier that can navigate the body following some form of systemic administration. Intravenous delivery is the most common route since all tissues in the body receive a constant blood supply. For many cationic liposome and polymer gene delivery systems, application to the circulatory system mainly results in high, localized expression in the lung.[20,21] This effect is possibly due to aggregate formation with various blood components and subsequent filtration in the fine capillary beds of the lungs.[22] Endothelial cells in other organs, such as the liver and spleen, also accumulate a significant portion of the applied dose.[23] The lack of transfection in most tissues of interest is the result of the tight endothelial layer throughout the circulation. Only in the liver, spleen, bone marrow and some tumors does the endothelia have fenestrations large enough to permit some diffusive extravasation of particles up to $0.1 - 1$ μm in size.[24] The heavily restricted access to most underlying parenchymal cells remains an important unsolved issue for systemic gene therapy.

In addition to extracellular factors, many barriers at the cellular level must be overcome to achieve efficient gene incorporation and expression (Figure 1.3). Viral carriers have evolved to delivery their genomic contents with high efficiency into the cell nucleus. Non-viral vectors are less efficient and often limited at several levels within the cell, as reviewed previously.[12,25-27] Each of these barriers will be addressed briefly here, along with some of the design elements that have been employed in synthetic systems to overcome these cellular impediments.
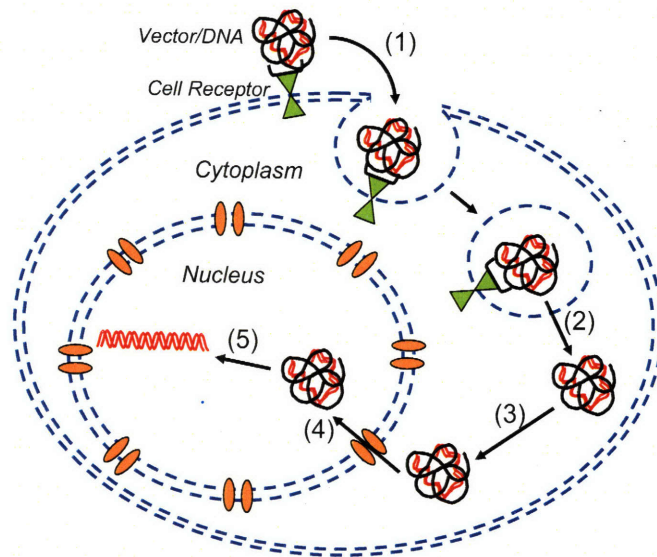
Figure 1.3 Cellular gene delivery barriers. (1) Cellular uptake, (2) endosomal escape, (3) cytosolic trafficking, (4) nuclear uptake and (5) DNA unpackaging.

(1) **Cellular Uptake.** Internalization into the target cell is the first step in the DNA transfection process. Only minimal amounts of free DNA are associated and endocytosed by cells due to electrostatic repulsions between the cell surface and the DNA. Most synthetic delivery systems are formulated with an excess positive charge to condense the plasmid and also non-specifically bind to the cell surface through charge interactions. This association is typically mediated by heparin sulfate proteoglycans that decorate the cell surface. After binding the cationic delivery system, endocytosis typically ensues for particulates less than 200 nm in diameter. In some cases, delivery to a specific cell type can be accomplished by conjugating a ligand to the delivery system that has a high affinity for a cell surface receptor.[28] This mechanism of receptor-mediated endocytosis has been exploited many times, mostly to target tumors using folate,[29] transferrin,[30] RGD peptides,[31] growth factors,[32] and antibodies.[33] Sugars are also widely used as targeting ligands since galactose- and mannose-bearing molecules have a high affinity for surface receptors on hepatocytes and macrophages, respectively.[34] The use of such targeting strategies can potentially improve the uptake levels into the cells of interest and favorably shift the biodistribution *in vivo*.

(2) **Endosomal Escape.** Cellular internalization of gene delivery systems usually occurs by endocytosis. As a result, the carrier-gene assemblies are contained within intracellular endosome compartments.[28] Acidification of the vesicle lumen occurs soon after endosome formation by the action of membrane spanning ATPase proton pumps, in which the pH drops from 7.4 to approximately 5. In these late stages, the endosome then fuses with lysosomes that also contain a low pH environment and a high concentration of nucleases. To avoid these harsh conditions, viruses activate fusogenic peptide domains in the low pH state to essentially pierce and burst the endosomal vesicle. Cationic liposome formulations can facilitate release by mixing with and disrupting the endomosal lipid membrane.[35] Polymeric systems typically make use of two separate mechanisms to promote escape. In the first case, viral peptides such as the HIV TAT sequence can be incorporated to disrupt the membrane.[36] Another common approach is to utilize tertiary amine functionalities, which most likely cause endosomal rupture by indirectly increasing the osmotic pressure during acidification.[37,38]

(3) **Cytosolic Trafficking.** Following release from the endosome, the DNA must be transported to the nuclease for transcription to occur. Due to the high viscosity of the cytoplasm and the cytoskeleton network, the diffusivity of plasmid DNA is less than 1% of its free solution value and is inversely related to the number of base pairs.[39] Another complicating factor is the presence of cytosolic nucleases, which degrade DNA with a half-life of 50-90 minutes.[40] Consequently, an appropriate delivery system must protect DNA from degradation and improve its transport through the cell. Recent evidence has been reported to suggest that non-viral systems, such as PEI, overcome these obstacles by actively transporting DNA along microtubules to the perinuclear region.[41]

(4) **Nuclear Import.** DNA can reach the inside of the nucleus by passing through nuclear pores or associating with chromatin during mitosis and nuclear breakdown. In the first instance, enhancer regions in the DNA (e.g., SV40) can aid in nuclear import through pores since they have a high affinity for transcription factors.[42] Nuclear localization sequences have also been covalently grafted to plasmids for this purpose and can result in a beneficial effect.[43] For either strategy, the DNA must pass through an open nuclear pore, which only facilitates the uptake of particles less than 26 nm in diameter.[44] A more likely mechanism for DNA uptake into the nucleus occurs by association with nuclear components during cell division. In this case, plasmid threading through the tight nuclear pores is bypassed by simply waiting for the nucleus to breakdown during replication, followed by diffusion and nuclear reformation around the DNA. Imaging and transfection performance of non-viral gene delivery vehicles at various stages in the cell cycle provide support for this mechanism.[45]

(5) **Vector Unpackaging.** The last and often overlooked step in transfection is the disassembly of the DNA from the carrier to permit transcription. The unpackaging process is a significant barrier for poly-L-lysine (PLL) transfections and may be limiting for other delivery systems.[46] In this respect, degradable materials may be particularly advantageous since they provide a clear mechanism for breakdown and release of the plasmid, although this has not been demonstrated definitively.

1.3.2 Viral Vectors

Several viruses have been used to transfer new genetic information into cells. The most common are retroviruses, adenoviruses, and adeno-associated viruses in which replication-deficient derivatives are constructed and loaded with a therapeutic gene. Characteristics of each virus type that are important for gene delivery are presented in Table 1.1. Each type has significant advantages that have contributed to their widespread use in gene therapy research. The properties, fabrication, and clinical attributes of many viral vectors have been described extensively.[47-51] In this section, only a brief overview of the main viral systems will be presented based on these reviews.

Table 1.1 Characteristics of viral vectors [adapted from Ponder[47]]

Virus	Genome	Infect[a]	Diameter	Capacity[b]	Expression	Titer
Retrovirus	7 – 10kb ssRNA	No[c]	100 nm	≤ 8 kb	Stable (integrates)	10^8 cfu/ml
Adenovirus	36 kb dsDNA	Yes	70 – 100 nm	8 – 35 kb	3 – 4 weeks (episomal)	10^{12} cfu/ml
AAV[d]	4.7 kb ssDNA	Yes	18 – 26 nm	≤ 4.5 kb	Stable	10^{10} cfu/ml

[a] Ability to infect non-dividing cells
[b] Maximum DNA carrying size
[c] Yes, for lentiviruses
[d] AAV – Adeno-associated virus

(1) **Retroviruses.** Present in essentially all vertebrates, retroviruses are enveloped viruses that contain a single-stranded RNA genome. Each viral particle has two copies of the RNA genome along with proteins required for reverse transcription and integration into the host cell chromosomes. The virion particle is approximately 100 nm in diameter and contains an even distribution of envelop proteins on the surface that define the vector tropism, or range of host cell types that it can infect. All retrovirus types contain at least the *gag, pol,* and *env* genes, flanked by long terminal repeats (LTR) on both sides of the genome. These three genes encode for the both the structural proteins (capsid, nucleocapsid, envelop glycoproteins) and important viral enzymes (reverse transcriptase, integrase, protease).

Viral infection begins with binding to a cell surface receptor via the envelop glycoprotein. The viral RNA enters the cytoplasm and is converted in double-stranded DNA by reverse transcriptase. The DNA genome is then transported to the nucleus and inserted into the host chromosome by the viral integrase enzyme. For oncoretroviruses such as the murine leukemia virus (MLV), this only occurs in dividing cells whereas lentiviruses (e.g., HIV) can infect non-dividing cells as well. Retroviruses for gene therapy are made replication-deficient so that new virions cannot be manufactured by the host cell. This is usually accomplished by deleting the essential *gag, pol,* and *env* genes and replacing them with a therapeutic gene. These removed viral genes are incorporated into a "packaging" cell line genome that can be used to produce viral particles. Typically, the LTRs and other adjacent sequences are retained since they are necessary for viral DNA synthesis and integration after infection.

Retroviruses have been used extensively in gene therapy research and clinical trials. The ability of lentiviruses to transfect non-dividing cells has made them particularly useful in *ex vivo* therapies on stem cells, lymphocytes, hepatocytes, myoblasts, endothelial cells and smooth muscle cells. Currently, there are a few specific concerns related to the clinical use of retroviral vectors: (1) limited tropism, (2) insertional mutagenesis after infection, and (3) generation of wild-type virus during production.

(2) **Adenoviruses.** Originally isolated from a patient with an acute respiratory infection, adenoviruses have become a highly researched vector for gene delivery. Each virion contains a 36 kb double stranded DNA genome housed inside a protein capsid that is 70 – 100 nm in diameter. Adenoviruses contain 30 – 40 genes along with inverted terminal redundancies and packaging signals that are needed for genome replication and virus assembly. Most replication-deficient vectors used for gene therapy replace the multiple early (E) genes with a promoter and therapeutic gene. Packaging cells containing the missing E genes are then used for virus assembly and production.

The infection cycle for adenoviruses begins with a binding event between fiber proteins in the virus capsid and the coxsackie adenovirus receptor on the cell surface. This interaction triggers clathrin-coated pit endocytosis of the virus. The exposure of protein VI and partial virus disassembly are sufficient to lysis the

endosomal membrane and release fragmented virus into the cytoplasm. Interactions with dynein result in microtubule-mediated transport of the virus to the nucleus and subsequent incorporation of the viral genes. Deletion of the E genes ensures that viral replication cannot occur.

The use of adenoviral vectors in gene therapy clinical trials has notably increased over the years. Unlike other viruses, genetic modification of the capsid and fiber proteins can be used to more clearly define the cell specificity for infection. They can also accommodate a large genome which is beneficial for carrying and transmitting large therapeutic genes. Furthermore, their DNA is maintained episomally, substantially reducing the possibility of cancer development from insertional mutagenesis. Although promising, several issues still limit the effectiveness and use of adenoviral vectors which include strong immune responses towards the vector and the cells it infects, development of tolerance to the vector, and the potential for wild-type virus production.

(3) **Adeno-Associated Viruses.** First identified as a contaminant in cell cultures, adeno-associated viruses (AAV) are now promising vectors for gene delivery due to their non-pathogenic nature and inability to self-replicate. The 4.7 kb single-stranded DNA genome of the AAV consists of the *rep* and *cap* genes flanked by inverted terminal repeats (ITR). These genes are typically contained in the packaging cell line and replaced by a therapeutic gene in the viral genome. Co-infection with an adenovirus provides the necessary proteins for AAV assembly.

Similar to lentiviruses and adenoviruses, AAVs are capable of transfecting non-dividing cells. The small (18 – 26 nm) virion particle enters the cell through receptor-mediated endocytosis. Cellular proteins then convert the ssDNA into dsDNA, which can be maintained episomally for long periods of time. These characteristics have made AAVs useful for transfecting hematopoietic stem cells *ex vivo* and hepatocytes, neurons, retina, lung, and cardiac cells *in vivo*.

The major limitation with AAVs is their small carrying capacity, which is limited to approximately 5 kb. An additional issue confronting the administration of these virions is the presence of circulating antibodies in most people that can neutralize the virus. Along the same lines, a single injection of an AAV elicits a strong humoral response against the capsid, making it difficult to effectively administer multiple rounds of therapy. Futhermore, these vectors suffer from the same setbacks as other virus systems which include the potential for insertional mutagenesis and reversion to the wild-type.

1.3.3 Non-Viral Vectors

The problems of immunogenicity, insertional mutagenesis, and potential reversion to the wild type associated with viruses have provided motivation for the design and use of alternative delivery strategies. As mentioned above, DNA alone has a low ability to transfect cells, mainly due to its poor cellular uptake and susceptibility to nucleases. Increased efficiency *in vivo* has been accomplished using physical methods such as electroporation, ultrasound, and hydrodynamic pressure. Despite the still low and transient expression, naked DNA administration is currently involved in 17% of clinical

trials because of its relative safety and potential for a therapeutic effect at low expression levels. For most systemic applications, DNA must be coupled to a carrier to improve its pharmacokinetics and effectiveness. Two broad classes of materials explored for this purpose are lipids and polymers. Typically cationic versions of each are used since they offer the ability to electrostatically bind DNA and can interact with cell surfaces to promote cellular uptake. The synthesis, properties, biodistribution, and transfection characteristics of both lipids[52-55] and polycations[56-59] have been reviewed extensively. In this section, the most common materials and their properties relevant to gene delivery will be highlighted based on these reviews.

(1) **Cationic Lipids.** Over 8% of gene therapy clinical trials worldwide involve the use of cationic lipids as a delivery system. For almost 20 years, many different lipophilic compounds have been developed and used for gene transfection. Cationic lipids generally consist of three distinct parts which include (1) a hydrophobic segment (usually long alkyl chains or cholesterol derivatives), (2) a cationic head group (amines with different degrees of substitution), and (3) a linker region to connect these two parts (e.g., esters, ethers, amides, carbamates). Based on this framework, many lipids have been developed, the most common of which are DOTMA, DOTAP, DOSPA, DOPE, DOGS, DMRIE, and DC-Chol. Some of the most effective liposomal formulations involve blends of these materials. For example, Lipofectamine is a 3:1 wt:wt mixture of DOSPA:DOPE and Lipofectin is a 1:1 wt:wt mixture of DOTMA:DOPE. The gene delivery efficiency is mainly dependent on the composition and even small structural changes to the lipid have been shown to have large effects. The conditions used to formulate lipoplexes (i.e., lipid-DNA assemblies) are known to impact delivery as well, since they have a direct effect on liposome properties such as size, charge, shape, and encapsulation efficiency. Their enhanced ability to deliver DNA into cells is attributed to the lipophilic nature, which can penetrate and disrupt cell membranes.

Cationic lipids are widely used as *in vitro* transfection agents due to their effectiveness in cell culture. However, the *in vivo* biodistribution and transfection characteristics of these materials are not as favorable. For some of the most common reagents such as Lipofectin and LipofectACE, lipoplexes are rapidly cleared from the circulation and accumulate predominantly in the lung.[60] Redistribution typically occurs after a few hours in which DNA can be measured at high levels in the liver. Significant increases in the circulation half-life have been realized with "stealth" liposomes, which incorporate PEG to sterically block serum proteins and reduce uptake by phagocytic cells.[61,62] In combination with antibodies and other targeting agents, these materials show some ability to deliver genes in a cell specific manner, which may be useful for cancer gene therapy.[63]

Several limitations currently impede the development and clinical use of cationic lipids for gene therapy. First, these materials have high inherent toxicity due to their cationic charge and lipophilicity, both of which can disrupt cell membranes to cause significant damage or interact with cellular proteins.[64] Furthermore, the lipid structure is not readily amenable to chemical modification to easily alter or otherwise improve the safety and efficacy. Various *in vivo*

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challenges also need to be addressed which include their unfavorable biodistribution and immune responses towards both the PEG[65,66] and antibody[67,68] extensions.

(2) Cationic Polymers

For over 20 years, new cationic polymers have been discovered and synthesized to improve gene transfer into cells (Figure 1.4). By nature of their positive charge, many polycations spontaneously interact with plasmid DNA to form nanoscopic polyplexes that can be readily endocytosed. Since this self-assembly process with DNA is not driven by polymer-polymer interactions, the macroscopic properties of a polycation-based delivery system (e.g., size, charge, shape) can be tightly controlled. The main advantage of these materials, however, is the relative ease in synthesizing and chemically modifying the structure to improve the gene delivery properties. Along with the physical properties of the polyplexes, these attributes have been largely exploited to develop targeted systems with enhanced serum stability and gene delivery efficiency.

poly-L-lysine   histidine-PLL   dendrimers

linear PEI   branched PEI   chitosan

poly(4-hydroxy-L-   poly[α-(4-aminobutyl)-   poly(β-amino ester)
proline ester)   L-glycolic acid]
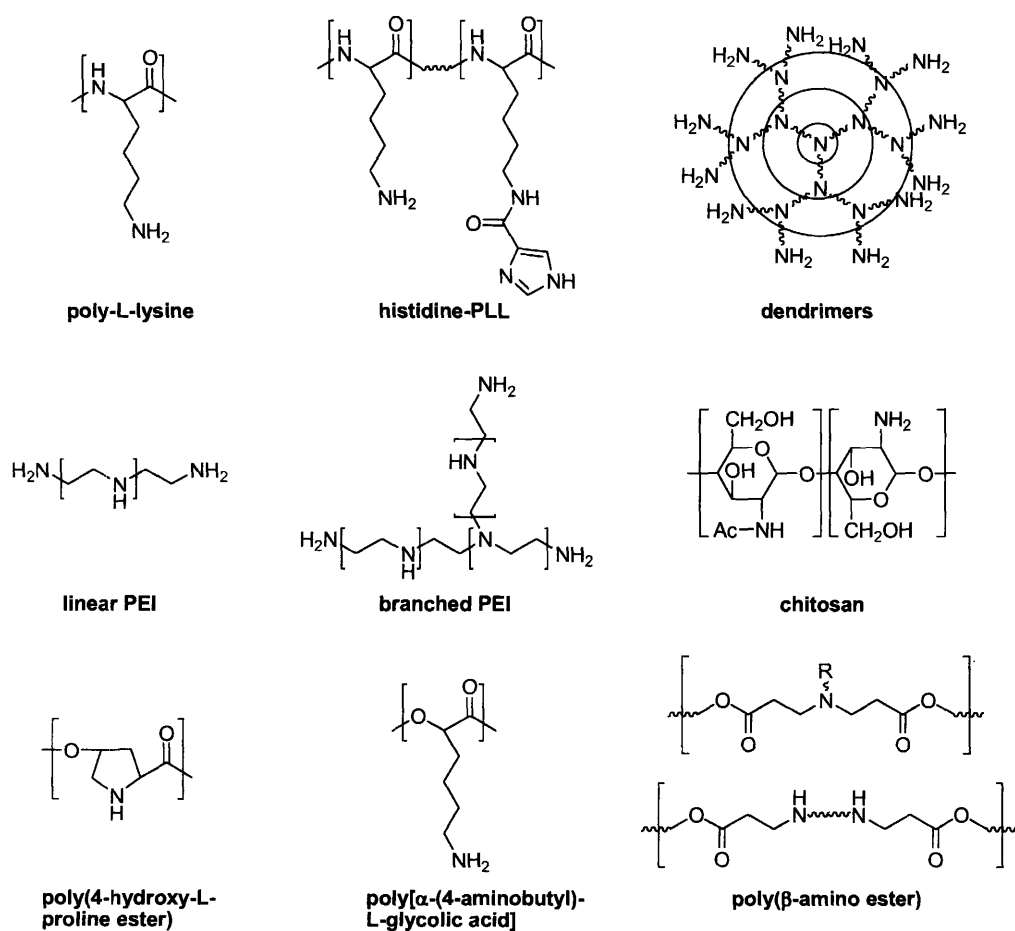
Figure 1.4 Structures of cationic polymers used for gene delivery

Most cationic polymers synthesized and used for gene therapy applications contain primary, secondary, tertiary, and/or quartanary amines in the polymer backbone and/or as part of pendant side chains. The type of amine and its neighboring functionalities have substantial effects on the pKa, charge density, and nucleophilicity of the polymer. In general, primary and secondary amines have very high pKa values and can act as strong nucleophiles. This latter property is frequently exploited to covalently conjugate drug molecules,[69] targeting ligands,[70,71] and polyethyleneglycol chains[72,73] to cationic polymers with these functionalities. Tertiary amines are generally less nucleophilic but can have pKa values around the physiological range. This property is advantageous for intracellular delivery because these amines can buffer endosomal compartments where the influx of protons causes acidification and potential DNA degradation. In addition, amine binding of protons leads to the formation of an osmotic gradient between the endosome and cytosol that can cause vesicle swelling and rupture. This mechanism of action, known as the proton sponge effect,[37] has been useful for the cytoplasmic delivery of proteins, DNA, and antisense oligonucleotides.

Polyethylenimine (PEI) is perhaps the most widely used and developed polycation for gene transfection.[74] This material can be prepared as either linear (ExGen500, jetPEI)[75] or branched,[76] depending on the starting materials and method of polymerization. A wide molecular weight range is commercially available, with polymers 5 – 25 kDa in size having the highest transfection efficiency. The poor performance of higher molecular weight polymers is mostly due to their excessive toxicity[77] while smaller polymers are ineffective at condensing DNA into nanoscale complexes that can be endocytosed.[78] The endosomal escape properties of 25 kDa PEI are likely related to the protonation behavior. Approximately 20% of the PEI nitrogen atoms are protonated at pH 7.4 outside the cell, whereas 45% are protonated at pH 5.0 in the acidified endosomes.[79] The high cationic charge density also affects the self-assembly of PEI with plasmid DNA. Depending on the conditions, polyplexes with spherical, globular or rod-like shapes can be formed with diameters less than 100 nm,[80] where each assembly contains approximately 3.5 plasmids bound to 30 PEI molecules.[81] The physicochemical properties of the complexes have made them useful for gene delivery, with most recent studies focusing on improving the specificity of transfection.[74]

Poly-L-lysine (PLL) was one of the first synthetic materials tested for gene delivery. The high positive charge density and available molecular weights enable this polymer to effective condense plasmid DNA into complexes less than 100 nm in diameter.[82] Since it is a peptide, PLL is considered biodegradable, a property that is particularly beneficial for *in vivo* use. However, this material is very cytotoxic by nature of its high cationic charge density.[83] Furthermore, the PLL gene delivery efficiency is very low, most likely due to its inability to facilitate endosomal escape. This barrier can be overcome to a modest extent by either (1) adding an endosomolytic agent such as chloroquine,[84] (2) combining with fusogenic peptides,[85] or (3) grafting imidazole functionalities to some of the primary amine side chains.[86]

Many other polycationic materials from both natural and synthetic orgins have been used and developed for gene delivery (Figure 1.4). In addition to PLL, other cationic peptides are still being developed such as polyornithine, polyarginine, polyhistidine and various combinations thereof.[87] Peptides derived from the active regions of viral proteins are also used alone or in conjunction with other delivery systems to boost transfection levels.[36,88] Cationic sugars, such as chitosan[89] and diethylaminoethyl-dextran,[90,91] have also found application as transfection reagents and have the potential advantage of high biocompatibility and biodegradability. Along with natural and synthetic biomolecules, dendrimeric polycations have been developed and show high transfection efficiencies relative to other non-viral vectors (Table 1.2).

Table 1.2 Comparison of gene delivery systems[adapted from Merdan et al.[92]]

Vector	DNA dose (μg/well)	Viability	Expression (% of linear PEI)
Linear PEI	0.75	79	100
Branched PEI	0.75	85	39
Superfect dendrimer	0.75	84	134
Lipofectin	0.4	63	40
Lipofectamine	0.4	61	176
Cellfectin	0.4	67	8

Recent advances in polymer-mediated gene delivery have involved the development of hydrolytically degradable vectors. In addition to offering all of the advantages listed above for polycations, degradable materials are potentially less cytotoxic since their charge density is reduced over time. Furthermore, polymer hydrolysis provides a clear, spontaneous and potentially tunable mechanism for polymer-DNA disassociation following nuclear uptake. Examples of hydrolysable polymers that have been developed include polyphosphoesters,[93] polyphosphazenes,[94] poly(4-hydoxy-L-proline ester),[95] poly(α-[4-aminobutyl]-L-glycolic acid),[96] and poly(β-amino ester)s.[97] Of these materials, the poly(β-amino ester)s have been developed most extensively and demonstrate highly effective gene delivery with good biocompatibility.

1.4 Poly(β-amino ester)s

1.4.1 Synthesis and Properties

Poly(β-amino ester)s are readily synthesized by the conjugate addition of either a primary amine or bis-secondary amine to a diacrylate compound,[97] as shown in Figure 1.5. The resulting linear polycations have tertiary amines in the backbone that are separated by two methylene units from ester bonds. The choice of diacrylate and secondary amine monomers allows for control over the chemical structure of the polymer backbone.
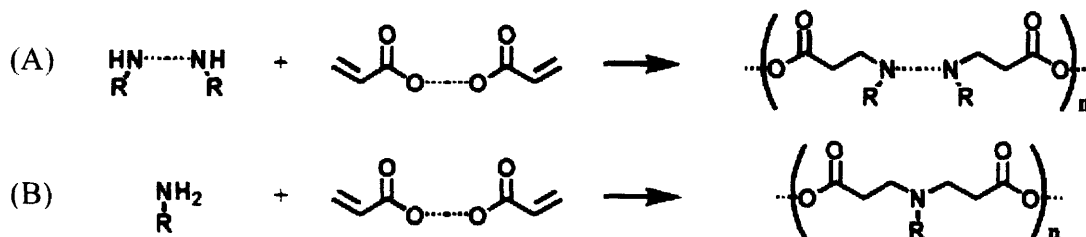
Figure 1.5 Reaction of diacrylates with (A) secondary amines and (B) primary amines to synthesize poly(b-amino ester)s.

Polymers formed from primary amine monomers have the additional advantage of pendant side chains, which allow for increased flexibility in polymer design. Due to their lack of reactivity to diacrylates, functionalities such as alcohols, ethers, aromatics, amides, tertiary amines and some heterocycles have been incorporated into the polymer side chains using the appropriate amine monomers. This greatly increases the number of potential building blocks and has permitted the generation of many structurally diverse polymers that could be useful for a range of biomedical applications.

The synthesis of many poly(β-amino ester)s can be conveniently carried out by direct mixing of the monomers in the absence of a solvent. This is possible since many amine and diacrylate monomers are liquids or solids with melting points only slightly above room temperature. However, for some monomer combinations the resulting polymer is an overly hard gel-like material with low solubility in organic solvents. For these cases, polymerizations are conducted using a minimum quantity of an anhydrous organic solvent. In general, reaction rates and the resulting degree of polymerization are enhanced at higher temperatures, by extending the reaction time, and for solvent-based reactions, by increasing monomer concentrations. The polymers formed usually have weight average molecular weights between 1,000 and 61,000 Da (relative to polystyrene standards) with polydispersities ranging between 1.5 and 7.0.[98] In the pure state, many of these polymers are orangish viscous liquids with high solubility in most organic solvents (e.g., DMSO, CH_2Cl_2, THF, DMF, acetone, ethanol), very low solubility in ether and hexanes, and a pH-dependent solubility profile in aqueous buffer solutions (insoluble at pH's greater than or equal to physiologic with modest solubility at acidic pH's). The latter property has made this class of polymers particularly useful for the intracellular deliver of DNA and the pH-dependent release of DNA encapsulated in poly(β-amino ester) microparticles.

Degradation of poly(β-amino ester)s proceeds through hydrolytic cleavage of ester bonds in the backbone to generate the corresponding bis(β-amino acid) and diol fragments. These products were confirmed by ^{1}H NMR and the spectra showed no evidence for the retro-Michael reaction to regenerate amine and acrylate groups.[97] This degradation pathway is important because (1) changes in the polymer structure can have a direct, controllable affect on rate of hydrolysis, and (2) free acrylate functionalities that are DNA-alkylating agents and potentially carcinogenic are not formed. Despite the large

number of poly(β-amino ester)s synthesized thus far, the degradation rate for only a few has been examined.[97] For these polymers the rate of hydrolysis was slower at pH 5.1 than at pH 7.4. The half-life for hydrolysis was approximately 7-8 hours at pH 5.1 while almost complete degradation occurred within 5 hours at pH 7.4. By selecting more hydrophobic monomers, it is envisioned that the polymer degradation rate can be substantially prolonged and even tightly controlled as can be done with other hydrolysable, ester-based polymers such as poly(lactic-co-glycolic acid) (PLGA).

1.4.2 DNA Delivery Studies

Initial studies by Lynn and Langer[97] established the utility of poly(β-amino ester)s for gene delivery. The synthesis and degradation kinetics of three model polymers were examined along with their ability to form nano-complexes with DNA sufficient for endocytosis. Gel electrophoresis experiments demonstrated that DNA could be effectively immobilized by complexing it with an equivalent or small excess of polymer. This indicates that charge neutralization of polyanionic DNA could be achieved with these polymers despite a sterically crowded environment surrounding the tertiary amines and their relatively low pKa values. Consistent with the electrophoresis experiments, zeta-potential and particle sizing measurements showed that simple electrostatic associations between poly(β-amino ester)s and DNA result in their spontaneous self-assembly into cationic particles with diameters less than 200 nm. In particular, one of the polymers studied formed complexes with DNA on the order of 50-150 nm that remained stable over an 18-hour period. Therefore, even though polymer degradation proceeds with a half-life of only a few hours, substantial resistance to hydrolysis is achieved by association with plasmid DNA. In addition to favorable DNA binding properties, all three polymers were found to be non-toxic to NIH3T3 cells at concentrations up to 100 ug/ml. This is in contrast to PEI which resulted in almost complete loss of cell viability over the same concentration range. All of these results taken together illustrate the potential for poly(β-amino ester)s to be developed into effective non-viral gene therapy vectors and have provided motivation for the synthesis and testing of more polymer structures.

The potential for the high throughput discovery of poly(β-amino ester) gene delivery vectors was demonstrated by synthesizing a 140-member polymer library and characterizing polymer/DNA complex size, surface charge, cellular uptake, cytotoxicity, and transfection efficiency. Screening of these polymers for transfection of COS-7 fibroblast cells identified two polymers that mediate gene delivery 4-8 times higher than PEI and at comparable levels to Lipofectamine 2000, a leading commercially available lipid-based vector. The low activity of most polymers in the library may have been due in part to the negative surface potentials of their complexes with DNA. Anionic particles typically do not interact favorably with the negatively charged cell surface and, therefore, show limited cellular uptake. It was also shown that polymer/DNA complex size is important for cell uptake and transfection, with the best polymers forming complexes with DNA that are less than 250 nm to permit endocytosis. Both of these particle characteristics, namely size and surface charge, are the direct result of the polymer/DNA ratio used, which was fixed at 20:1 by weight.

Akinc et al.[99] expanded on this work to show that end functionality and nonstructural factors (e.g., molecular weight, polymer:DNA) substantially affect the transfection properties of poly(β-amino ester)s. To examine these factors, polymers were synthesized at a variety of amine:diacrylate molar ratios. This synthetic strategy led to the formation of either amine- or acrylate-terminated polymer chains with a range of molecular weights between 3,350 and 18,000 Da, depending on the monomer ratio. In this way the effects of molecular weight and end chain functionality on transfection ability could be systematically examined. It was shown that the highest levels of transfection were observed with high molecular weight (> 13,000 Da) amine-terminated polymers. Furthermore, results were largely dependent on the polymer:DNA ratio used and varied considerably for each polymer. The lack of transfection found with low molecular weight polymers was attributed to their inability to form stable polymer/DNA complexes, as determined by agarose gel electrophoresis. Acrylate-terminated polymers were poor transfection agents primary due to the low cellular uptake of their polymer/DNA complexes, and to a lesser extent because of their cytotoxicity levels. In general, both uptake and viability were found to decrease as the molecular weight of acrylate-terminated polymer decreased. This study established that the best transfection agents are relatively high molecular weight amine-terminated polymers and the optimal polymer:DNA ratio needs to be determined empirically for each polymer.

To further explore structure-function relationships governing gene delivery with these polymers, Anderson et al.[100] developed semi-automated, high-throughput methods to synthesize a library of 2350 poly(β-amino ester)s and screen for their capacity to transfect COS-7 cells at several polymer/DNA ratios. Initial broad-based screening and more-refined optimization procedures lead to the discovery of 46 polymers that transfect better than PEI and 26 that are superior to Lipofectamine 2000. Furthermore, a few key structural features were found to be common among the most effective polymers. In general, the combination of hydrophobic diacrylates with hydrophilic amines gave the best results. The diacrylates usually consisted of an aliphatic or aromatic chain bridging the esters while the amines were either bis-secondary or primary amines containing alcohol or imidazole groups. While this highlights the effect of chemical structure on transfection activity, the influence of polymer molecular weight and chain-end groups were not examined.

To better optimize gene delivery and study the structure-function relationships that govern polymeric gene delivery, a 486 second-generation poly(β-amino ester) library was synthesized and characterized.[98] The monomers for this library are shown in Figure 1.6. Previous work established that polymers formed from these monomers were the most effective transfection agents. Further diversity was generated by synthesizing each polymer at several amine:diacrylate ratios between 0.6 to 1.4, resulting in a distribution of molecular sizes and either amine or acrylate-terminated chains. Gene delivery was assessed on COS-7 cells over a range of polymer:DNA ratios and the results of the most effective polymers are presented in Figure 1.7. Several polymers in the library were found to be more efficient DNA delivery vectors than PEI and Lipofectamine 2000. Importantly, a clear convergence in structure was seen among the top performing
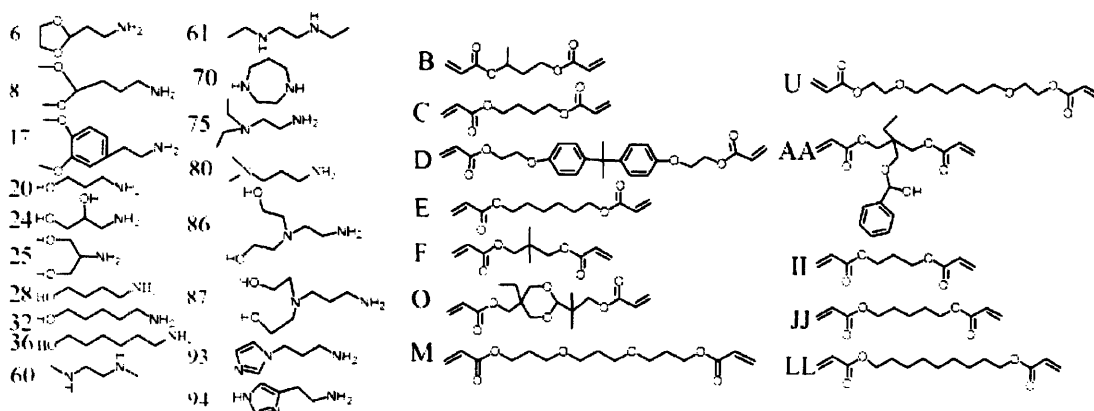
Figure 1.6 Monomers used to synthesize effective poly(β-amino ester)s.

polymers: the top 9 polymers had alcohol side chains and the three best polymers differed by only one carbon, either in the backbone or side chains. Also, the majority of effective polymers were formed using a 1.1 or 1.2 molar excess of amine over diacrylate. The excess of amine monomer allowed for the synthesis of amine-terminated polymer chains and had a direct effect on molecular weight. The most effective polymers all had molecular weights greater than 10,000 Da, again emphasizing the importance of this physical property on transfection as demonstrated previously. In addition, these polymers formed complexes with DNA that were smaller than 150 nm, with the top three forming particles that were 71, 79, and 82 nm in diameter. In general, it was concluded that high molecular weight (> 10,000 Da) and small polymer/DNA complex size (< 150 nm), alone or taken together, are both necessary but not sufficient properties to enable high transfection levels. It is also clear that the alcohol side chains confer an important advantage to the polymer, however the nature and mechanism of their participation in the gene delivery process is currently unknown.

The best performing polymer discovered from previous studies, C32, was subsequently tested for *in vivo* gene delivery in mice with prostate cancer xenographs.[101] Following intratumor injection, it was found that C32 delivers a reporter gene with four-fold higher effectiveness to tumor cells than the current state-of-the-art polymer, *in vivo* jet-PEI. Conversely, C32 complexation with plasmid DNA inhibited its transfection of the surrounding muscle tissue, indicating that this polymer may be ideal for localized delivery to prostate tissue. The C32 polymer also demonstrated good biocompatibility with no evidence of tissue damage following either intratumor or intramuscular injection. Furthermore, analysis of blood protein and metabolite levels were normal, indicating that the kidneys, liver or muscle tissue were not damaged. These results are in contrast to PEI which resulted in significant myocyte damage and calcification following an intramuscular injection. Finally, C32 delivery of a prostate specific anti-cancer DNA encoding the diphtheria toxin chain A resulted in suppressed tumor growth and tumor size reduction in 40% of treated mice.

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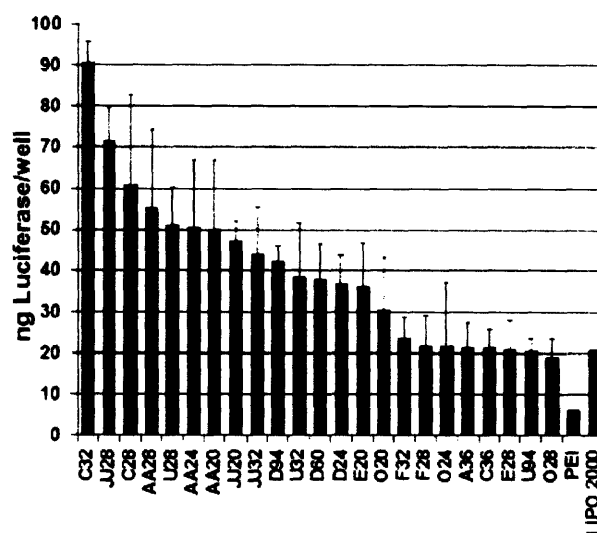
Figure 1.7 *In vitro* transfection levels of poly(b-amino ester)s. Expression levels for the luciferase reporter protein are shown for each polymer at its optimal polymer:DNA weight ratio on COS-7 cells.

1.5 Thesis Introduction

It is clear that the lack of a safe and efficient DNA delivery system is currently the major impediment preventing the routine use of gene-based drugs to treat cancer and other diseases. Viral vectors appear to be the most effective agents to date and their continued development may eventually lead to beneficial treatments. However, several major issues must be addressed and overcome for viruses to gain widespread use, which are primarily (1) the potential for insertional mutagenesis which can lead to carcinogenesis, (2) the strong and potentially deadly immune responses toward the virus and the cells it infects, (3) the potential for wild-type reversion, (4) the limited carrying capacity and (5) low viral production levels. The current non-viral alternatives suffer from reduced transfection efficiencies and many are substantially toxic. In addition, most materials are not degradable and could accumulate in tissues to cause significant damage after repeated administration.

The poly(β-amino ester)s represent a class of materials that are particularly promising for development into a safe and efficient gene transfer system. First, many polymers of this type have been synthesized and are more effective than most other state-of-the-art non-viral gene delivery systems. Also, they are degradable in the time frame of several hours to days, which can allow for (1) improved biocompatability and (2) a mechanism for DNA unpackaging inside the cell. While many poly(β-amino ester)s have been developed, none are known to be effective for systemic and/or cell-specific delivery of nucleic acids. Most importantly, chemical methods to modify these materials have not

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been addressed to date, which are non-trivial and need to be established for their subsequent development into targeted, efficient delivery systems.

This thesis addresses the current limitations of poly(β-amino ester)s and presents (1) fast and reliable methods to chemically modify these materials, and (2) the discovery and characterization of new polymers that can mediate highly effective *in vitro* and systemic *in vivo* transfection. Following this introductory chapter, the next chapter outlines the objectives of this thesis and the rationale used to achieve these goals. The subsequent three chapters present distinct approaches to design, synthesize and functionalize poly(β-amino ester)s. Characterization of polymer gene delivery properties is presented along with the *in vivo* ability of these polyplexes to systemically deliver reporter genes. Finally, the thesis concludes with summary chapters that outline the main contributions of this work and future directions for developing more effective polymers that may be clinically useful.

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Chapter 2 – Thesis Objectives

The primary objective of this thesis is to present new methods for the synthesis and functionalization of poly(β-amino ester)s to enhance their gene delivery properties. Currently, these cationic degradable polymers have shown significant potential as transfection agents and possess several advantages over many other delivery systems that include (1) degradation into low molecular weight products, (2) low cytotoxicity, and (3) easy, inexpensive synthesis. Due to the commercially availability of many amine and diacrylate monomers, large libraries of poly(β-amino ester)s have been constructed and screened to identify many polymers with transfection efficiencies that surpass the best commercially available vectors. Superior effectiveness has also been realized *in vivo* for the treatment of prostate cancer by intratumoral injection of polyplexes. In contrast to local administration, the *in vivo* systemic effectiveness of these polymers has been rather limited. As shown in later sections, the transfection levels of the best poly(β-amino ester) to date is not equivalent to or elevated over a jet-PEI formulation. As a result, it would be beneficial to chemically modify these materials to improve their effectiveness and expand the structural and functional diversity of the current poly(β-amino ester) library. Since all studies to date have only focused on the synthesis and testing of new poly(β-amino ester)s from commercially available reagents, this thesis presents a series of chemistries to (1) synthesize new polymers with enhanced reactivity, and (2) modify existing polymers to improve their performance. In addition to improved transfection properties, these methods can be useful for the development of poly(β-amino ester)s as targeted gene delivery systems. In this respect, this thesis presents the synthesis and evaluation of the galactosylated poly(β-amino ester)s for hepatocyte-specific delivery, which could be useful for the gene-based correction of metabolic diseases and various forms of liver cancer. In light of these goals, three specific objectives have been identified and satisfied:

2.1 Synthesis of Thiol-Reactive Poly(β-amino ester)s

Poly(β-amino ester)s are synthesized using a new amine monomer, 2-(pyridyldithio)-ethylamine (PDA). The resulting polymers contain pyridyldithio functionalities in the side chains that display fast, selective reactivity towards thiolated ligands through a disulfide exchange reaction. The 2-mercapopyridine by-product has strong absorbance at 343 nm, which permits the reaction kinetics and level of side-chain substitution to be monitored colorimetrically. In addition, since the newly incorporated side-chain functionalities are linked through disulfide bonds, the PDA polymers have the potential to breakdown inside the cell due to the high, cytoplasmic concentrations of glutathione. This chemistry can be useful to couple peptides, antibodies and other thiol-containing

molecules to poly(β-amino ester)s to improve their cell-targeting properties and enhance cellular transfection.

2.2 Synthesis of End-Modified Poly(β-amino ester)s

End-modified poly(β-amino ester)s are synthesized using a two-step approach that involves initial preparation of acrylate-terminated polymers followed by an amine-capping step. Due to the wide, commercial availability of amine reagents and the specificity of the amine-acrylate reaction, many different functional groups can be incorporated at the terminal segments by choice of the appropriate amine molecules, without the need for complicated protection chemistries. This approach is useful to (1) systematically assess the effects of terminal amine structure and functionality on polymer function, and (2) rapidly end-modify a large collection of poly(β–amino ester)s to optimize their gene delivery properties.

2.3 Development of Galactosylated Poly(β-amino ester)s for Targeted Delivery to Hepatocytes

Three approaches are examined to conjugate galactosylated ligands to poly(β-amino ester)s for targeted hepatocyte delivery. First, end-modification with lactose is examined since the terminal amine is known to have an important effect on cellular uptake. Second, C32-Galactose copolymers are prepared to couple the high transfection efficiency of C32 with the potential targeting properties of the galactose molecule. And finally, a combined end- and side-chain conjugation strategy is devised in which a series of end-modified poly(β-amino ester)s are prepared containing 100% galactose side chains.

Chapter 3 – Synthesis of Thiol-Reactive Poly(β-amino ester)s for Gene Delivery

3.1 Summary

Many obstacles to DNA delivery exist at both the systemic and cellular levels, impeding the routine application of non-viral gene therapy for the treatment of inherited and acquired genetic diseases. To address these challenges, it would be advantageous to have a polymer delivery system that can bind DNA and remain amenable to chemical modification to improve and optimize its performance for gene therapy applications. With this goal in mind, this chapter presents novel, multifunctional biodegradable polymers capable of simple graft modification that demonstrate low cytotoxicity and the ability to respond to intracellular conditions. To this end, poly(β-amino ester)s were synthesized using a novel amine monomer, 2-(pyridyldithio)-ethylamine (PDA). These cationic degradable polymers contain pyridyldithio side chains that are capable of reacting with high specificity towards thiol ligands. This is demonstrated using mercaptoethylamine (MEA) and the thiol peptide RGDC, a ligand that binds with high affinity to integrin receptors on angiogenic endothelial cells. Both polymer derivatives displayed strong DNA binding as determined using electrophoresis and dye exclusion assays. In addition, the MEA based polymer and plasmid DNA were shown to self-assemble into cationic complexes with effective diameters as low as 100 nm. Furthermore, the DNA binding ability was substantially reduced in response to intracellular glutathione concentrations, which may aid in DNA unpackaging inside the cell. These complexes also displayed low cellular toxicity and were able to mediate transfection at levels comparable to PEI in human hepatocellular carcinoma cells. These results suggest that the PDA-based poly(β-amino ester)s may serve as a modular platform for polymer mediated gene delivery.[1]

3.2 Introduction

Cationic polymers have emerged as promising materials for the intracellular delivery of DNA.[2] Some polycations have demonstrated the ability to electrostatically bind DNA and condense it into nanoparticles sufficient for cellular uptake.[3] However, no material is capable of overcoming all obstacles to efficient gene delivery including selective cellular uptake, endosomal escape, cytoplasmic trafficking, and nuclear import.[4] Each of these barriers has been addressed in the design of DNA delivery systems, usually individually and rarely in combination.

Current research into the development of an effective polycation system involves the synthesis and testing of new cationic materials or the modification of existing ones to improve their performance. Cationic polymers such as poly-L-lysine (PLL), polyethylenimine (PEI), poly(amido amine)s (PAMAMs), and chitosan are frequently employed as base materials of the delivery system due to their inherent positive charge that can facilitate DNA binding.[5,6] Using these materials as a platform, various functional groups have been grafted to the polymers to enhance their delivery properties. For example, cell specific delivery has been demonstrated by conjugating mainly peptide ligands such as growth factors,[7,8] RGD sequences,[9,10] transferrin,[11] and antibodies[12,13] to the polymer chains. Viral mechanisms to promote endosomal escape have also been incorporated into these polycationic materials using peptide transduction domains, such as the HIV TAT sequence and influenza virus hemagglutinin subunit HA-2.[14,15] Also, nuclear localization sequences derived from intracellular proteins have been grafted to the vector to direct DNA to the nucleus.[16,17] Despite all of these approaches, no single polycation delivery system currently integrates all of these functions together to form one highly efficient, virus-like vector.[18]

The current non-viral delivery systems suffer from several disadvantages and limitations. The most important is low transfection efficiencies relative to viral vectors.[19] In addition, many are non-degradable and do not provide a mechanism for elimination and DNA release.[20] The high cationic charge densities present in the most common polymers, such as PLL and PEI, result in substantial cytotoxicity.[21,22] In the case of PLL and other protein based systems, immune responses can be problematic, especially for repeat injections.[23] From a chemistry standpoint, material modifications are often time consuming, involve difficult and/or multi-step purifications, and may employ reaction conditions at high temperatures and pH's that are potentially damaging to peptides and antibodies. Furthermore, these reactions are usually performed using amine functionalities in the polymer which can significantly lower the charge density and DNA binding properties of the delivery system.[24] Given all of these limitations, it would be beneficial to have an alternative material that can bind DNA, displays good cell compatibility, and is readily amenable to chemical modification to optimize its delivery performance.

Recently there has been a new initiative to develop degradable cationic polymers that can mediate gene delivery.[18] One promising class of such materials are the poly(β-amino

ester)s. These polymers are cationic by nature of their tertiary amines and degradable through ester bonds in the backbone.[25] They can be readily synthesized by the conjugate addition of primary amines or bis-secondary amines to diacrylate compounds.[25] Due to the large number of commercially available monomers, libraries of structurally diverse poly(β-amino ester)s have been synthesized and tested for gene delivery.[26-28] Structure-function studies have shown that the combination of hydrophobic diacrylates and amino-alcohols resulted in polymeric vectors with DNA delivery efficiencies that are superior to PEI and Lipofectamine.[29]

In a continued effort to develop degradable polycations for gene delivery, this chapter presents the synthesis of new poly(β-amino ester)s that can serve as cationic degradable scaffolds for building in a variety of structural and functional enhancements. These polymers are synthesized using a novel primary amine monomer, 2-(pyridyldithio)-ethylamine (PDA). The poly(β-amino ester)s that result contain pyridyldithio groups in the side chains that display fast and selective reactivity with thiol groups, without altering the charge density of the polymer backbone. Such reactivity can be useful for conjugating cell targeting peptides, viral transduction domains, and other synthetic peptides into a single polymer chain to improve its gene transduction efficiency. An added benefit of this reaction mechanism is that a molecule of 2-mercaptopyridine (2-MP) is released for each thiol molecule reacted. This compound has a strong absorbance at 343 nm which enables the side chain conversion to be followed colorimetrically.[30] In addition to reactive side groups, the polymer end chains consist of reactive acrylates that can be used to couple amine compounds.

As one potential application of the flexibility of this approach, the attachment of mercaptoethylamine (MEA) and the RGDC peptide to PDA poly(β-amino ester)s is demonstrated. Both polymers were shown to condense DNA and, for the MEA derivatives, form cationic DNA complexes with effective diameters around 100 nm. The MEA based polymer was also shown to be responsive to intracellular glutathione concentrations in which a significant decrease in DNA binding was observed. To test the generality of the approach, three different PDA-based poly(β-amino ester)s were synthesized, along with their MEA derivatives, and tested for transfection on human hepatocellular carcinoma cells. In all cases, the transfection efficiency was greatly improved by MEA side chain substitution and rival that observed for optimized PEI formulations.

3.3 Experimental Procedures

3.3.1 Materials

Polyethylenimine (water free, $M_w \sim 25$ kDa, $M_n \sim 10$ kDa), 2,2'-dipyridyl disulfide (98%), mercaptoethylamine (98%), mercaptoethylamine hydrochloride (~95%), 2-mercaptopyridine (99%) (2-MP), reduced L-glutathione (99%), and 1,6-hexanediol ethoxylate diacrylate (U) were purchased from Sigma-Aldrich (St. Louis, MO). 1,4-Butanediol diacrylate (99+%) (C) and 5-amino-1-pentanol (32) were from Alfa Aesar (Ward Hill, MA). Neopentyl glycol diacrylate (>85%) (F) was from Scientific Polymer

Products, Inc.(Ontario, NY). All chemicals were used as received without any further purification. A 25 mM sodium acetate buffer solution pH 5.2 (NaAc buffer) was prepared by diluting a 3 M stock solution (Sigma-Aldrich). Peptide arginine-glycine-aspartate-cysteine (RGDC) was purchased from Bachem Bioscience, Inc. (King of Prussia, PA). Peptides GRGDC and GRDGC were synthesized by the Biopolymers Laboratory at MIT and HPLC purified. PicoGreen was purchased from Molecular Probes (Eugene, OR). pCMV-Luc and pCMV-EGFP plasmid DNA stock solutions (1 mg/ml in water) were obtained from Elim Biopharmaceuticals (Hayward, CA). The MTT Cell Proliferation Assay, Bright Glo Luciferase Assay Kits, and recombinant Luciferase protein were from Promega Corporation (Madison, WI). White and black polystyrene tissue culture treated 96-well plates and half area polystyrene 96-well plates were obtained from Corning Costar. Clear polystyrene tissue culture treated 96-well plates were obtained from Becton Dickinson (Bedford, MA).

3.3.2 Cell Culture

FOCUS hepatocellular carcinoma (HCC) cells were a kind gift from Professor Dane Wittrup and grown at 37°C, 5% CO_2 in Minimum Essential Medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 0.1 mM non-essential amino acids, 100 units/ml of penicillin/streptomycin, and 1 mM sodium pyruvate. All HCC culture reagents were purchased from Invitrogen Corporation (Carlsbad, CA). Human umbilical vein endothelial cells (HUVECs) were obtained from Cambrex (Walkersville, MD) and grown at 37°C, 5% CO_2 in EGM-2 media supplemented with SingleQuot kits. HUVECs were passaged according to the manufacturer's instructions and used for transfection experiments before the sixth passage.

3.3.3 Methods

All ^{1}H NMR was conducted on a Varian Unity spectrometer (300 MHz). The structures of all compounds and polymers synthesized, along with their ^{1}H NMR spectrum, are shown in Appendix A. Gel permeation chromatography (GPC) was performed as described previously[31] to determine weight and number average molecular weights of the polymers relative to polystyrene standards.

Synthesis of 2-(Pyridyldithio)-ethylamine Hydrochloride (PDA*HCl). The synthesis of PDA*HCl was performed following a method similar to that reported by Ebright et al.[32] Briefly, 2-mercaptoethylamine hydrochloride (2.288g, 20 mmol) was dissolved in 17.5 ml of methanol and added dropwise to a stirred solution of 2,2'-dipyridyl disulfide (8.815 g, 40 mmol) dissolved in 41.6 ml of methanol containing 1.6 ml glacial acetic acid. The reaction was kept under an argon atmosphere to minimize free thiol oxidation. After 48 hours, the mixture was concentrated under reduced pressure to give approximately 10-15 ml of yellow oil. The product was precipitated by the addition of 100 ml cold ether and purified by redissolving in 20 ml methanol and precipitating with 100 ml cold ether six times to give a white powder. ^{1}H NMR (D_2O): δ (ppm) 3.1 (t, J = 6.26 Hz, 2H), 3.3 (t, J = 6.26 Hz, 2H), 7.3 (m, 1H), 7.7 (m, 1H), 7.8 (m, 1H), 8.4 (m, 1H).

Synthesis of 2-(Pyridyldithio)-ethylamine (PDA). Sodium hydroxide (384.7 mg; 9.6 mmol) was dissolved in 1 ml of water and quickly added to 1.95 g of 2-(pyridyldithio)-ethylamine hydrochloride (8.8 mmol) dissolved in 2 ml of water. The solution was briefly vortexed and allowed to stand for 15 minutes. The free base 2-(pyridyldithio)-ethylamine (PDA) phase separated and was isolated by draining from the tube bottom. ^{1}H NMR (CDCl$_3$): δ (ppm) 1.6 (bs, 2H), 2.8 (m, 2H), 3.0 (m, 2H), 7.1 (m, 1H), 7.6 (m, 2H), 8.5 (m, 1H).

Synthesis of Poly(β-amino ester)s. PDA-based polymers were synthesized by adding 187 mg (1 mmol) of freshly isolated PDA to 1 mmol of either C, F, or U diacrylate in a Teflon-lined screw cap vial with a magnetic stir bar. The mixture was stirred at 60°C for 2 days after which time the polymer was stored at -20°C until used for each experiment. Letter designations for the diacrylates correspond to those reported by Anderson et al.[27] Weight average molecular weights and polydispersities (given in parenthesis) were determined to be 4,041 (1.35) for C-PDA, 4,363 (1.40) for F-PDA, and 4,210 (1.38) for U-PDA. PDA polymer stock solutions at 100 mg/ml were made by dissolving 20 mg of polymer in 200 μl DMSO and used for all subsequent experiments unless otherwise noted.

Polymer C32 was prepared by mixing 2.3688 g of C with 1.4786 g of 32 in a Teflon-lined screw cap vial with a magnetic stir bar. The mixture was stirred at 90°C for 24 hours and stored at room temperature. A 100 mg/ml C32 solution in DMSO was prepared fresh for all experiments.

Synthesis of PDA Poly(β-amino ester) derivatives. A C-PDA-MEA polymer solution at 100 mg/ml was prepared by dissolving 20 mg of C-PDA in 200 μl of DMSO containing 20 mg/ml MEA. C-PDA-RGDC polymer solution at 100 mg/ml was prepared by dissolving 20 mg of C-PDA in 200 μl DMSO containing 23.4 mg RGDC. C-PDA-GRGDC and C-PDA-GRDGC polymer solutions at 100 mg/ml were prepared by dissolving 10 mg of C-PDA in 100 μl DMSO containing 7.5 mg of GRGDC or GRDGC, respectively. The C-PDA-MEA solution was always prepared 30 minutes before usage while the C-PDA-peptide solutions were made one day prior to allow for a 24 hour reaction period before using. These reaction times were necessary to ensure complete side chain conversion. These solutions were used in all subsequent experiments unless otherwise noted.

Characterization of polymer side chain reactivity. Concentrated solutions of the thiol reagents mercpatoethylamine (MEA) and RGDC were each prepared at 20 mg/ml in DMSO. A series of ten 1:2 dilutions in DMSO was performed to generate MEA and RGDC dilution sets. Both thiol dilution sets were plated at 150 μl/well in a 96-well plate. The C-PDA polymer was dissolved in DMSO to a concentration of 1 mg/ml and plated at 100 μl/well. Using a 12-channel pipettor, an equivalent volume of thiol solution (MEA or RGDC) was added to the C-PDA polymer solution and mixed briefly by pipetting. The mixture absorbance was measured at several time intervals using a Molecular Devices SPECTRAmax PLUS384 absorbance plate reader at a 343 nm wavelength.

Agarose Gel Electrophoresis of Polymer/DNA Complexes. Polymer solutions at 100 mg/ml in DMSO were diluted accordingly into NaAc buffer and a 25 μl aliquot was added to 25 μl of pCMV-Luc DNA (60 μg/ml in NaAc buffer) and mixed vigorously. The solutions were left undisturbed for 5 minutes after which time 10 μl of loading buffer was added to each. The loading buffer consisted of 10% Ficoll 400 in 25 mM HEPES buffer. Polymer-DNA solutions were then diluted 4-fold into TAE buffer and 20 μl of these solutions were added to the wells of a 1% agarose gel containing ethidium bromide. Gel electrophoresis of the complexes was performed in TAE buffer at 60V for 3 hours. Imaging of the gel was performed using a Kodak Electrophoresis Documentation and Analysis System 120.

Polymer-DNA Binding Assay with PicoGreen. All of the following manipulations were carried out in a black 96-well plate. Polymer solutions at 100 mg/ml in DMSO were diluted according into NaAc buffer and a 50 μl/well aliquot of each was added to 50 μl/well of pCMV-Luc DNA (12 μg/ml in NaAc buffer). The solutions were mixed vigorously and allowed to sit undisturbed for 5 minutes to allow for polymer-DNA complexation. After this time 100 ul/well of PicoGreen solution was added. PicoGreen working solution was made by diluting 60 μl of the purchased stock into 12 ml NaAc buffer. After 5 minutes, the plate fluorescence was measured on a Perkin Elmer Victor 3 plate reader using a standard FITC filter set (excitation 485 nm, emission 535 nm). The relative fluorescence (RF) was calculated using the following relationship:

$$RF = (F_{sample} - F_{blank})/(F_{DNA} - F_{blank})$$

Experiments were also conducted in the presence of L-glutathione essentially as described above. However, a 100 μl/well aliquot of the PicoGreen-polymer-DNA solution was added to a 100 μl/well glutathione solution (0, 0.2, or 20 mM) in phosphate buffered saline (PBS; 10 mM phosphates, 137 mM NaCl, pH 7.4) prior to measurement of the plate fluorescence.

Polymer/DNA Complex Size and Surface Charge Measurements. A C-PDA-MEA polymer solution at 100 mg/ml in DMSO was prepared as described above and diluted accordingly in NaAc buffer immediately before use. To prepare polymer:DNA complexes, a 100 μl aliquot of diluted polymer was added to a 100 μl aliquot of pCMV-Luc DNA (60 μg/ml in NaAc) and pipetted vigorously. Complexation was allowed to proceed undisturbed for 5 minutes after which time 150 μl of the sample was diluted into 1.8 ml of PBS. Polymer:DNA complex size and surface charge were measured on a ZetaPALS dynamic light scattering detector (Brookhaven Instruments Corporation, Holtsville, NY; 15 mW laser; 676 nm incident beam, 90° scattering angle). Effective particle diameters were calculated from the autocorrelation function using the MAS option of the BIC particle sizing software assuming a log normal distribution. The solution viscosity and refractive index were assumed equal to pure water at 25°C. Zeta potentials were calculated from the measured electrophoretic mobilities of the complexes using the BIC PALS zeta potential analysis software and the Smoluchowsky model for aqueous dispersions.

Measurements of Polymer Cytotoxicity. HCC cells were plated at 15,000 cells/well in clear 96-well plates and grown overnight. A 29 mg/ml solution of 2-MP in DMSO was prepared. Polymer and 2-MP solutions in DMSO were diluted accordingly into NaAc buffer and a 25 μl aliquot of each was mixed vigorously with a 25 μl aliquot of pCMV-Luc DNA (60 μg/ml in NaAc buffer) in a half-area 96-well plate. The solutions were left undisturbed for 5 minutes after which time a 30 μl aliquot was added to 200 μl of Opti-MEM in a separate clear 96-well plate. The media over the cells was then removed with a 12-channel aspirator wand and followed by the addition of 150 μl/well of polymer-DNA complex solution in Opti-MEM. Complexes were incubated over the cells for one hour after which time they were aspirated off and replaced by 105 μl/well of fresh cell culture media. Metabolic activity of the cells was measured 24 hours later using the MTT Cell Proliferation Assay Kit following the supplied instructions.

Transfection of Hepatocellular Carcinoma (HCC) cells. HCC cells were plated at 5,000 cells/well in opaque 96-well plates and allowed to adhere overnight. C-PDA, F-PDA, and U-PDA polymer solutions in DMSO were prepared as described above. A 100 μl sample of each was diluted into 900 μl of NaAc buffer to produce a 10 mg/ml aqueous solution. A 10 mg/ml aqueous C-PDA-MEA polymer solution was prepared by diluting 100 μl of 100 mg/ml C-PDA into 900 μl of NaAc buffer containing 10 mg/ml MEA. Aqueous solutions of F-PDA-MEA and U-PDA-MEA were prepared in a similar manner using the base polymers F-PDA and U-PDA, respectively. The 10 mg/ml aqueous polymer solutions were diluted accordingly into NaAc buffer and a 25 μl sample of each was mixed vigorously with 25 μl of pCMV-Luc DNA (60 μg/ml in NaAc buffer) in a half-area 96-well plate. The solutions were left undisturbed for 5 minutes after which time a 30 μl aliquot was added to 200 μl of Opti-MEM in a separate clear 96-well plate. The media over the cells was then removed with a 12-channel aspirator wand and followed by the addition of 150 μl/well of polymer-DNA complex solution in Opti-MEM. Complexes were incubated over the cells for one hour after which time they were aspirated off and replaced by 105 μl/well of fresh cell culture media. Cells were allowed to grow for three days at 37°C, 5% CO_2 and then analyzed for luciferase protein expression.

Luciferase expression was analyzed using Bright-Glo assays kits. Briefly, 100 μl/well of Bright-Glo solution was added to the cell plates. The plates were gently agitated to promote mixing for 2 minutes. Luminescence was then measured on a Mithras Luminometer using a 1% neutral density filter and a one second per well counting time. A standard curve was generated by performing an identical assay with dilutions of recombinant Luciferase protein.

Transfection of Human Umbilical Vein Endothelial Cells (HUVEC) cells. HUVEC cells were plated at 75,000 cells/well in 24-well plates and allowed to adhere overnight. C-PDA-GRGDC and C-PDA-GRDGC were prepared on the same day as cell plating as described above. On the following day, 50 μl of C-PDA-GRGDC or C-PDA-GRDGC polymer solution was mixed with 50 μl of C32 polymer solution and used for the transfection. The C32 control solution was made by mixing 50 μl of C32 polymer solution with 50 ul of DMSO (no C-PDA-peptide). Polymer solutions in DMSO were

diluted into NaAc buffer to a final C32 polymer concentration of 2.5 mg/ml. Polymer-DNA complexes were formed by vigorously mixing 100 μl of diluted polymer with 100 μl of pCMV-EGFP DNA (50 μg/ml in NaAc buffer). Solutions were left undisturbed for 5 minutes and then 125 μl was diluted into 1 ml of cell culture media. The media over the cells was then removed by aspiration and followed by the addition of 750 μl/well of polymer-DNA complex solution. Complexes were incubated over the cells for two hours after which time they were aspirated off and replaced by 500 μl/well of fresh cell culture media. Cells were incubated for two days at 37°C, 5% CO_2 and then analyzed for GFP protein expression.

HUVEC cells were analyzed for GFP protein expression using a Becton Dickinson FACSCalibur flow cytometer equipped with an argon ion laser capable of exciting GFP (488 nm). Fluorescence emission was filtered using a 535 nm band pass filter. Propidium iodide was used to identify and exclude dead cells. Both an FL1 vs. FL2 density plot and FL1 histogram were generated from 10,000 cells and used to analyze cellular fluorescence. Detector amplitude and gain for the GFP (FL1) channel were adjusted so that the untreated cell population peak centered between 10^0 and 10^1. Experiments were performed in triplicate for each polymer system.

3.4 Results

3.4.1 Monomer Synthesis

The synthesis of the amine monomer, 2-(pyridyldithio)-ethylamine hydrochloride (PDA*HCl), proceeds via the reaction between mercaptoethylamine hydrochloride (MEA*HCl) and 2,2'-dipyridyl disulfide as shown in Figure 3.1. Following the protocol outlined above, the PDA*HCl is found to be 93-94% pure by ^{1}H NMR analysis (Figure A1). The only detected by-product is the cystamine dihydrochloride formed from the reaction between MEA*HCl and the PDA*HCl product. This side reaction is favored towards the end of the dropwise MEA*HCl addition when there is a high concentration of PDA*HCl product (order 1 equivalent) and a reduced concentration of 2,2'-dipyridyl disulfide (order 1 equivalent). By increasing the 2,2'-dipyridyl disulfide concentration and/or decreasing the MEA*HCl concentration, the purity of the PDA*HCl product can be increased and the formation of cystamine*2HCl can be reduced. By using a five-fold
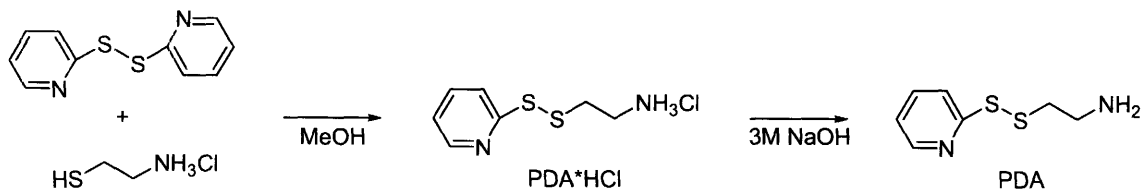

Figure 3.1 Synthesis of 2-(2-pyridyldithio)-ethylamine (PDA) by first reacting 2,2'-dipyridyl disulfide with mercaptoethylamine*HCl, followed by conversion of the hydrochloride salt (PDA*HCl) to the free base form (PDA) using concentrated sodium hydroxide.

or ten-fold molar excess of 2,2'-dipyridyl disulfide over MEA*HCl, the purity can be increased to 97% and 99%, respectively. Such increases in product purity can be particularly important if the intended use of PDA is as a monomer in a step-growth polymerization, where equimolar concentrations of reagents are necessary to produce high molecular weight polymers. Furthermore, it is critical to minimize the cystamine concentration in this case since it can act as a crosslinking agent by nature of its diamine functionality. In order for the PDA*HCl to be used in this fashion or in any applications which require the free nucleophilic amine, the hydrochloride must be removed.

The PDA*HCl can be converted to the corresponding free amine base by treating it with a strong base. In effect, application of a base stronger than PDA itself will cause an HCl transfer or a reaction between the base and the HCl. If sodium hydroxide is used, the reaction between it and the HCl generates NaCl and water with concomitant production of the PDA free amine. If an organic base is used such as triethylamine, the HCl is essentially transferred from the PDA to the base to create a new base-HCl salt (e.g., triethylamine hydrochloride).

Two different methods have been explored to generate the free amine, both using sodium hydroxide as the added base. In the first instance, PDA*HCl is dissolved in a minimum volume of water and mixed briefly, but vigorously, with a concentrated NaOH solution. This action causes the free amine to be generated immediately since NaOH is a very strong base. By using a minimium volume of aqueous solutions, the PDA phase separates as a yellow liquid from the water. Since it has a higher density, it concentrates at the bottom of the tube and can be removed by pipetting or draining out of the tube bottom. This procedure results in the formation of intact PDA with approximately 36% wt. water as the main contaminant, as determined by peak integration in the ^{1}H NMR spectrum ($\delta = 1.6$ ppm for water in CDCl$_3$). Attempts to remove the excess water by distillation cause rapid degradation of the PDA, as evidenced by a deep red-black color change and thin-layer chromatography (TLC) analysis (see below). In addition, the use of drying agents such as magnesium sulfate, sodium sulfate, and 3 Å molecular sieves can cause color changes and precipitates to form, indicative of PDA degradation.

The second method developed to isolate PDA is similar to that reported by Sigurdsson et al.[33] In this procedure, PDA*HCl is again treated with NaOH but in a larger volume of water. Under these conditions, the PDA formed is miscible with water and is subsequently extracted into an organic solvent such as dichloromethane or ether. The organic phase is then isolated, dried using magnesium sulfate, filtered, and concentrated under reduced pressure to give the PDA product. Before vacuum distillation the PDA/dichloromethane solution is clear and yellow. As the dichloromethane is removed and the PDA is concentrated in vacuum, a precipitate begins to form due to the spontaneous degradation of PDA. The ^{1}H NMR analysis of this precipitate shows two triplet peaks closely spaced from one another and appearing around 2.6-2.8 ppm, indicative of two methylene units with nitrogen and/or sulfur heteroatoms on either or both sides. Therefore, the precipitate may be cystamine or some other molecule containing the non-aromatic portion of PDA (see below). This is further supported by the ^{1}H NMR spectrum of the final filtered PDA product after complete removal of the

dichloromethane which shows a higher content of pyridine hydrogens relative to the ethylamine hydrogens.

In general, the PDA product is unstable and cannot be isolated in the pure state using either method, a finding supported by Sigurdsson et al.[33] Storage of newly formed, crude PDA at room temperature results in the formation of precipitates and a darkening from yellow to a deep orange and finally dark red/black. The stability is greatly enhanced by storing at -20°C while relatively rapid degradation is observed at elevated temperatures. TLC analysis of freshly isolated PDA shows a distinct spot close to the starting line along with an additional spot close to the solvent front, suggesting the presence of 2,2'-dipyridyl disulfide. A heated PDA sample shows a decrease in intensity for the starting line spot. In addition, the 2,2'-dipyridyl disulfide spot remains and a third spot appears at the level of 2-mercaptopyridine. None of these compounds are a deep red color suggesting that other more complex and unidentified pyridine derivatives are most likely present. Similar color transitions are observed with the related pyridine compound 2-(2-pyridyl)-ethylamine, indicating that certain pyridyl reactions or rearrangements may be occurring.

The exact degradation pathway(s) for PDA are unknown but it is suspected it may occur through a disproportionation reaction, as evidenced by TLC and ^{1}H NMR (Figures 3.2, A2, A3). This form of rearrangement occurs for other structurally similar unsymmetrical disulfides such as 2-(phenyldithio)-ethylamine[34] and substituted benzyl 2-(n-decylamino)-ethyl disulfides.[35] These compounds have a tendency to revert into their symmetrical disulfide counterparts. In the case of PDA, this implies that the major degradation products are 2,2'-dipyridyl disulfide and cystamine. This reaction is accelerated by heat, ambient light, bases, and the presence of amines.[34] Furthermore, depending on the solubility of the final symmetrical disulfides, the reaction rate is also increased by Le Chatelier's principle. This is particularly evident for PDA isolation from organic solvents as mentioned above, where the aliphatic portions of the molecule precipitate out during solvent evaporation.
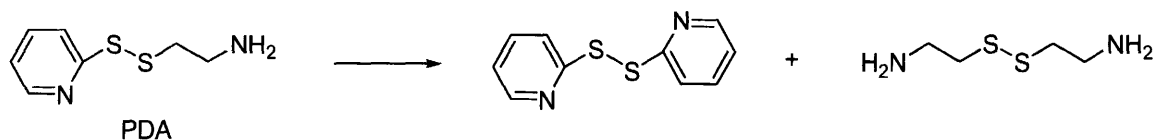
Figure 3.2 Disproportionation of PDA into the symmetrical disulfides 2,2'-dipyridyl disulfide and cystamine.

The first step in the disproportion pathway is believed to be an intramolecular reaction in which the free amine induces cleavage of the disulfide bond to generate the corresponding thiol ions.[35] A catalytic degradation then proceeds as the thiol ions react with the parent PDA molecules and regenerate themselves in the process. In the end, complete disappearance of the unsymmetrical PDA occurs with the concomitant generation of its symmetrical counterparts, namely 2,2'-dipyridyl disulfide and

cystamine. The latter is a diamine disulfide that, if present during the polymerization, can lead to substantial crosslinking. From the ^{1}H NMR spectrum of the PDA product, there is evidence of some disproportionation and the formation of symmetrical disulfide molecules. Peak integration indicates that there is an approximate 3.5:1 molar ratio of PDA to cystamine. Much lower ratios (i.e., higher cystamine content) were observed following the alternative isolation procedures mentioned above. The phase separation method followed here minimized PDA breakdown through its brief exposure to base and the use of equimolar neutralization conditions. Furthermore, the PDA product was used immediately upon isolation before any significant transformation could occur.

While the disproportionation mechanism is certainly feasible, it cannot be ruled out that other reactions may be occurring during PDA degradation. Various displacement reactions can occur at a sulfenyl sulfur in a molecule.[36] For example, amine compounds can react at this position to generate sulfenamides.[37] If the reaction is conducted with heating, homolytic cleavage of the sulfur-nitrogen bond can occur to eventually generate sulfonamides.[37] In addition, several by-products can form, most notably is the amine hydrosulfate, which precipitates out of organic solvents but can readily dissolve in water.[37] Because of this solubility behavior, it is suspected that the precipitate seen in PDA isolation may be a PDA or cystamine hydrosulfate, although this has not been confirmed. Overall, the complete degradation pathway(s) of PDA is complex and beyond the scope of this study.

3.4.2 Polymer Synthesis

The synthesis of PDA-based poly(β-amino ester)s was carried out by direct mixing of PDA with diacrylates (Figure 3.3). Equimolar monomer concentrations were used, assuming pure PDA, and the reaction temperature was maintained at 60°C to promote polymerization and minimize PDA degradation. Higher temperatures and excess molar equivalents of PDA over diacrylate resulted in substantial crosslinking due to the

C: R = (CH$_2$)$_4$
F: R = CH$_2$C(CH$_3$)$_2$CH$_2$
U: R = (CH$_2$)$_2$O(CH$_2$)$_6$O(CH$_2$)$_2$

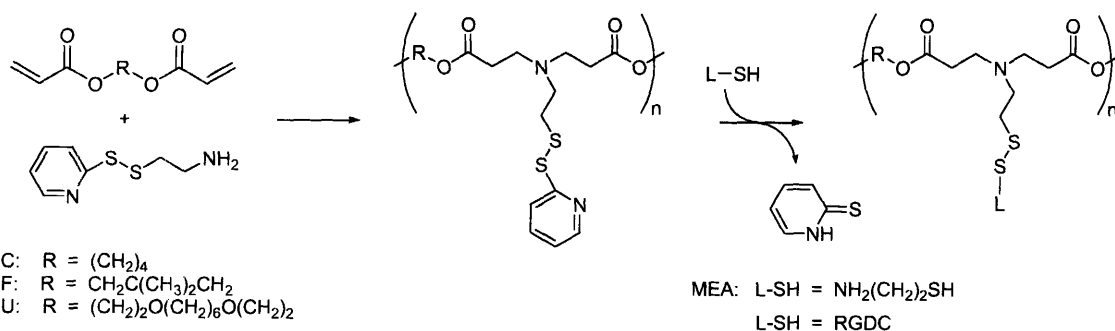MEA: L-SH = NH$_2$(CH$_2$)$_2$SH
L-SH = RGDC

Figure 3.3 Synthesis of PDA poly(β-amino ester)s by reaction of PDA with diacrylate monomers. Polymer side chains can react with thiolated ligands, such as mercaptoethyl-amine (MEA) and RGDC, to release a molecule of 2-mercapopyridine (2-MP). The strong absorbance of 2-MP at 343 nm allows the side chain reaction to be quantified colorimetrically.

generation of cystamine from PDA disproportionation. The optimal reaction conditions lead to weight average molecular weights (relative to polystyrene) around 4 kDa. The molecular weights were limited due to water contamination in the PDA that lead to an imbalance in monomer ratios.

Despite the inherent instability of PDA, the ^{1}H NMR spectrum of the PDA polymers revealed several peaks that are consistent with intact, thiol-reactive poly(β-amino ester)s (Figures A4 – A6). First, multiplet peaks were observed at 2.4 and 2.7 ppm and correspond to hydrogens on the newly formed methylene bonds between the tertiary amine and the esters. In addition to GPC measurements, this peak assignment provides evidence for polymerization through the anticipated reaction between the PDA amine and diacrylates. Second, protons in the pyridyldithio side chain showed four characteristic peaks in the 7 – 8.5 ppm range. No peaks were observed below 7 ppm that would indicate the presence of 2-MP from disulfide hydrolysis or disproportionation. Therefore, the polymer is intact and should display thiol-specific reactivity in the side chains. Finally, due to the imbalance in monomer ratios, an excess of diacrylate over PDA resulted in several peaks around 6 ppm. Similar peaks have been seen in other poly(β-amino ester)s generated using excess diacrylate and correspond to acrylate functionalities at the ends of the polymer chains.[31] This structural feature can allow for photo-polymerization or the conjugation of nucleophilic molecules to the polymer terminus while thiol-containing ligands can be linked specifically to the side chains.

Further analysis of the polymer ^{1}H NMR spectra reveals branched structures (Figures A4-A6). Partial disproportionation of PDA apparently occurs during its isolation and polymerization, leading to the formation of the cystamine crosslinking agent. Peak integration and comparison of pyridyl and ethyl side chain protons indicates that 70% of the polymer consists of PDA side chains, with the balance composed of cystamine crosslinks. Treatment of C-PDA with MEA leads to a 35% reduction in the molecular weight, indicating that these crosslinks may be reducible by thiolated molecules.

3.4.3 Side-Chain Reactivity

The reactivity of C-PDA polymer side chains to the thiol compounds MEA and RGDC was investigated as a function of concentration and time. Reaction with MEA resulted in a degradable cationic poly(β-amino ester) with pendent primary amines linked to the main chain through disulfide bonds. The RGDC compound was used to demonstrate the attachment of peptide compounds useful for targeting endothelial cells and other integrin expressing cell types.[38] In both cases, the reaction was carried out in DMSO instead of aqueous buffer solutions to prevent polymer hydrolysis. As shown in Figure 3.3, each thiol molecule reacts with a pyridyldithio side chain of the polymer to release a corresponding 2-MP molecule. This one-to-one correlation and the strong 2-MP absorbance at 343 nm permitted the progress of the reaction to be conveniently followed colorimetrically. These results were also confirmed by ^{1}H NMR for each PDA polymer (Figures A7 – A9). Reaction with excess MEA caused the disappearance of the three characteristic pyridyldithio side chain proton between 7 and 8.5 ppm and the formation of 2-MP (6.8 – 7.6 ppm).

51

The reaction kinetics of MEA and RGDC with the C-PDA polymer are shown in Figure 3.4. The absorbance of 2-MP was measured and converted into the percent of side-chains reacted using a 2-MP calibration curve. At low concentrations, both molecules demonstrate a linear increase in conversion with thiol concentration. Nonlinearity becomes apparent when the side chain conversion passes 60%, at which point the thiol concentration is approximately equal to the pyridyldithio concentration. In both cases, an approximate 2-fold molar excess of thiol over pyridyldithio groups is necessary to attain complete conversion, regardless of the reaction time. While the concentration dependence is very similar, the MEA and RGDC compounds show very different rates of reaction. MEA reached equilibrium at every concentration tested within 30 minutes. In fact, the reaction appeared instantaneous based on the immediate yellow color change of the solution upon mixing, but time points early than 30 minutes were not taken to verify this. Conversely, RGDC demonstrated much slower kinetics, taking over 28 hours to approach completion at each concentration tested. Full side chain conversion could be obtained under 30 minutes but it required an almost 30-fold molar excess of RGDC over pyridyldithio side groups (data not shown). Similar reaction kinetics and ligand concentration dependencies were seen with the F-PDA and U-PDA polymers, indicating that these effects are general for PDA poly(β-amino ester)s (Figure A10).

(A)   (B)
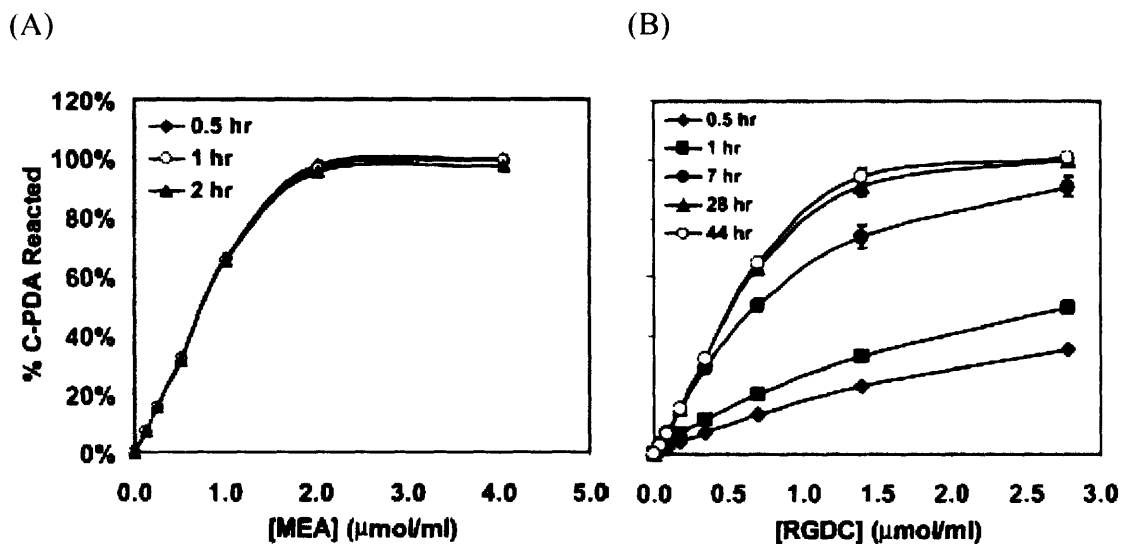

Figure 3.4 Side chain reactivity of the C-PDA polymer with (A) MEA and (B) RGDC in DMSO. In both cases, a 1 mg/ml polymer solution was mixed with an equal volume of either of an MEA or RGDC solution. Absorbance measurements were taken at the indicated time intervals and converted into side chain conversion using a 2-MP calibration curve. Data are expressed as mean values ($\pm$ SD, n=3).

3.4.4 Polymer-DNA Binding

An important requirement for an effective gene delivery system is the ability to interact with plasmid DNA. Polycationic materials that have high molecular weights and charge densities can bind negatively charged DNA through electrostatic interactions.[3] For the C-PDA based polymers, qualitative assessments of such interactions were made using an agarose gel electrophoresis shift assay. In this experiment, the migration of C-PDA, C-PDA-MEA, and C-PDA-RGDC polymer DNA complexes through the gel was compared to free DNA. The results are shown in Figure 3.5 for a range of polymer:DNA weight ratios. As shown, the free DNA migrated through the gel and resolved into two distinct bands, which correspond to the supercoiled and nicked-circular forms of the plasmid. Complexation of the C-PDA polymers with DNA retarded its movement to varying degrees depending on the polymer type and side chain substituent. For both the base C-PDA polymer and C-PDA-RGDC, partial inhibition of DNA migration is seen at all polymer:DNA ratios. As the polymer:DNA ratio is increased, more localization of DNA can be seen adjacent to the loading wells with a corresponding decrease at the levels of unbound DNA. This trend indicates that increasing amounts of polymer are necessary to more effectively bind DNA but full complexation cannot be realized even at high polymer:DNA ratios. On the contrary, the C-PDA-MEA polymer displays very effective DNA binding at all ratios. For each condition there appear sharp, distinct bands close to the loading wells with no apparent free DNA. The enhanced ability of this C-PDA derivative to bind DNA is the result of its primary amine side chains that significantly increase the cationic charge density of the polymer.
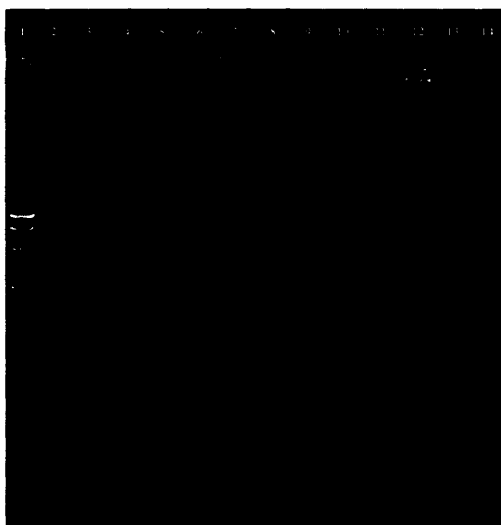
Figure 3.5 Agarose gel electrophoresis of polymer-DNA complexes. Lane assignments are as follows: (1) DNA ladder; (2) pCMV-Luc DNA; (3)-(6) C-PDA-RGDC/DNA complexes at polymer:DNA ratios of (3) 20:1, (4) 40:1, (5) 60:1, and (6) 100:1; (7)-(10) C-PDA/DNA complexes at polymer:DNA ratios of (7) 20:1, (8) 40:1, (9) 60:1, and (10) 100:1; (11)-(14) C-PDA-MEA/DNA complexes at polymer:DNA ratios of (11) 20:1, (12) 40:1, (13) 60:1, and (14) 100:1.

A more quantitative assessment of polymer-DNA interactions was made by performing a dye exclusion assay. In this experiment, the PicoGreen dye was incubated with unbound DNA and polymer-DNA complexes. The dye's fluorescence is significantly enhanced only when it intercalates between the bases of DNA. High fluorescence it typically produced with free plasmid but significant reductions can occur when a cationic polymer binds the DNA and shields it from dye penetration.[39] Such fluorescence reductions relative to free DNA are shown in Figure 3.6 for each C-PDA derivative as a function of the polymer:DNA ratio. For the base C-PDA polymer, a gradual reduction in the relative fluorescence (RF) is seen as the polymer:DNA ratio is increased. The extent of polymer-DNA complexation appears to be low, even at high polymer:DNA ratios. The weak interactions are most likely the result of the low molecular weight and hydrophobicity of the polymer, which greatly limits its solubility and propensity to bind DNA. The C-PDA-RGDC polymer also displayed limited DNA binding up to a ratio of 10:1, after which a significant decrease in the RF occurred. At ratios only exceeding 100:1, near complete inhibition of dye penetration is seen. The highly cationic C-PDA-MEA polymer demonstrated a greater capacity to interact with and shield plasmid DNA. Dye intercalation could be significantly reduced at polymer:DNA ratios at low as 5:1. At ratios greater than 10:1 almost complete complexation of the plasmid occurs. Therefore, the C-PDA polymer and its derivatives display a general increase in DNA binding and association as the polymer:DNA ratio is increased, consistent with the observed electrophoretic mobilities of the complexes. Furthermore, increased hydrophilicity and charge density appear to mediate stronger DNA binding at lower polymer:DNA ratios, as expected. These results are compared to PEI in which the high solubility, molecular weight, and charge density enable this polymer to complex DNA at much lower ratios.
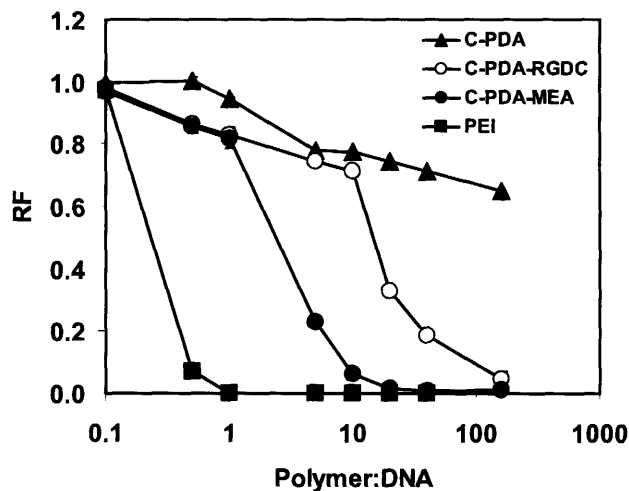

Figure 3.6 Polymer-DNA binding with PicoGreen. Polymer-DNA complexes in NaAc buffer were mixed with an equal volume of PicoGreen in NaAc buffer and the fluorescence was measured. Fluorescence intensities of polymer-DNA complexes are expressed relative to free DNA and as a function of the polymer:DNA weight ratio. Data are expressed as mean values (± SD, n=4).

The PicoGreen exclusion assay was also performed in the presence of L-glutathione for C-PDA-MEA polymer DNA complexes. Glutathione is a thiolated tripeptide present at high concentrations in the cytosol (up to 10 mM)[40,41] and at much lower amounts in the extracellular spaces (~4.5 uM in plasma).[42] It is capable of mediating disulfide bond cleavage through disulfide exchange reactions.[43,44] Therefore, it was speculated that this peptide could induce partial C-PDA-MEA breakdown by cleaving the disulfide bonds and separating the primary amine side chains from the polymer backbone. The consequence of this reaction was expected to be a reduction in the polymer charge density and a corresponding decrease in the electrostatic association with DNA. The results of this assay are shown in Figure 3.7 for C-PDA-MEA polymer DNA complexes at a 40:1 ratio incubated with 0, 0.1, and 10 mM glutathione. At the highest concentration, a very rapid RF increase occurs during the first 2 hours while a more gradual rise proceeds for the next 2 to 8 hours. The overall change in RF is from approximately 0 to almost 0.5. Complete DNA unbinding is not realized because the polymer still contains tertiary amines in the backbone that can remain associated with the DNA. A much smaller RF effect is seen at the lower glutathione concentration tested. In this case, a much slower rise in RF occurs over the 12 hour incubation period up to a maximum of 0.1. Therefore, strong DNA binding is still observed at 0.1 mM glutathione, which is over 20 times higher than the blood plasma levels. This result is potentially important for subsequent *in vivo* administration and indicates that substantial decreases in polymer-DNA binding can be expected to occur in the cytosol and not in transit to the target cells.
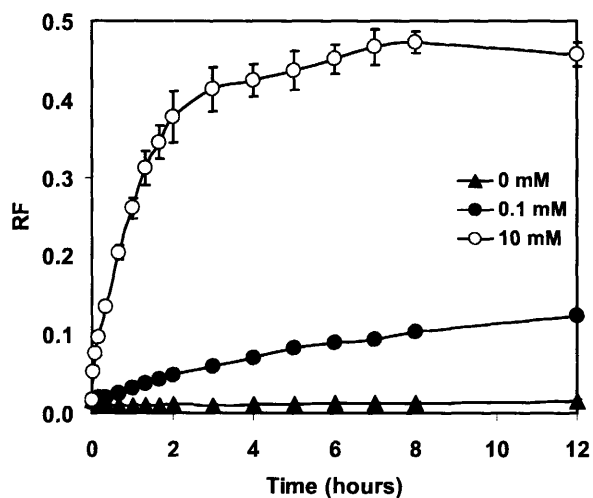
Figure 3.7 Polymer-DNA binding with PicoGreen in the presence of L-gluathione. Polymer-DNA complexes in NaAc buffer were mixed with an equal volume of PicoGreen in NaAc buffer and subsequently mixed with a solution of glutathione in PBS buffer. Fluorescence intensities of polymer-DNA complexes are expressed relative to free DNA as a function of the polymer:DNA weight ratio and glutathione concentration. Data are expressed as mean values (± SD, n=4).

3.4.5 Polymer-DNA Complex Properties

The size and surface charge of C-PDA-MEA polymer DNA complexes were measured using dynamic and phase analysis light scattering (Table 3.1). Effective particle diameters of approximately 100 nm were formed at 20:1 and 40:1 polymer:DNA ratios. Higher and lower amounts of polymer tended to give larger nanoparticles. In all cases, the complexes formed had positive surface charges in the range of 10-30 mV without a clear correlation to the polymer:DNA ratio. The small size and cationic charge of the C-PDA-MEA polymer DNA complexes suggests that they are sufficient for cellular endocytosis.[5,45] For comparison, PEI polymer DNA particles had effective diameters around 74 nm with a surface charge of approximately 10 mV.

Table 3.1: Size and surface charge measurement of polymer-DNA complexes.

Polymer	Polymer:DNA	Effective diameter (nm)	Zeta potential (mV)
C-PDA-MEA	100	183.3 ± 29.1	18.87 ± 3.90
	60	133.0 ± 16.0	30.21 ± 4.04
	40	100.8 ± 6.2	10.61 ± 2.14
	20	104.2 ± 2.9	28.31 ± 5.29
	10	255.4 ± 15.2	22.23 ± 0.96
PEI	10	74.4 ± 20.4	10.42 ± 0.89

3.4.6 Polymer Cytotoxicity

Cell compatability studies were performed by incubating C-PDA based polymer DNA complexes on the cells and assaying for metabolic activity one day later. The results for C-PDA and C-PDA-MEA are shown in Figure 3.8 along with PEI for comparison. The C-PDA polymer showed minimal toxicity up to a 40:1 polymer:DNA ratio but induced substantial cell death at very high ratios. In contrast, the more cationic C-PDA-MEA polymer caused little toxicity over the entire range, with a maximum of approximately 30% cell death at 75:1 polymer:DNA ratio. The more toxic effects of C-PDA might be attributed to the hydrophobicity of the polymer that could perturb or lyse the lipophilic cell membranes. In addition to the polymers, the toxicity profile of 2-MP was also measured. Concentrations of 2-MP were set to those at which this molecule is present in polymer-DNA samples at the given weight ratios. As shown, this small molecule has excellent biocompatibility even at very high concentrations. Because of the low 2-MP toxicity, it may be possible to perform thiol ligand conjugations to the C-PDA polymer without necessarily purifying the polymer product from the 2-MP released. Compared to the C-PDA polymers, PEI resulted in higher toxicity levels at almost all polymer:DNA ratios. Cell viability decreased in an approximately linear fashion as the ratio was increased.
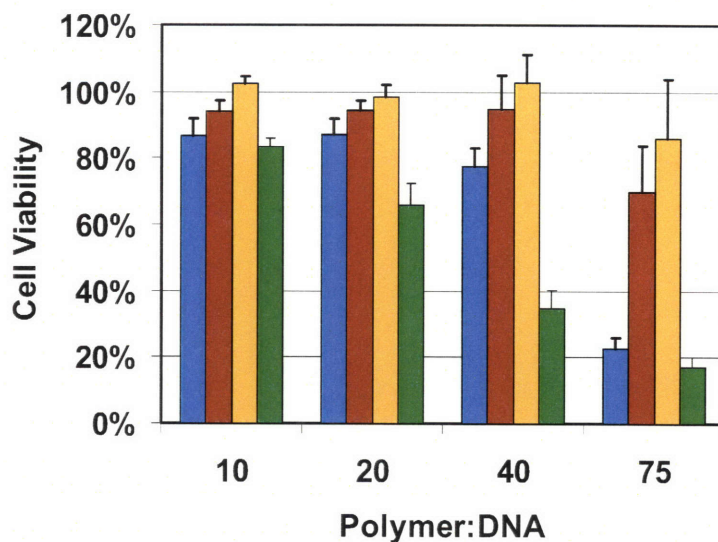
Figure 3.8 Cytotoxicity of polymer-DNA complexes for C-PDA (blue), C-PDA-MEA (red), 2-MP (yellow) and PEI (green). HCC cells were exposed to polymer-DNA complexes for one hour and analyzed for metabolic activity 24 hours later using the MTT Cell Proliferation Assay. Toxicity of 2-MP solutions were also measured at concentrations in which this molecule is present in polymer-DNA samples at the given weight ratio. The percent cell viability is shown as a function of the polymer-DNA weight ratio for each sample. Data are expressed as mean values ($\pm$ SE, n=4).

3.4.7 HCC Transfections

The ability of PDA based polymers to delivery DNA into HCC cells was determined by performing a luciferase reporter gene transfection. In addition to the C-PDA polymer, PDA polymers made with the F and U diacrylates were also tested (Figure 3.3). These diacrylate monomers have been used in previous studies and can result in poly(β-amino ester)s with good DNA delivery properties.[29] MEA derivatives were formed for each polymer and tested along with the unmodified PDA base polymers. The results are shown in Figure 3.9 for each polymer at four different polymer:DNA ratios. The transfection levels for the base polymers C-PDA, F-PDA and U-PDA were all very low and not higher than the untreated control. However, protein expression was significantly enhanced for all of the MEA derivatives. In the case of F-PDA-MEA, an order-of-magnitude increase in Luciferase expression was seen at the higher polymer:DNA ratios over F-PDA. The U-PDA-MEA and C-PDA-MEA polymers displayed an even greater effect with expression levels over 300-fold higher than naked DNA. These polymers show transfection levels equal to optimized PEI, the best commercially available polymer transfection reagent. All of these results suggest that these are a new effective class of poly(β-amino ester)s. In addition to the pyridyldithio functionalities of the base polymers, the amine side chains in the MEA polymer derivatives can also be used to easily conjugate ligands for cell targeting, but with the added benefit of efficient transfection.
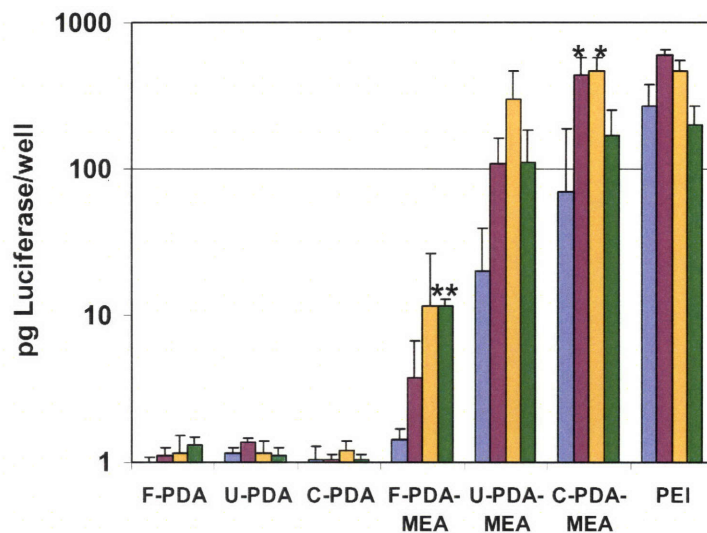
Figure 3.9 Transfection of HCC cells. PDA and PDA-MEA polymers were complexed with pCMV-Luc DNA at 20:1 (blue), 30:1 (red), 40:1 (yellow) and 60:1 (green) polymer-DNA weight ratios and incubated with HCC cells for one hour. Luciferase expression levels were measured three days later. The relative light units measured were converted to mass of expressed protein using a Luciferase calibration curve. Data are expressed as mean values as a function of both the polymer and polymer:DNA weight ratio ($\pm$ SD, n = 3). Statistical significance of MEA polymers over the base PDA polymers was determined by a t-test (two-tailed, unequal variances) with $p < 0.05$ (*) and $p < 0.01$ (**).

3.4.8 HUVEC Transfections

Transfection experiments with HUVEC cells was performed to test the potential enhancement and selectivity of C-PDA poly(β-amino ester)s containing RGD side chains. These cells are known to express the appropriate integrin receptors that can bind and internalize RGD-containing drug delivery systems.[46,47] Transfections were performed using C-PDA-GRGDC and a polymer with a scrambled peptide sequence, C-PDA-GRDGC, to assess the specificity of delivery. Polymers were complexed with GFP-encoded plasmid DNA and the transfection was performed as with the HCC cells, but scaled up five-fold for flow cytometry analysis. The GFP-based method with flow cytometry was used for HUVEC transfections because experiments using luciferase gave very low protein expression levels with large errors (data not shown). The GFP method proved more sensitive, provided fluorescence output for each individual cell, and allowed for cell viability to be assessed with propidium iodide incubation.

Initial experiments with C-PDA-GRGDC complexed to plasmid DNA and delivered to HUVECS showed no GFP expression, regardless of the polymer:DNA ratio (data not shown). As a result, transfection experiments were repeated with 1:1 wt/wt blends of C-

PDA-GRGDC and C32, a very effective polymer for non-specific DNA delivery to HUVECs.[48] The objective was to couple the inherent DNA delivery capabilities of C32 with the potential cell targeting ability of the RGD-containing polymer. Results of these transfections are presented in Figure 3.10 for both C-PDA-GRGDC and C-PDA-GRDGC blends with C32 and C32 alone, with the amount of C32 kept constant for each experiment. For each polymer system, plots of the forward scatter versus FL3 (PI), FL1 versus FL2, and the FL1 histogram are shown to assess cell viability and fluorescence.

Untreated HUVEC cells showed the anticipated high cell viability and very low fluorescence in both FL1 and FL2 (between 10^0 and 10^1 in both channels with very few cells outside this range). Setting up the appropriate gates gave the percentage of GFP positive cells to be 0.23% using the FL1 versus FL2 density plot and 1.0% according to the FL1 histogram. Cells transfected with C32/GFP-DNA complexes demonstrated very good viability as well. Slight shifts can be seen in the cell population towards lower forward scatter and higher PI fluorescence. In addition, a sizable shift occurs in FL1 with a smaller, but noticeable shift in FL2. More importantly, the histogram shows a small trail of cells with very high fluorescence above 10^2, which represents high GFP expression levels for a small population of cells. This effect is diminished when C-PDA-GRGDC is blended into the delivery system. Shifts can still be seen in FL1 and FL2 and appear to be higher, on average, than that observed with C32. However, this result is not indicative of increased GFP expression and is an artifact of increased cellular auto-fluorescence, since the FL1 and FL2 levels are almost equivalent. Furthermore, almost identical results were seen with C-PDA-GRDGC blends with C32 and DNA. Taken together, the RGD moiety does not appear to enhance transfection or increase the specificity of delivery by simply conjugating it to PDA poly(β-amino ester)s through a short linker. The lack of a favorable effect is due to an insufficient number and/or inefficient presentation of RGD effector ligands on the nano-complex surface. Increased spacing between the ligand and complex using inert polymers, such as polyethyleneglycol, may improve serum stability and allow for more favorable RGD-integrin interactions.

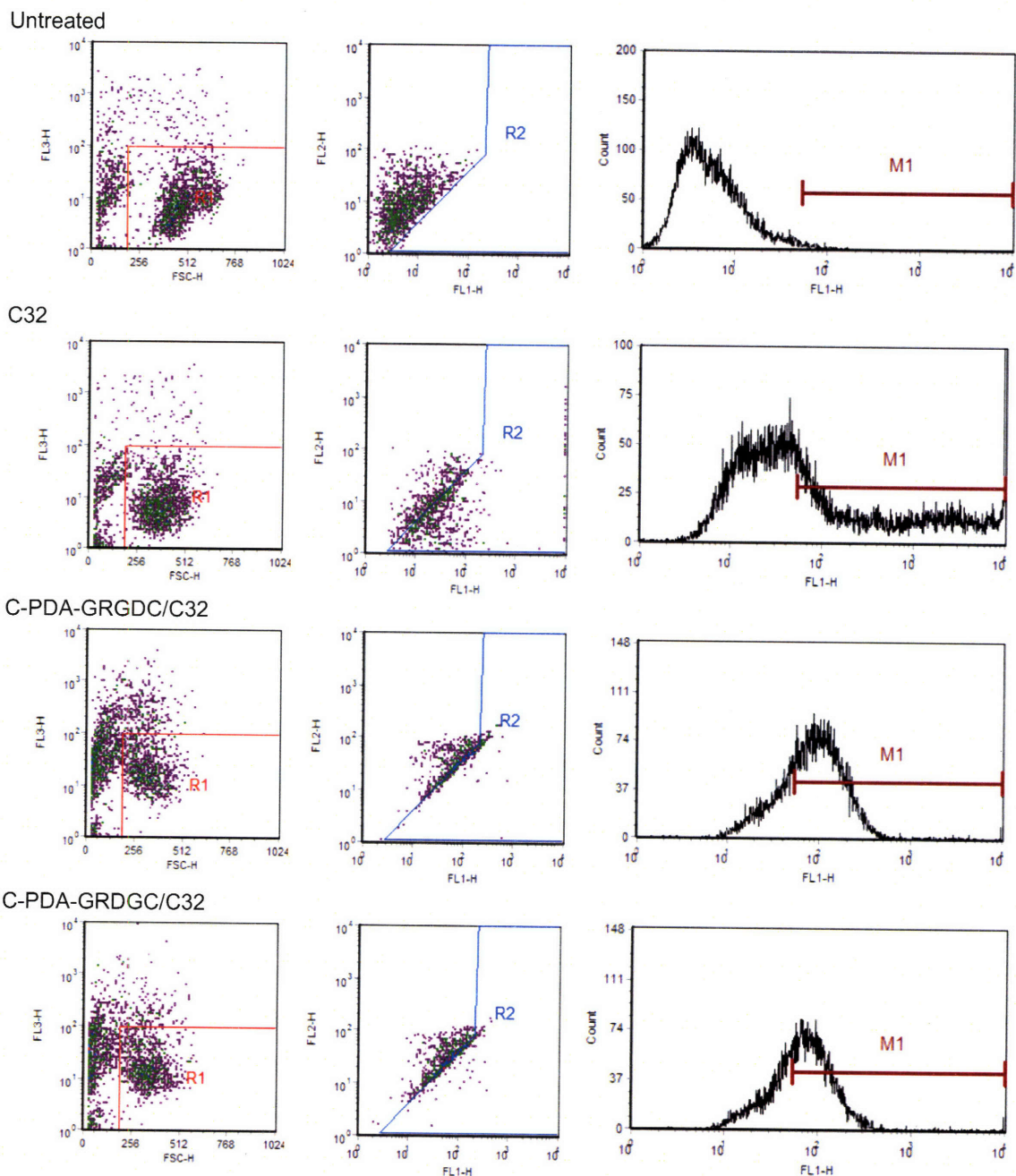**Figure 3.10** Transfection of HUVEC cells by C32 and C32 blended in a 1:1 weight ratio with either C-PDA-GRGDC or C-PDA-GRDGC. Polymer and polymer blends were complexed with pCMV-EGFP DNA in a 50:1 weight ratio and incubated on the cells for two hours. GFP expression was determined two days later by flow cytometry analysis. Plots of FL3 vs. forward scatter, FL2 vs. FL1, and the FL1 histogram are shown for each polymer transfection.

3.5 Discussion

For gene therapy to be clinically viable, a safe and efficient delivery system needs to be developed. The shortcomings of viral vectors have elicited the exploration of non-viral alternatives such as cationic lipids and polymers. In order for these materials to be effective, they must overcome the cellular barriers to gene delivery. These include DNA condensation for endocytosis, cellular association and uptake, endosomal escape, cytoplasmic transport, nuclear import, and DNA unpackaging. *In vivo* administration presents additional challenges that include serum stability, avoidance of the reticulo-endothelial system, resistance to nucleases, and specific delivery to the target cell population.[49]

To address these challenges, a new class of poly(β-amino ester)s have been synthesized with thiol-reactive side chains using the amine monomer 2-(pyridyldithio)-ethylamine. The long-term vision is that these polymers could serve as a modular platform for characterizing and enhancing the polymer functionality, which could include the attachment of (1) thiolated targeting ligands such as peptides and proteins to provide tissue and cell specific delivery without altering the charge density of the backbone, (2) inert polymers (e.g., PEG) to improve serum stability, and (3) peptide transduction domains, nuclear localization sequences and other functionalities to overcome cellular transfection barriers. Ultimately, this level of convenience and design flexibility may prove valuable in constructing a delivery system that is capable of overcoming obstacles to efficient gene transfection.

Although the PDA molecule can be used to synthesize a poly(β-amino ester) with such a flexible design, this unsymmetrical disulfide suffers from several limitations that are linked to its inherit propensity to disintegrate. Most notably is the cystamine by-product that is generated from PDA disproportionation. This diamine disulfide is produced in high yields at elevated temperatures and can lead to crosslinked polymers that have no solubility and little use as DNA carriers. Therefore, polymer synthesis using PDA is often a trade-off between generating low molecular weight oligomers or high molecular weight, crosslinked polymers.

In light of the PDA instability and the difficulty using this monomer in high temperature polymerizations, other thiol reactive compounds were explored that may offer improved stability for poly(β-amino ester) synthesis or modification (Figure 3.11). One alternative monomer that displays thiol reactivity similar to PDA is 5-(2-aminoethyl)dithio-2-nitrobenzoic acid (ADNB). This compound is easily synthesized by the reaction of cysteamine with Ellman's reagent.[50,51] The reaction is performed in an aqueous buffer and the resulting ADNB product precipitates out upon formation, allowing it to be easily isolated by filtration or centrifugation. Also, it undergoes a thiol-disulfide exchange reaction to release the colorimetric compound 5-thio-2-nitrobenzoic acid, which has a higher molar extinction coefficient than 2-MP, potentially allowing for more sensitive detection of thiol ligand attachments to ADNB-based poly(β-amino ester)s. And unlike
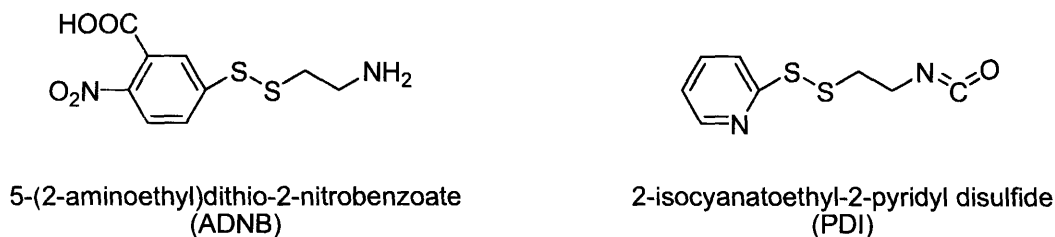
5-(2-aminoethyl)dithio-2-nitrobenzoate
(ADNB)

2-isocyanatoethyl-2-pyridyl disulfide
(PDI)

Figure 3.11 Structures of other pyridyldithio compounds that were unsuccessfully used to construct poly(β-amino ester)s with thiol-reactive side chains.

many other unsymmetrical disulfides, ADNB is fairly stable and resistant to disproportionation because it is a solid at room temperature (mp 219-220°C).[50] However, it was discovered that this compound has very poor solubility properties which limit its utility for polymer synthesis. It has almost no solubility in all common organic solvents, especially DMSO, DMF, dichloromethane, chloroform, and THF which are commonly used for poly(β-amino ester) synthesis. As a result, it was not possible to synthesize ADNB-based poly(β-amino ester)s, despite the attractive properties of this monomer.

In addition to generating thiol reactive amines for polymer synthesis, it is also possible to construct compounds with similar thiol reactivity that can be grafted to poly(β-amino ester)s in a post-polymerization reaction. To this end, the compound 2-isocyanatoethyl-2-pyridyl disulfide (PDI) was synthesized by reacting PDA with trichloromethyl chloroformate.[33] This molecule has a pyridydithio functionality, identical to PDA, but contains an isocyanate group instead of a primary amine. Isocyanates are known to react with nucleophiles such as amines and hydroxyl groups. Therefore, this compound could be theoretically attached to C32 and other hydroxyl-containing poly(β-amino ester)s to give these polymers thiol reactivity. In addition, it has been noted that PDI has very good stability properties,[52] perhaps due to the lack of a free amine that can catalyze the intramolecular disproportionation as described above. However, preliminary attempts so far have been unsuccessful to graft PDI to the C32 side chains. The ^{1}H NMR of the polymer product showed very little side chain substitution when PDI was reacted with C32 in CDCl$_3$ overnight at room temperature. The low levels of substitution may be due to the low reactivity of aliphatic isocyanates and/or primary hydroxyl groups. Higher temperatures or the use of poly(β-amino ester)s with amine side chains may be more appropriate for PDI.

Despite the difficulty in using PDA and the lack of a suitable alternative, low molecular weight PDA-poly(β-amino ester)s could be synthesized and demonstrated very useful properties. Similar to other poly(β-amino ester)s, these PDA-based polymers are degradable by hydrolysis of the backbone ester bonds and have tertiary amines to facilitate DNA binding.[25] Furthermore, the side chains consist of pyridyldithio functionalities that are highly reactive with thiol groups through a disulfide exchange reaction.[53] This reaction can be performed over a wide pH range and with very fast colorimetric quantification of the conversion due to 2-MP release.[30] The 2-MP generated

following thiol conjugation is a good leaving group due to its resonance stabilization and does not react back with the side chains.[53]

As one application of PDA polymer modification, we demonstrate the reaction of the pyridyldithio side groups with the thiol compound MEA. The reaction proceeded almost instantaneously and resulted in the formation of poly(β-amino ester)s containing primary amines in the side chains that are linked to the backbone through disulfide bonds. Furthermore, NMR analysis shows the disappearance and reaction of polymer acrylates with MEA, demonstrating the potential to couple nucleophilic molecules at the polymer terminus. These polymers displayed strong DNA binding over a range of polymer:DNA ratios as determined by electrophoresis and dye exclusion assays. Particle sizing and surface charge measurements indicated that the polymer-DNA complexes were cationic with effective diameters as low as 100 nm. These features suggest that the particles are sufficient for association with the negatively charged cell membrane and subsequent endocytosis. Although the polymers are cationic, cytotoxicity was minimal, even at high polymer:DNA ratios.

The MEA-based poly(β-amino ester)s developed here are among only a few degradable polymers with amine side chains to have been synthesized. Lim et al[54,55] have demonstrated the synthesis of poly(4-hydroxy-L-proline ester) (PHP ester) and poly[a-(4-aminobutyl)-L-glycolic acid] (PAGA) and the utility of these polymers for gene delivery. Both polymers are degradable via ester hydrolysis and contain either secondary (for PHP ester) or primary (for PAGA) amines in side chains. Transfection levels were elevated over poly-L-lysine but required the presence of chloroquine to enable endosomal escape. Presumably, the MEA-based polymers circumvent that requirement since they are capable delivering high transfection levels without exogenous endosomal buffering agents. This could be a result of the tertiary amines in the backbone of the polymer that may act through a proton sponge mechanism to facilitate endosomal release, similar to PEI.[56]

Another advantage of the MEA-based poly(β-amino ester)s over other degradable polycations synthesized thus far is its response to glutathione. Using a dye exclusion assay, it was shown that polymer-DNA binding is significantly reduced in the presence of glutathione. Intracellular glutathione concentrations are sufficient to break the polymer disulfides and decrease the cationic charge density, thereby weakening the strength of DNA binding. Furthermore, lower concentrations of glutathione that are found outside the cell were shown to have a minimal effect. These results indicate that the MEA-based polymer delivery system should be relatively stable in the extracellular space, but responsive to intracellular conditions in which partial unpackaging is triggered. A similar response has also been seen by Pichon et al[57] with a poly-lysine derivative that contained disulfide linked primary amine side chains. Theoretically, complete dissociation of the amine side chains could be accomplished, resulting in a neutral polymer with little DNA binding capacity. For the C-PDA-MEA polymer, almost 50% restoration of dye fluorescence was obtained, indicating an increase in the amount of unbound DNA. The fluorescence was not restored to the levels of fully unbound DNA since the polymer also has tertiary amines in the backbone that still enable some DNA binding. This interaction

is purely electrostatic in nature since the ^{1}H NMR of the MEA polymers do not show any acrylate groups that could react with the DNA. Although the unpackaging effect is more limited, the tertiary amines confer the potential advantage of endosomal escape, as mentioned above.

The ability of the PDA-based polymers to deliver DNA was tested on a human hepatocellular carcinoma cell line. All of the base PDA polymers with pyridyldithio side chains were ineffective, regardless of the diacrylate used in the polymerization. This result is not surprising since structure-function studies on poly(β-amino ester)s have shown that polymers formed from hydrophobic primary amines rarely have the capacity to transfect cells.[27] Despite the minimal performance, these polymers offer many advantages as described above. Although the base PDA polymers were ineffective, gene delivery could be markedly enhanced by changing the side chain functionality. In particular, MEA substitution resulted in elevated transfection levels for all three polymers tested. These results extend previous structure-function studies to now include poly(β-amino ester)s with primary amine side chains as effective DNA delivery polycations.[27,29] For the case of C-PDA-MEA and U-PDA-MEA, the transfection levels were almost as high as that observed with an optimal PEI formulation. While the mechanism behind this transfection increase is unclear, both solubility and DNA binding significantly improved with MEA substitution.

The reaction of RGDC to the C-PDA polymer side chains was used to demonstrate the potential to attach thiolated ligands for the development of a targeted delivery system. The RGD peptide sequence has been shown to bind with high affinity to the $\alpha_v\beta_3$ integrin receptor that is overexpressed on the surface of angiogenic endothelial cells.[38,58] Therefore, incorporation of integrin-binding ligands in the polymer side chain may promote specific targeting of the polymer-DNA complexes to the tumor vasculature, as observed for other delivery systems.[59] It is shown that RGDC can be grafted to all of the polymer side chains in a one-day reaction using a small excess of peptide. The reaction can be conveniently carried out in DMSO due to the high solubility of the peptide in this organic solvent. Elevated temperatures and the use of catalysts are not necessary because of the high reactivity of the pyridyldithio group towards thiol ligands. This may prove a valuable mechanism for the conjugation of sugars, proteins, and antibodies ligands that are heat-sensitive or unstable in certain aqueous buffers. With RGDC side chains, the polymer displayed the ability to bind and condense plasmid DNA, especially at high polymer:DNA ratios. Unfortunately, RGD substitution did not enhance transfection, either alone or in blends with C32. Although a limited set of conditions was explored, there is no evidence that continued optimization would improve C-PDA-GRGDC performance, both in terms of overall transfection and cell-specificity of delivery. Since the ligand density is high, the failure of this polymer may be due to the arrangement and presentation of the RGD ligands. To more effectively promote ligand-receptor interactions, it may be necessary to increase the spacing between the ligand and polymer-DNA complex surface. In addition, the surface charge needs to be neutralized or negative to minimize non-specific electrostatic interactions.

To achieve cell specific delivery, these results suggest that more fundamental studies of the effects of polymer modification on gene delivery properties need to be performed. Not only is ligand presentation important for interacting with the receptor, but ligand grafting effects (in terms of attachment number, chemistry, and positioning) need to be understood so that transfection conditions can be optimized. The side chains are logical points for ligand attachment but, as the next chapter illustrates, the end group is a strong determinant of cellular uptake for poly(β-amino ester) polyplexes and may be a more appropriate site to attach ligands for targeted gene delivery.

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Chapter 4 – Synthesis of End-Modified Poly(β-amino ester)s for Gene Delivery

4.1 Summary

The development of poly(β-amino ester)s into clinically useful transfection agents requires the establishment of methods to modify and improve their *in vivo* performance. In addition to the side-chains, the polymer end segments are an attractive location to conjugate a variety of molecular enhancers since these sites contain unique chemical functionalities. However, while it has been shown that amine termination of poly(β-amino ester)s can significantly improve their transfection ability, the effects of the terminal amine structure on gene delivery are not known. An understanding of these effects is necessary to use end-chain conjugation as a strategy to modify and improve these polymers. With this goal in mind, this chapter presents (1) a general method to synthesize end-modified poly(β-amino ester)s and (2) a thorough characterization of the end amine structural effects on various gene delivery properties. End-modified poly(β-amino ester)s were synthesized using a rapid, two-step approach that involves initial preparation of acrylate-terminated polymer followed by a post-polymerization amine-capping step to generate end-functionalized polymers. Using a highly efficient poly(β-amino ester), C32+, it is shown that the terminal amine structure can greatly affect and improve polymer properties relevant to gene delivery. Specifically, the transfection levels can be increased by 30% and the optimal polymer:DNA ratio lowered 5-fold by conjugation of the appropriate end group. The most effective modifications were made by grafting primary diamine molecules to the chain termini. The added charge and hydrophobicity of some derivatives enabled strong DNA binding and the formation of polymer-DNA complexes less than 100 nm in diameter. In addition, the uptake levels were improved 5-fold over C32+, with the best polymer delivering 4000 plamsids/cell, suggesting that ligand conjugation to the chain ends may promote cell-specific delivery. Differences of single carbons and functional groups at the polymer ends affect all of these properties and can greatly increase the DNA binding, levels of endocytosis, and transfection. Intraperitoneal gene delivery in mice using end-modified C32 polymers resulted in transfection levels over one order-of-magnitude higher than jet-PEI and C32+ in several isolated organs. It is also demonstrated that the transfection efficiency of less effective poly(β-amino ester)s can be substantially improved by proper end-functionalizion with primary diamine molecules. These results suggest that end-modification of poly(β-amino ester)s, particularly with primary diamines, is a versatile strategy to improve their *in vitro* and *in vivo* performance.

4.2 Introduction

For poly(β-amino ester)s to be useful gene delivery agents, the specificity and effectiveness of these polymers needs to be improved. In this regard, the PDA-based poly(β-amino ester)s presented in the last chapter could serve as a versatile scaffold for attaching the necessary functionalities and achieving a high level of performance. The side chain chemistry is specific for thiolated ligands so that many peptides or small molecules could be grafted in a particular orientation to a single polymer chain. Despite this advantage and all others listed in the previous chapter, these particular poly(β-amino ester)s suffer from a few notable problems that currently limit their utility. First, due to the hydrophobicity of PDA, even low molecular weight polymers that are formed have very low solubility in aqueous solutions, complicating their use for attaching antibodies, sugars, and other hydrophilic ligands of interest that may be insoluble in organic solvents. In addition, simply conjugating these ligands does not insure targeted and/or efficient transfection, as demonstrated using an RGD peptide and HUVEC cells as a model system. As a result, it may be beneficial to refine and build off the structure of existing poly(β-amino ester)s that have proven to be highly efficient transfection agents. Also, understanding the effects of modifications on the functioning of these existing polymers may give insight on how to modify the PDA poly(β-amino ester)s to improve their performance.

All studies to date with poly(β-amino ester)s have only addressed the synthesis and structure-property relationships of new polymers and not the modification of existing ones.[1-6] However, these studies have yielded valuable information on the composition and structural factors that are important for transfection, which provides useful design criteria that can guide and aid in polymer modification. The molecular weight and chain end group, in particular, have been shown to have important effects on the delivery process.[5] In general, the polymer molecular weight must be high enough to facilitate DNA binding and nanoparticle formation but also low enough to permit DNA unpackaging inside the nucleus.[5,7] The functional group at the chain end has received little attention but has been shown to affect both cytotoxicity and cellular uptake of polymer-DNA complexes.[5] Although general conclusions cannot be made yet, the amine-terminated polymers studied to date have resulted in higher uptake and transfection levels relative to their acrylate-capped counterparts.[5,6]

In addition to structural factors, previous studies have provided important information on monomer composition that gives rise to highly efficient poly(β-amino ester)s. The most effective polymers are usually formed by combining hydrophobic diacrylates with primary amino-alcohols.[6] The best polymer, designated here as C32+, is synthesized by mixing 1,4-butanediol diacrylate (C) with 5-amino-1-pentanol (32) at a 1.2:1.0 amine:diacrylate mole ratio.[6] Polymerization is typically conducted at 90°C for 24 hours to yield aminopentanol-terminated polymer chains with a weight average molecular weight of approximately 18 kDa (relative to polystyrene standards).[6] This polymer has shown excellent biocompatibility *in vivo* and effectiveness as a gene delivery vector for the treatment of prostate cancer *via* intratumoral injection.[8]

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Although it is generally established that amine-terminated poly(β-amino ester)s are highly efficient transfection agents,[6] the type of amine molecule at the end of the polymer chain has not been examined. Studies to date have only focused on optimizing the amine:diacrylate ratio,[5,6] which only gives rise to amine-terminated chains with identical end and interior amines. Since an amine-terminus is important for transfection, it may be useful to systematically optimize its structure for various gene therapy applications. Furthermore, the end segments of the polymer chains present unique functionalities that may be useful to attach targeting ligands, peptide transduction domains, nuclear localization signals and other molecules to enhance delivery. It would be beneficial to have a general chemistry to accomplish this and an understanding of how certain modifications may impact the delivery properties of the polymer.

This chapter presents a generalized method for synthesizing poly(β-amino ester)s with terminal amines different from those used in the polymerization. These end-capped polymers are prepared by first synthesizing the acrylate-terminated polymer, using an excess of acrylate over amine monomer, followed by an amine capping step. This scheme results in an end-only attachment of chemically diverse amines without crosslinking polymer chains or cleaving backbone ester bonds through an aminolysis reaction. In addition, the reaction conditions were optimized so that the end-modified polymers could be synthesized and tested for gene delivery in a high throughput fashion, without the need for purification. Similar to polymer repeat units, end segments differing in a single additional carbon or functional group are shown to drastically affect the polymer delivery properties. In general, the terminal amine structure has a large influence on cytotoxicity, physical properties, and cellular uptake of polymer-DNA complexes. As a result, significant improvements in transfection efficiency have been made by proper end-modification of several base polymers, indicating that this may be a method for improving PDA poly(β-amino ester)s as well.

End-modification of poly(β-amino ester)s is also shown to greatly improve *in vivo* transfections. In particular, intraperitoneal gene delivery using end-modified C32 polymers resulted in protein expression levels over one order-of-magnitude higher than C32+ both overall and in several isolated organs. Polymers terminated with primary diamines proved most effective, with diaminopropane derivatives optimal for in vivo delivery. These results indicate that the terminal functionalities of poly(β-amino ester)s are important structural features that need to be optimized for each gene delivery application.

4.3 Experimental Procedures

4.3.1 Materials

Polyethylenimine (water free, $M_w \sim 25$ kDa, $M_n \sim 10$ kDa), 3-amino-1-propanol (99%), N,N'-dimethylethylenediamine (99%), anhydrous DMSO, and anhydrous THF were purchased from Sigma-Aldrich (St. Louis, MO). Jet-PEI was purchased from Polyplus Transfection (Illkirch, France). A 25 mM sodium acetate buffer solution pH 5.2 (NaAc buffer) was prepared by diluting a 3 M stock (Sigma-Aldrich). 1,4-Butanediol diacrylate

(99+%) and 5-amino-1-pentanol (97%) were from Alfa Aesar (Ward Hill, MA); Ethoxylated (2) bisphenol A diacrylate was from Scientific Polymer Products, Inc.(Ontario, NY); Amine capping reagents were purchased from Sigma-Aldrich, Alfa Aesar, Acros Organics/Fisher Scientific (Pittsburgh, PA), TCI America (Portland, OR), Molecular Biosciences (Boulder, CO), and Toronto Research Chemicals (Ontario, Canada). All chemicals were used as received without any further purification. PicoGreen and Redi-plate 96 PicoGreen dsDNA Quantification Kit were purchased from Molecular Probes (Eugene, OR). pCMV-Luc plasmid DNA stock solution (1 mg/ml in water) was obtained from Elim Biopharmaceuticals (Hayward, CA). gWIZ-β-gal plasmid DNA stock solution (5 mg/ml) was obtained from Aldevron (Fargo, ND).pCAG/luc plasmid DNA was prepared using a Qiagen maxi-plamid prep kit (Valencia, CA).[9] Luciferase GL3 Duplex siRNA was purchased from Dharmacon (Chicago, IL). The MTT Cell Proliferation Assay, Bright Glo™ Luciferase Assay Kits, and Dual Glo™ Luciferase Assay Kits were purchased from Promega Corporation (Madison, WI). White polystyrene tissue culture treated 96-well plates were obtained from Corning Costar. Clear polystyrene tissue culture treated 96-well plates were obtained from Becton Dickinson (Bedford, MA). Polypropylene 96-well deep-well plates were purchased from Sigma-Aldrich.

4.3.2 Cell Culture

COS-7 cells were obtained from ATCC (Manassas, VA) and grown at 37°C, 5% CO_2 in phenol red-free DMEM supplemented with 10% fetal bovine serum and 100 units/ml of penicillin/streptomycin. HepG2 cells were obtained from ATCC and grown in MEM supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 0.1 mM non-essential amino acids, 100 units/ml of penicillin/streptomycin, and 1 mM sodium pyruvate. Luciferase expressing HeLa cells were obtained from Alnylam (Cambridge, MA) and maintained in DMEM supplemented with 10% fetal bovine serum, 100 units/ml of penicillin/streptomycin, 500 ug/ml zeocin (Sigma-Aldrich), and 0.5 ug/ml puromycin. All cell culture reagents were purchased from Invitrogen Corporation (Carlsbad, CA) unless otherwise noted. All cell lines were grown at 37°C, 5% CO_2 atmosphere.

4.3.3 Mice

Six-week old male FVB/J mice were purchased from Jackson Laboratory (Bar Harbor, ME). Mice were 8 – 10 weeks of age at the time of nanoparticle administration. All procedures on mice were done in accordance with protocols approved by the Lankenau IACUC.

4.3.4 Methods

^{1}H NMR was conducted on a Varian Inova 500 MHz Spectrometer. Spectra for representative polymers are shown in Appendix B.

Synthesis of Acrylate-Terminated Poly(β-amino ester)s. Acrylate-terminated polymers were synthesized by mixing the appropriate monomers in a 1.2:1.0 molar ratio of

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diacrylate:amine. C32-Ac was prepared by mixing 793 mg of 1,4-butanediol diacrylate (4 mmol) with 344 mg of 5-amino-1-pentanol (3.3 mmol). D60-Ac was prepared by mixing 1443 mg of ethoxylated (2) bisphenol A diacrylate (3.4 mmol), 250 mg of N,N'-dimethylethylenediamine (2.8 mmol), and 1 ml of DMSO. C20-Ac was prepared by mixing 793 mg of 1,4-Butanediol diacrylate (4 mmol) with 250 mg of 3-amino-1-propanol (3.3 mmol). Polymerizations were performed in Teflon-lined screw cap vials under magnetic stirring at 90°C for 24 hours. ^{1}H NMR of C32-Ac (d_6-DMSO): δ (ppm) 1.2-1.4 (m, -NCH$_2$(CH_2)$_3$CH$_2$OH), 1.6 (bs -N(CH$_2$)$_2$COOCH$_2$CH_2- and CH$_2$CHCOOCH$_2$CH_2-), 2.3-2.4 (m, -COOCH_2CH$_2$N- and –NCH_2(CH$_2$)$_4$OH), 2.6-2.7 (m, -COOCH$_2$CH_2N-), 3.4 (bs, -N(CH$_2$)$_4$CH_2OH), 4.0 (bs, -N(CH$_2$)$_2$COOCH_2CH$_2$-), 4.1 (m, CH$_2$CHCOOCH_2CH$_2$-), 4.4 (bs, -N(CH$_2$)$_5$OH), 5.9 (m, CH_2CHCOOCH$_2$CH$_2$-), 6.1-6.2 (m, CH$_2$CHCOOCH$_2$CH$_2$-), 6.3-6.4 (m, CH_2CHCOOCH$_2$CH$_2$-).

Synthesis of Amine-Capped Poly(β-amino ester)s. **Method 1:** Acrylate-terminated polymers were dissolved in DMSO at 31.13% wt/wt. Amine capping reagents were dissolved in DMSO at 0.25 M. End chain capping reactions were performed by mixing 321 mg of polymer/DMSO solution with 800 μl of amine solution. Reactions were performed in eppendorf tubes with constant agitation overnight. Polymers were stored at -20°C until used for each experiment. ^{1}H NMR of C32-Ac capped with 5-amino-1-pentanol (C32-32) (d_6-DMSO): δ (ppm) 1.2-1.4 (m, -NCH$_2$(CH_2)$_3$CH$_2$OH), 1.6 (bs -N(CH$_2$)$_2$COOCH$_2$CH_2-), 2.2-2.4 (m, -COOCH_2CH$_2$N- and –NCH_2(CH$_2$)$_4$OH), 2.4-2.7 (m, -COOCH$_2$CH_2N-), 3.3 (t, J = 6.6 Hz, -N(CH$_2$)$_4$CH_2OH), 4.0 (bs, -N(CH$_2$)$_2$COOCH_2CH$_2$-).

Method 2: Acrylate-terminated polymer C32-Ac was dissolved in THF at 31.13% wt/wt. Amine capping reagents were dissolved in THF at 0.25 M. End chain capping reactions were performed by mixing 321 mg of polymer/THF solution with 800 μl of amine solution in Teflon-lined screw cap vials with magnetic stirring overnight. End-modified polymers were precipitated by the addition of 10 volumes of diethyl ether and centrifugation at 2500 rpm for 2 minutes. Polymers were washed with 5 ml of ether twice and dried overnight in a vacuum desiccator. Dried polymers were dissolved at 100 mg/ml in DMSO and stored at -20°C until used for each experiment.

In vitro Transfections. COS-7 (15,000 cells/well) or HepG2 (5,000 cells/well) cells were plated in white 96-well plates and allowed to adhere overnight. Polymers at 100 mg/ml in DMSO were diluted accordingly into NaAc buffer to concentrations that yielded the different polymer:DNA weight ratios. One hundred microliters of diluted polymer solution was mixed vigorously with 100 μl of DNA (60 μg/ml in NaAc buffer) in a 96-well polystyrene plate. The solutions were left undisturbed for 5 minutes after which time 120 μl of each was added to 800 μl of cell culture media in a deep-well polypropylene plate. The media over the cells was then removed with a 12-channel aspirator wand and followed by the addition of 150 μl/well of polymer-DNA complex solution. Complexes were incubated with the cells for one hour after which time they were aspirated off and replaced by 105 μl/well of fresh cell culture media. Cells were allowed to grow for three days at 37 °C, 5% CO$_2$ and were then analyzed for luciferase enzyme activity.

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Luciferase activity was analyzed using Bright Glo™ assays kits (Promega; Madison, WI). Briefly, 100 µl/well of Bright-Glo solution was added to the cell plates. The plates were gently agitated to promote mixing for 2 minutes. Luminescence was then measured on a Perkin Elmer Victor 3 plate reader using a 1% neutral density filter and a one second per well counting time.

Measurements of Polymer Cytotoxicity. Cytotoxicity measurements of polymer-DNA complexes were performed essentially as described for the transfection experiments except that cellular metabolic activity was measured after one day instead of Luciferase protein expression. The cytotoxicity of amine solutions was measured following the same protocol except that no DNA was used. In both cases, metabolic activity was quantified by first adding MTT reagent to the cell plates at 10 µl/well. The plates were incubated at 37°C for 2 hours. Detergent reagent was then added at 100 µl/well and the cell plates were left in the dark at room temperature for 4 hours. Optical absorbance was measured at 570 nm using a Molecular Devices SPECTRAmax PLUS384 absorbance plate reader and converted to percent cell viability relative to untreated cells.

Polymer-DNA Binding Assay with PicoGreen. Polymer solutions at 100 mg/ml in DMSO were diluted into NaAc buffer to a final concentration of 6 mg/ml. In a half area 96-well plate, 50 µl/well of diluted polymer was added to 50 µl/well of DNA (60 µg/ml in NaAc buffer). The solutions were mixed vigorously and allowed to sit undisturbed for 5 minutes to allow for polymer-DNA complexation. After this time, 100 µl/well of PicoGreen solution was added. PicoGreen working solution was prepared by diluting 80 µl of the purchased stock into 15.2 ml NaAc buffer. After 5 minutes, 30 µl/well of polymer-DNA-PicoGreen solution was added to 200 µl/well of DMEM media in black 96-well polystyrene plates. The plate fluorescence was then measured on a Perkin Elmer Victor 3 plate reader using a FITC filter set (excitation 485 nm, emission 535 nm). The relative fluorescence (RF) was calculated using the following relationship:

$$RF = (F_{sample} - F_{blank})/(F_{DNA} - F_{blank})$$

where F_{sample} is the fluorescence of the polymer-DNA-PicoGreen sample, F_{blank} is the fluorescence of a sample with no polymer or DNA (only PicoGreen), and F_{DNA} is the fluorescence of DNA-PicoGreen (no polymer).

Polyplex Size. Polymer solutions at 100 mg/ml in DMSO were diluted into NaAc buffer to the appropriate concentration. Concentrations were adjusted for each polymer so that the final polymer:DNA ratio was the same that produced the highest transfection. To prepare polymer:DNA complexes, 100 µl of diluted polymer was added to 100 µl of DNA (60 µg/ml in NaAc) and pipetted vigorously. Complexation was allowed to proceed undisturbed for 5 minutes after which time 150 µl of the sample was diluted into 1.8 ml of DMEM media. Polymer:DNA complex size was measured on a ZetaPALS dynamic light scattering detector (Brookhaven Instruments Corporation, Holtsville, NY; 15 mW laser; 676 nm incident beam, 90° scattering angle). Effective particle diameters were calculated from the autocorrelation function using the MAS option of the BIC

particle sizing software assuming a log normal distribution. The solution viscosity and refractive index were assumed equal to pure water at 25°C.

Cellular Uptake Assay. Uptake measurements of polymer-DNA complexes were performed essentially as described for the transfection experiments but using the β-galactosidase (β-gal) plasmid. Instead of quantifying protein expression levels after three days, total cellular DNA was isolated fours hours post-transfection using a DNeasy 96 Tissue Kit (Qiagen; Valencia, CA) following the manufacturer instructions. Total DNA was quantified using a Redi-Plate 96 PicoGreen dsDNA Quantification kit following the supplied instructions. The amount of β-gal DNA delivered was quantified using RT-PCR with a Taqman primer and probe set specific for the β-gal plasmid (Applied Biosystems; Foster City, CA). After activating the Taq enzyme at 95°C, 40 cycles of amplification were performed, with each cycle consisting of 95°C for 15 seconds, 60°C for one minute, followed by a fluorescent plate read using a Chromo4 Continuous Fluorescence Detector (MJ Research; Waltham, MA). Plasmid copy numbers were determined by comparing the RT-PCR cycle threshold values to a plasmid standard curve and analyzed using the Opticon Monitor 3 sofware package (MJ Research).

siRNA Delivery. HeLa cells (15,000 cells/well) stably expressing firefly and renilla luciferase proteins were plated in opaque 96-well plates and allowed to adhere overnight. Polymers at 100 mg/ml in DMSO were diluted accordingly into NaAc buffer to concentrations that yield the different polymer:RNA weight ratios. Twenty five microliters of diluted polymer solution was mixed vigorously with 25 μl of RNA (30 μg/ml in NaAc buffer) in a 96-well polystyrene plate. The solutions were left undisturbed for 20 minutes after which time 30 μl of each was added to 200 μl of cell culture media in a deep-well polypropylene plate. The media over the cells was then removed with a 12-channel aspirator wand and followed by the addition of 150 μl/well of polymer-RNA complex solution. Complexes were incubated over the cells for one day (37°C, 5% CO_2) after which time they were aspirated off and assayed for luciferase expression using the Dual Glo^{TM} Luciferase Assay following the manufacturer instructions. Luminescence was measured on a Perkin Elmer Victor 3 plate luminometer using a one second per well counting time. The percent knockdown (%KD) was calculated for each polymer in quadruplicate using the following equations:

$$\%KD = 1 - (F_f)_p / [n*(F_f)_u]$$
$$n = (F_R)_p / (F_R)_u$$

where $(F_f)_p$ is the measured firefly fluorescence of a polymer sample, $(F_f)_u$ is the measured firefly fluorescence of the untreated cells, $(F_R)_p$ is the measured renilla fluorescence of a polymer sample, and $(F_R)_u$ is the measured renilla fluorescence of the untreated cells.

Intraperitoneal Administration. Diluted polymer solutions were prepared by mixing 20 – 30 μl of polymer stock solution (100 mg/ml in DMSO) with 70 – 80 μl of 50 mM NaAc buffer. To prepare polymer/DNA complexes, 100 μg pCAG/luc DNA in 50 μl H_2O was mixed with 50 μl diluted polymer. Following a 5 minute incubation at room temperature,

20 μl of a 30% (w/v) solution of glucose in PBS was added to the polymer/DNA mixture (5% glucose final concentration). The resulting 120 μl volume was injected immediately into the peritoneal cavity of a mouse using a 28G 1/2" insulin syringe.

Intravenous Administration. Diluted polymer solutions were prepared by mixing 10 – 15 μl of polymer stock solution (100 mg/ml in DMSO) with 85 – 90 μl of 50 mM NaAc buffer. To prepare polymer/DNA complexes, 100 μg pCAG/luc DNA in 50 μl H_2O was mixed with 50 μl diluted polymer. Following a 5 minute incubation at room temperature, 20 μl of a 30% (w/v) solution of glucose in PBS was added to the polymer/DNA mixture (5% glucose final concentration). A 28G 1/2" insulin syringe was used to inject complexes at a slow rate into the tail vein.

Optical Imaging. Optical bioluminescence imaging was performed on whole bodies of mice and on harvested organs 6 hr following injection of polymer/DNA complexes using an IVIS imaging system as previously described.[8] LIVE IMAGE acquisition and analysis software was used to quantify luciferase expression levels from the optical images. Statistical differences between end-modified C32 polymers and C32+ were determined using Kruskal-Wallis tests with Dunn's multiple comparison post tests for each tissue.

4.4 Results

4.4.1 Polymer Synthesis

Amine-capped polymers were prepared using C32 as a common backbone. This polymer was synthesized in previous studies using a 1.2:1.0 amine:diacrylate molar ratio to generate aminopentanol-terminated polymer chains (designated C32+ to signify a C32 positive control).[6] High throughput synthesis and screening studies have identified this combination of monomers to yield the best performing poly(β-amino ester) to date for gene delivery.[6] Therefore, these two monomers were selected to design the initial set of next generation amine-terminated polymers. The synthesis involved an initial polymerization with excess diacrylate monomer to produce acrylate-terminated C32 polymer (C32-Ac), followed by an amine-capping step to yield C32 polymers terminated with different amine compounds (C32-X), as outlined in Figure 4.1.

The synthesis of C32-Ac was performed by mixing the C and 32 monomers in a 1.2:1.0 molar ratio at 90°C for 24 hours (Figure 4.1(A)). This ratio was selected since C32+ and many other top performing, amine-terminated polymers are made at the inverse ratio using excess amine.[6] It was hypothesized then that the exact opposite ratio may be optimal so that the relative number of interior to terminal units is approximately preserved, with the end-capping step causing a very small change to this balance. Since the terminal amine is very small relative to the polymer chain, the diacrylate:amine ratio selected also controls the final molecular weight.[5] For many poly(β-amino ester)s, molecular weights greater than 10 kDa are usually most effective and can be achieved using a 1.2:1.0 molar monomer ratio.[6] For the C32-acrylate polymer (C32-Ac), the
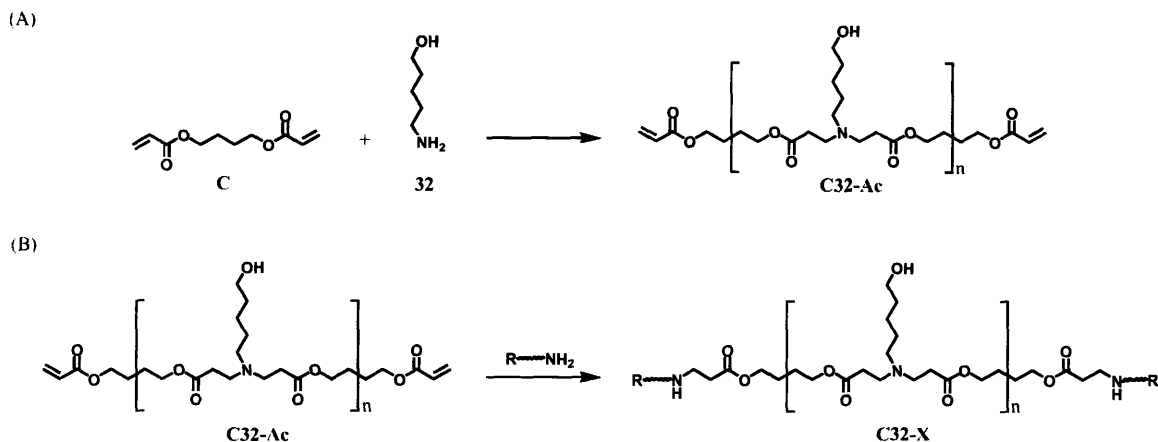
77

(A)

(B)

Figure 4.1 Synthesis of end-modified poly(β-amino ester)s. (A) Synthesis of acrylate-terminated C32 polymer (C32-Ac) by 1.2:1.0 diacrylate:amine polymerization. (B) End-capping of acrylate-terminated C32 with different amine molecules.

weight-average molecular weight is approximately 8,800 Da, relative to polystyrene standards, with a 1.9 polydispersity index. Assuming that each amine-acrylate combination designates a unit, either a repeat unit in the backbone or terminal unit, then 2 of the 16 units (12.5%) in an average length chain are terminal units. This implies that the ends make a non-negligible contribution to the size and functionality of the polymer.

The structure of the C32-Ac polymer was verified by ^{1}H NMR and showed several characteristic acrylate peaks between 5.9-6.4 ppm at the chain ends (Figure B1). Furthermore, butyl protons adjacent to ester bonds resolved into two peaks, one large peak at 4.0 ppm representing protons in the repeat unit of the polymer, and one small peak at 4.1 ppm due to protons near the terminal acrylates.

Amine-terminated C32 polymers for *in vitro* screening experiments were generated by reacting C32-Ac with amine compounds in DMSO as shown in Figure 4.1(B). The end-capping reaction occurs via an amine-acrylate Michael addition, identical to that used in the polymerization.[1] Since the acrylate functionality has no detectable reactivity towards hydroxyls, ethers, tertiary amines, amides, aromatics, and some types of heterocycles, all of these functionalities can be incorporated at the chain ends using the appropriate amine reagents.[2,4] In general, the amine capping reagents were selected for structural diversity, DMSO solubility and biocompatibility (Figure 4.2). In this way, the effects of hydrophobicity/hydrophilicity, carbon chain length, and functional groups (e.g., hydroxyl, primary amine) at the amine terminus could be systematically assessed. Capping reactions were performed by mixing 500 mg/ml C32-Ac solution in DMSO with a 0.25 M amine solution in DMSO. The volumes of each solution were adjusted so that the final polymer concentration was 100 mg/ml and the amine-capping reagent was in a 4-fold molar excess over acrylate functionalities. The reaction proceeded to completion overnight at room temperature as shown by ^{1}H NMR (Figure B2). Higher amine
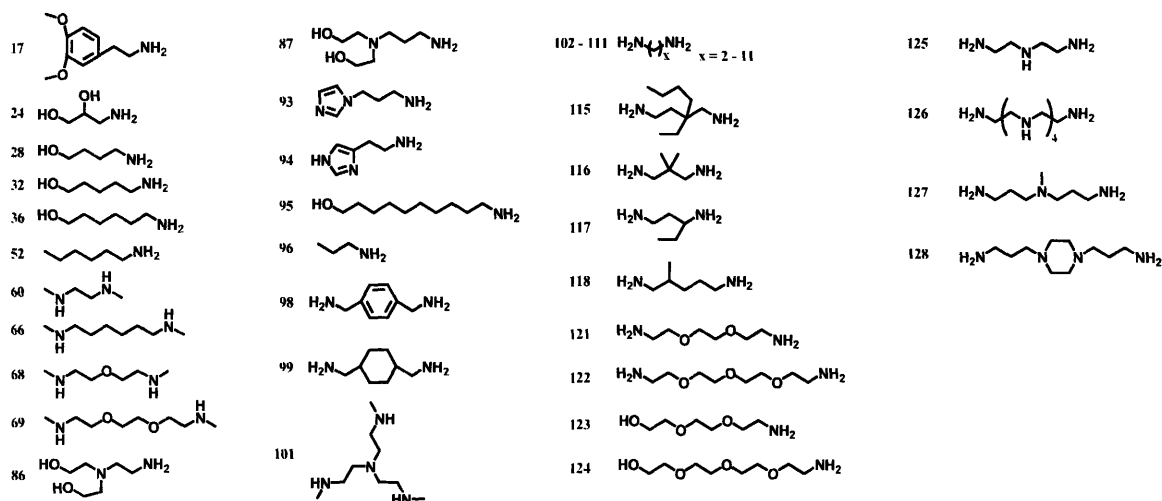
78

Figure 4.2 Structures of amine capping molecules.

concentrations and temperatures were found to cause aminolysis of the polymer ester bonds (Figure B3). The excess of amine was necessary to cap the polymer chain and prevent crosslinking due to newly formed secondary amines, as determined by ^{1}H NMR and GPC measurements. In addition, the excess amine not incorporated at the chain ends did not interfere with subsequent polymer transfections (see Figure 4.3) or affect cell viability (data not shown). As such, these reaction conditions allowed for a high throughput synthesis of amine-capped C32 polymers in DMSO without the need for purification. By conducting the reaction at a 100 mg/ml concentration in DMSO, polymers could be diluted into sodium acetate buffer and used directly in transfection assays without any DMSO induced toxicity.

Amine-terminated polymers for *in vivo* gene delivery experiments were synthesized by a slightly modified route that permitted isolation of pure polymers. Although the excess amine capping reagents were not cytotoxic in cell culture experiments, they can have adverse effects on health and viability when injected into mice. As a result, polymers were synthesized as outlined above but with an additional step to remove excess amine. For the amine-capping step, the reaction was conducted in THF using the same concentrations and reaction time as the DMSO-based protocol. Pure polymers were then isolated by precipitation in diethyl ether, which effectively extracted out any excess, unreacted amine and the THF solvent, as determined by ^{1}H NMR (Figure B4).

4.4.2 Polymer Transfections

A preliminary cell culture transfection using a positive control C32 modification verified the two-step synthesis conditions. Polymer C32-32, prepared by capping C32-Ac with aminopentanol (#32), is structurally identical to C32+ and functions just as effectively. The transfection results of these polymers (C32-32 and C32+), along with C32-Ac and a
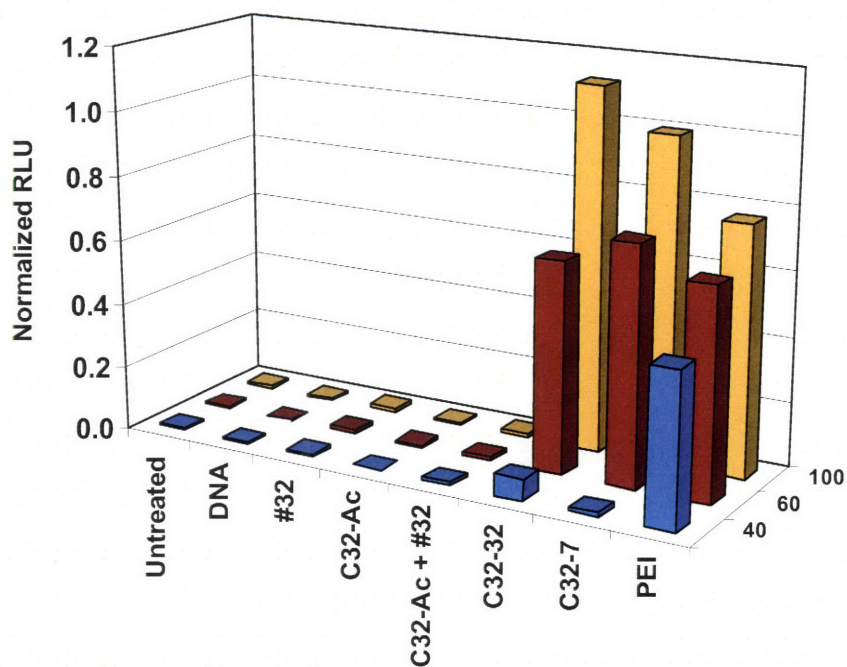
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Figure 4.3 Control transfections. Luciferase protein expression, normalized to C32+ at a 100:1 ratio, for polymers and control conditions as a function of the polymer:DNA ratio. Conditions from left to right: Untreated cells, naked DNA, #32 (excess #32 amine without polymer), C32-Ac (acrylate-terminated C32 polymer), C32-Ac + #32 (blend of C32-Ac with the amount of #32 amine used in the end-capping reaction, without sufficient time for end-chain coupling), C32-32 (C32 polymer end-capped with amine #32, structurally equivalent to C32+), C32+ (prepared in one step using a 1.2:1 amine:diacrylate ratio), PEI (25 kDa).

few additional control conditions, are shown in Figure 4.3. As with other acrylate-terminated polymers, C32-Ac has a very low capacity to transfect cells.[5] End-capping this polymer with amine #32 greatly improved its transfection to the level of C32+ at several polymer:DNA ratios. Importantly, simply combining C32-Ac with excess amine #32 did not improve transfection, indicating that a covalent reaction and modification is necessary to alter the polymer effectiveness. Furthermore, amine #32 alone was insufficient to transfect cells, which suggests that excess amine in each polymer sample should not contribute to or appreciably affect the polymer transfection ability.

The transfection ability of end-modified C32 polymers was quantified using a high throughput, 96-well plate based protocol. Each polymer was tested at five polymer:DNA ratios since this parameter is known to have a marked effect on polymer-mediated transfection.[5] COS-7 fibroblasts were selected as a model cell line for the delivery of a DNA plasmid encoding the firefly luciferase reporter protein. Unlike previous poly(β-

amino ester) studies, transfections were performed in cell culture media to identify polymers with high activity in the presence of serum proteins. Such considerations are especially important for *in vivo* applications where there is an abundance of extracellular proteins that can interact with and affect the delivery properties of polymer-DNA complexes.[10,11]

The transfection results for amine-capped C32 polymers are shown in Figure 4.4. The average luciferase expression levels, measured in relative light units, are given for each polymer as a function of the polymer:DNA weight ratio. The wide distribution in measured RLUs indicates that the terminal amine has a large effect on poly(β-amino ester)-mediated transfections. It appears that the most effective polymers have hydrophilic end groups containing primary or tertiary amines. Polymers demonstrating the lowest transfection levels generally consist of a hydrophobic end group containing either an alkyl chain or aromatic ring. In terms of overall transfection levels, five polymers showed an improvement over C32+ while the top 14 polymers were more effective than PEI.

Perhaps the most important result is that very subtle structural differences in just the terminal amine can have a large effect on polymer transfection efficiency. This is most evident by comparing the C32-36 and C32-52 polymers. The C32-52 polymer, which contains a six-carbon alkyl chain extending from the terminal secondary amine, has a maximum transfection only twice that of naked DNA. In contrast, the C32-36 polymer is 34-fold more effective than C32-52, but only differs in a single hydroxyl group on carbon-6 at the chain end. In fact, the C32-36 polymer is half as effective as C32+, demonstrating that a single functional group, in this case a terminal hydroxyl, can significantly alter the polymer delivery properties. A similar effect can be seen between the C32-95 and C32-110 polymers, which consist of terminal decylamines containing either a hydroxyl group or primary amine on carbon-10, respectively. In this case, substituting the terminal hydroxyl for an amine improves the transfection performance by over one order-of-magnitude. This same substitution pattern also changes the optimal polymer:DNA ratio. Comparing two highly efficient polymers, C32-122 and C32-124, the former displays very high RLU output at a 20:1 ratio, whereas the latter requires 5-fold more polymer (i.e., a 100:1 ratio) to achieve the same effect. A similar trend is also seen between the C32-36 and C32-106 polymers. Therefore, amine capping molecules with hydroxyls and primary amines are most effective, with the latter being optimal at 5-fold lower polymer:DNA ratios in general.

Polymers terminated with primary diamine molecules had the highest transfection efficiency, as determined by both highest RLU output and lowest optimal polymer:DNA ratio. In general, polymers capped with primary diamine compounds can maintain high levels of DNA delivery at much lower polymer:DNA ratios than mono-amine terminate polymers. Specifically, the C32-102 polymer had a very similar transfection profile to C32+, with a maximum occurring at the highest polymer:DNA ratio of 100:1, but had an overall 30% higher RLU output. This demonstrates that a simple modification at the chain ends can significantly improve the delivery performance. Primary diamine capping also lowered the optimal polymer:DNA ratio substantially in many cases. Seven primary
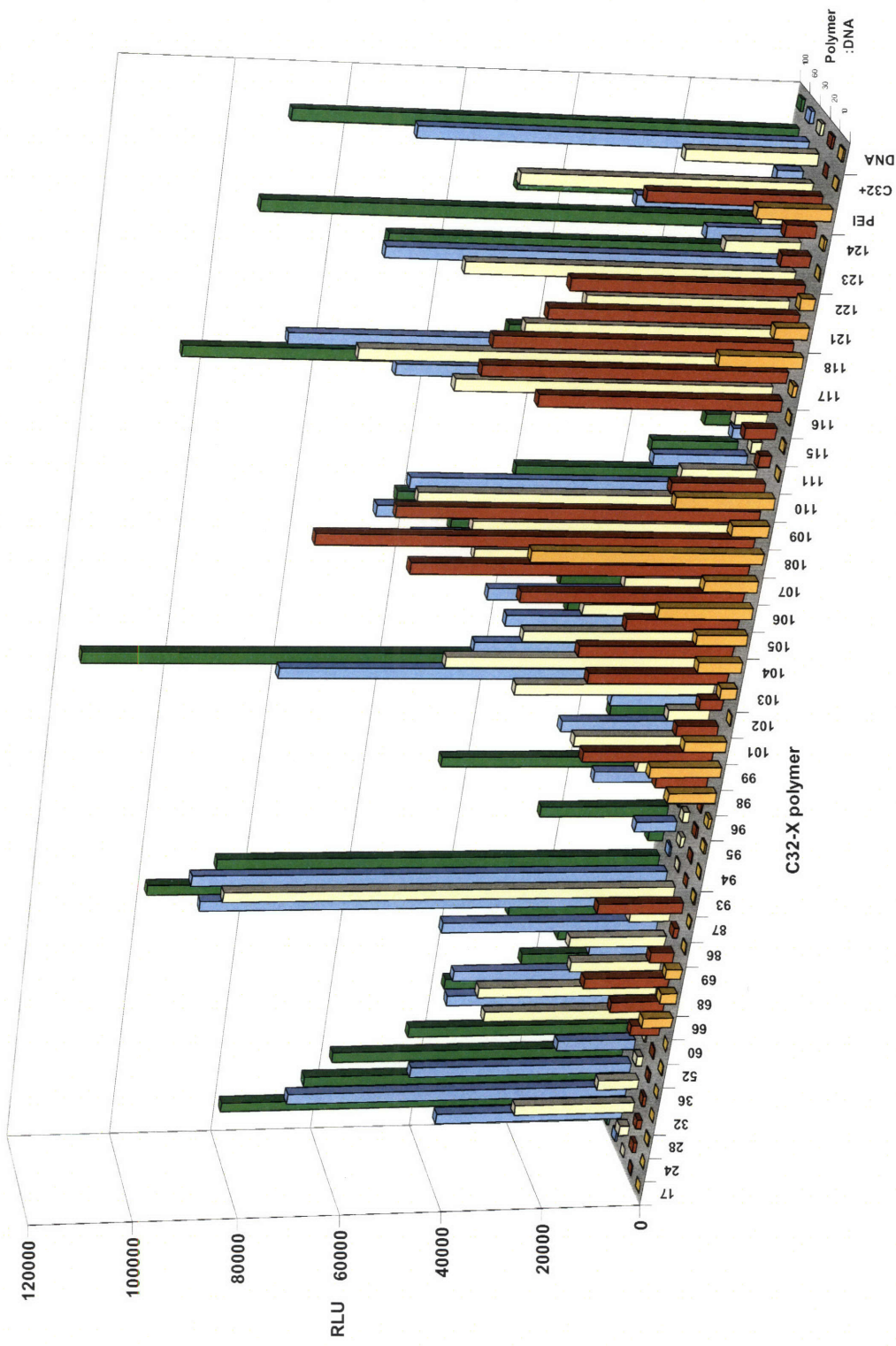
Figure 4.4 Transfection of COS-7 cells. The relative light units (RLU) of luciferase reporter protein expressed are shown for each end-modified C32 polymer (C32-X) at five polymer:DNA weight ratios (10, 20, 30, 60, and 100:1).

amine-terminated polymers had optimal polymer:DNA ratios of 20:1 while one polymer, C32-110, had a maximum RLU at a 10:1 ratio. The transfection profile at the 20:1 ratio for diamine capped polymers, C32-102 through C32-111, appears to be a skewed bell-shaped curve with a maximum occurring at the C32-108 polymer. This indicates that larger alkyl chains bridging the diamine functionalities are generally more effective than their short-chain counterparts, with an optimum of eight carbons. The C32-108 polymer had an optimal transfection at a 20:1 ratio that is almost as high as that for C32+, which requires a 100:1 ratio. Such a significant reduction in the amount of polymer needed to mediate high levels of transfection has important implications for *in vivo* delivery, where the amount of polymer injected needs to be limited to minimize toxic side effects.

4.4.3 Cytotoxicity

Many polycations have been shown to elicit considerable cell toxicity that may limit their utility as gene delivery vectors.[12,13] The biocompatibility of cationic polymers is determined by a number of factors that include molecular weight, charge density, type of amines, polymer structure (linear, branched, dentritic), and chain flexibility.[14-19] In general, high molecular weight polymers with a high density of primary and/or secondary amines usually result in substantially cytotoxicity.[20] PEI and PLL are examples of such polymers and bring about significant cell damage by compromising the cell membrane, as determined by the cytosolic release of lactate dehydrogenase following exposure.[21] Several poly(β-amino ester)s have been shown to be less toxic than PEI, presumably due to their lower molecular weights, degradability and the lack of primary or secondary amines.[8] It was suspected that the reduced transfection of primary amine end-modified polymers at high polymer:DNA ratios may be due to increased cytotoxic activity.

The cytotoxicity of end-modified poly(β-amino ester)s was evaluated using the MTT assay. This colorimetric test is based on the ability mitochondrial reductase enzymes in viable cells to reduce 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide to a purple formazan. New polycationic materials for biomedical applications are frequently tested for their effects on cellular proliferation using this assay.[20] Toxicity of end-modified polymers was assessed by performing the same transfection experiment, but assaying for metabolic activity instead of luciferase expression. All polymers were tested at the highest 100:1 polymer:DNA weight ratio, which corresponds to an approximate 400 ug/ml concentration of polymer on the cells. Toxicity analysis at such a high polymer concentration may explain the differences in polymer transfection at high polymer:DNA ratios, and simultaneously assess polymer biocompatibility under very aggressive conditions that may be important for their future use and development.

The cytotoxicity levels of end-modified C32 polymers are shown in Figure 4.5. The percentage of viable cells is displayed as a function of the amine-terminated polymer. Positive and negative control conditions are shown to the far right for PEI and naked DNA. At such high polymer concentrations, PEI is known to be very cytotoxic,[8] as reflected by the low 3% cell viability. In contrast, both C32+ and the acrylate-terminated C32 polymer (C32-Ac) show no significant affects on the growth and metabolism of
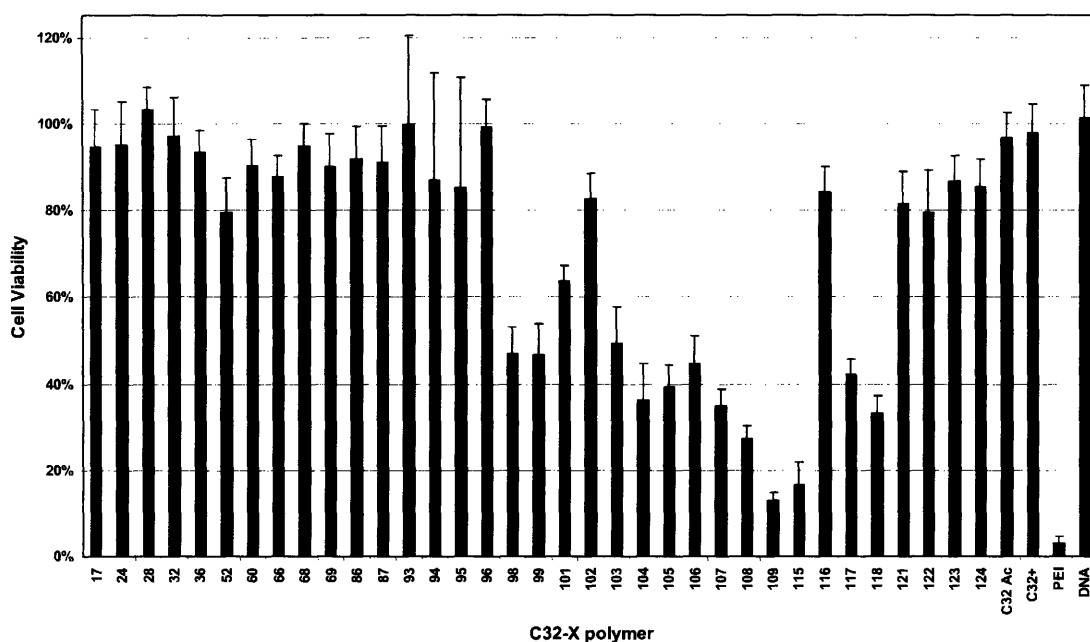
Figure 4.5 Cytotoxicity levels of end-modified C32 polymers (C32-X) measured using the MTT assay. PEI, C32+, C32-Ac, and free DNA toxicity are shown on the far right.

COS-7 cells. This result for C32-Ac is somewhat surprising though since other acrylate-terminated poly(β-amino ester)s have shown considerable toxicity at this concentration.[5]

The majority of end-modified C32 polymers show good biocompatibility. This is especially true of all polymers capped with mono- primary amine reagents, regardless of the functional groups extending from the amine. Aromatic, alkyl, hydroxyl, secondary and tertiary amines, and imidazole functionalities at the chain end points do not appear to invoke any adverse effects. Therefore, elevated cytotoxic effects do not sufficiently explain the low transfection ability of polymers terminated with the more hydrophobic amines. In contrast to the polymers capped with primary monoamines, polymers terminated with primary diamine molecules compromise cell viability to varying extents. While the increased charge is a determining factor, the overall toxicity is also strongly dependent on the hydrophobicity of the end group. In general, increasing the size of the alkyl chain bridging the amine groups increases the toxicity, as is evident by comparing the C32-102 through C32-109 polymers. Furthermore, C32-121, a polymer containing a terminal polyethyleneglycol amine with an eight atom spacer between amine groups, is much less toxic than the corresponding alkyl derivative, C32-108. This indicates that both the spacing between amines, and the degree of hydrophobicity in the terminal amine spacer, are important determinants of end-amine toxicity.

These significant cytotoxic effects, in large part, explain the decreasing transfection ability of most primary diamine capped polymers at the higher polymer:DNA ratios. The

additional charge, in conjunction with increased hydrophobicity, may be particularly damaging to the cell membranes since both properties are known to disrupt lipid bilayers.[22]

4.4.4 Plasmid DNA Binding

An important requirement for cationic transfection agents is the ability to bind and condense plasmid DNA for cell entry.[23] In general, higher molecular weight polymers with increased cationic charge density can form more stable complexes with plasmid DNA at low polymer:DNA ratios.[14,24,25] While strong electrostatic interactions are important to effectively condense and deliver the DNA, the polymer must possess a mechanism to unbind from the DNA once inside the nucleus.[7] For this reason, the poly(β-amino ester)s may be particularly advantageous since they undergo hydrolysis with short half-lives,[1] which may aid in DNA packaging.

The binding and condensation of DNA by polycations is often monitored using an agarose gel electrophoresis assay.[26] This assay can be used to adequately determine the minimum polymer:DNA ratio required for plasmid condensation but does not provide any information on the accessibility of the DNA. In contrast, dye binding assays provide a quantitative measure of the polymer-DNA binding event at all polymer:DNA ratios.[27-29] As a result, a PicoGreen dye penetration assay was utilized to determine the degree of plasmid condensation by the end-modified poly(β-amino ester)s. In this assay, polymer-DNA complexes are formed in a manner similar to their preparation for transfection experiments. The complexes are then mixed with a PicoGreen dye solution, diluted into cell culture media, and the solution fluorescence is measured. The dye exhibits fluorescence only when it intercalates between the DNA base pairs. High fluorescence is typically seen with free plasmid, but significant reductions can occur for polymer-DNA complexes in which the DNA is partially shielded from dye penetration. The magnitude of this fluorescence reduction relative to free DNA correlates to the degree of polymer-DNA condensation.[5,30]

The DNA binding levels for each end-modified polymer are shown in Figure 4.6. Fluorescence measurements relative to free DNA are given at the optimal transfecting polymer:DNA ratio for each polymer. With the exception of mono-amine PEG terminated polymers, all end-modified materials displayed some measurable level of DNA binding. In general, increased cationic charge at the polymer end groups increased the polymer-DNA binding affinity. This effect is most noticeable by comparing the results of the PEG amine-capped polymers. The mono-amine capped polymers, C32-123 and C32-124, displayed no measurable binding. However, the substitution of a single primary amine for a hydroxyl at the chain ends (C32-121 and C32-122) leads to increased polymer-DNA binding and less dye penetration. This result indicates that a single functional group only at the very end point of the polymer can bring about large changes in polymer function. Similar conclusions were reached when assessing the overall transfection ability of the polymer but now are seen at just one part of the delivery process.
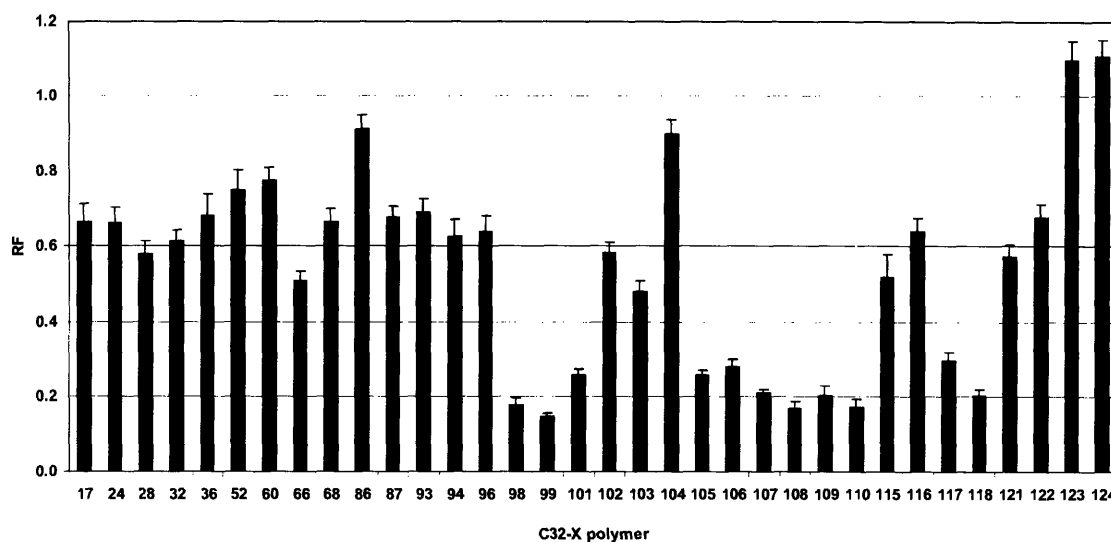
Figure 4.6 Polymer-DNA binding measured using a PicoGreen assay. Fluorescence reductions relative to free DNA (RF) are shown for each end-modified C32-polymer (C32-X) at the optimal transfecting polymer:DNA ratio for each polymer.

Perhaps the most noticeable trend in the data is that polymers terminated with primary diamine molecules are most effective at condensing and binding DNA. Additional secondary or tertiary amines at the chain ends were not as effective to increase the DNA binding ability of the polymer, possibly due to pKa differences or a more sterically crowded environment that may prevent their electrostatic interaction with DNA.[31,32] Similar to the cytotoxicity data, more effect is seen with increased terminal hydrophobicity in addition to the added positive charge. In general, smaller relative fluorescence is seen as the alkyl chain length is increased between terminal amine groups, as is evident by comparing polymers C32-102 through C32-110. These results are supported by lower DNA binding affinity of polymers terminated with the more hydrophilic primary ethyleneglycol amines (C32-121 and C32-122).

4.4.5 Polyplex Sizing

Simple electrostatic interactions between polycations and negatively charged DNA can often lead to their spontaneous self-assembly into cationic polymer-DNA nanoparticles (i.e., polyplexes).[33,34] The physical properties of these complexes are particularly important for their subsequent uptake into cells. Complexes with a positive surface charge and a diameter less than 200 nm are usually sufficient for cellular endocytosis.[3] These properties are dependent upon a number of polymer characteristics and the polymer-DNA mixing technique.[35-39] Since the terminal amine has demonstrated significant effects on the DNA binding ability of polymers, it should also affect the physical properties of the resulting polymer-DNA complexes.
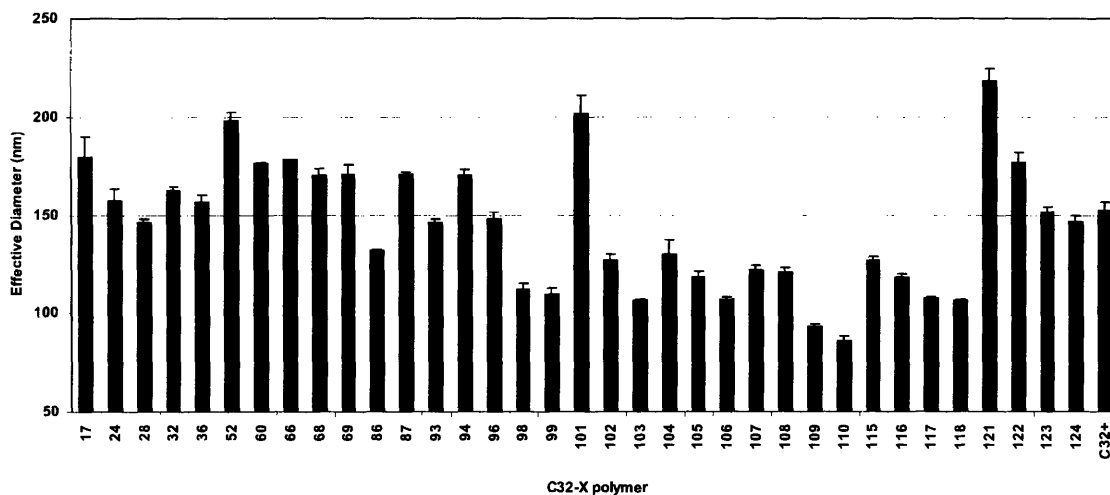
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Figure 4.7 Polymer-DNA complex size measured by dynamic light scattering. The effective diameter of complexes are shown for each end-modified C32 polymer (C32-X) at the optimal transfecting polymer:DNA ratio for each polymer.

The effective diameter of complexes formed between end-modified poly(β-amino ester)s and plasmid DNA were measured using dynamic light scattering. Polymer-DNA complexes were formed at the optimal transfecting polymer:DNA ratio for each polycation and then diluted into cell culture media prior to each measurement. Concentrations, solution compositions, and polymer-DNA complexing procedures in each step were identical to those used in the transfection assay. In this way, the nanoparticle physical properties measured in this experiment reflect the actual particle properties in the transfection screen.

Average diameters of the polymer-DNA complexes are presented in Figure 4.7 for each end-modified C32 polymer. The average diameter varied between 85 to 220 nm, demonstrating the crucial effects of terminal amine structure on the physical properties of polymer-DNA complexes. Also shown on the far right is the average diameter of the C32+ complexes, which is determined to be 152 nm. In a previous study, C32+ complexes were diluted into HEPES buffer and subsequently measured to be 79 nm in diameter.[6] This difference in particle size illustrates the large effect of serum proteins to disrupt or interact with cationic polymer-DNA complexes. Increases in polymer-DNA complex size in the presence of serum have been seen in studies with other polycations such as PLL and PEI, and is a well known effect on polymer-DNA properties.[10,11] Importantly, the C32+ complex diameter is still below the threshold for endocytosis and maintains high transfection levels.

All end-modified polymers formed complexes with effective diameters in a suitable range for cellular uptake. Only two polymers, C32-101 and C32-121, formed complexes

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with diameters slightly above 200 nm. The former material consists of highly charged chain end groups whereas the latter contains a short PEG diamine at the chain end points. In general, the PEG terminated polymers (C32-121 to C32-124) formed larger complexes with diameters between 150 to 220 nm. Despite the large size and weak DNA binding of these polycations, they can still deliver DNA with relatively high efficiencies. This effect is also true of most polymers terminated with mono-primary amine molecules. These polymers, shown on the left side of Figure 4.7, mostly result in complexes with diameters greater than 150 nm and lower DNA binding ability than primary diamine capped polymers. Although a general conclusion cannot be made, it is interesting to note that some polymers with very low transfection efficiencies (e.g., C32-117, -52, -101) also form relatively large complexes, suggesting that their physical properties may not be conducive to uptake.

Similar to the DNA binding data, particle sizing appears to be more favorable for polymers capped with primary diamine molecules. For almost all of these polymers, their complexes with DNA have diameters between 85-130 nm. The more hydrophilic PEG diamines, C32-121 and C32-122, are the exception, illustrating the importance of a hydrophobic alkyl chain space between amines at the terminus. Although the trend is not as pronounced as that for the DNA binding, it appears that the polyplex diameter decreases as the alkyl chain length increases. This is especially true at the long chain lengths where C32-109 and C32-110 form the smallest complexes that are less than 100 nm in diameter. These polymers assemble into smaller complexes with DNA compared to those terminated with additional secondary and/or tertiary amines, again illustrating the benefits of primary amines at the chain ends over these other amine functionalities. Consequently, it appears that polymers terminated with alkyl primary diamine molecules have the strongest DNA binding characteristics and assemble into the smallest polymer-DNA complexes.

4.4.6 Plasmid DNA Uptake

Differences in the physical properties of polyplexes can naturally lead to differences in the rates and levels at which they are endocytosed into cells. Previous studies with poly(β-amino ester)s have shown that smaller complexes with high cationic surface charge are more favorable for cellular uptake.[3] In addition, amine termination has been shown to promote higher cellular internalization over the corresponding acrylate-terminated polymer.[5] In light of these findings and the terminal amine affects on polymer-DNA properties, the uptake levels of end-modified C32 polymers were measured. Although previous studies with poly(β-amino ester)s used a novel fluorescence-based technique,[40] we choose to use a DNA extraction and RT-PCR amplification protocol to quantify the amount of endocytosed DNA.[41] This method provides (1) high sensitivity due to the PCR amplification, (2) linearity over several orders-of-magnitude, (3) the ability to quantify DNA uptake without pre-labeling the plasmid, and (4) a high-throughput, 96-well plate format to simultaneously and rapidly analyze all polymers. For this experiment, transfections were performed following the standard protocol using a β-galactosidase (β-gal) plasmid. This DNA was isolated from the cells after a four hour incubation period and amplified using RT-PCR. The total
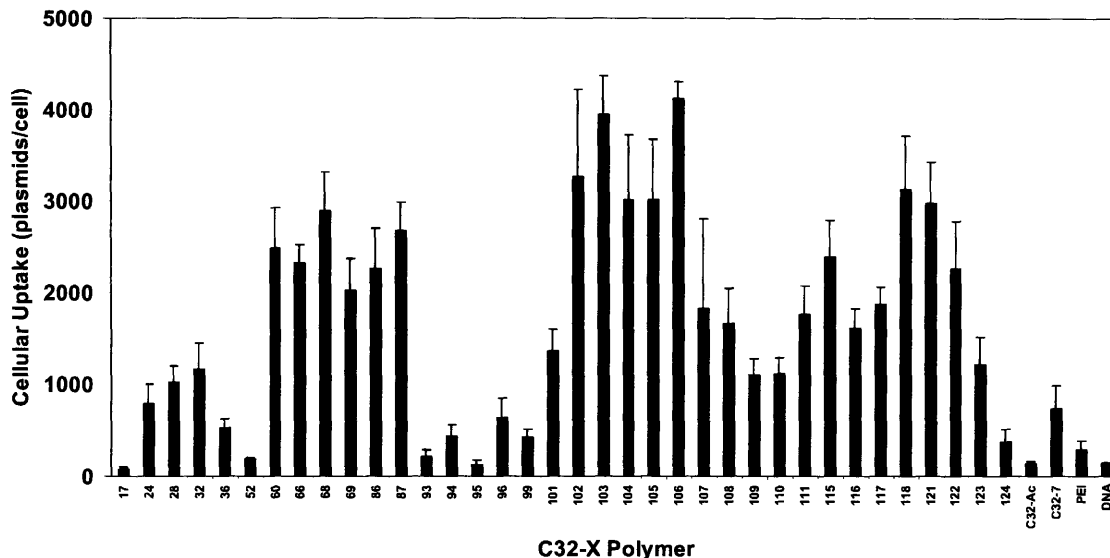
Figure 4.8 Plasmid DNA uptake into COS-7 cells. DNA uptake levels are shown in number of plasmids endocytosed per cell for each end-modified C32 polymer (C32-X).

number of β-gal DNA plasmids harvested for each sample was calculated using a standard curve and normalized to the number of COS-7 cells.

The DNA uptake levels for each end-modified polymer are shown in Figure 4.8. The results are expressed as the number of plasmids endocytosed per cell for each polymer at its optimal transfecting polymer:DNA ratio. Also shown is the low uptake level of free plasmid DNA, which is most likely due to its large size and high anionic charge density that repels the cell surface. Positive control polymers C32+ and PEI are also shown to the far right, both of which increase DNA uptake by approximately 5-fold over the free plasmid. On the other hand, plasmid condensation with the C32-Ac polymer did not improve uptake to any measurable level, which explains the inability of this polymer to mediate transfection. The large difference between C32+ and C32-Ac, both in terms of uptake and transfection, highlight the importance of amine termination to improve the C32 polymer delivery properties.

The results in Figure 4.8 demonstrate that the type of amine at the chain ends has a considerable effect on the endocytosis of C32 polymer-DNA complexes. The uptake levels varied over two orders-of-magnitude among the end-modified polymers, with polymer C32-106 mediating the highest plasmid internalization that is 30-fold greater than free DNA. The most obvious trend in the data is the improved uptake that occurs for polymers with additional terminal amines. This is evident for polymers containing extra secondary and tertiary end amines (C32-60 through C32-87) and mostly for those with an additional primary end amine (C32-102 through C32-122). These results suggest that

conjugation of targeting ligands to the chain ends may be a promising strategy to achieve cell specific delivery.

The differences in uptake between each polymer also explain some important differences in their transfection efficiencies. First, many polymers that are poor transfection agents also displayed very low uptake (e.g., C32-17, -52, -93, -95). This indicates that the extra charge alone at the chain end points (compared to C32-Ac) is not sufficient to promote C32-DNA endocytosis. Specific functional groups at the chain ends, such as hydroxyls and amines, have an enhanced capacity to interact with cell surfaces and increase uptake as compared to more hydrophobic terminal segments. For example, the transfection differences between C32-36 and C32-52 are largely related to their differences in uptake. This comparison demonstrates that a single functional group in the polymer chain, in this case a terminal hydroxyl, can have a large effect on cell interactions and endocytosis. Extending the comparison further, C32-106 differs from these two materials by a terminal primary amine. The results show that this single substitution at the terminal amine carbon-6 can increase uptake by over 20-fold. This effect is even more surprising considering that the polymer:DNA ratio used for C32-106 is 20:1, 5-fold less than that used for C32-36 and C32-52. In general, the increased uptake by the polymers capped with primary diamines largely explains their increased effectiveness at reduced polymer:DNA ratios. The overall transfection levels may not be substantially improved over C32+ and other non-primary amine polymers because the terminal functionalities may have important effects on other downstream gene delivery barriers such as endosomal escape, cytosolic trafficking, or nuclear import.

4.4.7 Variation of Internal Composition on Transfection

Terminal amine modifications to the C32 polymer are shown here to have a large effect on several gene delivery properties. In particular, differences in the end amine structure have resulted in significantly improved DNA binding, the formation of much smaller polymer-DNA complexes, enhanced cellular endocytosis of these complexes, and increased transfection efficiencies, especially at the lower polymer:DNA ratios. Since the terminal amine can affect and improve the C32 polymer performance, such modifications may also alter the transfection profiles and gene delivery properties of other poly(β-amino ester)s. Furthermore, given that the terminal amine has a large affect on cellular uptake, simple amine-capping may be an effective means to promote cell specific delivery.

Two additional poly(β-amino ester)s, D60 and C20, were synthesized and end-capped to assess the combined effects of internal polymer structure and amine termination on transfection efficiency (Figure 4.9(A)). The former is an effective gene delivery polymer with a structure very different from that of C32.[6] Comparing the transfection efficiencies between end-modified derivatives of both D60 and C32 polymers can lead to the identification of terminal amine molecules that may be effective for any poly(β-amino ester), irrespective of the internal sequence. Conversely, the C20 polymer is a very poor transfection agent but is structurally very similar to C32, differing only in the length of the alcohol side chain.[6] Optimization of the C20 terminal amine is useful to demonstrate
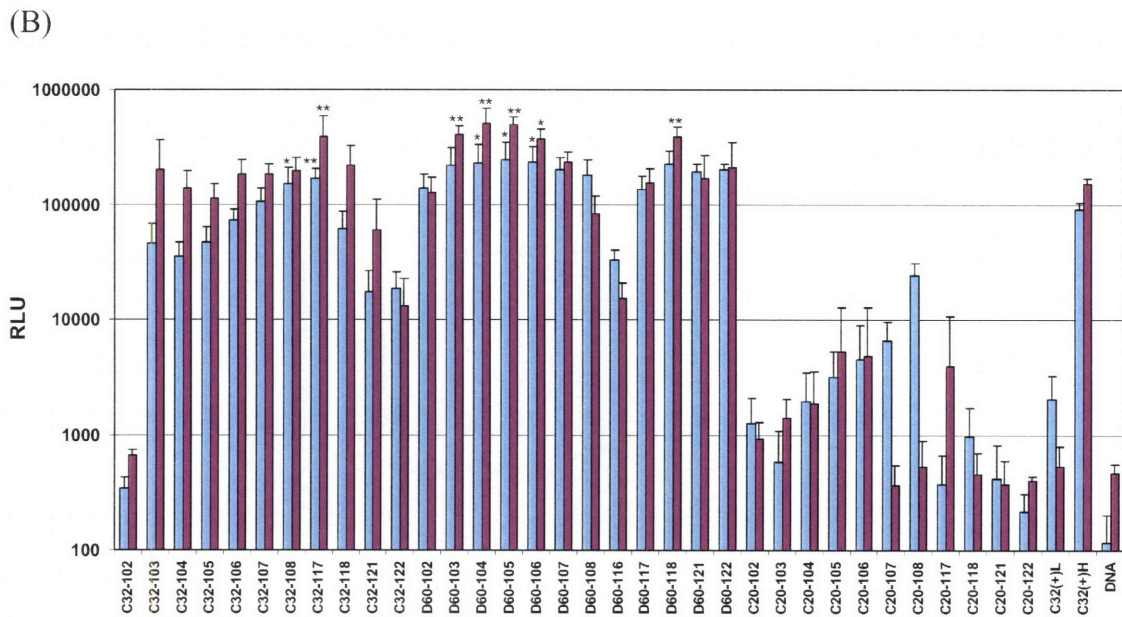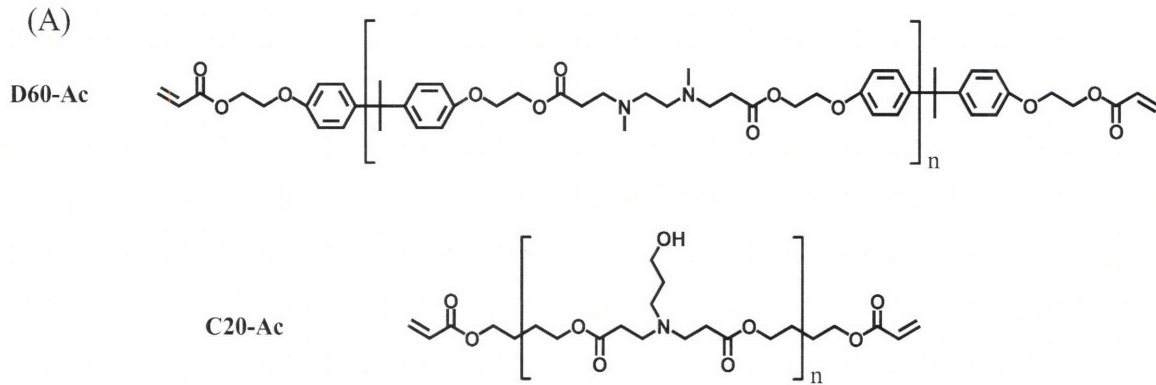
Figure 4.9 (A) Structures of acrylate-terminated D60 and C20 polymers. (B) Transfection of COS-7 (blue bars) and HepG2 (red bars) by end-modified C32, D60, and C20 polymers at a 20:1 polymer:DNA ratio. The C32+ polymer was tested at a low 20:1 (C32+L) and high 100:1 (C32+H) polymer:DNA ratio. Statistical differences between modified polymers and C32H were determined for each cell line using a one-way ANOVA with a Dunnett multiple comparison test (* $p < 0.05$; ** $p < 0.01$).

the potential conversion of an otherwise ineffective poly(β-amino ester) into one that has the capacity to transfect cells. These polymers were end-capped with the primary diamine molecules that produced the most effective C32 modifications in terms of both overall transfection and lower optimal polymer:DNA ratio. Transfection screens of all three polymer types (C32, D60, and C20) were performed on COS-7 and HepG2 cell lines. The latter was included because (1) it is a human cancer cell line frequently used to test new polymers for gene delivery, (2) transfection of liver cells has therapeutic

relevance, and (3) it provides a second cell line to evaluate potential targeting effects of each polymer. Lastly, all transfections were carried out at a 20:1 polymer:DNA weight ratio.

The transfection levels for the end-modified C32, D60, and C20 polymers are shown in Figure 4.9(B). Measured RLUs from the expression of the luciferase reporter protein are given for each polymer for both the COS-7 (blue bars) and HepG2 (red bars) cell lines. Also shown on the far right is the transfection using the positive control C32 polymer at a low (C32+L) and high (C32+H) polymer:DNA ratio, and naked DNA. It can be seen that all of the end-modified C32 and D60 polymers, with the exception of C32-102, are more effective than the control C32 polymer at the same polymer:DNA ratio (i.e., C32+L) for both cell types. As a result, all of these end-modified derivatives are more efficient vectors, indicating that chain termination with primary diamine molecules is a useful way to improve the delivery efficiency. Although the mechanism underlying this effect is unclear, the increased charge density appears to be an important factor, suggesting that more cationic amine molecules could improve the efficiency in general.

Most end-modified C32 polymers demonstrate the ability to transfect both cells lines, indicating that these materials may be effective delivery systems for a variety of cells. Both C32-108 and C32-117 were statistically more effective than C32+H on COS-7, while only C32-117 was better on the hepatocytes. In general, C32 polymers terminated with primary alkyl diamines (C32-103 through C32-108) were more effective than those with PEG spacers (C32-121 and -122), indicating that a degree of hydrophobicity at the chain ends is preferential for these polymers. For both cell types it appears that at least a three carbon spacer between terminal amines is necessary to obtain effective gene delivery with C32 polymers at the 20:1 ratio. The C32-103 efficiency is 130- and 300-fold higher than C32-102 on the COS-7 and HepG2 cell lines, respectively. This result demonstrates that a single additional carbon at the chain ends can alter the transfection levels by two orders-of-magnitude.

In addition to the C32 polymers, many of the end-modified D60 polymers were highly effective gene delivery vectors. In fact, the measured RLUs for five of these polymers were statistically higher on hepatocytes at the reduced 20:1 ratio than C32+ at its optimum 100:1 ratio (C32+H). The best performing polymer, D60-105, has a transfection level almost 3-fold higher than C32+H. Three of these polymers (D60-103, 104, 105) were also statistically better than C32+H on COS-7 fibroblast cells, indicating that they could be very effective transfection reagents on other cell types. Unlike the end-modified C32 polymers, highly efficient D60 polymers were formed with both alkyl and PEG terminal diamines. And for a given amine capping molecule, the D60 polymers appear to outperform the C32 polymers on both cell lines. This suggests that even though previous studies have identified C32 as the superior polymer (by optimization of the monomer ratio only),[6] it is necessary to concurrently optimize both the interior sequence and end-amine structure to arrive at the most efficient and effective poly(β-amino ester).

In comparison to the C32 and D60 polymers, all of the C20 modifications were much less effective. Nevertheless, the C20 gene delivery efficiency could be remarkably improved

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by proper end-functionalization. The measured RLUs for the most effective modified polymer, C20-108, was over two orders-of-magnitude higher than that observed with C20-122 and naked DNA. The C20-108 effectiveness was still 3- to 4-fold less than the optimal C32 transfection level, but was never tested over a range of polymer:DNA ratios where it may have better performance. Regardless, the conversion of a completely ineffective polymer into a material with gene delivery capabilities by end-modification is an important result for the future development of poly(β-amino ester)s. It suggests that the transfection ability of a polymer, which could be compromised by interior conjugation of targeting ligands, PEG chains or other molecules, can be potentially restored or improved by end-chain functionalization.

Some differences in polymer transfection could be seen between the COS-7 and HepG2 cell lines. The most significant difference occurred for the C20-108 polymer, which was two orders-of-magnitude more effective in COS-7 cells over the HepG2 cells. A similar but less dramatic effect was seen with the C20-107 polymer, suggesting that C20 termination with long alkyl diamines may be a possible means to target fibroblasts. For all other polymers, including the C32 and D60, most transfection differences between the cell lines were within an order-of-magnitude for each polymer. The inability to achieve a high level of cell specific delivery is not surprising given that none of these end amines or polymer sequences have an obvious mechanism to preferentially bind to a given cell type.

4.4.8 Delivery of siRNA

In addition to DNA delivery, some poly(β-amino ester)s have shown the ability to deliver siRNA to down-regulate protein expression (unpublished data). Initial experiments with a previous polymer library have specifically identified polymer AA28 as a promising candidate for further development. As a result, we synthesized an acrylate-terminated AA28 base polymer (Figure 4.10(A)) and explored the effects of amine end-capping on AA28 siRNA delivery. A select group of primary diamine capping reagents was used along with the highly charged 101 compound. Four additional capping reagents were included that contain multiple amines (125 through 128) to assess the effects of highly charged ends on siRNA-mediated knockdown. As a model system, we delivered firefly-luciferase siRNA to a HeLa cell line that stably expresses both firefly and renilla luciferase proteins. The decrease in firefly levels was used to quantify knockdown while any decreases in renilla levels were used to measure and correct for cytotoxic effects.

The percent knockdown of firefly luciferase for each end-modified AA28 polymer at its optimal polymer:siRNA ratio is shown in Figure 4.10(B). Similar to the DNA delivery experiments, the end-amine structure of the polymer has a large effect on its siRNA delivery efficiency. The most effective polymer discovered, AA28-126, can mediate 75% knockdown of the firefly luciferase level. This efficiency is equal to that seen with Lipofectamine, one of the most effective cationic lipid formulations for siRNA delivery. Interestingly, the AA28-126 polymer derivative contains the most cationic end group. In fact, the percent knockdown appears to increase as the charge density is increased at the chain ends, indicating that this property may be generally important for siRNA delivery with poly(β-amino ester)s.
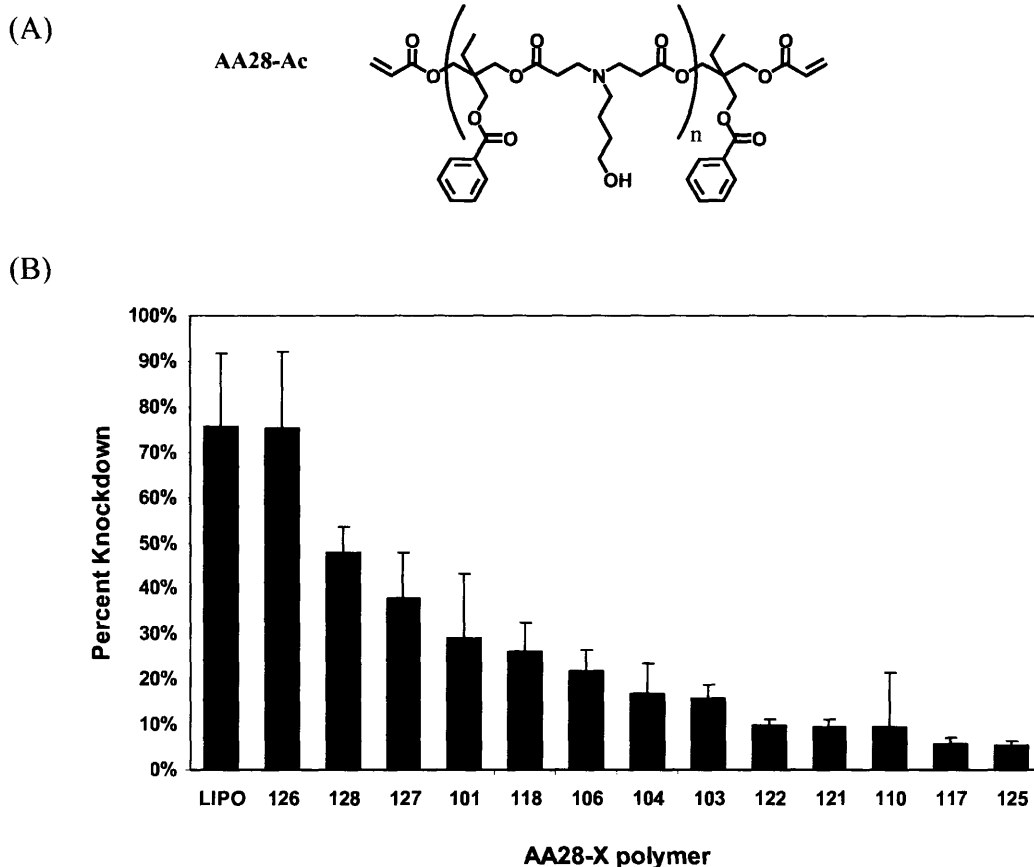
93

(A)

AA28-Ac

(B)

Figure 4.10 (A) Structure of acrylate-terminated AA28 polymer. (B) siRNA delivery with AA28 poly(β-amino ester)s. Percent knockdown of firefly luciferase in HeLa cells is shown for each end-modified AA28 polymer (AA28-X) at its optimal polymer:siRNA ratio.

Although only one polymer and a small subset of capping molecules were tested, several effective end-modified polymers have been discovered for siRNA delivery. With the large pool of base polymers and the availability of many amine molecules, a wide array of structurally diverse poly(β-amino ester)s can be prepared using the end-modification and screening strategy. Since this small test of AA28 end capping produced several strong hits, a much larger library of materials could lead to the identification of many poly(β-amino ester)s capable of high DNA and siRNA delivery efficiencies.

4.4.9 Intraperitoneal Gene Delivery

Based on cell transfection experiments, a smaller subset of polymers were selected for *in vivo* gene delivery in mice. For these experiments, polymer-DNA complexes were made by mixing the appropriate polymer mass with luciferase DNA. diluting into PBS containing glucose, and immediately injecting into the intraperitoneal cavity. A high

DNA dose of 100 µg was injected to maximize gene delivery and reporter protein expression. This high DNA dose along with the small injection volume and limited solubility of poly(β-amino ester)s in aqueous buffers limited the polymer:DNA weight ratio to 30:1. Mice were sacrificed six hours following the injection, at which time reporter protein expression levels were visualized and quantified in whole body mouse images and harvested organs using an IVIS imaging system.

Since the *in vitro* transfection ability may not directly correlate to *in vivo* performance, an initial screening experiment was performed with nine polymers selected for structural diversity and on the basis of their *in vitro* transfection characteristics (luciferase expression levels, cytotoxicity, and optimal polymer:DNA ratio). Most of the polymers selected for *in vivo* transfection consisted of primary diamine end groups since these polymers were among the most effective at the low polymer:DNA ratios used for injection. Polymers selected included those terminated with a tertiary amine (C32-87), secondary amine (C32-66), aromatic primary amine (C32-99), linear alkyl primary amine (C32-103, -106), branched alkyl primary amine (C32-116, -117) and short PEG primary amine (C32-121, -122).

All of the polymers tested *in vivo* resulted in significant gene delivery and protein expression in the abdominal region. Representative whole mouse images are shown in Figure 4.11 for each polymer along with jet-PEI and C32+ positive controls. Although jet-PEI is considered an optimal *in vivo* transfection reagent, it resulted in relatively low levels of diffuse gene expression. This is in contrast to the high gene expression patterns in the lungs that typically occur following an IV injection route.[42] These results are consistent with those reported by Aoki et al.[43] in which intraperitoneal injections of PEI-DNA complexes gave low levels of protein expression only in the spleen, stomach and muscle, without any detectable lung expression. The C32+ positive control poly(β-amino ester) demonstrated higher expression after intraperitoneal injection compared to jet-PEI. It also showed a diffuse pattern of expression throughout the abdomen but was accompanied by a few additional focal points of high expression.

With the exception of C32-87, all end-modified C32 polymers proved more effective than jet-PEI in whole body mouse scans. The most effective polymers were C32-103, -116, -117, and -122. Even though a limited set of polymers were tested, a convergence in structure seems apparent. The most effective polymers (C32-103, -116, -117) are terminated with primary diamine molecules that contain a three carbon spacer between amine functionalities, but differ in the degree and pattern of substitution at the interior carbons. Polymer C32-116 appears to display high transfection levels throughout the abdomen whereas C32-117 appears to be more localized. Polymer C32-103 falls between these two extremes as the protein expression levels appear to occur in a few large connected clusters.

Quantitative measurements of the luciferase reporter protein expression were made from images of whole mice and harvested organs using the LIVE IMAGE acquisition and analysis software. These results are shown in Figure 4.12 for jet-PEI, C32+, and the four best end-modified C32 polymers. Median transfection levels are shown with statistical
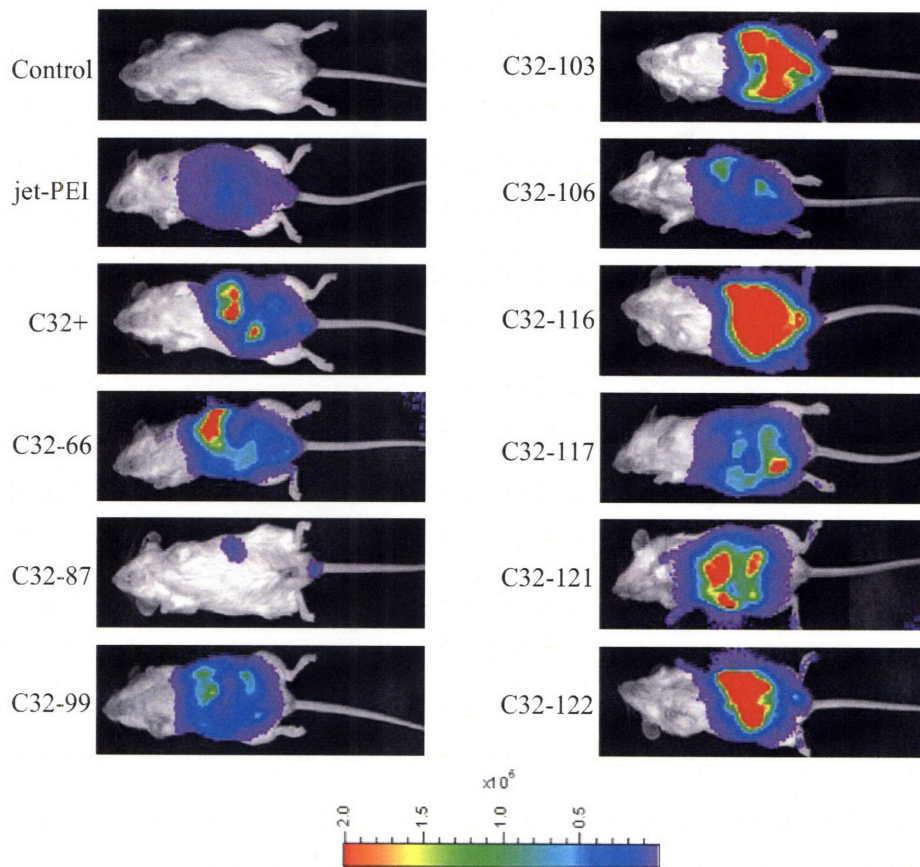
Figure 4.11 Whole body optical images of luciferase expression in FVB/J mice 6 hr after intraperitoneal injection of polymer-DNA complexes. Images show the highest expression obtained for each polymer. Control mouse was injected with 120 μl of 50mM NaAc buffer, pH5.2. Pseudocolor images representing emitted bioluminescence are superimposed over grayscale images. RLUs/pixel are indicated in the color scale bar.

differences relative to C32+ determined using the Kruskal-Wallis test and Dunn's multiple comparison post test ($p < 0.05$) for each tissue. Consistent with the whole body images, mice injected with jet-PEI had low total expression levels that were only one order-of-magnitude higher than the untreated control mice. Similar differences were seen in the fat, spleen, and stomach while all other harvested organs had expression levels that were only slightly elevated over untreated mice. These results are supported by those of Aoki et al.[43] in which the spleen and stomach were the only abdominal organs transfected following intraperitoneal injection of PEI. A very similar *in vivo* response was seen with the C32+ vector, in which none of the measured levels proved to be statistically higher than jet-PEI in any organ.
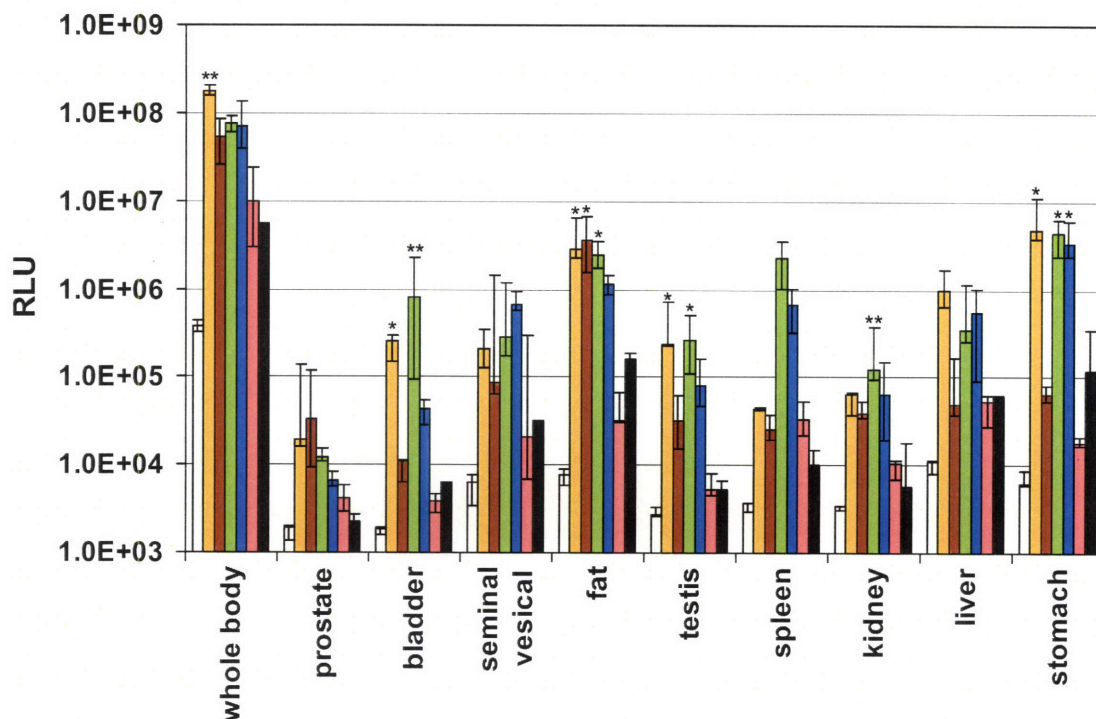
Figure 4.12 Quantification of luciferase expression in whole body and individual organs 6 hr after intraperitoneal injection of polymer-DNA complexes in FVB/J mice. Results are expressed as median transfection levels for a buffer control (white), C32-103 (yellow; n = 3), C32-116 (red; n = 5), C32-117 (green; n = 4), C32-122 (blue; n = 4), C32+ (pink; n = 5), and jet-PEI (black; n = 5). Statistically significant differences between modified C32 polymers and C32 in each tissue were determined using the Kruskal-Wallis test with Dunn's multiple comparison test (* p < 0.05; ** p < 0.01).

Intraperitoneal gene delivery using the amine-terminated C32 polymers was significantly more effective than the C32+ and jet-PEI polymers. Whole body and individual organ transfection levels are shown in Figure 4.12 for the best four polymers. Overall expression levels observed in mice treated with amine-capped C32 polymers were over two orders-of-magnitude higher than the untreated mice, with C32-103 resulting in the highest expression at almost 500-fold higher. These polymers were also more efficient than jet-PEI, with whole body transfection levels greater by at least one order-of-magnitude. Furthermore, the amine-capped polymers were between 6 – 18 fold more effective than C32+ on average. While the reasons for their superior performance relative to jet-PEI is unclear, the improved transfection of primary diamine terminated C32 polymers over C32+ is largely due to their effectiveness at the lower polymer:DNA weight ratios that are used.

Amine-terminated polymers also proved more efficient on an individual organ basis. For each tissue analyzed, at least one polymer was found to transfect one order-of-magnitude

higher than jet-PEI. In fact, C32-117 was capable of mediating these differences in every organ except for the prostate and liver, in which it was only 5-fold higher. These polymers also showed higher expression levels relative to C32+. Order-of-magnitude differences can be seen for at least one polymer in every tissue, with statistically significant improvements occurring in the bladder, fat, testis, kidney and stomach. The most notable differences occurred in the stomach, where reporter protein levels were over two orders-of-magnitude higher for most amine-capped polymers. These results demonstrate that altering the functionality at the ends of poly(β-amino ester)s can substantially improve their *in vivo* gene delivery performance.

The levels of gene expression in each organ varied considerably among the amine-terminated polymers. The C32-103, 116, and 117 polymers differ very slightly in end-group structure but resulted in very different transfection levels in several tissues. The most notable differences occurred in the bladder, spleen, liver and stomach, where reporter protein expression levels varied over an order-of-magnitude among some polymers. With the exception of liver, C32-117 resulted in median transfection levels 80-fold higher than C32-116 in these tissues. These results demonstrate that very subtle structural differences at the chain ends may have significant effects on the tissue distribution of polymer-DNA complexes. This suggests that end modifications may be a useful strategy to design targeted delivery systems, provided that the attachment of the targeting agent does not compromise the transfection ability of the polymer.

4.4.10 Intravenous Gene Delivery

The ability of C32 polymers to mediate gene delivery via an intravenous route was investigated by administering tail vein injections of polymer-DNA complexes. For these experiments, only half the quantity of polyplexes were injected compared to the intraperitoneal delivery route. Initial experiments showed that this reduced dose is the maximum that can be tolerated by mice. Similar to the intraperitoneal experiments, the luciferase plasmid was used to enable optical imaging and quantification of gene expression in whole body mouse images and harvested organs. Images and luciferase quantification were performed six hours after the injections.

An initial screening experiment was performed with the same nine polymers (C32-66, 87, 99, 103, 106, 116, 117, 121, 122) used in the intraperitoneal screens. Polymer C32-108 was also tested however this polymer needed to be withdrawn from the study since mice became significantly ill shortly following injection. For the nine polymers tested, tail vein injection of the polymer-DNA complexes resulted in relatively low levels of gene expression. Whole body images showed that most expression occurred in the side, chest and upper leg for most polymers (Figure 4.13). Similar to the intraperitoneal delivery studies, C32-103 and C32-117 emerged as the most efficient polymers for intravenous delivery. This suggests that C32 polymers terminated with propyl diamines may be optimal for *in vivo* DNA delivery in general. Interestingly, subsequent analysis confirmed that the gene expression from these polymer transfections was localized to the spine and hip bones. These results indicate that C32-103 or -117 may be useful in the gene-based treatment of late-stage prostate cancer, where metastasis frequently occurs to
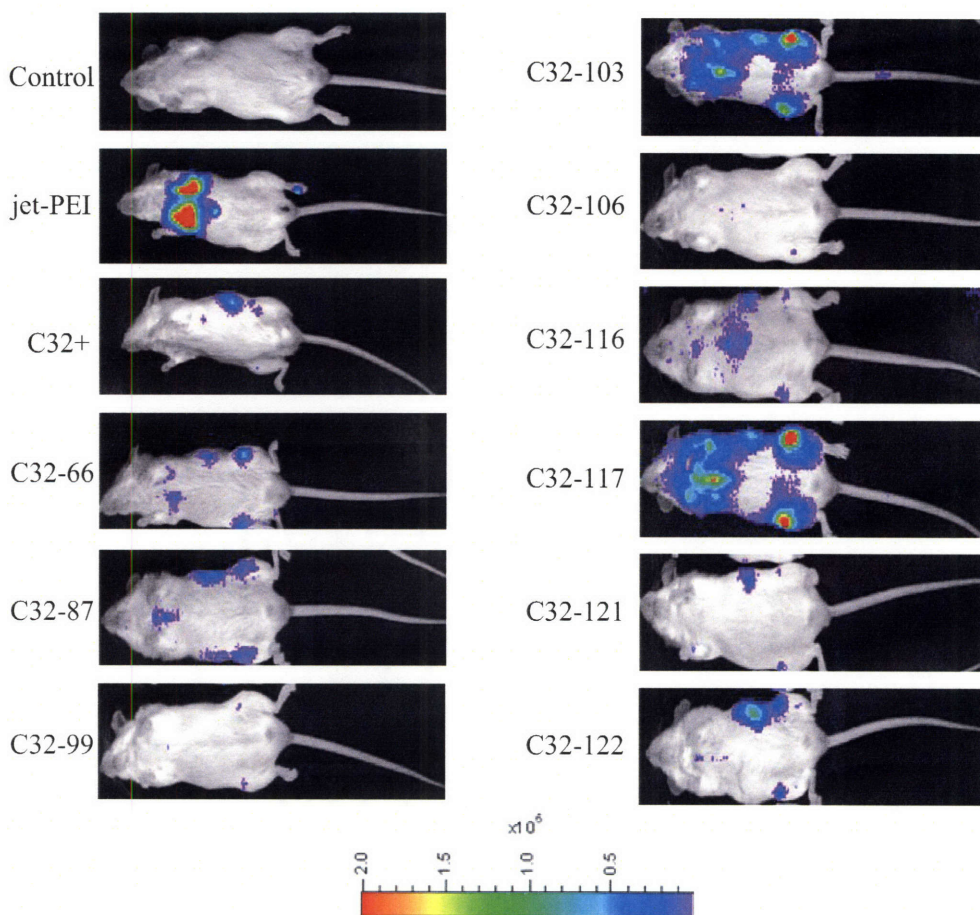
Figure 4.13 Whole body optical images of luciferase expression in FVB/J mice 6 hr after intravenous injection of polymer-DNA complexes. Images show the highest expression obtained for each polymer. Control mouse was injected with 120 μl of 50mM NaAc buffer, pH5.2. Pseudocolor images representing emitted bioluminescence are superimposed over grayscale images. RLUs/pixel are indicated in the color scale bar.

the spine and pelvis.[44] However, it needs to be noted that there was significant difficultly encountered in reproducing these transfection patterns.

Quantitative measurements of transfection in whole body images and harvested organs following intravenous injection were performed for C32-117, C32+ and jet-PEI. The measured RLU values as a function of the transfected organ for each polymer are shown in Figure 4.14. Similar to the intraperitoneal experiments, IV injection of jet-PEI resulted in overall expression levels that were approximately one order-of-magnitude higher than the untreated control mice. Most of the expression occurred in the lungs with lower levels appearing in the fat and liver. On the contrary, both C32+ and C32-117 showed low overall gene expression. The spleen was the only organ found for both polymers to have significantly higher transfection levels compared to the untreated mice. Polymer

C32-117 had additional expression detected in the lungs at levels elevated over control mice, but significantly less than those mediated by jet-PEI.
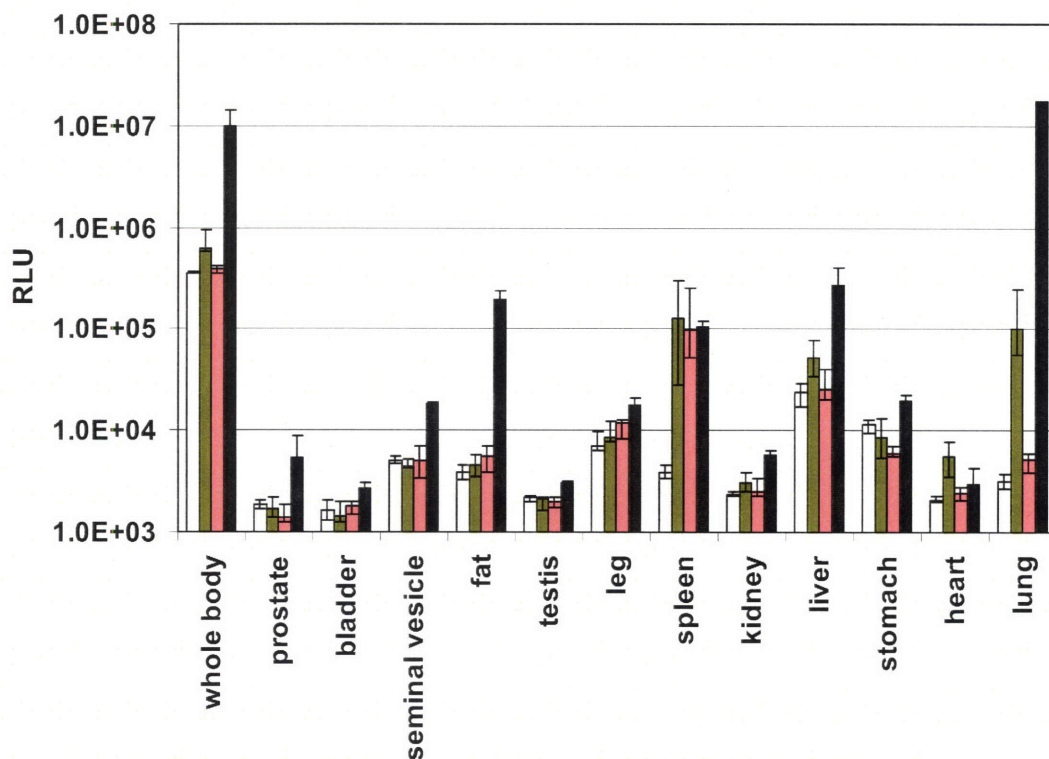

Figure 4.14 Quantification of luciferase expression in whole body and individual organs 6 hr after intraperitoneal injection of polymer-DNA complexes in FVB/J mice. Results are expressed as median transfection levels for a buffer control (white), C32-117 (green; n = 10), C32+ (pink; n = 6), and jet-PEI (black; n = 4).

4.5 Discussion

This chapter presents a general method for the functionalization of poly(β-amino ester)s through a selective end chain modification. The approach involves an initial synthesis of acrylate-terminated polymer chains followed by the conjugation of aminated molecules to these acrylate end groups. One significant advantage of this approach is the specific reactivity of the acrylates with amines, which permits many other functional groups such as hydroxyls, ethers, amides and aromatics to be incorporated in the side chains and end groups without any complex protection/deprotection steps.[2,4] This specificity in acrylate reactivity, coupled with the commericial availability of many different amine molecules, makes it possible to produce many end-modified variants of a single diacrylate-terminated polymer. In addition, the synthetic conditions employed for the amine

capping step were optimized at room temperature, in the absence of a catalyst, and with only a slight excess of amine so that the final end-modified polymers can be used without purification. As a result, this approach enables the parallel synthesis of many structurally diverse, end-modified polymers under conditions that allow them to be easily incorporated into high throughput protocols for assessing gene delivery efficiency.

Although an end-modification strategy is particularly advantageous for the development of functionalized poly(β-amino ester)s, few studies report similar methods for modifying other polycationic vectors. For the most common polymers such as PLL and PEI, ligands are usually conjugated to the backbone and/or side chains since these sites contain a high density of reactive amine nucleophiles.[45-47] End-modifications have been performed on these polymers but the high reactivity of interior functionalities usually necessitates a multi-step synthetic approach in which the inner amines are protected during the end chain reaction.[48] In these cases, almost all gene delivery applications to date have focused on the conjugation of polyethyleneglycol chains to improve blood stability and/or targeting agents to promote cell specific delivery.[48-51] A systematic analysis relating the end group structure to transfection efficiency has not yet been performed for most polycationic delivery agents.

Despite the paucity of other end-modified studies, it is demonstrated here that the terminal functionality greatly affects the transfection efficiency of poly(β-amino ester)s. Hydrophilic amines containing hydroxyl or additional amine groups proved to be the most effective capping agents. For the C32 polymer, transfection efficiency was improved by almost 30% by conjugating ethylenediamine to the ends (C32-102) instead of aminopentanol (C32+). Perhaps the most significant finding is that the optimal polymer:DNA weight ratio is lowered by choice of end group. In particular, polymers synthesized with primary diamine end functionalities exhibited optimal transfection at ratios as low as 10:1. These ratios are comparable to those used with PEI, however, the end-modified poly(β-amino ester)s have significantly lower charge density, less cytotoxicity, and higher transfection efficiencies.[6,8] These considerations are particularly important for *in vivo* gene delivery applications. Lower ratios minimize the polymer necessary for effective delivery and, therefore, minimize cytotoxic effects mediated by the polymer. In addition, the limited solubility of poly(β-amino ester)s in aqueous solutions requires a low polymer:DNA ratio for *in vivo* delivery.

The terminal amine structure is also shown to have a large effect on many polymer properties that are relevant to gene delivery. In general, amine termination with primary diamine molecules containing an aliphatic chain spacer results in end-modified polymers with the most favorable properties. Specifically, the polyplex size can be reduced from 150 to 86 nm in diameter by conjugating diaminodecane (#110) to the C32 ends. In addition, the cellular uptake of polyplexes was improved 5-fold (800 – 4000 plasmids/cell) by end-termination with several primary diamines. This increased level of endocytosis is most likely due to increases non-specific electrostatic interactions between the polymer and cell surface since (1) the additional primary amine at the ends adds a significant contribution to the overall cationic charge of the system, (2) similar increases in uptake were observed for secondary and tertiary amine capping agents, and (3)

polymer screening on two cells type, COS-7 and HepG2, did not reveal any significant differences that would suggest a potential ligand-receptor interaction. In any case, the large influence of the polymer end group structure on cellular uptake indicates that the terminal modification is a logical strategy for developing a targeted gene delivery system.

Very high transfection levels were observed following an intraperitoneal injection of polymer-DNA complexes. End-modified polymers resulted in over an order-of-magnitude higher reporter protein expression than both jet-PEI and C32+ in terms of whole body delivery and in several isolated organs. Perhaps the most surprising finding was that very subtle structural differences in the end-amine significantly altered the tissue expression pattern. This effect was most evident between the C32-117 and C32-116 polymers, were the former displayed over 80-fold higher delivery to the bladder, spleen and stomach. The only difference between these two diaminopropane end-capping reagents is the ethyl versus dimethyl branching.

Intraperitoneal gene delivery may be general strategy to treat an array of genetic diseases. One of the most widespread and heavily studied applications is cancer. Ovarian, pancreatic, and gastric cancers may be particularly amenable to this approach.[52-54] There is generally a poor prognosis associated with these cancers due to (1) the difficulty in diagnosis, (2) the lack of symptoms in the early stages, and (3) the generally low response to chemotherapeutic treatments.[55,56] In the late stages, metastasis occurs in which the cancer can spread throughout the peritoneum. Several studies have shown a positive response and suppression of peritoneal cancer growth using both viral and non-viral gene delivery approaches.[43,57-60] The end-modified poly(β-amino ester)s may be advantageous for these applications over the current delivery systems since they offer degradability, low cytotoxicity, and high expression relative to other non-viral systems. These characteristics are especially important for metastatic cancer in which multiple injections and several rounds of treatment may be necessary to eradicate the cancer. Despite their high transfection of several organs, tissue or tumor specificity may be engineered into the delivery system by conjugating targeting ligands and/or using tissue-specific promoters.

While intraperitioneal gene delivery was very effective with the end-optimized poly(β-amino ester)s, a weaker effect was observed following intravenous administration. The only notable difference was an improvement in lung delivery by C32-117 over C32+. While the reasons for their reduced delivery properties are not definitively established, it is most likely related to the serum stability of the polymer-DNA complexes. In vitro screening was performed in cell culture media containing 10% serum. Although the polymers proved highly effective under these conditions, their transfection efficiency in the absence of serum proteins is 4-fold higher. As a result, it may be that the higher concentration of negatively charged proteins in the circulation could shield, disrupt, or otherwise unfavorably interact with polymer-DNA complexes and prevent delivery. Grafting polyethyleneglycol or other inert polymers may be a beneficial strategy to improve poly(β-amino ester) serum stability, as shown for other polycationic materials.[61,62] The reduced transfection may actually be advantageous for the development of a targeted delivery system following intravenous injection. Since the

end-modified polymers are very efficient transfection agents, coupling targeting ligands and/or PEG chains could promote very high cell specific delivery via the circulation with very little delivery to non-target tissues. In other words, it may be easier and more beneficial to improve only delivery to the target tissues instead of having to concurrently reduce delivery to non-target tissues.

There are many parameters that can be varied to generate additional end-modified poly(β-amino ester)s that may be more optimal for gene therapy. In most of the analysis conducted here, the focus was restricted to one polymer (C32), one acrylate:amine ratio (1.2:1.0), and a small set of amine compounds that have proven useful in the synthesis of efficient poly(β-amino ester)s. In principle, other base polymers, monomer ratios, and amine-capping molecules could be explored since all of these parameters are known to greatly impact transfection efficiency. Alterations to the base polymer structure may be a particularly advantageous route for further development since (1) there are many commercially available diacrylate and amine monomers to generate structural diversity, and (2) previous studies have shown that small structural differences among polymers greatly influence their transfection characteristics.[4,6] Proof-of-concept experiments were performed to support this idea by end-capping the D60 polymer. A previous study has shown that even at its optimal amine:diacrylate ratio of 1:1, the transfection efficiency of this polymer is only 42% of that measured with C32+.[6] However, end-capping with primary diamines, as opposed to the #60 bis-secondary amine, resulted in D60 transfection efficiencies that are now almost 3-fold higher than C32+. This would suggest that it may be necessary to systematically optimize both the interior sequence and end amine concurrently, as opposed to sequentially as was done here by using the C32 polymer from a previous study.

In the extreme case, it may even be possible to convert otherwise ineffective polymers into highly efficient vectors simply by conjugating the appropriate amine capping molecule to the polymer ends. Proof-of-concept experiments to support this idea were performed by synthesizing and end-capping C20 polymers. Although this polymer is structurally similar to C32, it has a very low transfection efficiency at all amine:diacrylate ratios.[6] However, its effectiveness could be improved by over two orders-of-magnitude by end modification with a few different primary diamines (#107 and #108), reaching approximately 30% of the C32+ efficiency. This implies that it may be worthwhile to re-evaluate previously overlooked polymers with proper optimization of the terminal amine. Such a strategy may then sizably increase the library of potentially useful polymers for gene therapy applications. In addition, it could be a valuable method for the development and optimization of poly(β-amino ester)s that contain targeting ligands, serum stabilizers, or other functional groups linked to the polymer side chains.

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Chapter 5 – Development of Poly(β-amino ester)s with Galactose Ligands for Targeted Delivery to Hepatocytes

5.1 Summary

Gene delivery to hepatocytes could be a useful approach to treat a wide array of metabolic disorders and various forms of liver cancer. For these applications, it would be desirable to have a targeted system to improve hepatocyte transfection and minimize extrahepatic delivery. In light of this goal, this chapter presents three unique strategies to synthesize galactosylated poly(β-amino ester)s for targeted hepatocyte delivery and an evaluation of their transfection properties. Galactose was selected as the targeting ligand since it is known to bind with high affinity to the asialoglycoprotein receptor (ASGPR) on the hepatocyte cell surface. Using several highly effective end-modified poly(β-amino ester)s, end-chain conjugation of galactose molecules completely diminished the transfection activity, suggesting that either (1) the sugar is not binding to the ASGPR and facilitating uptake, and/or (2) these sugar-modified end groups are ineffective at overcoming other downstream gene delivery barriers. As a result, a second approach was devised that consisted of generating C32-galactose copolymers. The objective here was to couple the high transfection activity of C32 with the targeting effects of the galactose ligands. As expected, polymers with 20 – 30% of the side chains galactosylated maintained high transfection activity in hepatocytes with no measurable delivery to ASGPR-negative HeLa cells. However, this effect was determined to be non-specific since (1) high transfection levels were obtained on a second ASGPR-negative mesothelial cell line, and (2) excess free ligand did not reduce transfection relative to non-targeted polymers. As a result, a third approach was developed in which poly(β-amino ester)s were synthesized with 100% galactose side chains. A small library of polymers was prepared with variations in the backbone structure and terminal amine functionality. Some materials displayed high transfection levels in hepatocytes but also in the mesothelial cells. Taken together, all of these results suggest that simple galactose conjugation is an ineffective means to achieve hepatocyte specific delivery using poly(β-amino ester)s. In the future, higher affinity ligands such as multivalent galactose compounds and ASGPR antibodies need to be used and conjugated in such a way to maintain poly(β-amino ester)s activity.

5.2 Introduction

For gene therapy to be an effective and routine treatment option, a delivery system needs to be designed that can transport DNA to the target site in the body and efficiently incorporate it into desired cells.[1] In this respect, poly(β-amino ester)s have shown promise and possess several advantages over other delivery systems that motivate their continued development.[2-5] Relative to most other commonly used polycations, these polymers offer degradability, low cytotoxicity, and superior effectiveness as *in vitro* transfection agents.[2-6] Currently, however, the ability of poly(β-amino ester)s to delivery DNA *in vivo* is rather limited. Modest results have been realized with local administration of polymer-DNA complexes, where certain derivatives have shown some efficacy to retard the growth of cancer after intratumoral injection of polyplexes.[5] Systemic administration has met with less success though. Very low transfection levels are typically observed after intravenous injection of polyplexes, suggesting that the poly(β-amino ester) delivery systems are unstable in the circulation (see Chapter 4). Intraperitoneal injection of some polymer derivatives has resulted in notable transfection of several abdominal organs, but never in an organ or cell-specific fashion (see Chapter 4). The development of poly(β-amino ester)s into targeted delivery systems would provide an important advance in their development as a systemically applied therapy. Consequently, the challenge and objective then is to couple their effective tranfection characteristics with a new ability to recognize and localize to specific cell types.

The previous two chapters presented new chemical methods to design, modify, and functionalize poly(β-amino ester)s that may be useful for their further development into targeted delivery systems. Specifically, the novel PDA polymers and various derivatives thereof can serve as a platform for incorporating many high levels of functionality.[7] The thiol-reactive side chains are particularly advantageous as they enable the fast and selective covalent attachment of cell binding ligands such as peptides and antibodies, without compromising the charge density of the polymer backbone. The end-modification approach represents an alternative but complementary strategy to attach targeting agents. The reaction proceeds through an amine-acrylate addition so that any aminated molecule can be bound to the polymer terminus. Importantly, the terminal amine has been shown to strongly affect the cell uptake of polyplexes,[8] suggesting that end-chain optimization may be a favorable approach to achieve targeted or cell-specific delivery.

The liver is a particularly attractive organ for targeted gene therapy due to its critical involvement in normal digestive functions and the widespread occurrence of life-threatening disorders that arise from hepatic abnormalities.[9,10] Since it receives the entire blood supply from the intestine, the liver is designed to detoxify the blood and metabolize carbohydrates and lipids that come from oral ingestion.[11] Furthermore, it plays a central role in synthesizing blood proteins, including albumin and most of the globulins, which maintain the blood osmotic pressure, transport cholesterol and triglycerides, and participate in blood coagulation.[11] Genetic mutations and viral infection of the underlying hepatocytes are the cause of many devastating and ubiquitous diseases such as

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familial hypercholesterolemia, hemophilia, mucopolysaccharidoses, tyrosinemia, orthinine transcarboxylase deficiency, hepatitis and various forms of liver cancer.[12-14]

The first application of targeted gene therapy to the liver can be traced back almost 20 years to the pioneering work of Wu and Wu.[15] In this study, poly-L-lysine polyplexes were directed to hepatocytes by the action of polymer bound asialoorosomucoid protein, a ligand that binds with high affinity to the cell surface asialoglycoprotein receptor (ASGPR). Approximately 85% of the injected DNA payload was recovered in the liver, which was completely abolished when free asialoorosomucoid was co-injected, suggesting that a ligand-receptor binding event is necessary for delivery. Since this study, many others have been published using other polycation delivery systems and hepatocyte specific ligands.[16-21] Although conflicting results appear in the literature, some studies suggest that a single galactose unit is the only element in the ligand that is needed to bind to the ASGPR.[22-24]

This chapter presents the synthesis of galactose-bearing poly(β-amino ester)s and an evaluation of their ability to selectively transfect hepatocytes. Since no single approach has demonstrated high levels of cell-specific expression, the three main strategies that were attempted are described in detail which include the design of (1) sugar-terminated poly(β-amino ester)s, (2) C32-galactose copolymers, and (3) combined end- and side-chain galactose-modified polymers. For each approach, this includes the rationale, list of experimental procedures and analysis of the main results.

5.3 Antibody Staining of HepG2 and HeLa Cell Lines

Before developing a liver-directed delivery system, it is necessary to first confirm the presence and uniqueness of the ASPGR to the hepatocyte surface. The HepG2 hepatocellular carcinoma cell line was used as the positive control cell type since it is known to express the ASGPR at high levels.[25] The HeLa cell line, an epithelial adenocarcinoma isolated from the cervix of a patient, was used as a negative control cell line since it has been reported to express low or undetectable amounts of the ASGPR surface protein.[22] The primary set of experiments then consisted of surface staining both the HepG2 and HeLa cells to establish that the specific HepG2 cell preparation used in the subsequent targeting experiments (and not the HeLa cells) contained high amounts of surface accessible ASGPR.

5.3.1 Antibody Staining Protocol

FACS buffer (FB) was prepared by adding 10 ml of FBS to 500 ml of Hank's buffered salt solution (Invitrogen; Carlsbad, CA). All antibodies (Ab) were obtained from Cell Sciences (Canton, MA) and diluted in FB to a final working concentration of 10 μg/ml. HepG2 and HeLa cells were grown to confluence, trypsinized, aliquoted and centrifuged (2000 rpm, 5 minutes) so that each sample preparation contained 500,000 cells. The media supernatant was aspirated and the cells were resuspended in one of four different antibody solutions (100 μl): (1) FB (untreated); (2) ASGPR Ab; (3) Control-IgG1 Ab; (4) FB. Cell/antibody solutions were incubated on ice in the dark for 30 minutes,

followed by a wash cycle. The wash consisted of the addition of 900 µl FB, centrifugation, aspiration, resuspension in 1 ml FB, centrifugation and aspiration. The cell pellets for preparations (1) – (4) above were then resuspended in 100 µl of the following solutions: (1) FB (untreated); (2) Anti-IgG1-PE Ab; (3) Anti-IgG1-PE Ab; (4) CD120-FITC Ab. Solutions were incubated on ice in the dark for 30 minutes, followed by a wash cycle as described above. The final cell pellets were resuspended in 100 µl of FB containing 5 µg/ml propidium iodide. Flow cytometry analysis was performed on the cells to determine the cellular fluorescence.

5.3.2 Results

Histograms depicting the cellular fluorescence of antibody-stained HepG2 and Hela cells are shown in Figure 5.1. For each preparation, the cell counts are given as a function of the individual cell fluorescence (artibrary light units). Untreated control cells for both lines were centered at the left between $10^0 - 10^1$ light units (blue; condition #1). Cells stained with the ASPGR primary Ab, and a secondary phycoerthrin (PE) Anti-IgG1 Ab
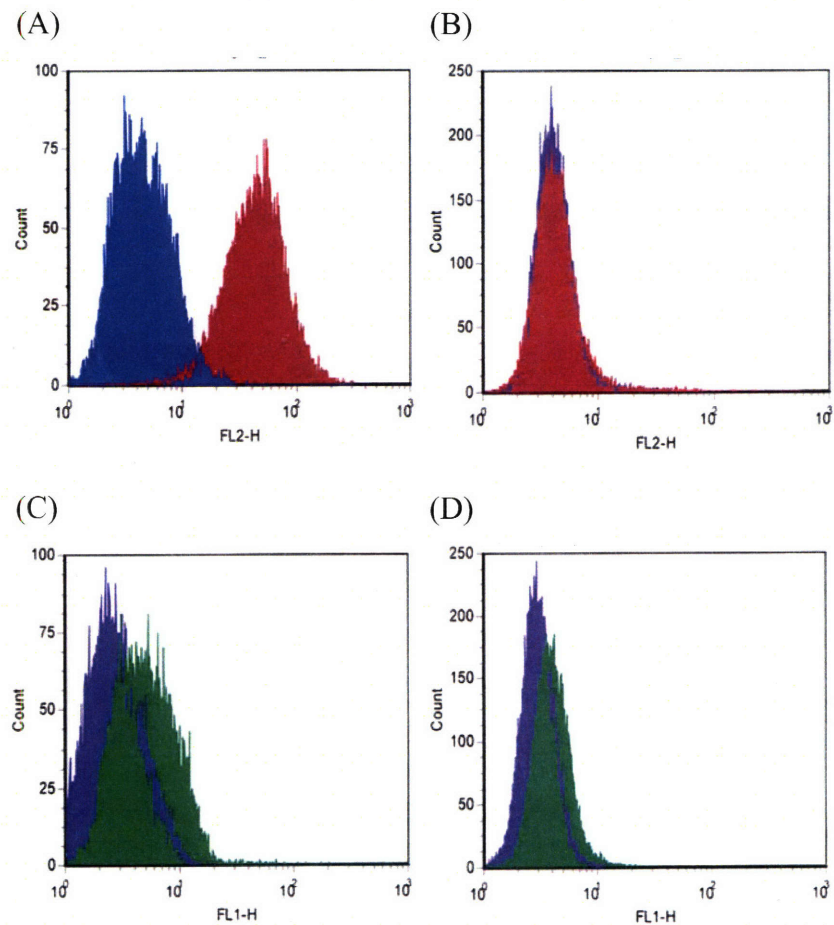
Figure 5.1 Antibody staining of (A) HepG2 and (B) HeLa cells to determine ASGPR levels. Antibody staining of (C) HepG2 and (D) HeLa cells verifying the expression of the CD120a surface protein for both cell lines. Histograms show the cell counts as a function of cellular fluorescence (FL1 = FITC; FL2 = PE).

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that binds to the primary Ab to provide a fluorescence signal, clearly show that only the HepG2 cells contain the ASGPR (Figure 5.1A,B; red; condition #2). In fact, the histogram peak shifts over an order-of-magnitude for the HepG2 cells with very few stained cells overlapping the untreated population, indicating that almost every cell expresses high ASGPR levels. Conversely, the HeLa cells show no detectable shift in the cell fluoroesence over the untreated control under the same antibody staining conditions, indicating that either the ASGPR is not expressed or that it is expressed at levels lower than the detectable threshold. To prove that the ASPGR binding was not due to the IgG1 portion of the Ab, a control staining experiment was performed in the same manner but using an artibrary IgG1 Ab instead of the ASPGR Ab (condition #3). As expected, there was no mesurable shift in the either cell population over the untreated cells (data not shown), indicating that the increased fluorescence observed with the HepG2 cells is not due to non-specific IgG1 binding to the hepatocyte surface. In addition, a separate Ab staining experiment was performed using a fluorescent CD120a-FITC Ab (green; condition #4). This antibody binds to the CD120a protein that is expressed on the surface of epithelial cells. Consequently, this staining condition should be positive for both cell types to prove that the HeLa cells can be stained, and that the negative result from the ASGPR Ab experiments is not due to an inaccessible or otherwise unstainable surface. As shown in Figure 5.1C,D, both cells demonstrate a small positive shift in fluorescence as expected.

These results collectively confirm that (1) almost 100% of the HepG2 cell population contains surface accessible ASGPR and (2) there is no detectable ASGPR expression on the HeLa cell surface. Therefore, these are adequate positive and negative control cell lines, respectively, that can be used to evaluate the targeting effects of poly(β-amino ester)s containing ASGPR-binding ligands.

5.4 Sugar-Terminated Poly(β-amino ester)s

The last chapter presented the effectiveness of end-modified polymers, and the large effect that the terminal amine has on cell uptake of polymer-DNA complexes. As a result, this section examines the end-conjugation of lactose ligands for targeted delivery to hepatocytes via the ASGPR.

5.4.1 Synthesis of Lactose-Conjugated Poly(β-amino ester)s

The conjugation of lactose to the terminal amines of poly(β-amino ester)s was carried out by mixing an NHS ester lactose derivative with polymers in DMSO. Briefly, end-modified polymers were prepared following the DMSO protocol outlined in the last chapter. The final working concentration was 100 mg/ml polymer in DMSO, which corresponds to 50 mmol/ml of end-amine groups (given a number average molecular weight of approximately 4 kDa). To eppendorf tubes, mono-(lactosylamido) mono-(succinimidyl) suberate (Pierce Biotechnology; Rockford, IL) was added between 1.5 – 3.0 mg along with the addition of polymer solution. The volume of polymer solution added was adjusted so that the concentration of lactose ligand was 50 mmol/ml, exactly a

1:1 molar equivalent with polymer end-groups. The solution was vortexed for two hours and assayed immediately for transfection on HCC cells as described in the last chapter.

5.4.2 Synthesis of Galactose-PEG Poly(β-amino ester)s

Galactose-PEG-NH$_2$ polymers were synthesized following a two-step procedure. Briefly, either 36.2 mg of 1-amino-1-deoxy-β-D-galactose (1-AG; Sigma-Aldrich; St. Louis, MO) or 43.2 mg of 2-amino-2-deoxy-D-galactopyranose hydrochloride (2-AG; Sigma-Aldrich) was dissolved in 1 ml of DMSO containing 60 ml triethylamine and added to 356.0 mg or 355.4 mg of FMOC-PEG-NHS (Nektar; Huntsville, AL), respectively. The reaction was mixed for three hours at room temperature. FMOC-PEG-AG polymer was precipitated by dropwise addition of the DMSO solution to 40 ml of diethyl ether. Polymer was isolated by centrifugation and washed with 20 ml ether. The pellet was redissolved in 2 ml of DMF and 500 μl of piperidine was added to remove the FMOC protecting group. The mixture was stirred for 30 minutes followed by precipitation in excess ether to isolate the AG-PEG-NH$_2$ polymers. Each polymer was then dissolved in 10 ml of water, added to a 1000 MWCO dialysis tube, and dialyzed against pure water for two days. The final solution was then lyophilized to give 188.4 mg of 1-AG-PEG-NH$_2$ and 192.3 mg of 2-AG-PEG-NH$_2$. Each polymer was dissolved in DMSO to a concentration of 0.25 M.

Acrylate-terminated C32 polymer (C32-Ac) was synthesized as described in the previous chapter and made to 31.13 wt% in DMSO. End conjugation reactions were performed by mixing 208 mg of AG-PEG-NH$_2$ solution with 40 mg of C32-Ac solution overnight at room temperature. The transfection activity of both polymers was assayed as described in the last chapter.

5.4.3 Results

Lactose was conjugated to the terminal segments of end-modified poly(β-amino ester)s to target and potentially improve gene delivery to hepatocytes. The transfection results for several lactosylated polymers over a wide range of polymer:DNA ratios are shown in Figure 5.2. Measurements of the expressed luciferase reporter protein are given in relative light units (RLUs). Transfection levels of the non-lactosylated end-modified polymers (NL) are shown in the left column. These results show that conjugation of a short lactose molecule to the polymer end segments completely diminishes the gene delivery ability. However, it is not clear at which level of the transfection pathway that the inefficiency occurs. It is suspected that cell binding is likely compromised since the end-chain charge, which is important for uptake, is neutralized by lactose conjugation (i.e., the positively charged secondary amine is converted into an uncharged amide). While this is beneficial to reduce non-specific electrostatic interactions, it suggests that the lactose ligand is not interacting with and binding to the ASGPR on the hepatocyte surface.

The lack of binding to the hepatocyte receptor may be due to insufficient spacing between the terminal lactose molecules and the polymer/DNA complex surface. For this reason, polyethyleneglycol (PEG) is frequently used as a molecular extension to present cell-binding ligands and improve the targeting effects of polycation delivery systems.[26]
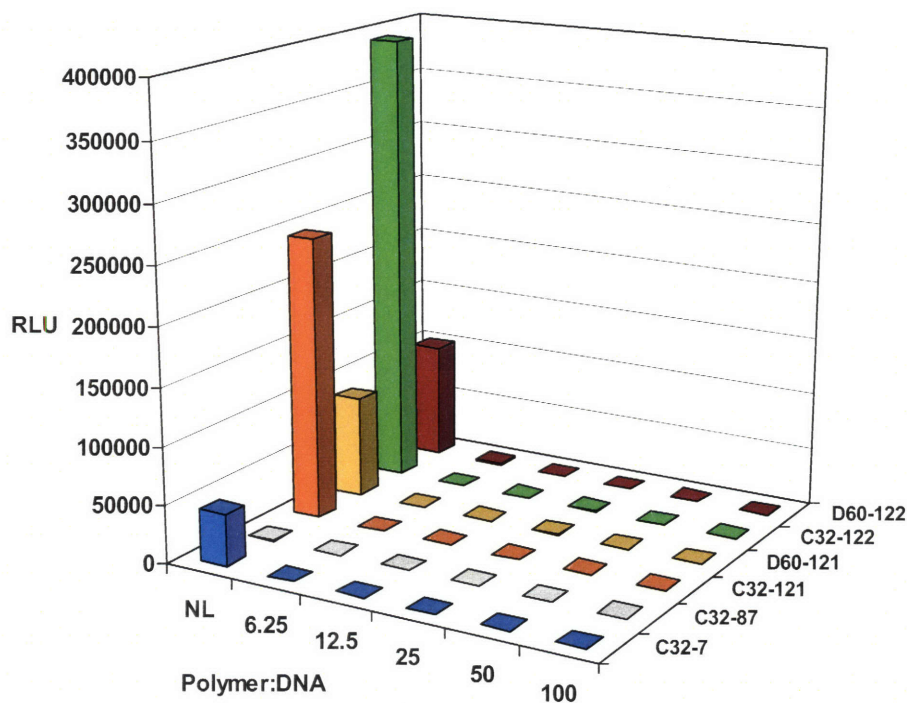
Figure 5.2 Transfection of hepatocyes with end-lactosylated poly(β-amino ester)s as a function of the polymer:DNA ratio. Non-lactosylated (NL) polymers are shown on the left at a 25:1 polymer:DNA ratio, except for C32-7 (100:1).

However, end-conjugation of galactose-PEG to C32-Ac did not improve the transfection ability to any measurable level (data not shown). Although uptake studies were not explicitly conducted, the extended galactose molecules may not be interacting with the ASGPR. Some studies report that galactose-bearing ligands must be multivalent (i.e., contain multiple galactose groups with strict spacing requirements) and presented in an appropriate conformation/orientation to effectively bind to the ASGPR,[27,28] although polycation targeting studies can indicate otherwise.[22-24] It must also be considered that these particular galactosylated end groups may be insufficient to overcome other cellular transfection barriers such as endosomal escape and nuclear import. In any case, for end-modified polymers to be effective as targeting agents, it is critical to (1) eliminate non-specific electrostatic interactions and uptake into non-target cells, (2) conjugate ligands at the appropriate density and orientation to improve target cell uptake through the desired ligand-receptor interaction, and (3) maintain or improve the target cell transfection capabilities. While it seems obvious to conjugate cell-binding ligands to the poly(β-amino ester)s to provide cell-specific uptake, to do so and maintain transfection activity is apparently non-trivial.

5.5 C32-Galactose Copolymers

The inability of end-modified sugar polymers to transfect hepatocytes suggested that (1) the ligand was not accessible for binding, and/or (2) the terminal structures explored are limited by downstream tranfection barriers such as endosomal escape, cytoplasmic transport, or nuclear import. As a result, it was reasoned that it may be more beneficial to utilize terminal amines that are known to be effective (e.g., aminopentanol) and conjugate galactosyl targeting ligands to the polymer side chains. Since studies with PEI indicate that low densities of lactose can increase target cell specificity,[23] a series of C32 copolymers were synthesized containing various amounts of the thiol-reactive 2-(2-pyridyldithio)-ethylamine (PDA) monomer. In this way, the inherent transfection activity of C32 can be potentially coupled with the reactivity of the PDA side chains, which can be used to attach targeting ligands.[7]

5.5.1 Synthesis of Thiol-Galactose

Thiolated galactose (Gal-SH) was prepared by mixing 63.3 mg of 4-aminophenyl-β-D-galactopyranoside (0.234 mmol; Sigma-Aldrich) with 32.6 mg of 2-iminothiolane hydrochloride (0.234 mmol; Sigma-Aldrich) in 1 ml of DMSO for one hour. The reaction proceeded to near completion as determined by both amine disappearance (using the fluorescamine assay) and thiol appearance (by reacting with 2,2'-dipyridyl disulfide and measuring the absorance of 2-mercapopyridine released). The final Gal-SH solution was used immediately for conjugation to C32-PDA copolymers.

5.5.2 Synthesis and Testing of C32-PDA and C32-Gal Copolymers

The PDA monomer was synthesized as outlined in Chapter 3 and freeze-dried overnight to remove the entrained water. A series of C32-PDA copolymers was synthesized by varying the relative amount of aminopentanol (32) and PDA amine monomers. Polymers were prepared at a 1.2:1.0 amine(total):diacrylate molar ratio, with the total amine amount composed of 0 – 50% mol PDA (balance is 32). Polymerization was performed using ~70 mg of diacrylate and the corresponding amount of amines.

Characterization of the C32-PDA copolymer side-chain reactivities toward Gal-SH was carried out as described in Chapter 3. For the preparation of C32-Gal polymers, C32-PDA polymer was dissolved in DMSO and mixed with a volume of Gal-SH solution so that the final polymer concentration was 100 mg/ml and the thiolated sugar was in 1.5 – 1.7 fold molar excess over PDA side chains. The reactions proceeded for two hours at room temperature. Transfection assays were performed immediately after synthesis as described in the previous chapter but using OptiMEM instead of cell culture media for polyplex dilutions. Ligand competition experiments were performed in essentially the same format except that asialofetuin (AF) or lactose monohydrate was dissolved in the OptiMEM before polyplex addition.

5.5.3 Results

A novel thiolated galactose derivative (Gal-SH) was synthesized by reacting an aminophenyl-galactose molecule with 2-iminothiolane (Figure 5.3). The use of a 1:1
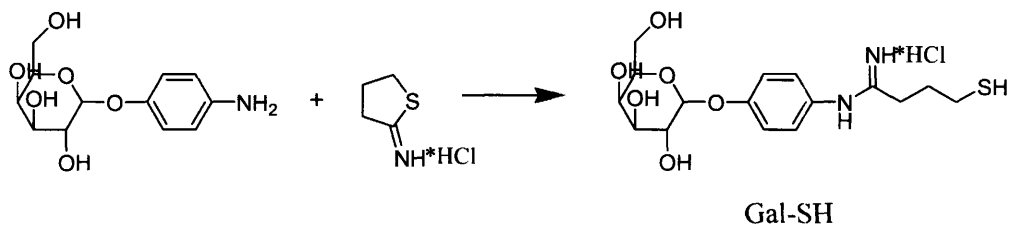
Figure 5.3 Synthesis of thiolated galactose (Gal-SH) by reaction of aminophenyl-galactose with 2-iminothiolane*HCl

molar ratio between reactants at concentrated conditions in DMSO enabled the conversion to reach 100% within one hour, as measured by amine disappearance and thiol formation. As a result, the newly formed Gal-SH product could be used directly for polymer conjugation without prior purification. In every instance, this molecule was made fresh and used within one hour before any measurable free thiol oxidation could occur to yield the symmetrical galactose disulfide.

C32-PDA copolymers were made by mixing both the aminopentanol and PDA amine monomers with 1,4-butanediol diacrylate. The PDA amount was varied between 0 – 50% of the total amine content, so that the final polymer always consisted of a 1.2:1.0 total amine:diacrylate ratio. In this way, each copolymer had amine terminated chains, regardless of the PDA content. Thiolated galactose was added to each copolymer to generate the corresponding C32-Gal copolymers (Figure 5.4)
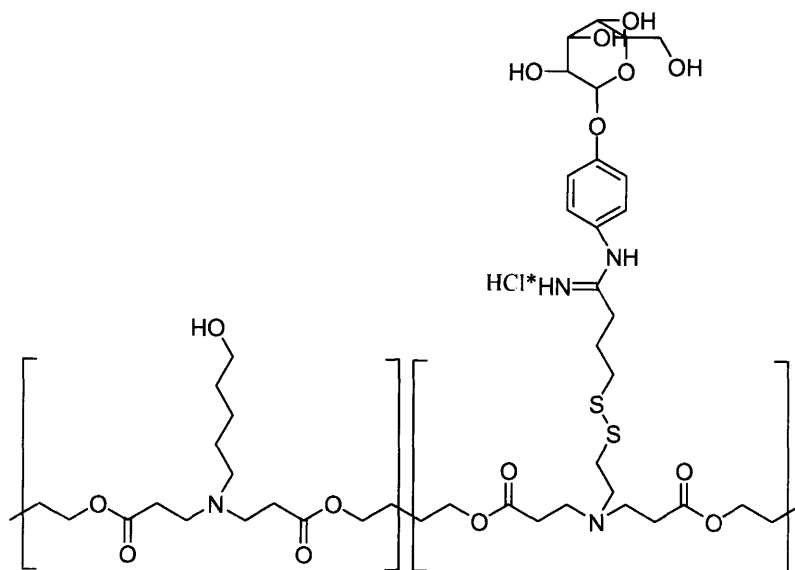
Figure 5.4 C32-Gal copolymer. A series of copolymers was synthesized containing 0 – 50% galactose side chains, the balance consisting of aminopentanol side chains.

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Reaction and conjugation of the Gal-SH sugar to the C32-PDA copolymer side chains was assessed using a simple colorimetric assay. Polymer solutions were mixed with Gal-SH solutions at varying concentrations to determined the minimum ratio of Gal-SH:PDA required to attain 100% side chain conversion. The absorbance of the 2-mercaptopyridine (2-MP) released was correlated to the amount of Gal-SH bound using a 2-MP calibration curve. The side chain conversion for each C32-PDA copolymer as a function of the Gal-SH concentration is shown in Figure 5.5. These results were obtained after 10 minutes and did not show any changes even after 60 minutes, indicating that the reaction has reached completion at each concentration. In addition, these reaction kinetics are similar to those observed between mercaptoethylamine and C-PDA,[7] reflecting both the high reactivity of the pyridyldithio functionality and accessibility (and nucleophilicity) of the ligand sulfhydryl group. For each copolymer, a linear rise in the absorbance and side chain conversion is seen as the Gal-SH concentration is increased. As expected, a maximum absorbance corresponding to 100% side chain conversion is reached for all copolymers, the level of which directly correlates to the PDA content. In all cases, however, a 1.5 – 1.7X molar excess of Gal-SH over PDA side chains is necessary for complete side chain conversion.

The transfection ability of each copolymer was evaluated on both HepG2 and Hela cells. These results are shown in Figure 5.6 for both the C32-PDA and C32-Gal copolymers. The transfection levels, measured in relative light units of luciferase reporter protein expression, are given for each polymer as a function of the polymer:DNA weight ratio. While the C32 polymer was highly effective on hepatocytes at ratios exceeding 20:1, its copolymers with PDA were less effective (Figure 5.6A). The transfection activity tended
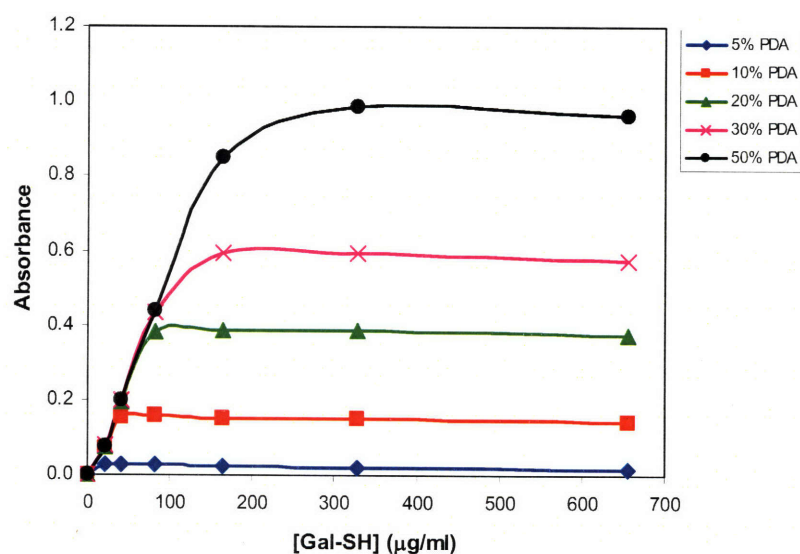

Figure 5.5 Side-chain reactivity of C32-PDA copolymers with the Gal-SH ligand in DMSO. Absorbance values, measured 10 minutes after Gal-SH addition, are shown for each copolymer as a function of the Gal-SH concentration.

to decrease at all polymer:DNA ratios as the percentage of PDA in the copolymer increased. These results are not surprising since pure C-PDA polymer does not measurably transfect hepatocytes.[7] Similar activity relationships are seen on HeLa cells, however, the RLUs are significantly lower (Figure 5.6B).

Side-chain substitution of C32-PDA copolymers with thiolated galactose altered the transfection profiles in both cell lines (Figure 5.6 C,D). Significant improvements can be seen with the 10% C32-PDA copolymer, in which the RLUs increased over an order-of-magnitude by Gal-SH conjugation. However, the activity remained unchanged in HeLa cells, suggesting that this low level of sugar substitution is not sufficient for cell targeting. On the other hand, higher levels of galactose attachment did show cell selectivity. The 20% and 30% C32-Gal copolymers, in particular, were the most promising, where both demonstrate significant transfection of hepatocytes with no measurable delivery to HeLa cells.
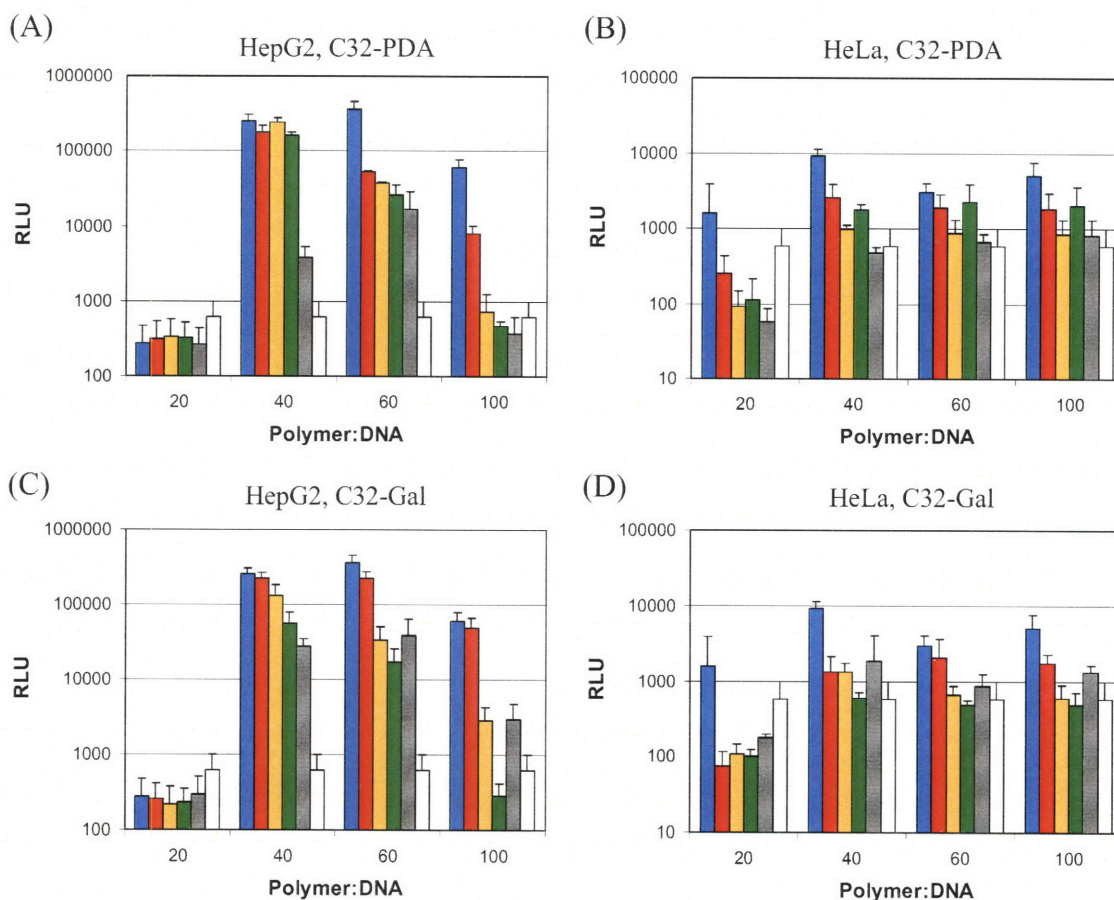
Figure 5.6 Transfection of HepG2 and Hela cells with C32-PDA and C32-Gal copolymers. Mean expression levels (in RLUs) are shown as a function of the polymer:DNA ratio (± SD, n=4). For each ratio, the %PDA or %Gal content of the copolymer is given as follows (from left-to-right): 0% (blue; C32), 10% (red), 20% (yellow), 30% (green), 50% (gray), untreated (white).

Ligand competition assays were performed to determine if the transfection of hepatocytes by C32-Gal copolymers was due to receptor-mediated uptake via the ASGPR. In these experiments, cells were transfected with copolymer-DNA complexes in the presence of either lactose or asialofetuin (ASF), both of which are natural ligands that are known to bind the ASGPR. The transfection results are shown in Figure 5.7 for C32, PEI, and 30% C32-Gal copolymer as a function of the competitor concentration. The measured RLUs were normalized to the competitor free condition so that changes from this condition could be more easily visualized and assessed. At low concentrations of either lactose or ASF, there was no significant decrease in the RLU for any polymer, suggesting that either (1) the transfection does not proceed via the ASGPR, or (2) the competitor concentration is too low to affect the uptake levels. As the competitor concentration is increased, the activity of all polymers decreases. Such changes would imply that the C32-Gal polyplexes are endocytosed via the ASGPR, as reported for other galactosylated polymers. However, the uniform decrease in both C32 and PEI confound the results since these polymers do not possess any galactosylated segments that could bind to the ASGPR. For these materials, their complexes with DNA may utilize endocytotic pathways that indirectly or inconsequentially involve the ASGPR, such that excess ligand makes these uptake routes inaccessible. Therefore, if the C32-Gal does not proceed through the ASGPR, then the inability of this polymer to transfect HeLa cells may simply be the result of the already low activity of C32 copolymers on this cell line.

To further examine the cell selectivity of the C32-Gal copolymers, transfections were conducted on a third cell line. For these experiments, an extrahepatic mesothelial cell line was transfected along with the HepG2 cells over a range of polymer:DNA ratios (Figure 5.8). Unlike the HeLa cells, transfection of the mesothelial cells was very effective for every C32-Gal copolymer. In fact, the measured RLUs were always within
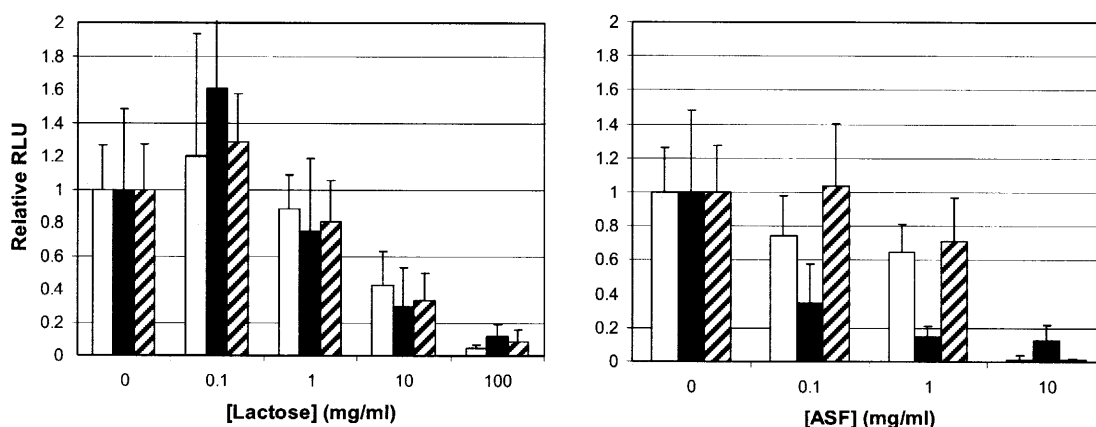

Figure 5.7 Ligand competition assay. HepG2 transfections were carried out using C32 (white), PEI (black) or 30% C32-Gal copolymer (hatched) in the presence of lactose or asialofetuin. Mean expression levels (normalized to competitor free levels) are shown for each polymer as a function of the competitor concentration (± SD, n=4).
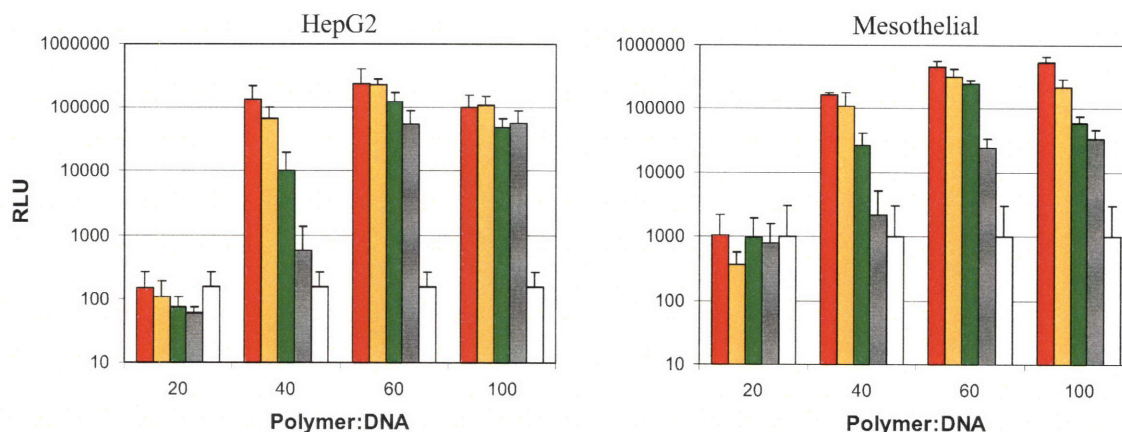
Figure 5.8 Transfection of HepG2 and mesothelial cells with C32-Gal copolymers. Mean expression levels (in RLUs) are shown as a function of the polymer:DNA ratio (± SD, n=4). For each ratio, the %Gal content of the copolymer is given as follows (from left-to-right): 10% (red), 20% (yellow), 30% (green), 50% (gray), untreated (white).

one order-of-magnitude between the cell lines. These results indicate that even if the copolymers are transfecting hepatocytes via the ASGPR, their physical properties do not prevent them from transfecting extrahepatic cell lines. Thus, these materials do not display the desired cell-selective delivery properties expected after galactose conjugation.

5.6 End-modified and Side-Chain Galactosylated Polymers

The PDA poly(β-amino ester)s are versatile platforms that can potentially be used to simultaneously optimize the backbone, side-chain, and terminal structures of a poly(β-amino ester) for certain gene applications.[7] Since PDA is a primary amine, the choice of diacrylate completely controls the backbone structure of the resulting polymer. In addition, the use of excess diacrylate over PDA yields acrylate-terminated chains that can be used to couple highly efficient terminal amine molecules. Finally, the PDA side chains display thiol-specific reactivity that can be used to conjugate a variety of thiolated ligands. By performing the amine end-capping reaction first, thiolated biomolecules can be attached selectively to the side chains. Furthermore, such coupling reactions can be performed on all side chain functionalities without necessarily altering the charge density and DNA binding of the polymer.

Given these advantages, a series of PDA poly(β-amino ester)s were synthesized that varied in backbone and end-chain structure. For each polymer, Gal-SH was conjugated to all side chains to examine the transfection activity of 100% galactose substituted polymers.

5.6.1 Synthesis of End-modified Galactose-Conjugated Polymers

The PDA monomer was synthesized as described in Chapter 3 and freeze-dried overnight to remove the entrained water. Acrylate-terminated C-PDA, U-PDA, and F-PDA polymers were also prepared as described in Chapter 3. Gal-SH was synthesized as described above.

Amine end-capping reactions were performed by dissolving acrylate-terminated polymers in amine solution (1.0 M in DMSO) to a final concentration of 500 mg/ml. Reaction tubes were agitated for four hours after which time a 5-fold (by volume) Gal-SH solution was added. Tubes were again agitated for 40 minutes to synthesize poly(β-amino ester)s containing 100% galactose side chains with varying internal (C, F, U) and terminal (103, 108, 122) composition. Transfection testing was performed immediately following synthesis as described in the last chapter except OptiMEM was used in place of cell culture media. Experiments were conducted on HepG2 and mesothelial cells, an alternate negative control cell line that does not express the ASGPR.

5.6.2 Results

As series of end-modified galactose polymers was synthesized using three PDA poly(β-amino ester)s as base materials (Figure 5.9). Acrylate-terminated base polymers were prepared by reacting PDA with an excess of C, F, or U diacrylate. These monomers were employed since they have been used in previous studies to synthesize highly effective poly(β-amino ester)s.[4,6] In addition, the structural differences between these three diacrylates provide some variation in the spacing between PDA or galactose side chains. Terminal amine modifications to these base polymer was performed with three different primary diamine molecules (103, 108, or 122), since these end-capping agents were found to be highly effective for other poly(β-amino ester)s (Chapter 4). Finally, reactions
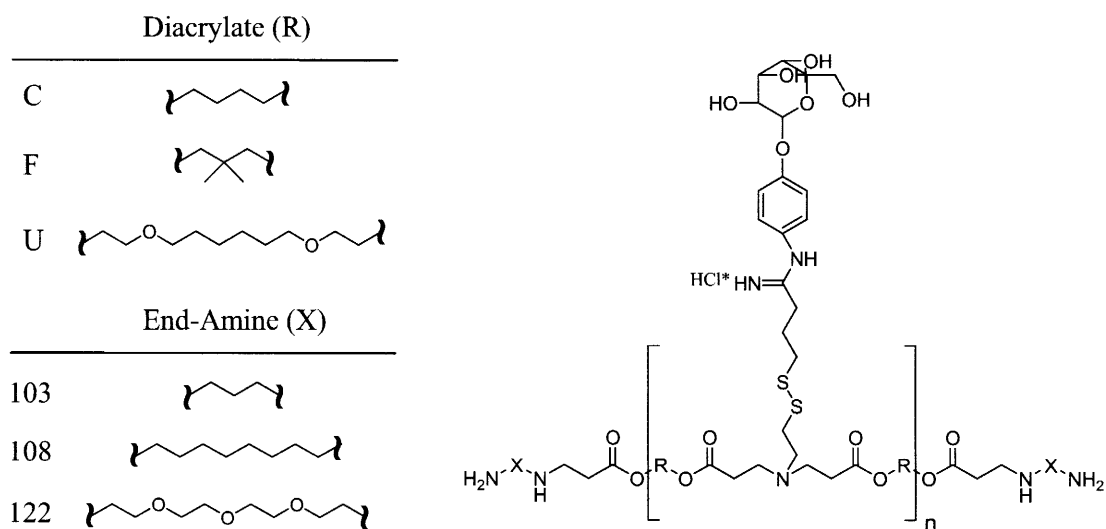

Figure 5.9 Structures of end-modified galactosylated poly(β-amino ester)s.

were performed with excess Gal-SH to incorporate galactose residues into all polymer side chains.

The transfection ability of the various galactosylated poly(β-amino ester)s is shown in Figure 5.10. The average RLUs are given for each polymer at four different polymer:DNA ratios. The most effective polymers discovered consisted of either the C or F diacrylate and the 103 or 108 amine end group. In fact, the maximum average RLU for the most effective polymer, F-Gal-103 (38,404), was 20% of the level measured for an optimal C32 formulation (195,417; not shown). Therefore, even though only three diacrylates and three end capping groups were evaluated, some polymers have been found that have significant transfection activity on hepatocytes.

Similar to the C32-Gal copolymers, the most effective galactosylated poly(β-amino ester)s found, C-Gal-103 and C-Gal-108, were assessed in a side-by-side transfection experiment on both HepG2 hepatocytes and mesothelial cells. The results are shown in Figure 5.11 for each polymer on both cell lines at a range of polymer:DNA ratios. These experiments show that even with 100% galactose side chain substitution and effective terminal amine groups, hepatocyte-selective transfection cannot be achieved with these poly(β-amino ester)s. For both polymers at all polymer:DNA ratios, the hepatocyte RLUs, while higher on average than the mesothelial RLUs, are not statistically different.
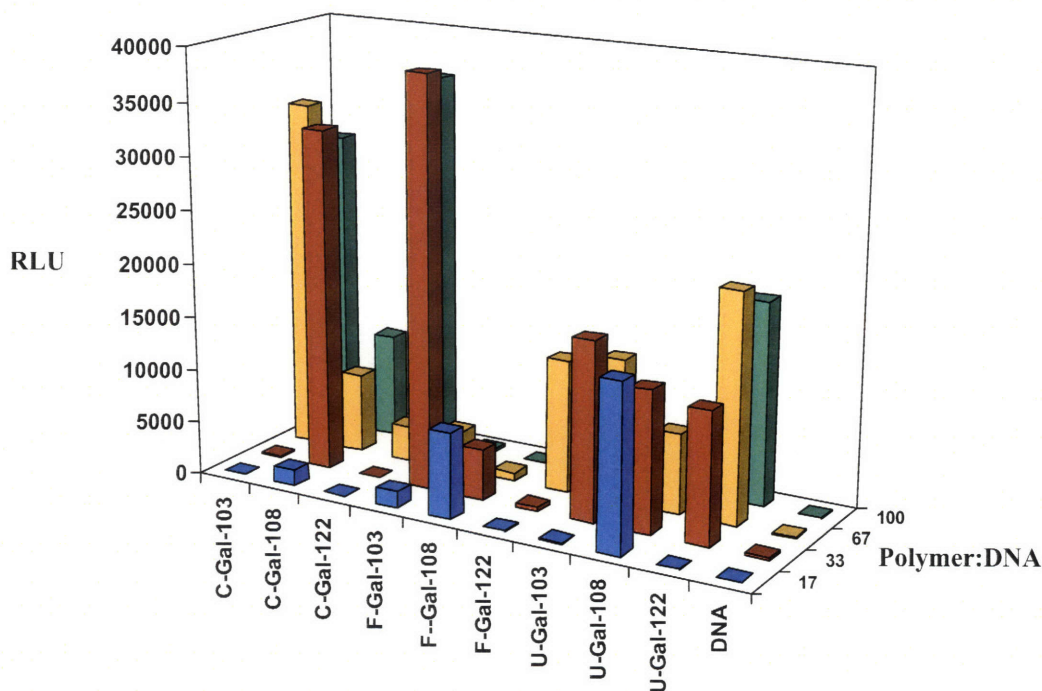

Figure 5.10 Transfection of HepG2 cells with end-modifed galactosylated poly(β-amino ester)s. Mean expression levels (in RLUs) are shown for each polymer as a function of the polymer:DNA ratio (n=4).
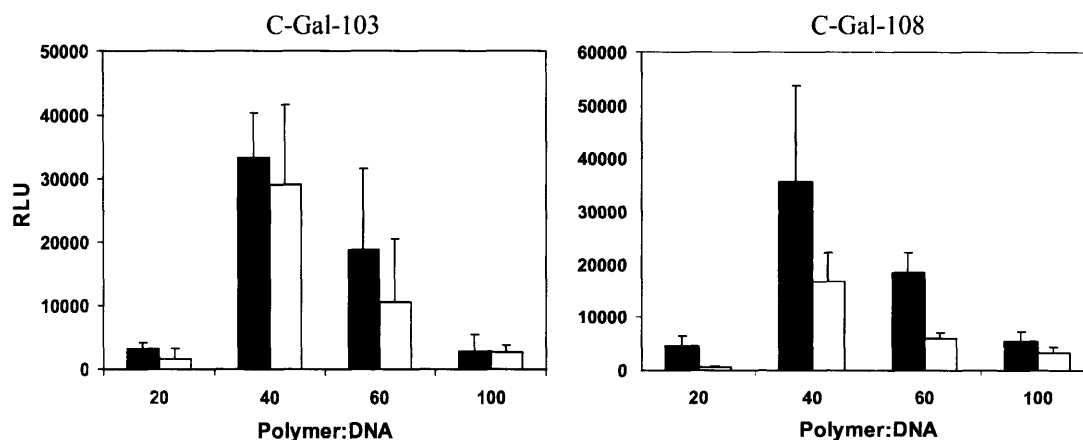
Figure 5.11 Transfection of HepG2 (black) and mesothelial (white) cells with C-Gal-103 and C-Gal-108. Mean expression levels (in RLUs) are shown for each polymer as a function of the polymer:DNA ratio (± SD, n=4).

5.7 Discussion

The introduction of new genes into hepatocytes has been extensively researched over the last 20 years. Expression of therapeutic RNA and/or protein in these parenchymal liver cells could be used to treat a variety of metabolic diseases and various forms of liver cancer.[9,10] For all of these applications, it would be desirable to have a non-viral delivery system capable of selectively and efficiently transfecting hepatocytes. This has been accomplished to varying degrees using common transfection agents, such as poly-L-lysine (PLL) or polyethylenimine (PEI), in combination with covalently attached targeting ligands that bind to liver cell surface receptors.[17,29] In the simplest case, galactose and lactose sugars have been used to attain high levels of hepatocyte-specific gene delivery via the ASGPR for several polycation transfection systems.[20,23]

One of the first delivery systems constructed for liver-directed gene therapy consisted of lactose-conjugated PLL.[22] This polymeric vector was synthesized by attaching an isothiocyanate lactose derivative to 35% of the PLL side chains. The remaining free amines were unmodified so that the polymer could electrostatically interact with plasmid DNA and form nanocomplexes sufficient for cellular endocytosis. Relative to bare PLL, the lactose-PLL polymer resulted in 60-fold higher transfection of hepatocytes. Importantly, low transfection levels were observed using a mannosylated-PLL polymer, indicating that delivery was proceeding in a ligand-specific fashion. Furthermore, only very low transfection levels were observed in ASGPR-negative HeLa cells using the lactose-PLL polymer, proving that delivery could be directed preferentially to hepatocytes. Since this initial study, several others have been published claiming to achieve similar liver-specific transfection by simply conjugating either galactose or lactose sugars to various polycations.[20,23,24]

Several strategies were examined to synthesize functional galactose- and lactose-containing poly(β-amino ester)s for targeted gene delivery to hepatocytes. Since the terminal amines have been shown to strongly affect cell uptake, the initial set of experiments consisted of selectively conjugating lactose ligands to the polymer end chains. This approach was an attempt to replace or modify the terminal amine, which facilitates uptake by non-specific electrostatic interactions, with a ligand capable of binding to a unique hepatocyte surface receptor. Unfortunately, for a series of highly effective end-modified poly(β-amino ester)s, end attachment of lactose drastically reduced the transfection ability of all polymers to the level of untreated cells. Several screening conditions were varied, including the DNA dose and polymer:DNA ratio, but no significant transfection was observed. The inability of modified polymers to transfect cells could be due to their ineffectiveness at any part of the transfection pathway. However, given the effects of end functionality on cellular uptake, the terminal lactose may not be adequately accessible or present at a sufficient density to mediate ASGPR binding and polyplex endocytosis. Some studies indicate that PEG spacers are necessary for ligand recognition by cell receptors and, for the ASGPR, multivalent sugar ligands are required that have much higher affinities than mono-galactose ligands.[17,18,27,29,30] Although some studies show improved targeted delivery using PEG extensions, no significant enhancement could be made with a PEG polymer. As a result, it may be beneficial to conjugate multivalent ligands that have a much higher affinity to the ASGPR compared to single galactose molecules.[27]

To incorporate higher ligand densities into a single polymer chain without compromising the end chain structure, a series of amine-terminated C32-PDA copolymers were prepared and subsequently modified to contain varying proportions of galactose side chains. Synthesis of C32-PDA copolymers was performed by mixing pure "C" diacrylate with an amine solution in a 1.2:1.0 amine:diacrylate molar ration. The amine solution consisted of a blend of the PDA and aminopentanol (32) monomers, containing between 0 – 50% by mole PDA. Subsequent reaction of each copolymer with a novel thiolated galactose ligand resulted in the formation of C32-galactose copolymers containing between 0 – 50% galactose side chains. In general, as the galactose content in the polymer was increased, the transfection levels in both hepatocytes and HeLa cells decreased. However, at 20 – 30% side chain substitution, significant expression could be measured in the hepatocytes while expression in the ASGPR-negative HeLa cells plummeted to the level of the untreated control. While this may be initially interpreted as a targeted delivery effect, subsequent transfections of an ASGPR-negative mesothelial cell line show similar expression levels to the HepG2 cells over a range of polymer:DNA ratios and galactose percentages. Furthermore, incubation with excess lactose or ASF protein uniformly decreased the transfection levels for C32, PEI and C32-Gal copolymers in a ligand competition experiment. These results strongly suggest that the enhanced transfection ability in hepatocytes over HeLa is not due to a ligand-receptor interaction, but simply due to the higher effectiveness of C32 polymers in the hepatocytes. More specifically, optimal C32 transfection of HepG2 cells results in RLU levels almost 600-fold higher than the untreated cells, as compared to the approximate 15-fold higher effect seen in HeLa cells. Therefore, if the activity of C32 is decreased as the copolymer content is increases, then it is not surprising that a copolymer formulation can be found

that diminishes the weak transfection ability on the HeLa cells while preserving relative high activity on hepatocytes.

Even in the extreme case where 100% of the polymer side chains contained galactose, a true targeted delivery effect could not be convincingly established. For these experiments, PDA poly(β-amino ester)s were combinatorially synthesized using three diacrylate monomers and three end capping molecules. Galactose ligands were subsequently attached to all of the PDA side chains to generate a small library of galactosylated poly(β-amino ester)s varying in backbone structure and end-chain functionality. The transfection levels of a few polymers were over two orders-of-magnitude higher than the untreated cell, similar to the C32-Gal copolymers. However, transfection levels in mesothelial cells were statistically equivalent, indicating that even fully substituted polymers cannot effectively target hepatocytes. This lack of cell specificity suggests that (1) ligands with a higher affinity to the ASGPR need to be used (e.g., multivalent lactose ligands), and (2) non-specific cell interactions, particularly those mediated by the terminal amines, need to be minimized.

Although many studies have been published demonstrating targeted gene delivery to hepatocytes, there are several studies that either contradicts these results or present non-targeted effects using simple sugar conjugation,[31-33] similar to the results observed here. For example, Kunath et al.[32] has shown that various lactosylated PEI polymers were all ineffective as targeted delivery vectors, opposite to the finding of Zanta et al.[23] Specifically, they found that regardless of the percent lactose conjugation, there were no statistical differences in transfection levels between hepatocytes and an ASGPR-negative fibroblast cell line. Furthermore, there were no significance differences between lactosylated-PEI and unmodified PEI, especially at low polymer:DNA ratios. Since the polymers used in both studies are identical, these contradictory results could be due to differences in polyplex formation, the transfection protocol, or other experimental procedures. Other studies have also been published with various polycations demonstrating an inability to target hepatocytes,[31,33] thereby lending some support to the negative results offered by Kunath et al.[32] Given these unfavorable findings, it is entirely possible that simple sugar conjugation to poly(β-amino ester)s is an ineffective means to confer hepatocyte-specific gene delivery.

From these experiments, it remains unclear how to develop poly(β-amino ester)s into targeted delivery systems for hepatocytes. Even though a complete loss of function was realized, it is still envisioned that end-chain optimization may be a promising strategy due to the large effect of the terminal amine on cell uptake. It may be necessary to utilize multivalent galactose ligands or an ASGPR antibody, either alone or in combination with PEG linkers.

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Chapter 6 – Thesis Conclusions

The main contribution of this thesis is to provide new chemical approaches for the synthesis and functionalization of poly(β-amino ester)s to enhance their gene delivery properties. This objective has been accomplished using several independent, but complimentary, approaches that include the development of new reactive monomers and strategies for modifying existing polymers.

One major focus of this thesis is on the design and use of a novel amine monomer, 2-(2-pyridyldithio)-ethylamine (PDA). The instability of this molecule prevented its isolation in the pure state. The major products of decomposition, namely 2,2'-dipyridyl disulfide and cystamine, contaminated the PDA preparation to some extent. In addition to affecting purity, cystamine induced partial crosslinking during polymerization that led to branched structures. Nevertheless, the PDA poly(β-amino ester)s demonstrated many properties that could be useful for the development of a highly efficient, targeted gene delivery system. In particular, thiolated ligands such as RGDC could be conjugated to the pyridyldithio side chains via a disulfide exchange reaction, without altering the charge density or compromising the DNA binding ability of the polymer. This reaction is highly specific for thiol functionalities and can occur over a broad pH range due to the nucleophilicity of the thiol group and the high reactivity of the pyridyldithio side chains. The 2-mercapopyridine by-product induces minimal cytotoxicity so that modified polymers may be used directly on cells without prior purification. Furthermore, this compound can be used to rapidly and conveniently to quantify the side chain conversion by absorbance measurements. And since the ligands are linked to the polymer backbone through disulfide bonds, it was shown that detachment or displacement could occur in the presence of glutathione, but only at intracellular concentrations. This suggests that partial breakdown of the polymer can occur in response to intracellular conditions, which may aid in DNA unpackaging inside the cell. These effects were demonstrated with a mercaptoethylamine-conjugated polymer but, theoretically, could be extended to other derivatives synthesized by a thiol reaction to PDA poly(β-amino ester)s. It was also shown that cationic polyplexes with diameters around 100 nm could be formed that transfected hepatocytes as effectively as polyethylenimine (PEI).

In addition to side chain reactions, this thesis presents methods for end-modifying poly(β-amino ester)s to attach targeting ligands and other functionalities to enhance gene delivery. A versatile two-step approach is provided that involves an initial synthesis of acrylate-terminated poly(β-amino ester)s followed by a post-polymerization amine capping step. Given the wide commercial availability of amine reagents, there are many possible end-modified derivatives that can be prepared from a single arcylate-terminated polymer. Furthermore, the specificity of the amine-acrylate reaction allows many

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different functional groups to be incorporated at the chain ends without the need for any complex, multi-step protecting group chemistries. Consequently, this end-modification strategy greatly expands the functional and structural diversity of the poly(β-amino ester) library. Importantly, synthetic conditions are given that so that end-modified polymers can be prepared rapidly in parallel, which enables the synthesis and screening of many derivatives in a short period of time.

Using this end-modification approach, the effects of terminal amine functionality on various gene delivery properties have been explored. The cellular transfection ability of poly(β-amino ester)s is particularly affected by the end-group choice. In general, hydrophilic amines with alcohol or pendant primary amines are the most effective end-capping agents, while more hydrophobic amines containing alkyl chains or aromatic rings are much less effective. Using C32 as a base material, its transfection efficiency could be increased by 30% and the polymer:DNA ratio lower 5-fold by conjugating primary diamine molecules to the chain ends. Unfortunately, cytotoxicity increases as well but is not unexpected since the cationic charge density is noticeably increased by this end-capping scheme. While there are many individual barriers to effective gene delivery, cellular uptake was found to be heavily affected by the end-chain structure. In fact, the low transfection efficiencies of many polymers could be explained by reduced endocytosis of their polyplexes. The most effective polymers, which contain primary diamine end groups, have the highest uptake levels due to the increased non-specific electrostatic interactions with the anionic cell surface. In addition to these cellular effects, the physical properties of the C32 polymers change dramatically depending on the terminal amine group. It appears that small polyplex diameters can be formed as the cationic charge and hydrophobicity at the polymer ends is increased. These same effects also increase the polymer affinity towards plasmid DNA. Therefore, altering the terminal amine functionality is a simple way to control and change the physicochemical properties of poly(β-amino ester)s. With respect to gene delivery, primary diamine molecules emerge as the most effective end-capping agents, resulting in the most favorable polyplex properties and transfection efficiencies.

The effectiveness of polymers terminated with primary diamines is also realized *in vivo* following systemic administration of polyplexes. Several such C32 derivatives out-perform the state-of-the-art C32 and PEI formulations, with expression levels over one order-of-magnitude higher overall and in several isolated organs following intraperitoneal injection. This elevated performance suggests that these materials may be useful as gene delivery vectors for the treatment of late-stage ovarian, pancreatic and gastric cancers that metastasize to the peritoneum. Much lower expression levels are observed after intravenous injection, with low expression occurring in the spleen and lung.

The methods for synthesizing new poly(β-amino ester)s and end-modifying existing ones were applied to develop a targeted gene delivery system. Galactosylated ligands, that have a high affinity for the asialoglycoprotein receptor (ASGPR), were conjugated to various polymers and evaluated for their ability to selectively transfect hepatocytes. Although the terminal amine greatly affects cell uptake, ligand conjugation to the ends of highly effective poly(b-amino ester)s completely diminished their activity on

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hepatocytes. While this effect could be due to inadequate interaction with the ASGPR and endocytosis, the modified polymers may be inefficient at overcoming other downstream transfection barriers. Consequently, a series of C32-galactose copolymers were prepared to couple the effectiveness of C32 with the targeting capabilities of the galactose ligand. Optimal percentages of galactose side chains were found that transfected hepatocytes selectively over ASGPR-negative HeLa cells. However, these effects were determined to be non-specific since (1) a third cell line, ASGPR-negative mesothelial cells, were transfected at high levels using all C32-gal copolymers, and (2) ligand competition experiments showed that the transfection levels could not be decreased relative to the non-targeted C32 and PEI polymers. Therefore, although these galactosylated polymers were ineffective as targeted systems, the use of higher affinity, multivalent ligands with less non-specific interactions could lead to the discovery of hepatocyte-directed polymers.

The work presented here provides a clear extension of previous studies with poly(β-amino ester)s and new approaches for their continued development. While past studies have focused on synthesizing new polymers from different commercially available amine-diacrylate combinations, this thesis presents new reactive monomeric species and strategies for post-polymerization modification. These chemistries and approaches have led to the discovery of highly effective, improved polymers for gene delivery and it is envisioned that their use will aid in the future development of poly(β-amino ester)s for targeted gene therapy.

Chapter 7 – Future Work

Over the past six years, poly(β-amino ester)s have emerged as an effective class of cationic polymers for gene therapy. The ultimate goal is to formulate materials that are efficient and useful vectors for the gene-based treatment of many inherited and acquired genetic diseases. Several studies have demonstrated this potential through the synthesis and testing of new poly(β-amino ester)s from commercially available starting monomers. As an extension of this work, this thesis presents new chemical methods and materials for the development of functionalized poly(β-amino ester)s with improved gene delivery properties. These approaches may be particularly useful in solving several problems that currently limit the widespread use of poly(β-amino ester)s for gene therapy.

(1) **Targeted gene delivery.** Gene delivery to a specific cell population can be useful to increase efficacy and minimize side effects to surrounding, non-target tissues. Given that the terminal amine has a large effect on uptake and transfection, end-modifications to poly(β-amino ester)s may be the best approach for achieving efficient, targeted delivery with these materials. Although ineffective using galactosylated ligands for targeted transfection of hepatocytes, this objective could be met simply by end-attaching ligands that have a high affinity for a specific cell surface receptor. In this case, the amine-acrylate reaction may be useful under the conditions present in Chapter 4. On the other hand, a more sophisticated approach could involve the synthesis of peptides or other oligomers from the chain ends. In fact, given the diversity of commercially available amine and diacrylates, and the tolerance of this chemistry to many functional groups, it may be possible to derive specific poly(β-amino ester) terminal "sequences" that confer cell specific delivery and high transfection. Other polymer chemistries could also be useful to generate end sequences including peptoids, poly(amido amine)s, and poly(sulfone amine)s. Since there are such a large number of potential sequences for any chemical approach, certain high throughput formats could be employed such as one-bead-one-compound (OBOC), iterative deconvolution, and positional scanning arrays. Regardless of the approach, it is necessary to screen materials on several cell types in parallel, after each iterative synthesis, to concurrently optimize the target cell specificity and transfection while minimizing non-targeted delivery.

Extending these concepts even farther, it is envisioned that the entire poly(β-amino ester) chain could be synthesized from a defined series of amine-acrylate reactions to generate whole polymer "sequences" that are highly effective. Different sequences could be designed that are optimal for certain gene therapy

applications, analogous to the sequence specificity of peptides for their intended uses.

(2) **Cellular transfection.** While poly(β-amino ester)s are highly effective transfection agents on many cell types, their performance can be rather limited when evaluated on non-dividing, primary cells. Although serious refinements could be made to the plasmid itself, it is almost certain that the delivery of the plasmid is inefficient and lacking as well. Therefore, central to the continued development of poly(β-amino ester)s as gene delivery vectors is a quantitative understanding of each cellular transfection barrier that limits their delivery in primary cells. In this regard, the PDA-based polymers could be useful for attaching fluorophores and other probes to track the cellular movement and localization of polyplexes. Following this analysis, these same polymers can be utilized as base materials for attaching a variety of enhancer molecules and improving the transfection capabilities.

(3) *In vivo* **effectiveness.** High-throughput synthesis and screening studies identified many end-modified poly(β-amino ester)s that have much improved *in vivo* effectiveness following systemic administration. In particular, C32-103 and -117 polyplexes result in high transfection levels in several abdomal organs following intraperitoneal injection. As a result, these materials could be useful for the gene-based treatment of late-stage ovarian or pancreatic cancer, in which significant metastasis occurs to the peritoneum. As a proof-of-concept, experiments are already in progress for treating metastatic ovarian cancer in a mouse model using C32-117 and a plasmid containing an ovarian-specific promoter. Expression of the diphtheria toxin A chain has shown significant extensions of the mouse survival rate compared to mice expressing a non-therapeutic luciferase protein. In the future, higher transfection of the ovarian cancer cells with much less delivery to non-target cells is clearly needed to improve the treatment efficacy.

In general, it is imperative to further increase the transfection levels and cell specificity following systemic administration. These functional improvements are especially needed following intravenous injection, in which only low expression levels are observed in the spleen and lung. Although no experiments were performed to identify the cause of their ineffectiveness, *in vitro* studies of the physical properties suggests that polyplex stability is compromised in the presence of serum proteins. In particular, polyplexes can aggregate substantially in these conditions due to charge interactions, which could cause them to get lodged in the fine capillary bed of the lung or spleen. Both the PDA and end-modification strategies could be used to design poly(β-amino ester)s that have greater resistance towards aggregation and electrostatic interactions with anionic proteins. Using these methods, it may be possible to attach polyethyleneglycol chains or other macrmolecules to improve polyplex stability in the circulation. In addition, targeting ligands could be incorporated to confer cell-specific delivery, as shown for many other polycation transfection systems.

(4) Microparticle delivery systems. In addition to polyplex formation, poly(β-amino ester)s have also been used in the fabrication of microparticles. These delivery systems are of special importance since they can physically encapsulate DNA or other hydrophilic drugs and passively target the payload to phagocytes such as macrophages and dendritic cells. Furthermore, their aqueous solubility properties allow for the pH-triggered release of their contents in response to the low pH environment inside endosomes. *In vivo* studies with poly(β-amino ester) microparticles have shown their ability to cause immune-mediate regression of tumors, presumably *via* delivery to dendritic cells. To further improve their properties and performance, PDA copolymers could be generated to retain the effectiveness of the parent material in the particle, and incorporate the advantages of molecular conjugation through the PDA moiety. Their use in a microparticle format could allow the attachment of peptides and other molecules that enable transfection of primary dendritic cells, which is currently non possible with these systems. And depending on the modifications, it may even be possible to fabricate materials that activate the dendritic cells, which is critical for up-regulation of co-stimulatory molecules and their subsequent activation of T-cells. Given the critical role of dendritic cells in the immune system, it seems feasible that the structural and functional characteristics of the microparticles could effectively control the ensuing immune response. Both the PDA and end-modifications approaches present here could be beneficial in this pursuit.

Appendix A – Supplementary material for PDA poly(β-amino ester)s

2-(2-Pyridyldithio)-ethylamine Hydrochloride (PDA*HCl) ^{1}H NMR
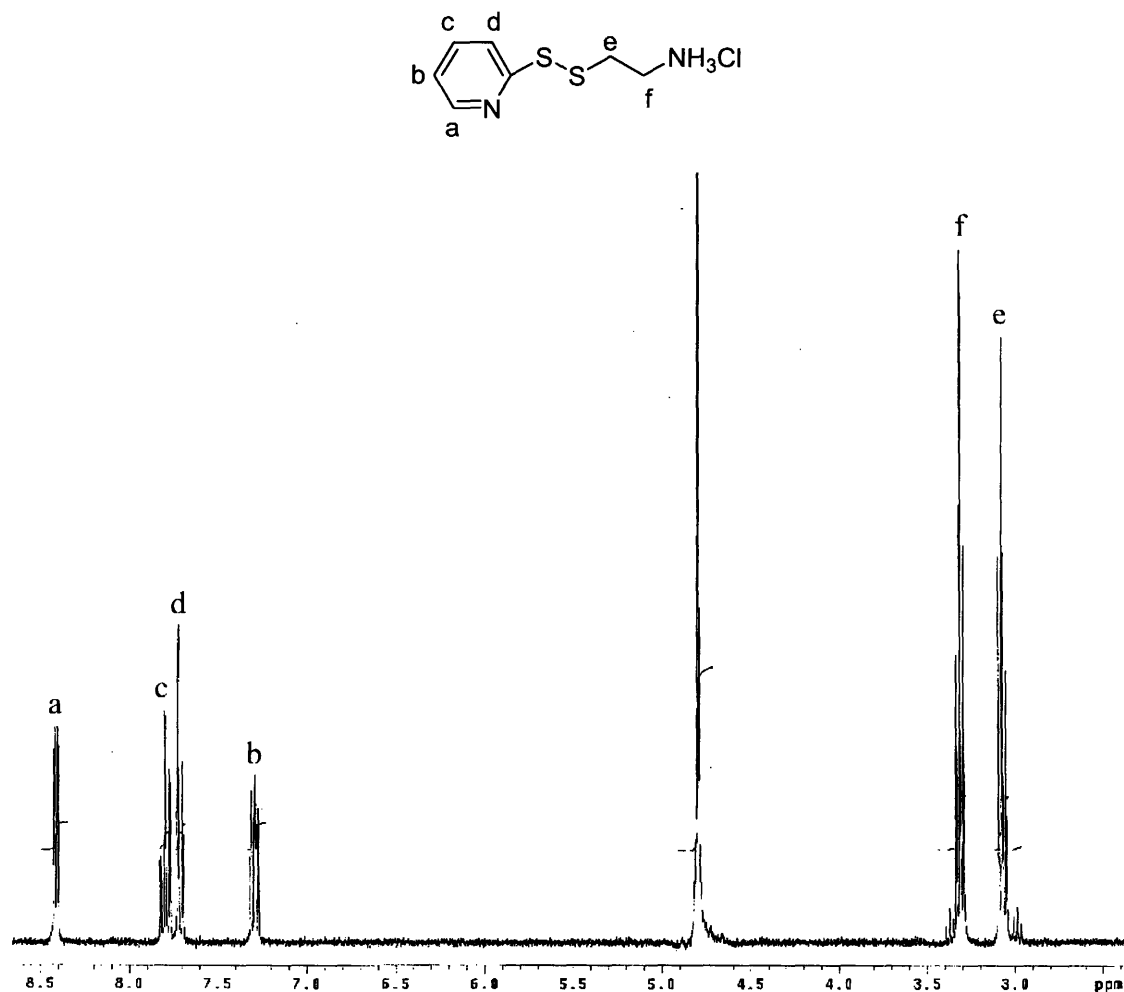
Figure A1 ^{1}H NMR of PDA*HCl in D$_2$O. This compound is 93-94% pure with the main cystamine*2HCl contaminant visible as two small triplet peaks at 3.0 and 3.4 ppm.
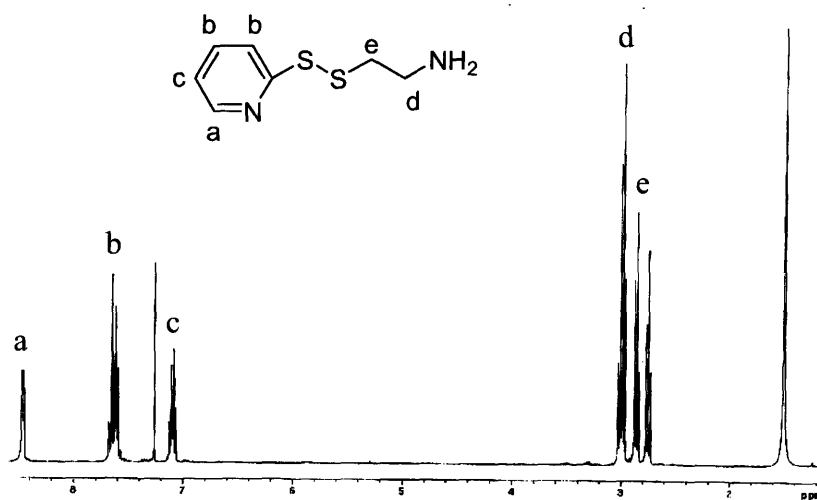
2-(2-Pyridyldithio)-ethylamine (PDA) Analysis
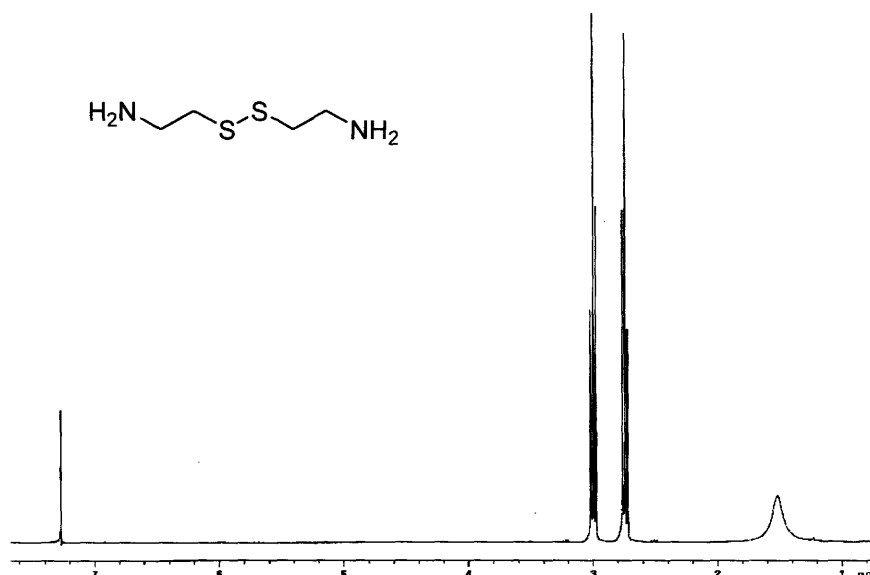

Figure A2: ^{1}H NMR of PDA in $CDCl_3$.

Figure A3 ^{1}H NMR of cystamine in $CDCl_3$.

The additional triplet peak at 2.8 ppm in the PDA spectrum is due to cystamine that is formed from PDA disproportionation. The second cystamine triplet peak overlaps with the PDA triplet at 3.0 ppm. Peak integration of the cystamine 2.8 ppm peak and the PDA triplet peak at 2.9 ppm indicates that there is an approximate 3.5:1 molar ratio of PDA to cystamine.

^{1}H NMR of PDA Poly(β-amino ester)s
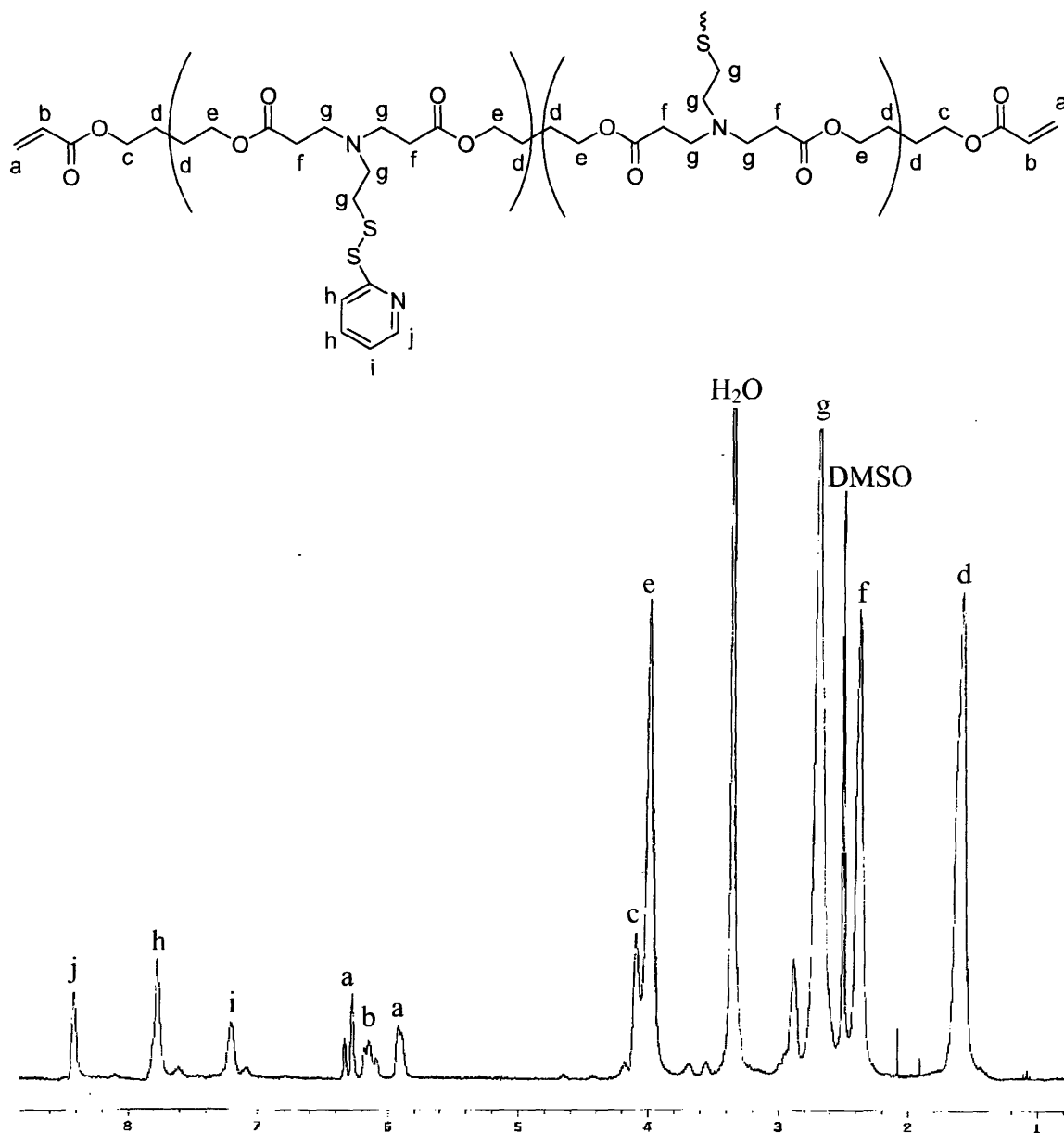
Figure A4: ^{1}H NMR of C-PDA in d_6-DMSO. For this polymer sample, and the F-PDA and U-PDA polymer NMR as well, a small preparation procedure was used to remove the excess 2,2'-dipyridyl disulfide. In a typical experiment, ~40 mg of polymer was dissolved in 100 µl of acetone and precipitated by the addition of 1 ml of ether. The polymer was then re-dissolved in 1 ml of d_6-DMSO and placed under vacuum (50 mbar) for 30 minutes to remove any residual ether and acetone. The percent crosslinks vs. pyridyl side chains was determined by peak integration of the pyridyl protons (h, i, j) compared to the aliphatic side chain protons (g) in the NMR.

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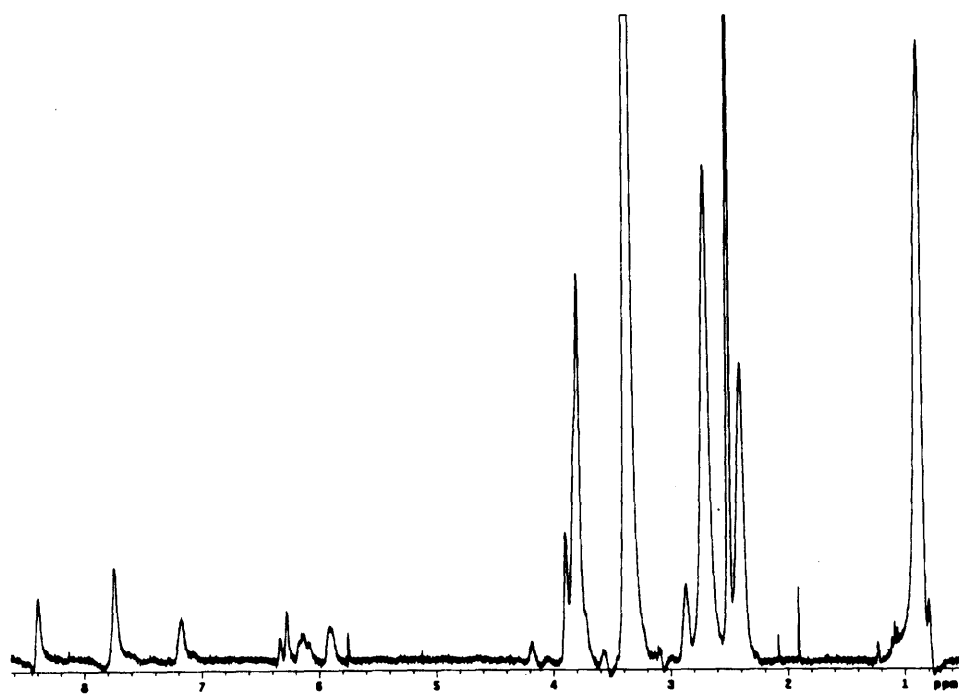
Figure A5: ^{1}H NMR of F-PDA in d_6-DMSO
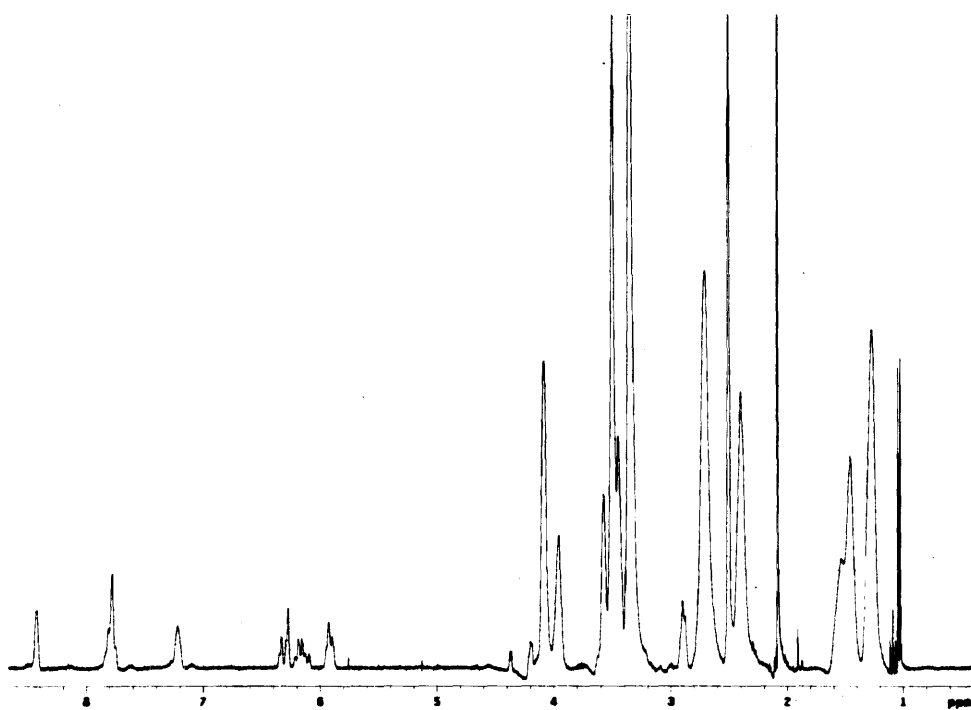
Figure A6: ^{1}H NMR of U-PDA in d_6-DMSO
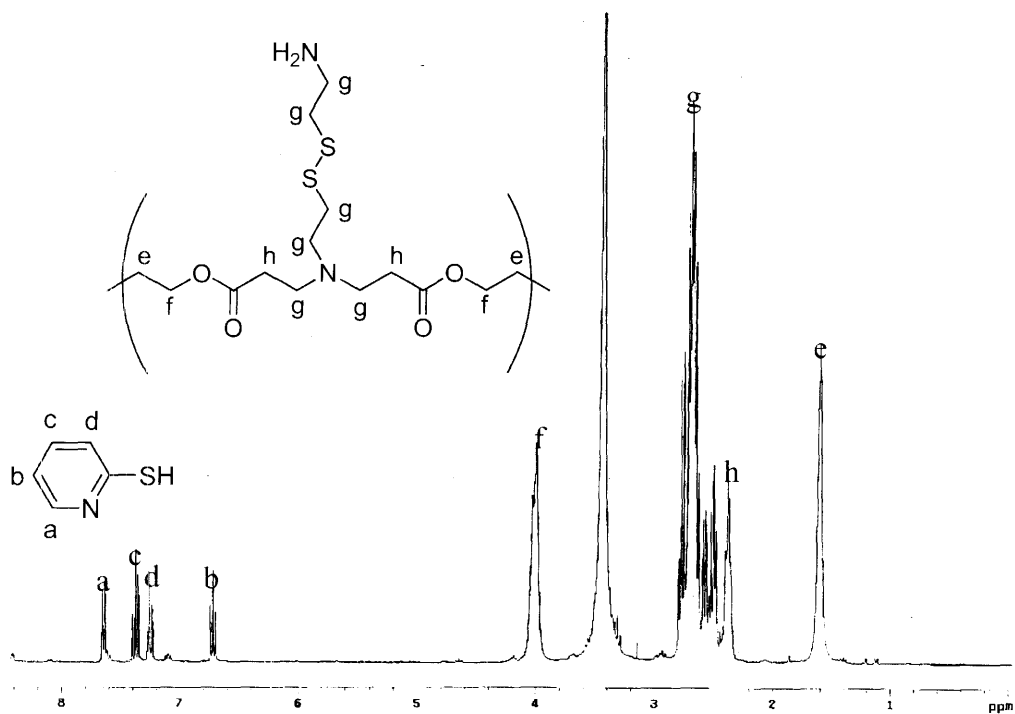
Figure A7: ^{1}H NMR of C-PDA-MEA in d$_6$-DMSO.
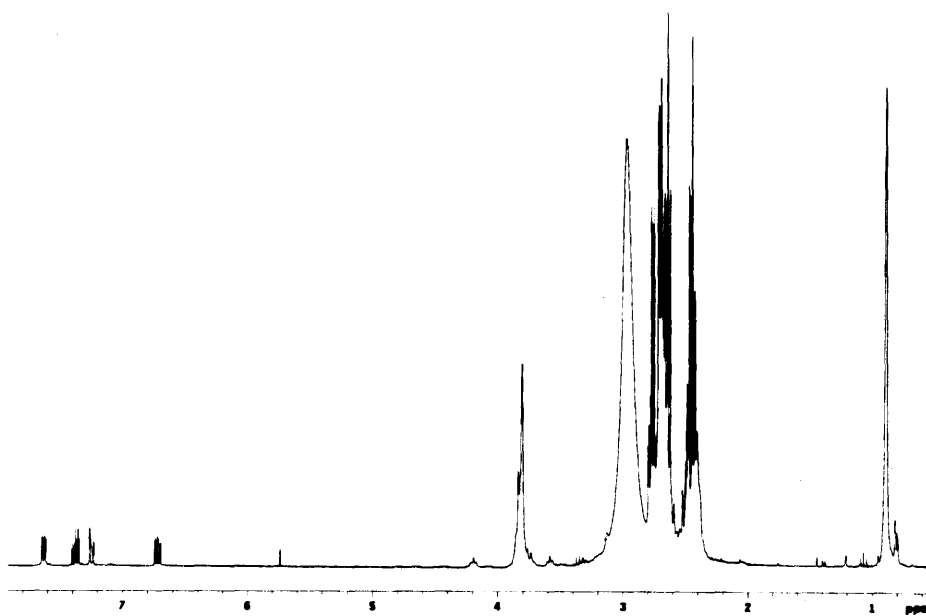
Figure A8: ^{1}H NMR of F-PDA-MEA in d$_6$-DMSO

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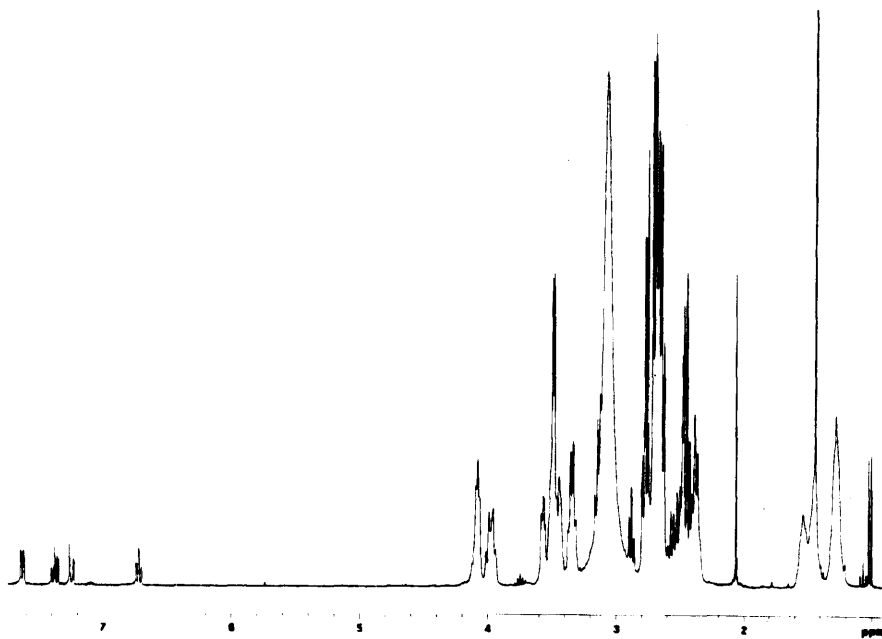
Figure A9: ^{1}H NMR of U-PDA-MEA in d_6-DMSO

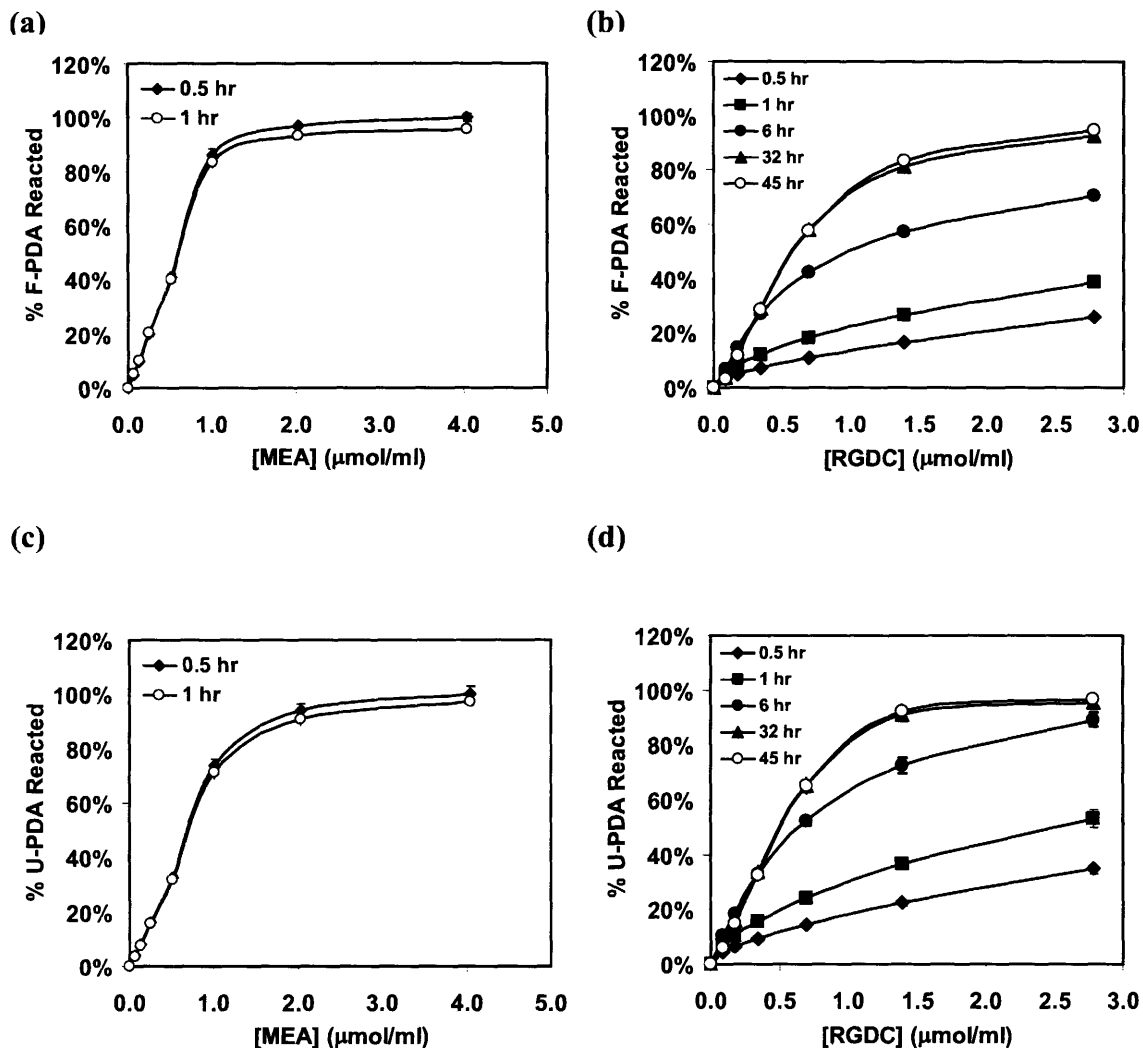## (3) Side chain reactivity of polymers

(a)

(b)

(c)

(d)

Figure A10: Side chain reactivity of the F-PDA polymer with (a) MEA, (b) RGDC, and U-PDA polymer with (c) MEA, (d) RGDC. Experimental conditions are the same as those given for the C-PDA polymer. These results, along with the NMR, illustrate that PDA-based poly(β-amino ester)s can be synthesized with any diacrylate monomer and react specifically at the PDA side chains with thiolated molecules.

Appendix B – Supplementary material for End-Modified poly(β-amino ester)s
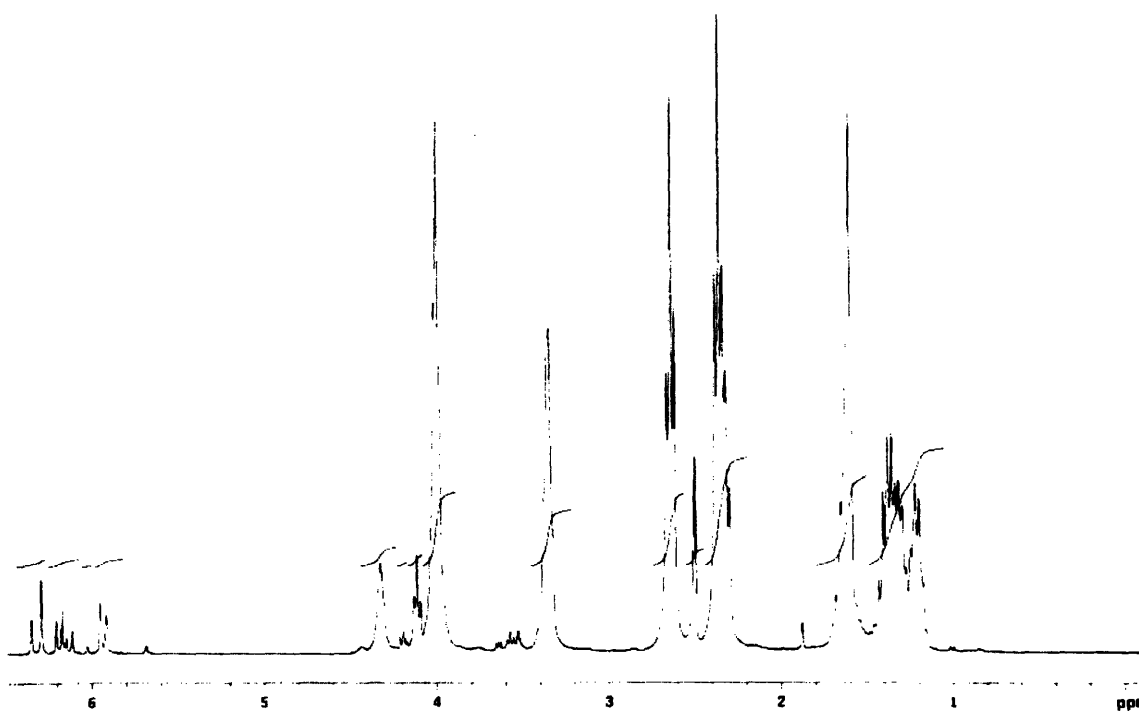
Figure B1 ^{1}H NMR of acrylate-terminated C32 (C32-Ac) polymer in d_6-DMSO. The polymer was synthesized by reacting 793 mg (4.0 mmol) of 1,4-butanediol diacrylate (C) with 344 mg (3.3 mmol) of 5-amino-1-pentanol in a Teflon-lined screw-cap vial with magnetic stirring at 90°C for 24 hours.
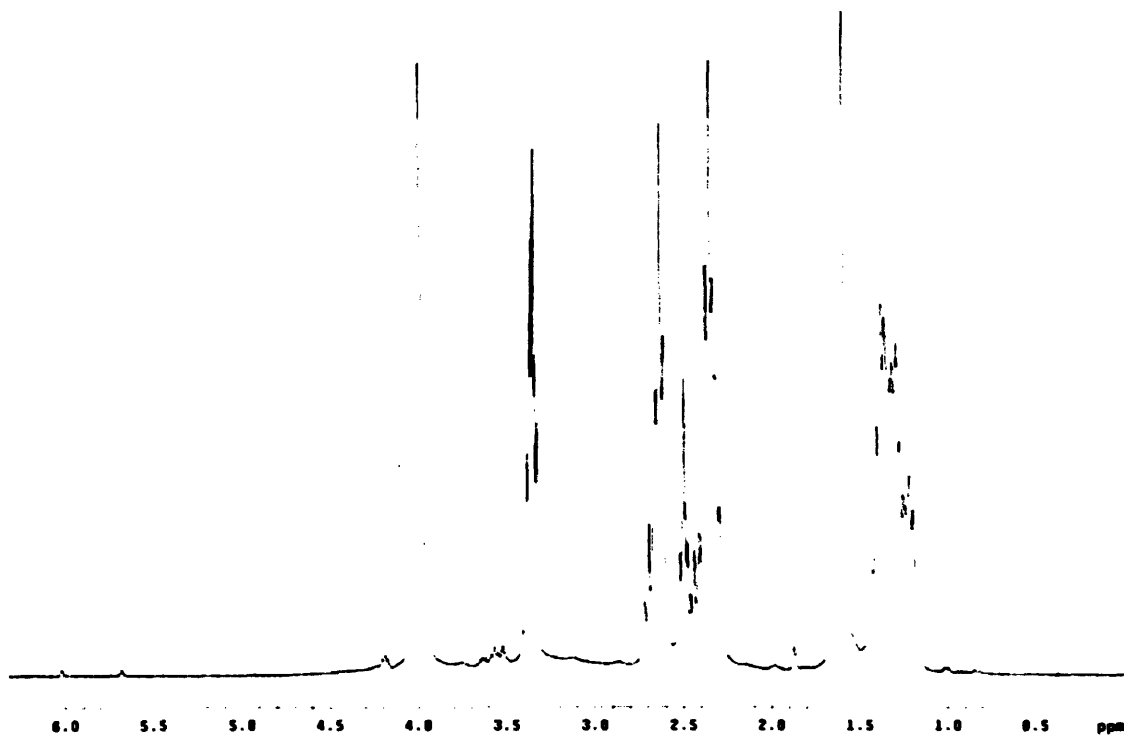
Figure B2 ^{1}H NMR of C32-32 (i.e., C32-Ac capped with 5-amino-1-pentanol) in d$_6$-DMSO. The end-capping reaction was performed by mixing 321 mg of polymer solution (31.13 wt% C32-Ac in DMSO) with 800 µl of 0.25 M aminopentanol in DMSO. Reaction was performed in an eppendorf overnight at room temperature with constant agitation.
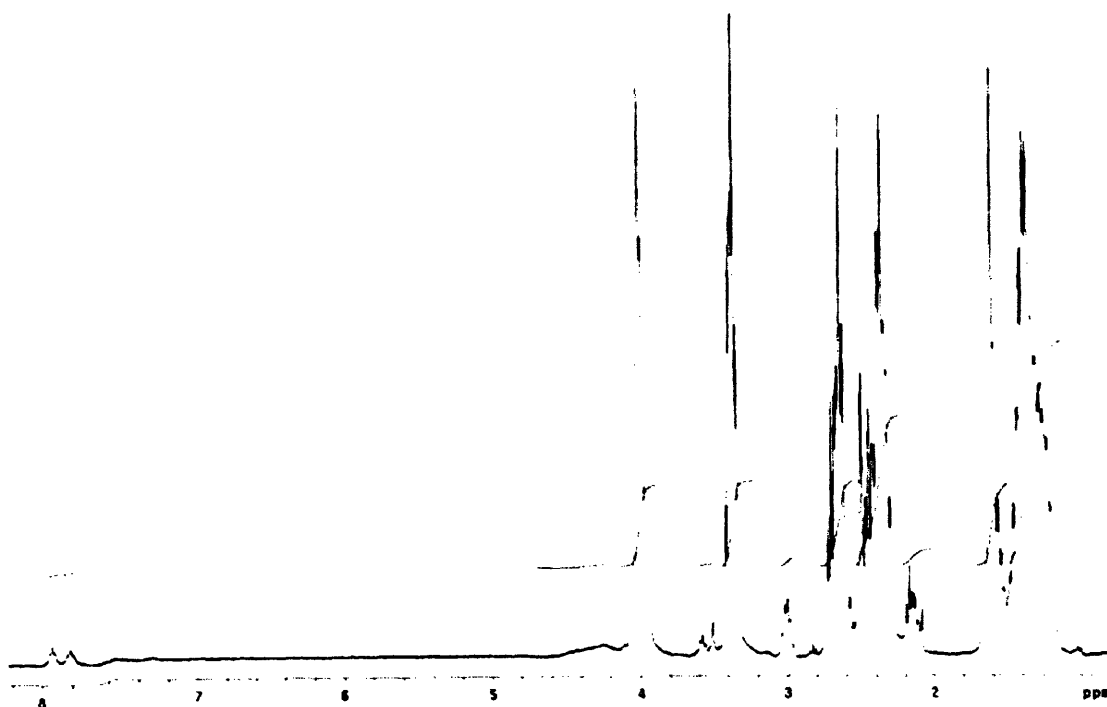
Figure B3 ¹H NMR of C32-32 (in d₆-DMSO) prepared at elevated concentrations and temperature. The end-capping reaction was performed by mixing 412 mg of polymer solution (67.15 wt% C32-Ac in DMSO) with 67 μl of aminopentanol. The reaction was performed in a Teflon-lined screw-cap vial with magnetic stirring at 90°C for 16 hours. The small peaks observed between 7.8-8.0 may be amides and suggest that these conditions cause aminolysis of the polymer ester bonds. Additional peaks at 3.0 and 2.1, which are not present in Figure B2, could be due to protons on carbons adjacent to the amide bond.
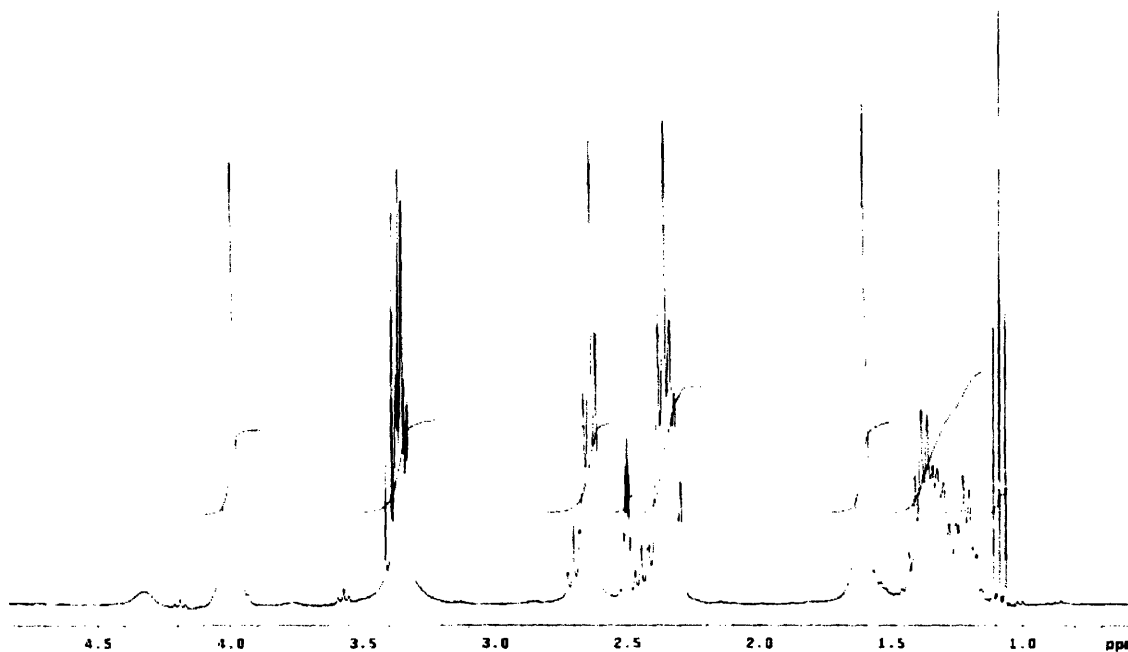
Figure B4 ^{1}H NMR of C32-32 (in d$_6$-DMSO) prepared in THF. The end-capping reaction was performed by mixing 321 mg of polymer solution (31.13 wt% C32-Ac in THF) with 800 μl of 0.25 M aminopentanol in THF. Reaction was performed in an eppendorf overnight at room temperature with constant agitation. Polymer was isolated by precipitation with 10 volumes of diethyl ether, centrifugation and washing with 5 ml of ether twice. Polymer was dried overnight and dissolved in d$_6$-DMSO for NMR. Purity was determined by comparing peak integration at 1.6 ppm (protons in the polymer backbone due to the diacrylate) and 1.2 – 1.5 ppm (protons in aminopentanol side chains). This analysis indicated that there was no free amine left in the polymer. There was residual ether in the polymer, as indicated by the triplet peak at 1.1 ppm and quartet peak at 3.4 ppm. Integration of the ether peak at 1.1 ppm and comparison to the polymer peak integration at 1.6 or 4.0 ppm indicated that there is approximately 3.6 wt% ether in the sample, so that the final polymer is 96.4% pure.