Investigation of Barriers to Non-Viral Gene Delivery and Design of Novel Polymer-Based Gene Delivery Systems

by

Akin Akinc

B.S.E., Chemical Engineering, Princeton University, 1998

Submitted to the Department of Chemical Engineering in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

at the

Massachusetts Institute of Technology

June 2003

© 2003 Massachusetts Institute of Technology. All rights reserved.

Signature of Author: ____________________________

Department of Chemical Engineering

May 19, 2003

Certified by: ____________________________

Robert S. Langer, Sc.D.
Germsheimen Professor of Chemical and Biomedical Engineering

Thesis Advisor

Accepted by: ____________________________

Daniel Blankschtein
Professor of Chemical Engineering
Chairman, Committee for Graduate Students
DISCLAIMER OF QUALITY

Due to the condition of the original material, there are unavoidable flaws in this reproduction. We have made every effort possible to provide you with the best copy available. If you are dissatisfied with this product and find it unusable, please contact Document Services as soon as possible.

Thank you.

Some pages in the original document contain pictures, graphics, or text that is illegible.
Investigation of Barriers to Non-Viral Gene Delivery and Design of Novel Polymer-Based Gene Delivery Systems

by

Akin Akinc

Submitted to the Department of Chemical Engineering in partial fulfillment of the requirements for the degree of Doctor of Philosophy

Abstract

The safe and effective delivery of therapeutic genes is the most significant challenge facing gene therapy today. Viral vectors remain the dominant approach for addressing the delivery problem; however, concerns regarding the safety of viral vectors have resulted in an increasing interest in non-viral vectors. Non-viral vectors offer the promise of improved safety, but because they have yet to match the functional sophistication of viral vectors, their transfection efficiencies have lagged those of viral vectors. The rational design of functional non-viral vectors requires a thorough understanding of both the cell’s sophisticated machinery and the vector material’s functional properties. We have developed a novel, flow cytometry based tool for investigating both the cellular uptake and lysosomal trafficking of non-viral vectors, two important barriers to efficient gene transfer. Using this and other tools we investigated the gene transfer properties of polyethylenimine (PEI), a highly effective non-viral vector material. We demonstrated that the transfection efficiency of this polymer is due to its ability to avoid lysosomal degradation as a result of its buffering capacity, providing quantitative validation of the proton sponge hypothesis. By studying the gene transfer properties of a library of polymeric vectors, we were able to elucidate new vector structure-function relationships. We also investigated the combined impact of non-structural factors—such as polymer molecular weight, polymer chain end-group, and polymer/DNA ratio—on gene transfer. The findings of these studies have lead to the development of non-viral vectors with transfection efficiencies surpassing those of PEI and Lipofectamine 2000, two of the best commercially available non-viral vectors.

Thesis Advisor: Robert S. Langer, Sc.D
Germeshausen Professor of Chemical and Biomedical Engineering
Acknowledgements

I would like to thank my advisor Bob Langer for providing me the opportunity to work in his extraordinary lab. I would also like to thank my thesis committee members Doug Lauffenburger, Jay Vacanti, and David Putnam for all their advice and generous support of this work.

Numerous lab members have contributed to this work, most significantly Dan Anderson and Dave Lynn. I sincerely thank them for all their assistance.

I would also like to acknowledge the role my parents and my wife Bridget have played in supporting this effort.
# TABLE OF CONTENTS

1. Introduction ............................................................................................................. 11

2. Background ............................................................................................................. 13
   2.1. Gene Therapy Overview and Historical Perspective ........................................ 13
   2.2. The Role of Gene Therapy in Medicine .............................................................. 15
   2.3. Gene Delivery: A Major Challenge ................................................................... 18
   2.4. Barriers to Effective Gene Delivery .................................................................. 19
   2.5. Gene Delivery Vectors .................................................................................... 22
       2.5.1. Viral Vectors ............................................................................................. 23
           2.5.1.1. Retroviral Vectors .......................................................................... 23
           2.5.1.2. Adenoviral and Adeno-Associated Viral (AAV) Vectors .............. 25
       2.5.2. Non-Viral Vectors ..................................................................................... 26
           2.5.2.1. Naked DNA ..................................................................................... 26
           2.5.2.2. Cationic Lipids .............................................................................. 27
           2.5.2.3. Cationic Polymers ........................................................................ 29
   2.6. References ......................................................................................................... 33

3. Design of an Assay to Measure the pH Environment of DNA Delivered
   Using Non-Viral Vectors ......................................................................................... 40
   3.1. Introduction ...................................................................................................... 40
   3.2. Materials and Methods .................................................................................. 41
       3.2.1. Materials .................................................................................................. 41
       3.2.2. Double-Labeling the Plasmid .................................................................. 42
       3.2.3. Preparation of Non-Viral Vectors .............................................................. 45
       3.2.4. Assay Protocols ..................................................................................... 45
       3.2.5. Flow Cytometry ..................................................................................... 46
       3.2.6. Transfection With GFP .......................................................................... 47
   3.3. Results and Discussion ................................................................................... 47
3.3.1. Principle of the Assay ............................................................... 47
3.3.2. Assay Methodology and Application ........................................... 49
3.4. Summary .................................................................................... 54
3.5. References ................................................................................ 56

4. PEI and the Proton Sponge Hypothesis ................................................. 58
   4.1. Introduction ............................................................................ 58
   4.2. Materials and Methods ............................................................ 60
       4.2.1. Materials ...................................................................... 60
       4.2.2. Luciferase Transfection Assays ......................................... 60
       4.2.3. Measurement of pH Environment of Delivered DNA and Cellular Uptake .......................................................... 61
       4.2.4. Flow Cytometry ............................................................... 62
       4.2.5. Polymer-DNA Binding Titrations ....................................... 62
   4.3. Results and Discussion ................................................................ 63
       4.3.1. Luciferase Transfections .................................................... 63
       4.3.2. Measurement of pH Environment ....................................... 65
       4.3.3. Cellular Uptake ............................................................... 66
       4.3.4. Vector Unpacking ............................................................ 67
   4.4. Conclusions ............................................................................ 69
   4.5. References .............................................................................. 70

5. Biophysical Characterization of a Degradable Poly(β-Amino Ester) Library ........................................ 72
   5.1. Introduction ............................................................................ 72
   5.2. Materials and Methods ............................................................ 73
       5.2.1. Materials ...................................................................... 73
       5.2.2. Cellular Uptake Experiments ............................................ 74
       5.2.3. Particle Sizing and Zeta Potential Measurements .................. 74
       5.2.4. Measurement of pH Environment of Delivered DNA .......... 75
       5.2.5. Flow Cytometry ............................................................. 76
       5.2.6. Measurement of Cytotoxicity ............................................ 76
5.3. Results and Discussion .................................................................................. 77
  5.3.1. Polymer Synthesis and Initial Screening ............................................... 77
  5.3.2. Evaluation of Intracellular Barriers to Transfection ............................. 79
  5.3.3. Analysis of Cellular Uptake .................................................................. 80
  5.3.4. Effective Diameter and Zeta Potential Measurements ....................... 82
  5.3.5. Analysis of pH Environment .................................................................. 85
  5.3.6. Analysis of Cytotoxicity ....................................................................... 87
  5.4. Summary .................................................................................................... 89
  5.5. References ................................................................................................. 91

6. Synthesis of Poly(β-amino esters) Optimized for Highly Effective Gene Delivery .......................................................................................... 93
  6.1. Introduction ................................................................................................. 93
  6.2. Materials and Methods .............................................................................. 94
    6.2.1. Polymer Synthesis ............................................................................... 94
    6.2.2. Gel Permeation Chromatography (GPC) .......................................... 95
    6.2.3. Luciferase Transfection Assays ............................................................. 95
    6.2.4. Measurement of Cytotoxicity ............................................................... 96
    6.2.5. Cellular Uptake Experiments ............................................................... 97
    6.2.6. GFP Transfections ............................................................................. 97
    6.2.7. Flow Cytometry .................................................................................. 97
  6.3. Results and Discussion ............................................................................... 98
    6.3.1. Polymer Synthesis ............................................................................... 98
    6.3.2. Luciferase Transfection Results ........................................................... 100
    6.3.3. Cytotoxicity ....................................................................................... 104
    6.3.4. Cellular Uptake .................................................................................. 107
    6.3.5. Enhancement of Transfection Using a Co-Complexing Agent .......... 111
    6.3.6. GFP Transfections ............................................................................ 114
  6.4. Summary ..................................................................................................... 117
  6.5. References ................................................................................................. 118
7. Conclusions .................................................................................................................. 120

8. Future Work .................................................................................................................. 122

Appendix: Measuring Buffering Capacity and Molecular Weight Effects ................. 124
LIST OF FIGURES

Figure 2.1. Gene therapy clinical trial protocols by disease ........................................ 16
Figure 2.2. The gene transfer process ........................................................................ 19
Figure 2.3. Gene therapy clinical trial protocols by delivery vector ......................... 23
Figure 2.4. Chemical structures of selected lipids ..................................................... 28
Figure 2.5. Chemical structures of selected cationic polymers ................................ 30
Figure 3.1. pH dependant emission spectrum of fluorescein ..................................... 43
Figure 3.2. Covalent double-labeling of plasmid DNA with fluorescein and Cy5 ...... 44
Figure 3.3. Median FL1 (fluorescein) signal divided by median FL4 (Cy5) signal as a function of pH ......................................................................................... 48
Figure 3.4. Overview of assay protocol ...................................................................... 50
Figure 3.5. Flow cytometry analysis ........................................................................... 50
Figure 3.6. Average pH environment of delivered plasmid DNA ......................... 52
Figure 3.7. Structures of polymers Poly-A and Poly-B ........................................ 53
Figure 4.1. Chemical structures of Me-PEI and Et-PEI ............................................ 63
Figure 4.2. Luciferase transfection results ................................................................. 64
Figure 4.3. Measured average pH environment of DNA delivered using the polymers PEI, Me-PEI, Et-PEI, and PLL ................................................................. 65
Figure 4.4. Cellular uptake relative to PEI ................................................................. 67
Figure 4.5. Assessment of polymer-DNA binding affinity by salt titration ............ 68
Figure 5.1. Diacrylate (A-G) and amine (1-20) monomers ...................................... 77
Figure 5.2. Transfection data as a function of structure ........................................... 78
Figure 5.3. Cellular uptake data as a function of structure ....................................... 81
Figure 5.4. Measured average pH ............................................................................. 86
Figure 5.5. Relative viability of cells treated with DNA/polymer complexes ........... 88
Figure 6.1. Control of polymer molecular weight and chain end-group by varying amine/diacrylate ratio ........................................................... 100
Figure 6.2. Luciferase transfection results for Poly-1 ............................................... 101
Figure 6.3. Luciferase transfection results for Poly-2 ............................................... 102
Figure 6.4. Cytotoxicity of Poly-1/DNA complexes .................................................. 105
Figure 6.5. Cytotoxicity of Poly-2/DNA complexes ........................................... 106
Figure 6.6. Relative cellular uptake level of Poly-1/DNA complexes .......... 108
Figure 6.7. Relative cellular uptake level of Poly-2/DNA complexes .......... 109
Figure 6.8. Enhancement of transfection activity of Poly-1 based delivery vectors through the use of co-complexing agents ............................................. 112
Figure 6.9. Enhancement of transfection activity of Poly-2 based delivery vectors through the use of co-complexing agents ............................................. 113
Figure 6.10. GFP gene transfer into COS-7 cells ............................................ 115
Figure 6.11. GFP expression by COS-7 cells transfected using Poly-1/PLL ......... 115
Figure 6.12. GFP gene transfer into different cell lines using Poly-1/PLL ......... 116
Figure A1. Synthesis of polylysine-\textit{graft}-imidazole acetic acid polymers ........ 124
Figure A2. Measured average pH environment using different MW PEIs ........ 125
Figure A3. Measured average pH environment using polylysine-\textit{graft}-imidazole acetic acid polymers ................................................................. 126
1. Introduction

Many of the molecular biological tools necessary for the conceptualization of gene therapy were developed as far back as the late 1960s and early 1970s. Since that time researchers have worked to address the technical issues required to make human gene therapy a reality. This effort culminated in the first clinical application of gene therapy in 1990, for the treatment of severe combined immunodeficiency (SCID) resulting from adenosine deaminase (ADA) deficiency. As a result of the positive momentum leading up to this first clinical trial, many in the field envisioned a gene therapy revolution in medicine occurring in the 1990s. Although the number of ongoing gene therapy clinical trials continues to grow, as of now, 13 years since the initiation of the first trial, there is still no federally approved gene-based therapy. The great progress expected by many to occur in the past decade has been stalled by ineffective gene delivery. Problems with existing delivery vectors have been more difficult to overcome than initially imagined, forcing a reassessment of both the timeline and scope of gene therapy in medicine.

Viral vectors continue to be the predominate means for delivering therapeutic genes to target cells, however safety and pharmaceutical concerns have led many in the field to look for non-viral alternatives. Both viral and non-viral vectors have their own unique set of advantages and disadvantages that make them particularly suitable for specific applications. Although viral vectors are likely to remain an important class of delivery system, the focus of this thesis is on non-viral delivery systems. Currently the gene transfer efficiencies of non-viral vectors lag those of viral vectors, but the development of an effective non-viral vector would likely lead to a more widespread
application of gene therapy technology. A highly efficient non-viral vector would target
the gene to the proper cell type, mediate cell entry, facilitate endosomal escape, direct
nuclear localization, and promote proper gene expression. To design a synthetic vector
for this purpose requires a thorough understanding of both the cell’s sophisticated
machinery and the vector material’s functionality. Because this understanding is largely
lacking, to date, non-viral vector design has been mainly empirical. Vector candidates
are introduced to the cell, and the resultant gene expression is measured. However, this
approach places the majority of gene delivery processes in a large black box. This thesis
is motivated by the hypothesis that a better understanding of these processes will lead to
the design of better gene delivery systems. As part of this approach, new tools were
developed to permit the study of specific steps in the non-viral gene delivery process.
These tools were then used to improve our understanding of non-viral vector
functionality. Finally, this understanding was used to develop novel, non-viral gene
delivery systems.
2. Background

2.1. Gene Therapy Overview and Historical Perspective

Gene therapy may be defined as the treatment of disease through the introduction of therapeutic genetic material into the cells of an organism. Currently, the dominant approach is not to correct the defective gene, but rather to provide a functional copy of the defective gene in order to ameliorate, if not cure, the disease phenotype. A defect in a gene can result in the absence of an important protein, leading to disease. By delivering a functional copy of the defective gene, the cell is provided with the genetic instruction set necessary to produce the missing protein, thereby treating the disease.

The foundations of gene therapy may go as far back as 1944 when O. T. Avery, C. M. MacLeod, and M. McCarty genetically transformed a bacterium by transferring DNA isolated from a genetically distinct bacterium.[1] The primary contribution of this work was to provide the first evidence for DNA as the carrier of genetic information, but it also demonstrated the possibility of altering the genetic makeup of an organism through gene transfer. In 1953 Watson and Crick deduced the structure of DNA and a month later postulated its mechanism of replication.[2] In 1961 F. Jacob and J. Monod put forth the concept of a polynucleotide “short-lived intermediate” acting as a messenger for protein synthesis (mRNA).[3] By 1966, largely through the work of both M. Nirenberg[4] and H. G. Khorana,[5] the entire genetic code was elucidated, firmly establishing the connection between DNA and protein. The next major step toward gene therapy occurred in the early 1970s, through the work of P. Berg, H. Boyer, and S. Cohen.[6, 7] Their work led to the development of recombinant DNA technology, a
process for splicing and recombining sections of DNA through the use of restriction enzymes and DNA ligase to generate novel genetic constructs. By this time, the advances made in molecular biology, in terms of both understanding and tools, led to the conceptualization of gene transfer as a potential means to treat disease.[8, 9]

With the conceptual framework in place, attention shifted to addressing the technical hurdles necessary to put gene therapy in practice. Early work had shown that the transfer of nucleic acids to cells in culture could be achieved through the use of viruses,[1] calcium phosphate precipitation,[10] DEAE-dextran,[11] and microinjection.[12] Although these techniques resulted in the transfer of genes in vitro, their application in vivo was largely unsuccessful due to poor transfection efficiencies. The development of the retroviral vector system in the early 1980s was a major advance toward successful gene therapy.[13-18] The system was comprised of a replication-deficient vector containing the therapeutic gene and a packaging cell line containing the genes necessary for the assembly and replication of the virion. The separation of viral genetic elements into two distinct pieces allowed the production of a transducing therapeutic vector while minimizing the possibility of wild-type viral infection.

During the early 1980s, the correction of genetic defects using gene therapy was demonstrated in lower organisms. In 1982 Spradling and Rubin used gene therapy to correct eye color in Drosophila embryos.[19] A genetic defect, resulting in the absence of the enzyme responsible for wild-type red eye color in Drosophila, produces flies with white eyes. Mutant flies treated with the gene coding for the missing enzyme acquired the wild-type eye color. Two years later the first successful application of gene therapy in mammals was reported.[20] Working with a mutant strain of mice with reduced growth hormone levels, Hammer et al. used gene therapy to increase the serum levels of growth hormone in these mice. The increased levels of growth hormone caused the dwarf mice to grow; however, over-expression of the hormone caused the animals to grow to one-and-a-half times their normal size. This work, while demonstrating the ability of gene therapy to alter disease phenotype in mammals, also highlighted the need for proper regulation of transferred genes.

The advances in in vivo gene therapy realized in the 1980s made possible the initiation of the first human gene therapy protocol in 1990.[21] The trial, lead by M.
Blaese and W. F. Anderson, used gene therapy to treat two patients with severe combined immunodeficiency (SCID) resulting from adenosine deaminase (ADA) deficiency. A genetic defect, resulting in the lack of the enzyme ADA, leads to the accumulation of a metabolite toxic to T lymphocytes. The destruction of T lymphocytes compromises immune function, manifesting as SCID. At various times, T lymphocytes were removed from the patients, treated \textit{ex vivo} with a retroviral vector carrying the ADA gene, and returned to the patient. The results of the study showed that transfer of the ADA gene did occur and that patients did demonstrate improved immune response. In addition, the retroviral vector used to transfer the ADA gene did not harm the patients in any observable manner. While the trial results were clearly promising as a whole, the trial also highlighted a number of problems. Gene transfer was both inefficient and inconsistent, and the ADA levels, T cell numbers, and immune responses were transient and highly variable.

2.2. The Role of Gene Therapy in Medicine

Gene therapy is expected to have a tremendous impact on the field of medicine, due both to the large number of diseases amenable to it and the relative ineffectiveness of many current interventions. In principle, any disease with a genetic component may be a candidate for gene therapy; however, certain genetic disorders are currently more amenable to gene therapy than others. Genetic diseases may be broken down into three main categories: chromosomal disorders, monogenic disorders, and multifactorial disorders. Chromosomal disorders result from a change in total mass of chromosomal material or an improper arrangement of chromosomes. A common example is Down syndrome, which is caused by an extra chromosome 21. While the symptoms of such disorders may be treated using gene therapy, it is unlikely that gene therapy will be able to provide cures for these conditions.

Monogenic disorders result from a defect in a single gene, and therefore, are the ideal disease candidates for gene therapy. Especially good disease candidates would involve genes with simple "always on" regulation, and would require only moderate recovery of gene expression levels to achieve a therapeutic effect. Monogenic disorders comprise nearly 4,000 known diseases including adenosine deaminase (ADA) deficiency,
sickle cell anemia, cystic fibrosis, Duchenne muscular dystrophy, hemophilia, phenylketonuria, and hypercholesterolemia.[22] In 1985 the efficacy of the latest surgical, dietary, and pharmacological interventions in the treatment of a sample of 65 well-understood monogenic diseases was studied. The findings were that treatment produced full recovery in only 12%, partial response in 40%, and no response in the remaining 48% of the diseases studied.[23] Gene therapy provides new hope where conventional approaches have failed, and in cases where effective interventions do exist, it offers the possibility of a less invasive treatment, free from painful surgery, restrictive diet, or pharmacological side effects.

Multifactorial disorders are the largest and most complex group of diseases, involving multiple genes or a combination of both genetic and environmental factors. Due to the complexity of these disorders, they will be challenging to treat using gene therapy, but in cases where current treatments are ineffective or undesirable, gene therapy may be pursued. In fact, over 60% of the gene therapy protocols in clinical trials are for the treatment of cancer, an acquired multifactorial disease (Figure 2.1).

![Figure 2.1. Gene therapy clinical trial protocols by disease.[24]](image-url)
A number of strategies have been employed to treat cancer using gene therapy, including stimulation of the immune system through expression of cytokines, tranfection of tumor cells with drug sensitivity genes, and replacement of tumor suppressor genes.[25-28]

Moving beyond genetic diseases altogether, gene therapy concepts have also been utilized in the field of immunology.[29-31] DNA vaccines have the potential to address a number of the limitations of traditional vaccine formulations. Traditional vaccines have relied on the administration of either live attenuated pathogens or non-living portions of pathogens. In the case of live attenuated vaccines, because the pathogen replicates and produces antigen inside the cell, both B cell and T cell activation is achieved. This confers broad immunity through the induction of both antibodies and cytotoxic T lymphocytes (CTLs). However, there are safety concerns associated with the use of live attenuated vaccines. There is the danger that the attenuated vaccine may cause disease in individuals with compromised immune systems or that the vaccine may mutate back to the disease causing form. In contrast, non-living inactivated vaccines offer improved safety, but since they do not result in intracellular antigen production, they only induce a B cell response. As a result, protective immunity is weaker and transient, requiring repeated booster injections. DNA vaccines are based on the transfer of genes encoding antigenic proteins. Because DNA vaccines are non-living, they are inherently safer than live attenuated vaccines, and because DNA vaccines result in the production of antigens inside the host cell, they give rise to both B cell and T cell activation. In addition to these advantages, DNA vaccines may be easier and less expensive to manufacture, store, and distribute.

As the field of gene therapy develops, there is likely to be an increasing use of the gene therapy paradigm for local drug delivery.[32, 33] By harnessing the cell’s protein making ability, drugs can be produced in situ, where they are more effective. Also, in certain cases, it may be more feasible to produce and deliver the genetic instructions for making a drug rather than producing and delivering the drug itself. For example, gene therapy approaches have been used to make the promising cancer drug endostatin, which has proven difficult to produce using recombinant technology.[34-36]
2.3. Gene Delivery: A Major Challenge

While the conceptual pieces necessary to begin the gene therapy revolution have been in place for some time, results have thus far fallen short of expectations. In 1995, five years after the initiation of the first gene therapy clinical trial, a NIH committee was formed to assess the current status of gene therapy research and provide recommendations.[37] The committee found that,

...clinical efficacy has not been definitively demonstrated at this time in any gene therapy protocol. ...Significant problems remain in all basic aspects of gene therapy. Major difficulties at the basic level include shortcomings in all current gene transfer vectors and an inadequate understanding of the biological interaction of these vectors with the host.

To this end the panel recommended that,

...efforts need to be applied to improving vectors for gene delivery, enhancing and maintaining high level expression of genes transferred to somatic cells, achieving tissue-specific and regulated expression of transferred genes, and directing gene transfer to specific cell types.

Currently, there are over 600 gene therapy clinical trials worldwide; however, problems continue to plague gene therapy. Thirteen years since the initiation of the milestone ADA-SCID trial, there are still no approved gene therapies. The safe and effective delivery of genes remains the main hurdle limiting the development of gene therapy.[38-42] Common problems include inefficient gene transfer and short-lived gene expression, but of perhaps greater concern are problems associated with the immunogenicity of delivery vectors and the possibility of mutagenesis due to random chromosomal integration of the therapeutic gene. These concerns were highlighted in September 1999 with the tragic death of Jesse Gelsinger, a patient participating in a trial at the University of Pennsylvania who was being treated for a rare metabolic disorder using an adenoviral vector-based therapy.[42, 43] Upon investigation of the case, it was concluded that the patient died from a violent immune and inflammatory response triggered by administration of the adenoviral vector. More recently, in 2002, it was discovered that a patient participating in SCID gene therapy trial in France had developed a rare form of leukemia.[44] In 2003, a second patient from the trial had also developed leukemia.[45] In both cases, chromosomal insertion of the retroviral vector-delivered gene has been implicated as the cause of the leukemia.
2.4. Barriers to Effective Gene Delivery

The challenge of achieving safe and efficient gene transfer is readily apparent when one considers the full set of criteria that a gene delivery vector must fulfill. Although there are a number of pharmaceutical issues that must be addressed for any therapeutic,[33, 46] the two most important criteria for a viable gene delivery vector are safety and efficacy. A safe vector must not elicit an immune or inflammatory response, must not be infectious, and must not cause mutagenesis due to improper gene insertion. While fulfilling these criteria, the vector must incorporate sufficient functionality to overcome the multitude of cellular and extracellular barriers to gene transfer. Although the specific functional requirements of a vector will depend on the mode of treatment (in vivo or ex vivo), the target cell, and route of administration—the main barriers to efficient gene transfer are common to all types of gene therapy (Figure 2.2).

![Gene transfer process diagram](image)

**Figure 2.2.** The gene transfer process.
Prior to reaching the target cell, an efficient gene delivery system must provide stability to the therapeutic gene in the extracellular environment, as nucleases in the blood and intrastitial fluids rapidly degrade plasmid DNA.[47-49] Protection of DNA has been achieved through the use of both viral systems that package the therapeutic gene and non-viral systems that complex DNA. Complexation limits the access of nucleases to the DNA, thereby increasing the stability of the DNA in the extracellular environment.

The first cellular barrier to gene delivery is cellular uptake. As part of this process, the vectors must first associate with the cell surface. This occurs through either non-receptor or receptor-mediated means. Most delivery vehicles carry a positive surface charge and are therefore attracted to the negatively charged cell membrane. Physical means have also been employed in vitro to increase cellular association by increasing the concentration of vectors at the cell surface. Two examples of this approach are centrifugation[50] and the anchoring of DNA to silica particles.[51] Receptor-ligand interactions have been used for both cellular targeting and for non-specifically increasing cellular association. In 1987 the work of Wu and Wu demonstrated that polylysine conjugated to asialoglycoprotein could effectively target hepatocytes in vitro.[52] One year later they were able to show effective liver targeting in vivo.[53] Other examples of receptor-mediated cellular targeting include the use of mannose ligands for macrophage targeting[54-57] and folate ligands for tumor targeting.[58-61] In contrast, transferrin conjugated delivery vectors have been used to non-specifically enhance cellular association through the ubiquitous transferrin receptor.[62-65] Cellular uptake of gene delivery vectors occurs principally via endocytosis, and this mechanism places a very important size restriction on the gene delivery vector. In general, particles larger than 150-200 nm are not efficiently taken up by endocytosis, with smaller particles more readily taken up by cells.[46, 66-68] Viral vectors, as well as cationic non-viral vectors, are able to satisfy this size requirement. The negatively charged plasmids used in non-viral systems have large hydrodynamic diameters (> 100 nm).[33] Cationic materials are able to shield the negative charges and compact the DNA.

Once internalized by endocytosis, vectors are contained in endosomal vesicles. These vesicles contain vacuolar ATPase proton pumps which lower luminal pH. Early endosomes (pH ~ 5.5-6.5) are the sites of sorting events which cycle some of the contents
(mainly receptors) back to the cell surface. Late endosomes (pH ~ 5.0-5.5) then fuse with lysosomes (pH ~ 4.0-5.0), the main degradative compartment of the cell. Any vectors unable to escape the endosomal/lysosomal trafficking pathway are presumably degraded in lysosomes.[69] Viruses have evolved to make use of the pH change in endosomes in order to escape trafficking to lysosomes.[70] They contain fusogenic peptides which change conformation upon a drop in pH, inducing fusion of the viral membrane with the endosomal membrane. This then causes the release of viral contents into the cytoplasm. Fusogenic peptides have also been incorporated into non-viral vectors;[71-75] however, concerns regarding the immunogenicity of these viral-derived peptides exist. Cationic lipids have also shown the ability to destabilize endosomal membranes. A mechanism for their escape from endosomes has been proposed by Xu and Szoka whereby the cationic lipids of the delivery vector interact with the anionic lipids of the endosome, causing membrane destabilization and subsequent DNA release into the cytoplasm.[76] Polyethylenimine (PEI), a polymer that mediates high levels of transfection, is thought to have a novel means for escaping endosomes.[77, 78] This polymer is believed to act as a “proton sponge”, buffering the acidic environment of the endosome. As the endosome continues to pump in protons, chloride ions also enter the vesicle to maintain electroneutrality. The increase in ionic concentration inside the endosome is thought to result in the osmotic swelling and eventual rupture of the endosome, releasing the DNA from the lysosomal trafficking pathway.

For ultimate gene expression to take place, the gene must be able to overcome the nuclear membrane and enter the nucleus. There are three possible routes to the nucleus.[79-81] First, the breakdown of the nuclear membrane during cell division allows DNA to diffuse into the nuclear regions of dividing cells. This is likely the main means of nuclear entry for most gene delivery systems. Second, molecules less than 10 nm can passively diffuse through nuclear pores. Due to the size of DNA, it is unlikely that it could enter the nucleus through this route. Third, particles less than 25 nm can be actively transported through the nuclear pore complex in its open state. It is possible that DNA could be “threaded” through an open nuclear pore. It has been shown that the use of nuclear localization signals (NLSs), short peptide sequences recognized by the nuclear pore complex, can improve transfection efficiency.[82]
Another important barrier to effective gene transfer, at least for non-viral vectors, may be appropriate unpacking of the vector.[83] In order for gene expression to take place, transcription factors must be able to access the DNA. There is therefore an important balance regarding the stability of a gene delivery vector. The vector must bind DNA tightly enough to protect it from nucleases, yet not bind so tightly that gene expression is hindered.

2.5. Gene Delivery Vectors

To overcome the many barriers to safe and effective gene transfer, a rather sophisticated gene delivery vector is required. The vectors currently being used can be classified as viral and non-viral gene delivery systems. They represent two distinct approaches to solving the problem of gene transfer. Because the virus has evolved to express its foreign genetic material in a host cell, it has much of the functionality required to overcome the cell’s barriers to gene transfer. The viral vector represents a top-down approach, where non-essential viral genes are stripped away and therapeutic genes are inserted to produce a delivery vector. However, the problems associated with viral systems have prompted some in the field to take a complementary bottom-up approach to the gene transfer problem. Starting from scratch, synthetic delivery systems are built to include the minimum set of functionality required to achieve effective gene transfer. Currently, the overwhelming majority of gene therapy clinical trials utilize viral vectors, but as non-viral systems become more sophisticated, they are likely to be more widely used (Figure 2.3). Both approaches have merit, and undoubtedly, both viral and non-viral systems will continue to be developed and used in the field of gene therapy. The distinct advantages and disadvantages of each type of delivery system make them particularly well-suited for different applications.
Figure 2.3. Gene therapy clinical trial protocols by delivery vector.[24]

2.5.1. Viral Vectors[84-91]

A large number of viral vector systems have been developed, but the most common ones have been based on retroviruses, adenoviruses, and adeno-associated viruses (AAVs). The fundamental concepts behind the production and use of these vectors are the same, but the specific characteristics of each system lead to differences in important features such as genetic insert capacity, ability to transduce non-dividing cells, ability to maintain sustained gene expression, and safety (Table 2.1).

2.5.1.1. Retroviral Vectors

Retroviral vectors have been around for two decades and are the most commonly used delivery vectors today (Figure 2.3). Most retroviral vectors are based on the Moloney murine leukemia virus (Mo-MLV). This virus is comprised of two identical strands of 8 kb linear RNA packaged in a protein core. Also packaged inside the viral core are the enzymes reverse transcriptase, protease, and integrase. The core is then wrapped in a glycoprotein envelope. In the first step of viral infection, the virion binds to
a receptor on the host cell surface. If the cell does not have a receptor that the virus recognizes, that cell is resistant to viral infection. The virus then fuses with the cell and sheds its glycoprotein envelope. Reverse transcriptase transcribes the RNA into DNA, and the protease releases the viral contents from the protein core. Integrate then inserts the DNA provirus into the host chromosome. Mo-MLV has no means for active transport across the intact nuclear membrane and can therefore infect only dividing cells.

The genome of the retrovirus contains three genes: \textit{gag} which encodes the core proteins, \textit{pol} which encodes the viral enzymes, and \textit{env} which encodes the envelope glycoprotein. In addition, the genome contains a sequence, \( \Psi \), which serves as the virion packaging signal. The genome of the retrovirus is separated into two distinct parts (therapeutic vector and helper or "packaging" cell line) in order to generate a replication deficient therapeutic vector. First, the \textit{gag}, \textit{pol}, and \textit{env} genes are removed from the retroviral genome, leaving the \( \Psi \) packaging signal intact. Next the therapeutic gene of interest is inserted, completing the genome of the replication deficient therapeutic vector. A second retroviral genome, with the \textit{gag}, \textit{pol}, and \textit{env} genes intact but lacking the packaging sequence \( \Psi' \), is integrated into a cell line to generate the helper cell line. The helper cell line is capable of producing all the required viral proteins, but since it lacks the \( \Psi \) sequence, it is unable to package new virions on its own. To produce the therapeutic retroviral vector, the therapeutic vector genome is integrated into the genome of the helper cell line. With the necessary viral proteins supplied by the helper cell line, and the existence of the \( \Psi \) sequence in the therapeutic vector genome, therapeutic retroviral vectors are produced. Since the packaged therapeutic vectors do not contain the \textit{gag}, \textit{pol}, and \textit{env} genes, they are able to infect cells only once and are unable to replicate outside of the packaging cell line.

The MoMLV retroviral vector is a relatively simple and proven system. This vector has been used extensively in \textit{ex vivo} therapies, but \textit{in vivo} transduction efficiency is quite poor. Applications are limited to the transduction of proliferating cells due to the vector's inability to cross intact nuclear membranes. A main advantage of this vector is that it results in stable transgene expression. However, this is achieved through random chromosomal integration, so the risk of insertional mutagenesis exists.
2.5.1.2. Adenoviral and Adeno-Associated Viral (AAV) Vectors

The adenoviral vector is based on a virus that has a 36 kb double-stranded linear DNA genome and can, therefore, carry a larger therapeutic insert than retroviruses. Adenoviral vectors have been shown to deliver genes effectively to a wide variety of cell types, both in vivo and ex vivo. In addition, they can transduce both dividing and non-dividing cells. The vector delivers a gene that is maintained episomally, thus circumventing the problems associated with random chromosomal integration. The main drawback of the adenoviral vector is that it elicits a strong immune and inflammatory response. This results in transient gene expression, and perhaps more significantly, represents a serious safety concern to the patient. Combined immunosuppressive therapy may improve safety, prolong gene expression, and allow for repeated administration of the vector.

The adeno-associated virus (AAV) has a 4.7 kb single-stranded linear DNA genome, and it is a replication deficient virus that can only replicate in the presence of a helper virus, such as adenovirus. AAVs have not been associated with disease in humans, even though up to 90% of all humans show evidence of prior AAV infection. The lack of pathogenesis makes AAVs particularly attractive for use as therapeutic vectors. AAV based vectors have demonstrated the ability to deliver genes to a variety of cell types, both dividing and non-dividing. Sustained gene expression is possible, and depending on the host cell, AAV vectors can integrate their genomes into a specific location on chromosome 19 or maintain them episomally. The most significant limitation of the AAV system is its relatively small therapeutic insert capacity.
Table 2.1. Comparison of common viral vectors

<table>
<thead>
<tr>
<th></th>
<th>Retrovirus (MoMLV)</th>
<th>Adenovirus (35 kb for &quot;gutless vectors&quot;)</th>
<th>Adeno-Associated Virus (AAV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Insert capacity</td>
<td>8 kb</td>
<td>8 kb</td>
<td>4.5 kb</td>
</tr>
<tr>
<td>Transduces dividing cells</td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>In vivo delivery</td>
<td>Poor</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Stable expression</td>
<td>+</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Chromosomal integration</td>
<td>+</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Oncogenicity</td>
<td>+</td>
<td>+</td>
<td>?</td>
</tr>
<tr>
<td>Immunogenicity</td>
<td>Low</td>
<td>High</td>
<td>Low</td>
</tr>
<tr>
<td>Disease caused by wild-type virus</td>
<td>Tumors, AIDS</td>
<td>Cold, conjunctivitis, gastroenteritis</td>
<td>No known</td>
</tr>
</tbody>
</table>

2.5.2. Non-Viral Vectors

Non-viral vectors are a diverse and growing class of gene delivery system, with widely varying degrees of sophistication, gene transfer efficiency, and mechanism of action. Developed as an alternative to viral gene transfer, non-viral vectors offer a number of potential advantages including: non-infectivity, non-oncogenicity, non-immunogenicity, possibility of repeated administration, and lower cost/ease of formulation. Currently, non-viral strategies fall into three major classes: naked DNA, cationic lipid-based, and cationic polymer-based.

2.5.2.1. Naked DNA

The direct administration of naked DNA (plasmid DNA in the absence of a carrier) has shown surprisingly good gene transfer in certain applications. The most successful application of naked DNA gene transfer has been the transfection of skeletal muscle, first demonstrated by Wolff and coworkers in 1990.[92] Since then other tissues have been transfected using naked DNA, notably skin[93] and liver.[94, 95] In general, gene transfer using naked DNA is an inefficient process; however, transfection efficiency has been increased by combining naked DNA injections with in vivo electroporation,[96, 97] ultrasound,[98, 99], and hydrodynamic pressure.[100] Naked DNA gene transfer has
been widely used in the field of DNA vaccines, an application where high levels of gene transfer are not required.[101-103]

2.5.2.2. Cationic Lipids

Liposomes are commonly used in the pharmaceutical industry to encapsulate and deliver drugs. However, encapsulation of DNA inside typical liposomes is highly inefficient due to insufficient interaction between the lipid (typically anionic) and DNA. This problem was overcome through the use of cationic lipids. In 1987, Felgner and coworkers described the use of the cationic lipid (N-[1-(2,3,3-dioleyoxy)propyl]-N,N,N-trimethylammonium chloride) (DOTMA) to complex plasmid DNA for transfection of mammalian cells in culture.[104] Since the work of Felgner and coworkers, many new synthetic cationic lipids have been developed for use as transfection agents (see Table 2.2 and Figure 2.4). These lipids have been used both alone and in mixtures, often in with neutral lipids cholesterol (Chol) and dioleoyl phosphatidyl ethanolamine (DOPE). These colipids are thought to enhance transfection efficiency by stabilizing the complexes and/or by promoting endosomal membrane destabilization.[105, 106] Compositions of some commonly used commercially available lipid reagents are listed in Table 2.3.

**Table 2.2.** Cationic lipids used for transfection (adapted from [107]).

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Chemical Name</th>
</tr>
</thead>
<tbody>
<tr>
<td>DOTMA</td>
<td>N-[1-(2,3-dioleyoxy)propyl]-N,N,N-trimethylammonium chloride</td>
</tr>
<tr>
<td>DOTAP</td>
<td>1,2-Dioleolxoy-3-(trimethylammonio)propane</td>
</tr>
<tr>
<td>DOGS</td>
<td>Dioctadecylamidoglycospermine</td>
</tr>
<tr>
<td>DODAB</td>
<td>Dioctadecyldimethylammonium bromide</td>
</tr>
<tr>
<td>DMRIE</td>
<td>1,2-Dimyristoyloxypropyl-3-dimethyloxyethylammonium bromide</td>
</tr>
<tr>
<td>DOSPA</td>
<td>2,3-Dioleolxoy-N-[(2-sperminecarboxamido)ethyl]-N,N-dimethyl-1-propanaminium</td>
</tr>
<tr>
<td>DC-Chol</td>
<td>3 β-[N-(N', N'-Dimethylaminoethane)carbamoy] cholesterol</td>
</tr>
</tbody>
</table>
Figure 2.4. Chemical structures of selected lipids.
Table 2.3. Commonly used commercial lipid reagents (adapted from [107]).

<table>
<thead>
<tr>
<th>Name</th>
<th>Composition</th>
<th>Vendor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lipofectin</td>
<td>DOTMA:DOPE 1/1 w/w</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>Lipofectamine</td>
<td>DOSPA:DOPE 3/1 w/w</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>LipofectACE</td>
<td>DODAB:DOPE 1/2.5 w/w</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>Transfectam</td>
<td>DOGS</td>
<td>Promega</td>
</tr>
<tr>
<td>DOTAP:Chol</td>
<td>DOTAP:Chol 1/1 mol/mol</td>
<td>Sigma-Aldrich</td>
</tr>
</tbody>
</table>

Cationic lipids have become the standard lab reagent for in vitro transfection and have shown promise for in vivo use as well. [46, 108, 109] Cationic lipids have been used to transfect the pulmonary epithelium through both intratrachial instillation and aerosol administration and the pulmonary endothelium via intravenous administration. Hepatic transfection has also been reported, however the majority of DNA-lipid complexes seem to be taken up by Kupffer cells and not hepatocytes.

2.5.2.3. Cationic Polymers

Cationic polymers are an attractive class of material for use as gene delivery vectors. Like cationic lipid systems they circumvent many of the problems associated with viral vectors; however, unlike lipids they have an almost limitless capacity for structural diversity and are much more easily modified. Cationic polymers can be small or large, linear or branched, flexible or rigid, hydrophilic or hydrophobic. Their capacity for functional modification has been utilized extensively, most commonly for the conjugation of ligands for targeted, receptor-mediated delivery. The structural diversity and capacity for modification exhibited by cationic polymers make them an ideal platform for the incorporation of functionality required to overcome the many barriers to efficient gene transfer.

There have been many novel polymer structures generated for gene therapy use, but the most frequently used polymer systems include polylysine (PLL), polyethyleneimine (PEI), and poly(amide amine) dendrimers (PAMAM) (Figure 2.5).
Figure 2.5. Chemical structures of selected cationic polymers.

These polymers (and the vast majority of all polymers used for gene transfer) contain cationic nitrogen atoms which interact with the negatively charged backbone of DNA. The polymer cation/DNA anion charge ratio (φ) controls many of the important properties of polymer/DNA complexes.[110, 111] At φ < 1, complexes typically have an overall negative surface charge. As φ approaches unity, large polymer/DNA aggregates are formed, and at φ > 1, positively charged, nanoscale complexes are often formed. Polymer/DNA conjugates, also referred to as "polyplexes", are typically used at φ > 1. The excess polymer provides increased protection from nucleases, and the positive
charge on the polypelex surface enhances its interaction with the negatively charged cell membrane, facilitating cellular uptake. This approach works quite well for serum-free in vitro applications, but has been met with problems in vivo due to the non-specific interactions of the positively charged polypelexes with serum proteins and non-target cells. One approach to addressing the problem of serum stability has been the use of poly(ethylene glycol) (PEG) modified cationic polymers.[112-114] The incorporation of the hydrophilic PEG chain is thought to increase polypelex solubility and decrease polypelex aggregation by limiting interaction with serum proteins. PEGylation will also decrease the interaction of the polypelex with the target cell surface, decreasing cellular uptake. This drawback can be tackled through the incorporation of ligands to take advantage of receptor-mediated mechanisms for cellular entry. As described in Section 2.4., this can be achieved either in a targeted manner by using a ligand specific to a receptor found only on the target cell, or in a non-specific manner by using a ligand for a ubiquitous receptor.

As polypelexes enter cells via either specific or non-specific endocytosis, endosomal escape is a significant barrier to efficient gene delivery. The poor transfection efficiency of PLL polypelexes is significantly enhanced in the presence of chloroquine, a weak base. Chloroquine is generally thought to increase transfection efficiency by destabilizing endosomes and inhibiting lysosomal enzymes by raising the pH of these acidic vesicles.[111] Another approach for enhancing the endosomal escape of PLL polypelexes, better suited for in vivo application, has been the use of viral-derived or synthetic fusogenic peptides.[71-73] These peptides undergo a conformational change upon acidification in endosomes to promote membrane destabilization. In contrast, polymers such as PEI and PAMAM mediate high levels of transfection efficiency even in the absence of chloroquine or fusogenic peptides. Both polymers contain amines with pK_a's in the physiological pH range, and as a result, both increase their levels of protonation as pH drops. For example, the protonation level of PEI increases from 20% to 45% as pH decreases from 7 to 5.[77, 78] These "proton sponges" are thought to absorb protons in acidifying endosomes, causing the vacuolar proton pump to continue bringing in protons in an attempt to acidify the endosome. As this occurs, chloride ions enter the endosome to maintain electroneutrality. The net effect of this process is an
increase in ionic concentration inside the endosome which thought to lead to osmotic swelling and endosomal rupture. The imidazole group (pK$_a$ ~ 6.2) has also been used to impart “proton sponge” character into cationic polymer systems.[115-117]

A more recent effort in cationic polymer gene delivery has been the design of degradable systems (see Figure 2.5). Although cationic polymers such as PLL and PEI are commonly used, they are also associated with significant levels of cytotoxicity.[118, 119] Degradable systems successfully reduce the cytotoxicity of polycations by degrading into less toxic metabolites. In addition to improved toxicity profiles, degradable systems should enhance vector unpackaging, another important barrier to effective transgene expression.[83] Examples of such polymers include poly(4-hydroxy-L-proline ester).[120, 121] poly[a-(4-aminobutyl)-L-glycolic acid] (PAGA),[122] and hyperbranched poly(amino ester) (PAE).[123, 124] PAGA is the ester version of the polyamide PLL and hyperbranched PAE resembles an ester version of the PAMAM dendrimer. Recently, our group has described the synthesis and characterization of another class of degradable cationic polymer, poly(β-amino esters), for use as gene delivery vectors.[125, 126]
2.6. References


3. Design of an Assay to Measure the pH Environment of DNA Delivered Using Non-Viral Vectors

3.1. Introduction

For effective gene delivery to take place, a vector must target the gene to the proper cell type, mediate cell entry, avoid lysosomal trafficking, direct nuclear localization, and promote proper gene expression. While much of this functionality is present in viral vectors, it is largely absent in current non-viral vectors. The rational design of such functionality into non-viral vectors will ultimately require a better understanding of the interactions of vectors with cells at each of these barriers. To date, non-viral vector design has been largely empirical with screening based on in vitro reporter gene assays. Although the reporter gene assay is useful for determining in vitro transfection efficiency, it is end-point focused and yields no information regarding intermediate vector-cell interactions. Considerable work has been done to quantify the barrier to targeted vector uptake[1-5] and the barrier to nuclear localization.[6-8] However, to the best of our knowledge, an assay to quantify the degree to which vectors avoid or escape lysosomes has not been reported.

Because DNA is degraded by lysosomal enzymes, the escape of vectors from the lysosomal trafficking pathway is an important step for efficient gene delivery.[9, 10] The use of polymeric vectors known as “proton sponges” is one area of interest for non-viral gene delivery.[11] These polymers contain amine groups with pKₐ values in the physiological pH range, resulting in their ability to buffer acidic endosomal vesicles. This buffering capacity is thought to promote the osmotic swelling and physical rupture of endosomes, assisting in the release of DNA into the cytoplasm prior to fusion with
lysosomes.[11] Polyethylenimine (PEI), a commercially available “proton sponge” polymer, has been shown to mediate high levels of transfection compared to polymers such as polylysine (PLL), which has no known mechanism for avoiding lysosomal trafficking.[12, 13]

We have developed a flow cytometry-based technique for measuring the pH environment of delivered DNA, which may serve as an indicator of the degree to which vectors avoid trafficking to acidic lysosomes. Focusing on the lysosomal barrier to gene delivery, this assay provides a unique tool for both identifying vectors that successfully avoid acidic lysosomes and for testing hypotheses regarding the lysosomal trafficking of non-viral vectors. This information may be useful for developing structure-function relationships for vectors mediating lysosomal escape.

3.2. Materials and Methods

3.2.1. Materials

Plasmid DNA (8 kb) was purchased from Elim Biopharmaceuticals (South San Francisco, CA). Psoralen amine, polyethylenimine (PEI) (MW = 25,000), and polylysine (PLL) (MW = 34,000) were purchased from Sigma-Aldrich (St. Louis, MO). Linear polyethylenimine (LPEI) (MW = 25,000) was purchased from Polysciences (Warrington, PA). Lipofectamine Plus was purchased from Invitrogen (Carlsbad, CA). The UV light source used was a 25 W handheld unit providing illumination at 365 nm. 6-(fluorescein-5-(and-6-)-carboxyamido)hexanoic acid, succinimidyl ester (NHS-Fl) was purchased from Molecular Probes (Eugene, OR) and FluoroLink Cy5 monofunctional dye (NHS-Cy5) was purchased from Amersham Pharmacia Biotech (Piscataway, NJ). NIH 3T3 cells were obtained from ATCC (Manassas, VA) and grown according to ATCC protocols. Opti-MEM and all other cell culture media and reagents were purchased from Invitrogen. All other materials were used as received without further purification.
3.2.2. Double-labeling the Plasmid

A critical first step for developing the desired assay was the generation of a delivery vector with a pH dependant emission signal. We chose to incorporate the signal into the DNA, as opposed to the DNA carrier, for two important reasons. First, measurement of pH at the plasmid is more relevant than measurement at the carrier molecule. Vector unpackaging can result in the localization of plasmid and carrier to different cellular environments. Since ultimate gene expression is driven by the plasmid, it is more relevant to discern the cellular location of the plasmid than the carrier. Second, the development of a separate dye conjugation strategy for each unique DNA carrier of interest is infeasible. For some carrier materials it would be simply time consuming, and for others lacking the appropriate functional groups necessary for conjugation, it would be impossible. Further, the modification of carrier materials with dyes may be expected to alter their gene transfer properties. Therefore, we chose to covalently label plasmid DNA with fluorophores that would allow for the determination of local pH.

There are a number of fluorophores with pH dependant emission spectra, most notably fluorescein which displays emission quenching at acidic pH (Figure 3.1). However, it is insufficient to simply attach fluorescein to plasmid DNA since there would be no way to normalize the signal for the quantity of labeled DNA in the cell. In other words, it would be impossible to discriminate a large amount of labeled DNA in a low pH environment (quenched emission) from a small amount of labeled DNA in a neutral pH environment. To resolve this issue, we labeled plasmid DNA with both a pH sensitive and a pH insensitive dye. The emission ratio of the two dyes is then only a function of pH.
Figure 3.1. pH dependant emission spectrum of fluorescein.[14]

Though there are a number of ways to covalently label DNA, one very important requirement for the conjugation chemistry is that the plasmid DNA-carrier material interactions be unaffected. This requirement prevents the use of certain dyes and certain conjugation techniques. For example, commonly used avidin-biotin chemistry cannot be employed for dye conjugation since the bulk and ionic charge of the avidin impacts the complexation of DNA with the carrier material. Protein dyes, such as phycoerythrin, can also disrupt this interaction. In addition to this requirement, there are other practical considerations that impact conjugation chemistry and dye choice. One dye must be pH sensitive and the other pH insensitive. Both dyes should have the appropriate excitation and emission wavelengths for the equipment used in the assay. Finally, the dyes should be spectrally isolated to prevent fluorescent resonance energy transfer (FRET). With these considerations in mind we chose to use the dyes fluorescein (pH sensitive, Ex 488/Em 530) and Cy5 (pH insensitive, Ex670/Em690), attaching them to DNA using psoralen photo-conjugation (Figure 3.2).
Figure 3.2. Covalent double-labeling of plasmid DNA with fluorescein and Cy5.

The contents of each vial of psoralen amine (12.5 μg) were dissolved in 20 μl of DMF and mixed with 100 μg of plasmid DNA (1 mg/ml in TE buffer). The psoralen amine and DNA mixture was reacted for 45 minutes using long UV light (365 nm) in a 96-well plate (120 μl per well). The 96-well plate was placed on ice during reaction with the light source resting directly on top of the plate. After reaction, excess psoralen amine was removed by ethanol precipitation of the DNA. The free amines on the plasmid were then labeled with succinimidyl esters of fluorescein and Cy5 (NHS-Fl and NHS-Cy5). The contents of the NHS-Fl vial (1 mg) were dissolved in 100 μl DMF and the contents of the NHS-Cy5 vial (0.2-0.3 mg, according to Amersham Pharmacia Biotech technical support) were dissolved in 400 μl water. Two vials of NHS-Fl (2 mg) were mixed with one vial of NHS-Cy5 (0.2-0.3 mg) and the mixture was added to 2 mg psoralen amine labeled DNA in reaction buffer (1 mg/ml, 0.1 M NaHCO₃, 1 mM EDTA, pH 9), resulting in a total volume of 2.6 ml. The reaction was carried out overnight at room temperature, protected from light. The double-labeled plasmid DNA was then purified by ethanol precipitating the DNA twice.
3.2.3. Preparation of Non-Viral Vectors

Polyplexes with PLL were formed by adding the double-labeled plasmid (100 μg/ml in 10 mM HEPES buffer) to the PLL (100 μg/ml in 10 mM HEPES buffer) at a DNA to polymer weight ratio of 1:1 while gently vortexing. Polyplexes with PEI and LPEI were formed in a similar manner by adding the plasmid to the PEI or LPEI (both 75 μg/mL in 10 mM HEPES) at a DNA to polymer weight ratio of 1:0.75. Lipoplexes with Lipofectamine Plus were formed according to Invitrogen protocols. Polyplexes with the poly(β-amino esters) Poly-A and Poly-B were formed by adding the plasmid to the polymers (both 2 mg/mL in 50 mM acetate buffer, pH 5) at a DNA to polymer weight ratio of 1:20, as this was the ratio used and reported by Lynn et al.[15] All polyplexes were given 30 minutes at room temperature to form in a total volume of 100 μl.

3.2.4. Assay Protocols

NIH 3T3 cells were seeded on 6-well plates at 4 x 10⁵ cells/well and grown for 24 hours, after which the growth medium in each well was removed and replaced with 0.5 ml Opti-MEM. Vectors were then added to the wells at a concentration of 5 μg DNA/well and placed in a 37 ℃ incubator for 30 minutes to allow for uptake. The loading medium was then removed and washed twice with PBS. Fresh growth medium was then added (2 ml/well) and the plates were returned to the 37 ℃ incubator. At various time points, a 6-well plate was taken out of the incubator and samples were prepared for flow cytometry analysis. Cells from each of the six wells were removed using trypsin and pelleted in six separate microcentrifuge tubes. Cells in two of the tubes were each resuspended in PBS + 2% FBS while cells in the other four tubes were resuspended in each of 4 intracellular pH clamping buffers (pH 5.0, 5.8, 6.6, and 7.4). These buffers were prepared by mixing 50 mM HEPES (pH 7.4) and 50 mM MES (pH 5.0) buffers (each also containing 50 mM NaCl, 30 mM ammonium acetate, and 40 mM sodium azide).[16] The cells were washed by pelleting and resuspending them once again in the appropriate buffers. One 6-well plate yielded one data point in duplicate. The fluorescein/Cy5 fluorescence ratio was measured for each of the six samples using a flow cytometer. The four intracellular pH clamped samples were used to generate a pH
calibration curve (linear), from which the fluorescein/Cy5 fluorescence ratios of the other two duplicate samples were converted to average pH values.

Evaluation of the six non-viral vectors at the 2 hour time point was done according to the same protocol as above with minor modifications. The main modification was a change in assay format from 6-well plates to T75 flasks to facilitate handling of the cells, with one T75 flask replacing one 6-well plate. Cells were seeded on T75 flasks at a concentration of 3 x 10^6 cells per T75 flask. The uptake of vectors (at 50 µg DNA per flask) was done in 3 ml Opti-MEM per flask. After 30 minutes, the loading medium was removed and 12 ml of fresh growth medium was added to the flask. Two hours after addition of the fresh culture medium, the cells were harvested and split into six aliquots. As in the previous case, two of the aliquots were used to obtain the data in duplicate and the other four were used to generate a pH calibration curve. The pH of the lowest intracellular pH clamping buffer used was 4.4 instead of 5.0 to reduce the extrapolation required for PLL vectors.

3.2.5. Flow Cytometry

Flow cytometry was performed with a FACSCalibur (Becton Dickinson) equipped with an argon ion laser to excite fluorescein (488 nm excitation) and a red diode laser to excite Cy5 (635 nm excitation). The emission of fluorescein was filtered using a 530 nm band pass filter and the emission of Cy5 was filtered using a 650 long pass filter. Distilled water was used as the sheath fluid, since we were concerned that PBS sheath fluid would alter the pH of the pH clamping buffers. The cells were appropriately gated by forward and side scatter and 30,000 events per sample were collected. Initially we gated the population to exclude cells that appeared not to have taken up labeled vectors, however we found no significant difference in pH values obtained by neglecting this gate. Therefore, we decided to eliminate this gate from the collection and analysis of the data. In preliminary experiments we also gated for viability using propidium iodide staining. Because we found greater than 97% viability in nearly all cases and because we were concerned about the possibility of propidium iodide interfering with the fluorescein and Cy5 signals, we decided to eliminate viability gating. The median FL1 (fluorescein) and FL4 (Cy5) values were used to determine the FL1/FL4 ratio for each sample. Ideally, the
FL1/FL4 ratio would be calculated for each gated event and the median value of this data set would be reported as the FL1/FL4 ratio of the sample. Unfortunately, the CellQuest software used to collect and analyze the data is unable to perform these operations. We reasoned that for a large enough sample (in our case 30,000 events), that (median FL1)/(median FL4) should closely approximate the median of FL1/FL4. To check this hypothesis we exported the raw data files for a couple of samples into Matlab. Using Matlab, FL1/FL4 was calculated for each event and the median value of this data set was determined. We compared this result to the (median FL1)/(median FL4) and found no significant difference.

3.2.6. Transfection With GFP

Cells were seeded in 6 well plates at 800,000 cells/well and were grown for 24 h prior to transfection. Non-viral vectors were prepared as previously described using the plasmid pEGFP-N1 (Clontech). Vectors were added to the cells at a concentration of 5 μg plasmid/well in 0.5 ml Opti-MEM. After 1.5 h at 37 °C, the transfection medium was replaced with fresh growth medium. After 48 h cells were harvested and assayed for GFP expression using the previously mentioned FACSCalibur flow cytometer.

3.3. Results and Discussion

3.3.1. Principle of the Assay

Bayer et al. have reported the measurement of endosomal pH using a flow cytometer to quantify the fluorescence of endosomes loaded with pH sensitive FITC-dextran and pH insensitive Cy5-dextran.[16] Using an intracellular pH clamping technique to generate a pH calibration curve, the ratio of FITC to Cy5 fluorescence emission was used to determine endosomal pH. We reasoned that, by incorporating pH sensitive fluorescent DNA into non-viral vectors, we could adapt that principle to measure the pH environment of delivered DNA. To prepare DNA with pH sensitive fluorescence emission, we covalently double-labeled plasmid DNA with fluorescein and Cy5. Using a flow cytometer, we measured the fluorescence of the DNA in buffers with
pH values ranging from 4.0-8.0 and found that the fluorescein/Cy5 emission ratio increased linearly by roughly 8X over this pH range (Figure 3.3).

![Graph](image-url)

**Figure 3.3.** Median FL1 (fluorescein) signal divided by median FL4 (Cy5) signal as a function of pH. Double-labeled DNA was layered on polylysine coated beads and fluorescence was measured in different pH buffers using a flow cytometer. The labeled plasmid was mixed with the polylysine modified beads and allowed to associate by interaction of the negatively charged DNA with the positively charged lysines on the surface of the beads. Polylysine coated beads were obtained by reacting polylysine with carboxylated 2 μm latex beads (Polysciences) using a carbodiimide protein coupling kit (Polysciences).

Low pH (4.5-5.0) is a defining characteristic of lysosomes.[17] By measuring the average pH environment experienced by delivered DNA, the degree to which a vector has avoided or altered lysosomal trafficking can be deduced. For example, vectors unable to escape lysosomes should yield average pH measurements between 4.5-5.0, while vectors able to escape lysosomes into the cytoplasm or other neutral pH compartments should yield average pH measurements near 7.0. An intermediate pH measurement would indicate an intermediate level of partitioning between low pH lysosomes and neutral pH compartments such as the cytoplasm. The interpretation of an average pH measurement is more complicated for vectors that have buffering capacity (such as PEI [11, 12]), as an
elevated pH measurement (ie. > 5.5) could arise from vectors escaping lysosomes into neutral pH compartments, buffering of endosomes and lysosomes, or some combination thereof. Although an ideal gene delivery vector would completely escape endosomes and lysosomes, a vector that simply buffers acidic vesicles may still be of interest. First, buffering of endosomes may prevent fusion with lysosomes.[18] Second, since lysosomal enzymes have maximum activity at acidic pH [17], buffering may decrease the rate of vector degradation, and thus increase transfection efficiency. In any case, the investigation of vectors that avoid lysosomal degradation should focus on vectors that show higher, rather than lower, average pH values.

3.3.2. Assay Methodology and Application

To test the feasibility of this approach we used two polymers, PEI and PLL, along with the NIH 3T3 cell line. The NIH 3T3 cell line was chosen since it is commonly used as a screening population for gene transfer using non-viral vectors. Polyplexes were prepared with the double-labeled plasmid at DNA:polymer weight ratios of 1:1 for PLL and 1:0.75 for PEI, as these ratios were found to optimize the balance between transfection efficiency and cytotoxicity. No significant difference in complex size was seen for polyplexes prepared (at the same DNA:polymer ratios) using labeled and unlabeled DNA, indicating that labeling did not significantly interfere with polymer-DNA interactions. Polyplexes were incubated with cells grown on 6-well plates in reduced serum medium at a concentration of 5 µg DNA per well for 30 minutes at 37 °C to allow for uptake by endocytosis. The cells were then washed twice with PBS, after which fresh culture medium was added, and the cells were returned to an incubator at 37 °C. We found that with the NIH 3T3 cell line this washing procedure was sufficient, but a more rigorous washing step may be required to remove surface-bound complexes from other cell lines. At various time points after addition of fresh culture medium, 6-well plates were removed from the incubator and cells were harvested for flow cytometry analysis (see Figures 3.4 and 3.5). Each 6-well plate yielded one data point in duplicate. Cells from four of the wells of the 6-well plate were used to generate a four point pH calibration curve (Figure 3.5-C), from which the fluorescein/Cy5 fluorescence ratios of cells in the other two wells were converted to average pH values. A comparison of
Figure 3.5-A to Figure 3.5-B shows that cellular autofluorescence was roughly two orders of magnitude less than the fluorescence of cells loaded with vectors prepared with double-labeled DNA.

I. Seed NIH 3T3 cells and allow them to grow to 50-80% confluence.

II. Prepare nonviral vectors and add to cells in OPTI-MEM. Allow cells to uptake vectors for 30 min at 37 °C.

III. Wash cells twice with PBS.

IV. Add fresh growth medium.

V. At various time points, trypsinize cells and split into six aliquots for flow cytometry analysis. Two of the aliquots provide duplicate data points, the other four are used to generate a FL/Cy5 emission vs. pH calibration curve.

VI. Flow cytometry analysis.

Figure 3.4. Overview of assay protocol.

Figure 3.5. Flow cytometry analysis. (A) density plot showing fluorescence of blank cells (FL4 detects Cy5 and FL1 detects fluorescein). (B) density plot showing the fluorescence of cells that have taken up non-viral vectors using double-labeled plasmid DNA. Median FL1 and median FL4 values are obtained from this data and are used to
As shown in Figure 3.6, the average pH experienced by both the PLL and PEI polyplexes dropped rapidly after uptake by cells, consistent with the acidification of endosomes. However, by 2 hours the average pH measurements for PLL and PEI stabilized to distinctly different values. The measured pH of PLL polyplexes settled to between 4.0-4.5 (these values were determined by extrapolation as the pH of the lowest intracellular pH clamping buffer was 5.0). This is consistent with lysosomal pH, suggesting very little escape of PLL polyplexes from the lysosomal trafficking pathway. On the other hand, the pH of PEI polyplexes leveled off at a value between 5.5-6.0, suggesting either appreciable escape of polyplexes from endosomes into the cytoplasm (consistent with the proton sponge hypothesis [11]) or buffering of lysosomes. To confirm that this technique could detect actual differences in cellular pH environment, we measured the pH of PLL polyplexes in the presence of 100 μM chloroquine. Chloroquine is a weak base that buffers acidic cellular vesicles and is commonly used to enhance the in vitro transfection efficiency of non-viral vectors.[19] Chloroquine was present in the transfection medium at the start of the experiment as well as in the growth medium added after 30 minutes. After 2-4 hours of chronic administration of chloroquine, the average pH experienced by the DNA increased from a low of nearly 4.0 to roughly 6.2.
**Figure 3.6.** Average pH environment of delivered plasmid DNA for PEI, PLL, and PLL + 100 μM chloroquine polyplexes as a function of time. Data was acquired in duplicate, all data points shown.

Next we evaluated six non-viral vectors at the 2 hour time point: Lipofectamine Plus, PEI, LPEI, PLL, and two degradable poly(β-amino esters) (Poly-A and Poly-B, see Fig. 3.7) recently reported by our laboratory.[15] Because the majority of the pH change measured for both PLL and PEI polyplexes occurred prior to 2 hours, we judged the 2 hour time point to be appropriate for identifying vectors that avoid low pH environments. These measurements were repeated four times, from which mean pH values and standard deviations were determined. The largest standard deviation calculated for the six different vectors was 0.15, and this value was taken to be the error in the pH measurement. We found that vectors with the highest *in vitro* transfection efficiencies (Poly-A and Poly-B) had substantially higher average pH values than vectors with the lowest transfection efficiencies (PLL and LPEI) (Table 3.1). This result implies that the
ability to avoid low pH environments inside the cell is indeed an important characteristic of vectors with high transfection efficiencies.

![Poly-A and Poly-B structures](image)

**Figure 3.7.** Structures of polymers Poly-A and Poly-B, both of which contain hydrolytically degradable ester groups. Poly-A is a proton sponge polymer containing the imidazole group ($pK_a \sim 6.5$).

<table>
<thead>
<tr>
<th>Vector</th>
<th>Avg. pH</th>
<th>Transfection efficiency $^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>PLL</td>
<td>4.5</td>
<td>0.07</td>
</tr>
<tr>
<td>LPEI</td>
<td>5.0</td>
<td>12</td>
</tr>
<tr>
<td>PEI</td>
<td>5.9</td>
<td>28</td>
</tr>
<tr>
<td>Poly-B</td>
<td>6.4</td>
<td>58</td>
</tr>
<tr>
<td>Poly-A</td>
<td>6.7</td>
<td>44</td>
</tr>
<tr>
<td>Lipofectamine Plus</td>
<td>7.1</td>
<td>31</td>
</tr>
</tbody>
</table>

$^a$Error ± standard deviation ($n = 4$)

$^b$% of viable cells positive for GFP expression
Of the six vectors evaluated, Lipofectamine Plus vectors were best able to avoid acidic environments, corresponding to a pH measurement of 7.1. This is consistent with the model proposed for lipoplex transfection whereby the cationic lipid destabilizes the endosomal membrane, releasing the plasmid into the cytoplasm.[10, 20] Both PEI and LPEI are thought to escape endosomes by way of the proton sponge mechanism. This mechanism may be used to justify the difference in measured pH for PEI (5.9) and LPEI (5.0) polyplexes. Although PEI and LPEI have identical chemical formulas, because the backbone of PEI contains both tertiary and secondary amines while the backbone of LPEI contains only secondary amines, the buffering capacity of LPEI on a weight basis is less than that of branched PEI. We have detected this difference in buffering capacity by performing acid titrations of PEI and LPEI solutions (unpublished results). Near neutral pH values (6.7 and 6.4, respectively) were measured for Poly-A and Poly-B polyplexes. While the elevated pH measured for Poly-A polyplexes may be attributable to the buffering imidazole group, the ability of Poly-B polyplexes to avoid low pH lysosomes is not satisfactorily explained using the current proposed mechanisms for endosomal or lysosomal escape. We are presently working to better understand the cellular trafficking of Poly-B polyplexes.

3.4. Summary

Through this work, we have developed a simple quantitative assay to measure the pH environment of DNA delivered using non-viral vectors. Data collected using this technique suggest that PLL polyplexes have low transfection efficiency because they are unable to avoid trafficking to lysosomes. By contrast, proton sponge vectors more successfully avoid acidic lysosomes, leading to improved transfection efficiencies. While we expect the general trends observed using the NIH 3T3 cell line to hold for many cell types, because lysosomal trafficking is likely to be cell line dependent, it is possible that different vectors will be more or less effective relative to one another in different cell types. Through the use of the assay described here, the lysosomal barrier to gene delivery can be studied more directly, and vectors that overcome this obstacle can be identified more reliably. Vectors successful in escaping lysosomes but unsuccessful in overcoming other barriers may show very poor levels of transfection, and therefore, may
be missed by transfection efficiency screens. The ability to identify and study such vectors could be useful for learning about vector-cell interactions and for discovering new mechanisms for avoiding lysosomal trafficking. We are currently using this assay to conduct structure-function studies to determine how factors such as molecular weight, buffering capacity, and polymer structure affect the ability of vectors to avoid acidic lysosomes.
3.5. References


4. PEI and the Proton Sponge Hypothesis

4.1. Introduction

The introduction of polyethylenimine (PEI) in 1995 represented a major advance toward functional, effective non-viral gene transfer.[1] The gene transfer efficiency of PEI was attributed to its unique ability to overcome a specific barrier to gene transfer, lysosomal degradation.[1, 2] The trafficking and subsequent degradation of vectors inside lysosomes is one of the main cellular barriers to effective gene transfer.[3] Viruses have evolved mechanisms for avoiding lysosomal trafficking by promoting the fusion of the viral envelope with the endosomal membrane, causing the release of the virus into the cytoplasm. The incorporation of inactivated virus particles or fusogenic viral peptides into non-viral vectors has lead to improved transfection efficiencies,[4-8] but potential immunogenicity of the viral components is of major concern. Chloroquine has also been used to enhance non-viral transfection efficiency.[9-14] Chloroquine is a small molecule drug that buffers acidic vesicles, inhibiting lysosomal enzymes and possibly altering normal lysosomal trafficking. However, the use of chloroquine in vivo is likely to be limited. In contrast to many non-viral vectors which require the use of viral components or chloroquine to achieve appreciable transfection efficiencies, PEI is able to mediate high levels of gene transfer on its own.[1]

PEI is a branched polymer containing primary, secondary, and tertiary amines in a 1:2:1 ratio. These amines have pKₐs spanning the physiological pH range, resulting in buffering capacity. The degree of protonation of the amines increases from roughly 20% to 45% as pH decreases from 7 to 5.[2] The “proton sponge” nature of PEI is thought to lead to buffering inside endosomes. The additional pumping of protons into the
endosome, along with the concurrent influx of chloride ions to maintain charge neutrality, results in an increase in ionic strength inside the endosome. This is then thought to cause osmotic swelling and physical rupture of the endosome, resulting in the escape of the vector from the degradative lysosomal trafficking pathway.[2] Since the identification of PEI as an effective gene delivery vector, numerous groups have worked to elucidate the mechanism of PEI-mediated gene transfer.[15-18] While the proton sponge hypothesis has not yet been definitively proven, it has been invoked to explain the relatively high transfection efficiencies of other proton sponges such as lipopolyamines,[2, 19] poly(amide amine) (PAMAM) dendrimers,[20] and various imidazole containing polymers.[14, 21, 22] The original hypothesis put forth in 1995 was that PEI buffering, leading to osmotic rupture and subsequent escape, occurred in lysosomes.[1] In 2000, Godbey et al. challenged the proton sponge hypothesis based on their findings of a lack of lysosomal involvement in PEI-mediated gene transfer.[16] However, three years earlier, J.P. Behr had proposed a version of the proton sponge hypothesis whereby PEI buffering, leading to osmotic rupture, was thought to occur in endosomes, prior to fusion with lysosomes.[2] This expression of the proton sponge hypothesis is now the dominant view on the topic and is consistent with the 1999 findings of Godbey et al.

Although there have been numerous prior studies on the mechanism of PEI mediated gene transfer, because the results have been qualitative and not quantitative, their findings have remained somewhat speculative. Here we attempt to elucidate the mechanism of PEI mediated gene transfer, with a focus on the proton sponge hypothesis, by quantitatively investigating the gene transfer properties of both PEI and N-quaternized versions of PEI. Quaternization of PEI amines prevents further protonization, and therefore, abolishes the proton sponge effect. According to the proton sponge hypothesis, because quaternized PEI vectors are not be able to buffer endosomes effectively, they should have much lower transfection efficiencies. We have demonstrated that quaternization of PEI results in a drastic decrease in transfection activity, and that this effect seems to be due to a quantitative difference in the ability to buffer acidic cellular compartments.
4.2. Materials and Methods

4.2.1. Materials

N-quaternized polymers permethyl-PEI (Me-PEI) and perethyl-PEI (Et-PEI) were synthesized as previously described (based on PEI with $M_n = 25,000$) [23] and generously provided by Dr. Mini Thomas (Department of Chemistry, MIT). PEI ($M_n = 25,000$), polylysine (PLL) ($M_n$ 34,000), bafilomycin A1, and chloroquine were purchased from Sigma-Aldrich (St. Louis, MO). Plasmid DNA containing the firefly luciferase reporter gene (pCMV-Luc) was purchased from Elim Biopharmaceuticals, Inc. (San Francisco, CA) and used without further purification. COS-7 cells were purchased from American Type Culture Collection (ATCC) (Manassas, VA) and grown at 37°C, 5% CO₂ in Dulbecco's modified Eagle's medium, 90%; fetal bovine serum, 10%; penicillin, 100 units/mL; streptomycin, 100 µg/mL. All cell culture media and reagents were purchased from Invitrogen (Carlsbad, CA). All other materials and solvents were used as received without additional purification.

4.2.2. Luciferase Transfection Assays

Transfection assays were performed in the following general manner. COS-7 cells were grown in opaque white 96-well plates at an initial seeding density of 15,000 cells/well in 200 µL of phenol red-free growth medium (90% Dulbecco's modified Eagle's medium, 10% fetal bovine serum, penicillin 100 units/mL, streptomycin 100 µg/mL). Cells were grown for 24 hours in an incubator at 37 °C, 5% CO₂ prior to transfection. Polymers/DNA complexes were prepared at polymer nitrogen/DNA phosphate (N/P) ratios of 10 for PEI, Me-PEI, and Et-PEI. An N/P ratio of 5 was used for PLL complexes. Complexes were prepared by adding DNA solution to polymer solution dropwise while gently vortexing. After 30 minutes incubation at room temperature, complexes were diluted in Opti-MEM, Opti-MEM + 200 nM bafilomycin A1, or Opti-MEM + 100 µM chloroquine. Complexes were then added to the cells at a concentration of 600 ng DNA/well in a total volume of 200 µL. After 4 hr incubation at 37 °C, 5% CO₂, complexes were removed and 105 mL of fresh growth media was added to each well. After 3 days, cells were analyzed for luciferase expression using Bright-Glo
assay kits (Promega, Madison, WI). Briefly, 100 μl of Bright-Glo solution was added to each well of the 96-well plate containing media and cells. Luminescence was measured using a Mithras Luminometer (Berthold, Oak Ridge, TN). A 1% neutral density filter (Chroma, Brattleboro, VT) was used to prevent saturation of the luminometer. A standard curve for Luciferase was generated by titration of Luciferase enzyme (Promega) into growth media in an opaque white 96-well plate.

4.2.3. Measurement of pH Environment of Delivered DNA and Cellular Uptake

COS-7 cells were seeded on 6-well plates at a concentration of 4 x 10^5 cells/well and grown for 24 hours in an incubator at 37 °C, 5% CO₂. Polymer/DNA complexes were prepared as described above except that fluorescein-Cy5 double-labeled plasmid[18] was used and the procedure was scaled up to 5 μg DNA/well. After 24 hours, the growth media was removed from the wells and complexes were added to the wells in 1 mL Opti-MEM. The plates were then returned to the incubator to allow for uptake of the fluorescently-labeled DNA. After 30 minutes, the loading medium was removed, and 2 ml of fresh growth medium was added to each well. Two hours after addition of the fresh culture medium, the cells in each well were harvested using trypsin and pelleted in three separate microcentrifuge tubes. One of the aliquots was used to obtain the data and the other two were used for pH calibration. Cells in sample vials were each resuspended in PBS containing 2% fetal bovine serum while cells in the other two vials were resuspended in each of two intracellular pH clamping buffers (pH = 4.4 and 7.4). The pH clamping buffers were composed of 50 mM MES (pH = 4.4) and 50 mM HEPES (pH = 7.4), each also containing 50 mM NaCl, 30 mM ammonium acetate, and 40 mM sodium azide. The cells were washed by pelleting and resuspending them once again in the appropriate buffers. The fluorescein/Cy5 fluorescence ratios of the samples were measured using a flow cytometer. The two intracellular pH clamped samples were used to convert the fluorescein/Cy5 fluorescence ratio of the sample cells into an average pH value. Cellular uptake information was simultaneous obtained through pH insensitive Cy5 fluorescence signal.
4.2.4. Flow Cytometry

Flow cytometry was performed with a FACSCalibur (Becton Dickinson) equipped with an argon ion laser to excite fluorescein (488 nm excitation) and a red diode laser to excite Cy5 (635 nm excitation). The emission of fluorescein was filtered using a 530 nm band pass filter and the emission of Cy5 was filtered using a 650 long pass filter. Distilled water was used as the sheath fluid. The cells were appropriately gated by forward and side scatter and 30,000 events per sample were collected. The median FL1 (fluorescein) and FL4 (Cy5) values were used to determine the FL1/FL4 ratio for each sample.

4.2.5. Polymer-DNA Binding Titrations

Polymer-DNA complexes were prepared as described above and diluted in water to yield a concentration of 300 ng DNA/100 μL solution. 100 μL of the aqueous solution containing the complexes was arrayed into each well of an opaque black 96-well plate. PicoGreen (Molecular Probes, Eugene, OR) solutions were prepared by diluting the supplied PicoGreen stock solution 200-fold in 10 mM Hepes buffer (pH 7.2) containing various concentrations of NaCl. The PicoGreen solutions were prepared such that the desired salt concentration was achieved upon addition of 100 μL PicoGreen solution to each well containing 100 μL of sample. After 10-15 minutes the plate was analyzed using a Mithras fluorescent plate reader (Berthold, Oak Ridge, TN). Data was collected using the FITC filter set (excitation 485 nm, emission 535 nm). Fractional dye exclusion was determined by the following relationship.

\[
Dye\ Exclusion = 1 - \frac{(F_{sample} - F_{blank})}{(F_{DNA\ only} - F_{blank})}
\]
4.3. Results and Discussion

4.3.1. Luciferase Transfections

According to the proton sponge hypothesis, abolition of the proton sponge character of PEI by N-quaternization should result in decreased transfection activity, and earlier experiments using the β-galactosidase reporter system have shown this to be the case.[23] In this work, we performed transfection experiments using PEI as well as N-quaternized versions of PEI, permethylated PEI (Me-PEI) and perethylated PEI (Et-PEI) (Figure 4.1).

![Chemical structure of Me-PEI and Et-PEI](image)

Figure 4.1. Chemical structures of Me-PEI and Et-PEI.

As our aim was to investigate the proton sponge hypothesis, transfections were also performed in the presence of chloroquine and bafilomycin A1. Chloroquine is known to buffer the pH of late endosomes/lysosomes and has been shown to improve the transfection efficiency of vectors such as polylysine (PLL),[9-11, 13] which are unable to escape the lysosomal trafficking pathway. Bafilomycin A1 is a specific inhibitor of the vacuolar ATPase proton pump found in early endosomes.[24, 25] The use of bafilomycin prevents the acidification of early endosomes, which in turn inhibits the formation of late endosomes.[26] Because bafilomycin inhibits proton transport into endosomes, it should inhibit the protonation of proton sponge vectors inside endosomes, and therefore, according to the proton sponge hypothesis, prevent endosomal escape. Transfection experiments were performed in 96-well plate format, using COS-7 cells and plasmid encoding the firefly luciferase reporter protein (pCMV-Luc) (600 ng/well). PEI,
Me-PEI, and Et-PEI were used at polymer nitrogen to DNA phosphate (N/P) ratios of 10. PLL was used at an N/P of roughly 5.

The data displayed in Figure 2 indicate that the transfection activity of PEI is reduced by nearly 2 orders of magnitude upon quaternization of the amines. In addition, the use of 200 nM bafilomycin decreased the transfection activity of PEI by roughly 200-fold. Bafilomycin was found to decrease transfection activity for Me-PEI, Et-PEI, and PLL as well, resulting in gene expression that was nearly identical for all four vectors. The use of 100 mM chloroquine slightly decreased the activity of PEI, but increased the activity of Me-PEI, Et-PEI, and PLL by a factor of 2-3. The finding that chloroquine does not enhance PEI transfection suggests very little involvement of late endosomes and lysosomes in the mechanism of PEI transfection. In contrast, the enhancement realized by the quaternized PEIs and PLL in the presence of chloroquine suggests that these vectors do traffic to late endosomes and lysosomes.

![Bar chart](chart.png)

**Figure 4.2.** Luciferase transfection results. Transfections were performed in Opti-MEM, Opti-MEM + 200 nM bafilomycin A1, and Opti-MEM + 100 μM chloroquine. (n = 8, error bars represent standard deviation).
4.3.2. Measurement of pH Environment

Determination of the local pH environment of delivered DNA can yield useful information regarding the likely intracellular location of the DNA. For example, vectors unable to avoid trafficking to lysosomes will experience a local environment of pH 4-5. In contrast, vectors that are able to escape endosomes into the cytoplasm will experience neutral pH environments. By measuring the average pH experienced by DNA delivered using a given vector, the intracellular fate of the DNA may be deduced. We have recently developed a flow cytometry based assay for this measurement, the details of which have been previously reported.[18] Briefly, the formation of polymer/DNA complexes using plasmid DNA labeled with both fluorescein (pH sensitive) and Cy5 (pH insensitive) allows the local pH environment of transfected DNA to be determined as a linear function of pH (in the range of pH from 4 to 8) by measuring the emission ratios of these two fluorophores. The fluorescence emission ratios of COS-7 cells incubated with labeled polymer/DNA complexes were measured using a flow cytometer and were used to calculate the average pH environment of the delivered DNA. The results of these experiments are displayed in Figure 4.3.

![Figure 4.3](image)

**Figure 4.3.** Measured average pH environment of DNA delivered using the polymers PEI, Me-PEI, Et-PEI, and PLL. (n = 3, error bars represent standard deviation).
The average local pH environment of DNA delivered using PEI was measured to be 6.1. For a proton sponge vector an elevated pH measurement could arise from either a certain fraction of vectors escaping into the cytoplasm (pH 7) and a certain fraction trafficking to acidic lysosomes (pH 4-5) or partial buffering of cellular vesicles, or some combination thereof. In any case, an average pH measurement of 6.1 indicates that the majority of vectors do not reside in acidic, unbuffered lysosomes. In contrast, the average local pH environment of PLL vectors was measured to be 4.6. This measurement is consistent with lysosomal pH, suggesting that PLL vectors are unable to escape lysosomal trafficking. N-quaternization of PEI resulted in significantly lower pH measurements, 5.4 for Me-PEI and 5.0 for Et-PEI, indicating that DNA delivered using these vectors resided in lower pH compartments within the cell, with perhaps only a small fraction in the cytoplasm. The improved performance of the quaternized PEIs over PLL could be due to the fact that Me-PEI and Et-PEI may still have a slight proton sponge nature as a result of incomplete N-quaternization. The extent of quaternization for Me-PEI and Et-PEI was 87% and 77%, respectively.[23] The obtained pH measurements as well as the above transfection results strongly suggest that buffering capacity plays a central role in the mechanism of PEI mediated gene transfer. However, in addition to endosomal escape, N-quaternization of PEI could impact other steps in the gene transfer pathway such as cellular uptake and vector unpacking leading to the observed differences in transfection activity. To more fully characterize the mechanism of PEI mediated transfection, we investigated these barriers as well.

4.3.3. Cellular Uptake

The flow cytometry based assay used to make pH measurements also supplies cellular uptake data. Measurement of pH requires analysis of both the pH sensitive fluorescein signal and the normalizing pH insensitive Cy5 signal. However, the relative cellular uptake of vectors can be determined by simply quantifying the Cy5 signal, which is only a function of the total amount of labeled DNA inside the cell. The uptake results displayed in Figure 4.4 show that Me-PEI and Et-PEI led to internalization extents that were 20% and 90% greater, respectively, than that of unmodified PEI. The fact that
cellular uptake is enhanced by alkylation indicates that the reduction in transfection activity resulting from quaternization of PEI is due to an effect downstream of the cellular uptake barrier.

Figure 4.4. Cellular uptake relative to PEI. (n = 3, error bars represent standard deviation).

4.3.4. Vector Unpacking

Appropriate unpacking of the vector has been postulated as another important barrier to effective gene transfer. In order for gene expression to take place, transcription factors must be able to access the DNA. Therefore, the vector must not bind DNA so tightly that gene expression is hindered. We initially suspected that the decreased transfection activity of quaternized PEIs may have been due to tighter binding of the polymer to the DNA, since quaternization results in a positive charge at every amine. To investigate the binding interactions between the DNA and the different polymers, salt titrations of the different complexes were performed in the presence of the dye PicoGreen. PicoGreen is a dsDNA-intercalating dye whose fluorescence is dramatically
enhanced upon binding to DNA. Increasing salt concentration can destabilize polymer-DNA complexes, allowing the dye access to the DNA. Under physiological salt concentrations (0.15 M) all complexes were found to be quite stable, with greater than 80% dye exclusion (Figure 4.5). However, the quaternized PEI complexes were significantly less stable than PEI complexes in the presence of higher salt concentrations. The 50% dye exclusion point was roughly 0.3 M, 0.4 M, and greater than 1.0 M for Et-PEI, Me-PEI, and PEI, respectively. This result contradicted our initial hypothesis; however, there are clearly other factors besides charge density that impact the polymer-DNA interaction. Specifically, in this case, it seems that steric differences between PEI, Me-PEI, and Et-PEI may play a role in the binding interaction between these polymers and DNA. Based on the above DNA binding experiments, it is unlikely that the decreased expression levels of the quaternized PEIs result from the inhibition of transcription due to poor vector unpacking.

![Graph](image)

**Figure 4.5.** Assesment of polymer-DNA binding affinity by salt titration. Binding affinity was determined by exclusion of the dsDNA intercalating dye PicoGreen.
4.4. Conclusions

In this work, we have used quantitative methods to study the mechanism of PEI mediated gene transfer. As part of this approach, N-quaternized PEIs and specific cell function inhibitors have been used to isolate specific steps in overall gene transfer process. We have found that the abolition of proton sponge character, by either N-quaternization of PEI or the use of bafilomycin A1, results in a dramatic decrease in transfection activity. The use of chloroquine, which leads to buffering of late endosomes and lysosomes, resulted in no effect for PEI and enhancement of transfection activity for N-quaternized PEIs and PLL. Cellular uptake measurements demonstrated that the decrease in transfection activity due N-quaternization is due to barriers downstream of vector internalization, and polymer-DNA binding experiments indicated that poor vector unpackaging was not the source of this decrease. Cumulatively, these data point toward the ability to mediate endosomal escape as the crucial difference between PEI and N-quaternized PEIs. The most direct evidence for this may be the measured average pH values for these vectors. DNA delivered using PEI had an average pH environment of 6.1, indicating that PEI vectors are able to avoid trafficking to acidic, unbuffered lysosomes. In contrast, the N-quaternized PEI vectors had average pH measurements of 5.4 and 5.0, suggesting that they were much less successful in avoiding degradative lysosomes. This study may represent the most complete validation of the proton sponge hypothesis to date. All of the results obtained in this study are consistent with the proton sponge hypothesis and indicate that PEI mediated transfection is efficient due to the avoidance of lysosomal trafficking.
4.5. References


5. Biophysical Characterization of a Degradable Poly(β-Amino Ester) Library

5.1. Introduction

Cationic polymers have been investigated broadly in the context of gene delivery, primarily due to the facility with which they form conjugates with negatively-charged strands of DNA, condensing it into nanometer-scale structures small enough to enter cells via endocytosis.[1] Although many polycations are effective at overcoming these early entry-based barriers to gene delivery, commonly used polymers such as polylysine and polyethylenimine (PEI) are also associated with high degrees of cytotoxicity.[2, 3] We and others have investigated the synthesis of biodegradable polycations for use as gene delivery agents, both from the standpoint of reducing the general toxicities of conventional materials as well as a potential means through which to control or trigger the release of transfected DNA inside the cell. Recent examples of biodegradable polycations introduced in the context of gene delivery include poly(4-hydroxy-L-proline ester),[4, 5] poly[α-(4-aminobutyl)-L-glycolic acid] (a polyester analog of polylysine),[6] poly(2-aminoethyl propylene phosphate),[7, 8] and degradable hyperbranched analogs of poly(amido amine) dendrimers.[9] As a class of materials, these biodegradable polycations are significantly less toxic than polylysine and PEI, and mediate the transfer and expression of genes to cells at levels that approach[4-8] or exceed[9] those using PEI,[10-12] a polymer generally considered to be the current standard and the example to which new polymers are often compared. These results suggest that the design of biodegradable polycations represents a reasonable approach to the development of safe and effective polymeric vectors.
The synthesis of poly(β-amino esters), a class of biodegradable polymers having both tertiary amines and esters in their backbones, synthesized via the conjugate addition of amine-based monomers to diacrylate compounds has been previously described (Eq 1). In a more recent paper, this work was extended to the parallel synthesis of a 140-member library of structurally unique biodegradable materials and the identification of new gene delivery vectors through rapid cell-based screening assays.[14] This approach led to the discovery of two polymers, **B14** and **G5**, that mediated gene expression at levels 4-8 times higher than that of polyethylenimine (PEI). In addition to providing a means for discovering new vectors for gene delivery, access to a library of structurally related polymers provides new opportunities to investigate structure/function relationships for a family of materials. Here, we analyze the resulting polymer/DNA complexes with respect to their size and surface charge, their ability to mediate cellular uptake, their ability to avoid trafficking to acidic lysosomes, and ultimately, their ability to mediate gene expression. Through this work we have identified several chemical features correlating with improved gene transfer properties.

### 5.2. Materials and Methods

#### 5.2.1. Materials

Polymers were synthesized as previously described.[14] PEI (Mₙ = 25,000) and PLL (Mₙ 34,000) were purchased from Sigma-Aldrich (St. Louis, MO). COS-7 and NIH 3T3 cells were purchased from American Type Culture Collection (Manassas, VA) and grown at 37º C, 5% CO₂ in Dulbecco's modified Eagle's medium, 90%; fetal bovine serum, 10%; penicillin, 100 units/mL; streptomycin, 100 µg/mL. All cell culture supplies
were obtained from Invitrogen (Carlsbad, CA). All other materials and solvents were used as received without additional purification.

5.2.2. Cellular Uptake Experiments

NIH 3T3 cells were seeded on 6-well plates at a concentration of $4 \times 10^5$ cells/well and grown for 24 hours, after which the growth medium in each well was removed and replaced with 0.5 ml Opti-MEM. DNA/polymer complexes employing fluorescein-Cy5 double-labeled plasmid[15] were prepared at a DNA/polymer ratio of 1:20 (w/w) in the following manner. Polymers were dissolved in 25 mM acetate buffer (pH = 5.0) to yield a concentration of 2 mg/mL. 50 μL of each polymer solution was pipetted into polypropylene microcentrifuge tubes. While gently vortexing, 50 μL of an aqueous solution of double-labeled DNA (100 μg/mL in HEPES buffer, pH = 7.2) was added dropwise to each microcentrifuge tubes containing polymer solution (100 μL total volume). Controls employing PEI and PLL were prepared in a similar fashion except that they were dissolved in acetate buffer at concentrations of 75 μg/mL and 100 μg/mL, respectively (resulting in DNA/polymer ratios of 1:0.75 (w/w) and 1:1 (w/w)). All complexes were incubated at room temperature for 30 minutes, after which they were added to the wells at a concentration of 5 μg DNA/well and placed in a 37 °C incubator for 30 minutes to allow for uptake. The loading medium was then removed and washed twice with PBS. Fresh growth medium was then added (2 mL/well) and the plates were returned to the 37 °C incubator to allow for uptake of any remaining surface-bound complexes. After 2 hr, cells from each of the wells were harvested using trypsin and pelleted in separate eppendorf vials. We found that with the NIH 3T3 cell line this procedure was sufficient to eliminate surface-bound complexes, but a more rigorous washing or stripping step may be required for other cell lines. Cells were washed twice and finally resuspended in PBS containing 2% fetal bovine serum. Each cell sample was analyzed by flow cytometry to determine the level of Cy5 fluorescence.

5.2.3. Particle Sizing and Zeta Potential Measurements

Particle sizing experiments and zeta potential measurements were made using a ZetaPALS dynamic light scattering detector (Brookhaven Instruments Corporation,
Holtsville, NY, 15 mW laser, incident beam = 676 nm). DNA/polymer complexes were prepared as described above, and each sample was diluted in 1.4 mL of HEPES buffer (10 mM, pH = 7.2). Correlation functions were collected at a scattering angle of 90°, and particle sizes were calculated using the MAS option of BIC's particle sizing software (ver. 2.30) using the viscosity and refractive index of pure water at 25 °C. Particle sizes are expressed as effective diameters assuming a lognormal distribution. Average electrophoretic mobilities were measured at 25 °C using BIC PALS zeta potential analysis software and zeta potentials were calculated using the Smoluchowsky model for aqueous suspensions.

5.2.4. Measurement of pH Environment of Delivered DNA

NIH 3T3 cells were seeded on T75 flasks at a concentration of 3 x 10^6 cells per flask and grown for 24 hr at 37 °C. The growth medium was then replaced with 3 mL Opti-MEM per flask. DNA/polymer complexes were prepared as described above except that the procedure was scaled up by a factor of 10 (0.5 mL DNA solution added to 0.5 mL polymer solution, total volume = 1 mL). The sample (50 μg DNA) was then added to the flask, and the flask was returned to the incubator. After 30 minutes, the loading medium was removed, and 12 ml of fresh growth medium was added to the flask. Two hours after addition of the fresh culture medium, the cells were harvested using trypsin and pelleted in six separate microcentrifuge tubes. Two of the aliquots were used to obtain the data in duplicate and the other four were used to generate a pH calibration curve. Cells in two of the vials were each resuspended in PBS containing 2% fetal bovine serum while cells in the other four vials were resuspended in each of 4 intracellular pH clamping buffers (pH = 4.5, 5.8, 6.6, and 7.4). These buffers were prepared by mixing 50 mM HEPES (pH = 7.4) and 50 mM MES (pH = 5.0) buffers (each also containing 50 mM NaCl, 30 mM ammonium acetate, and 40 mM sodium azide). The cells were washed by pelleting and resuspending them once again in the appropriate buffers. One T75 flask yielded one data point (one library member) in duplicate. The fluorescein/Cy5 fluorescence ratio was measured for each of the six samples using a flow cytometer. The four intracellular pH clamped samples were used to generate a pH
calibration curve (linear), from which the fluorescein/Cy5 fluorescence ratios of the other two duplicate samples were converted to average pH values.

5.2.5. Flow Cytometry

Flow cytometry was performed with a FACSCalibur (Becton Dickinson) equipped with an argon ion laser to excite fluorescein (488 nm excitation) and a red diode laser to excite Cy5 (635 nm excitation). The emission of fluorescein was filtered using a 530 nm band pass filter and the emission of Cy5 was filtered using a 650 long pass filter. Distilled water was used as the sheath fluid. The cells were appropriately gated by forward and side scatter and 30,000 events per sample were collected. The median FL1 (fluorescein) and FL4 (Cy5) values were used to determine the FL1/FL4 ratio for each sample.

5.2.6. Measurement of Cytotoxicity

Cytotoxicity assays were performed in triplicate in the following general manner. COS-7 cells were grown in 96-well plates at an initial seeding density of 15,000 cells/well in 200 μL of phenol red-free growth medium (90% Dulbecco's modified Eagle's medium, 10% fetal bovine serum, penicillin 100 units/mL, streptomycin 100 μg/mL). Cells were grown for 24 hours in an incubator, after which the growth medium was removed and replaced with Opti-MEM medium containing DNA/polymer complexes. Complexes were prepared as described above. After 1 hr incubation at 37 °C the Opti-MEM containing the complexes was removed and 100 μL of fresh growth medium was added to each well. After an addition 24 hr incubation period, cells were assayed for metabolic activity using the MTT Cell Proliferation Assay kit (ATCC, Manassas, VA). 10 mL of MTT Reagent was added to each well. After 2 hr incubation at 37 °C, 100 mL of Detergent Reagent was added to each well. The plate was then left in the dark at room temperature for 4 hr. Optical absorbance was measured at 570 nm using a microplate reader and converted to % relative to control (untreated) cells.
5.3. Results and Discussion

5.3.1. Polymer Synthesis and Initial Screening

Polymers were synthesized as previously described.[14] Diacrylate monomers A-G and amine monomers 1-20 were selected for the synthesis of the polymer library (Figure 5.1) based on representative degree of hydrophobicity, hydrophilicity, and either structural or functional character. Although, in principle, this set of monomers could be expanded to produce libraries of polymers numbering into the thousands, the size of this library (7 diacrylates x 20 amines = 140 polymers) was limited to facilitate the manual manipulation and characterization of monomers and polymers in proof-of-concept experiments.

![Figure 5.1. Diacrylate (A-G) and amine (1-20) monomers used in the synthesis of the poly(β-amino ester) library.](image)

Seventy-seven samples (representing 55% of the polymer library) were analyzed by gel permeation chromatography, indicating molecular weights ranging from 1000 to 50,000, relative to polystyrene standards.[14] A series of solubility and DNA-complexation assays were conducted to further characterize these polymers and identify the subset that
would be useful in subsequent assays. Seventy polymers were sufficiently water-soluble at a concentration of 2 mg/mL (25 mM acetate buffer, pH = 5.0) to be screened for DNA-binding capacity using an agarose gel electrophoresis retardation assay. Fifty-six of the 70 water-soluble polymers were found to interact with DNA sufficiently at a ratio of 1:20 (w/w, DNA/polymer) to retard the migration of DNA through an agarose gel; those polymers unable to interact sufficiently with DNA did not retard migration in this assay and were discarded from further consideration. The remaining 56 DNA-complexing polymers were screened for their abilities to transfect cells (Figure 5.2),[14] and formed the basis for our investigations of polymer structure/function relationships.

Figure 5.2. Transfection data as a function of structure for an assay employing pCMV-Luc (600 ng/well, DNA/polymer = 1:20). Light units are arbitrary and not normalized to total cell protein; experiments were performed in triplicate (error bars not shown). Black squares represent water-insoluble polymers, white squares represent water-soluble polymers that did not complex DNA. The right column (marked "**") displays values for the following control experiments: no polymer (green), PEI (red) (DNA/polymer = 1:1), and Lipofectamine 2000 (light blue) at 0.1, 0.2, 0.4, and 0.6 μL quantities. [14]
5.3.2. Evaluation of Intracellular Barriers to Transfection

In addition to the identification of new materials for use in gene delivery systems, access to a library of novel structures affords the opportunity to test and evaluate vector design criteria. For efficient gene transfer and expression to occur, a vector must be capable of overcoming or avoiding several important intracellular barriers to transfection.[1, 16] Two important requirements for efficient delivery are the ability to mediate cell entry (e.g., via endocytosis) and the ability to avoid intracellular trafficking to lysosomes, the main degradative compartment of the cell. Although the observation of gene expression in transfected cells is an important tool for evaluating new materials, it is merely an end-point result and provides no specific mechanistic information about the ability of a given material to overcome individual, intermediate barriers to transfection. For example, the transfection experiments above (Figure 5.2) clearly demonstrate that polymer/DNA complexes formed from polymers B14 and G5 are able to cross the cell membrane. However, the absence of observed expression using other polymers is not sufficient to conclude that these complexes are uptake-limited—i.e., these polymers may mediate cellular uptake, but could ultimately be limited by downstream barriers such as lysosomal compartmentalization and degradation, the inability to localize to the nucleus, or the inability to release DNA once inside the nucleus.[1, 17, 18]

While it is generally straightforward to measure the cellular uptake of polymer/DNA complexes, it is more difficult to quantify the extent to which these complexes avoid trafficking to lysosomes. To address these and related mechanistic questions, we recently developed a fluorescence-based flow cytometry assay that can be used to quantify levels of cellular uptake of polymer/DNA complexes and the average pH environment of delivered DNA.[15] By directly evaluating these processes, the impact of these barriers on gene transfer can be assessed individually. Such detailed information should yield a more complete biophysical understanding of these polymers in a cellular environment and could lead to the identification of new structural elements useful in the design of improved polymeric vectors. The following sections describe the application of these analytical techniques to the biophysical characterization of the 56 DNA-complexing members of the polymer library.
5.3.3. **Analysis of Cellular Uptake.**

The ability of different polymers to mediate the cellular uptake of DNA was measured by adapting a fluorescence-based flow cytometry protocol used to measure the pH environment of vector-delivered DNA.[15] Polymer/DNA complexes were prepared using plasmid DNA covalently labeled with two fluorescent labels, fluorescein (pH sensitive) and Cy5 (pH insensitive). To permit the direct correlation of cellular uptake data with the gene expression data outlined above, complexes were formed at the same DNA/polymer ratios used in previous transfection experiments (e.g., 1:20 w/w). Double-labeling was used to permit simultaneous measurement of particle uptake and pH environment (as described below); however only the signal corresponding to Cy5 emission was used for cellular uptake experiments. Labeled complexes were incubated with NIH 3T3 cells for 30 minutes at 37 °C to allow for uptake (see Materials and Methods for details), and the relative intensity of Cy5 fluorescence due to particle uptake was quantified by flow cytometry. The results of these uptake experiments are shown in Figure 5.3.
Figure 5.3. Cellular uptake data as a function of structure for an assay employing Cy5-labeled plasmid DNA (DNA/polymer = 1:20). Relative uptake level determined by median Cy5 fluorescence of cells (measured in FL4 channel of the flow cytometer); experiments were performed in duplicate (sample discrepancies were within 10%). Black squares represent water-insoluble polymers, white squares represent water-soluble polymers that did not complex DNA. The right column (marked "*".) displays values for the following control experiments: no polymer (green), PLL (red) (DNA/polymer = 1:1), and PEI (light blue) (DNA/polymer = 1:0.75).

The results in Figure 5.3 indicate that the majority of polymer/DNA particles prepared using the polymer library were uptake-limited, as only a small fraction of the polymer library was able to mediate high levels of cellular uptake under these conditions. As anticipated, polymers B14 and G5, previously identified as "hits" in the transfection assay, had cellular uptake levels significantly higher (18X and 32X, respectively) than that of "naked" DNA. The relative cellular uptake of particles formed from polymer A14 were equivalent to those measured using polymer B14, but levels of gene expression were consistently much lower in transfection experiments. This result suggests that particles formed from A14 are not uptake-limited, but may be limited by downstream
barriers to efficient transfection. Complexes prepared using polymers C14 and G14 demonstrated very high levels of uptake – 50X and 67X higher than “naked” DNA, respectively. The discrepancies between these high uptake levels and correspondingly low levels of gene expression could be related to downstream barriers to transfection, but are most likely linked to the apparent toxicity of these two polymers at the concentrations used in this assay.[14] Conversely, it is possible that the extremely high levels of uptake for particles prepared with these polymers could be responsible for the observed cytotoxicities, resulting in lower gene expression.

5.3.4. Effective Diameter and Zeta Potential Measurements

Particle size and surface charge both influence the internalization of polymer/DNA complexes by nonspecific endocytosis. Most cell types efficiently internalize particles less than 200 nm in size,[3, 17] and a positive surface charge may lead to a greater interaction with the negatively charged cell membrane, triggering endocytosis.[19] We analyzed the size and surface charge of DNA/polymer complexes prepared using members of the polymer library to investigate the relationships between polymer structure and these important physical factors. Particle size was determined by measuring the effective diameter of complexes using dynamic light scattering, and surface charge was quantified by zeta potential analysis. For these experiments, complexes were formed under the same conditions used in uptake experiments with the exception that unlabeled plasmid DNA was used. As we determined that the presence of fluorescent label did not alter the effective diameters or zeta potentials of resulting complexes (data not shown), unlabeled plasmids were used to conserve labeled DNA.

The results of the particle size measurements and zeta potential analyses of the DNA-complexing members of the polymer library are shown in Tables 5.1 and 5.2. The vectors with the highest uptake levels, namely A-C14, G14, A13, B13, and G5, all had measured effective diameters below 250 nm. Conversely, of the complexes with effective diameters greater than 1000 nm (A-E9, E1, and D11), none were internalized by cells at appreciable levels. The majority (~ 70%) of the complexes in the library had negative zeta potentials at DNA/polymer ratios of 1:20 (w/w) (as measured in 10 mM HEPES buffer, pH = 7.2), and this could explain the poor levels of uptake and expression.
for these polymers. Complexes formed from the family of polymers synthesized from amine monomers 10, 13, and 14 had positive zeta potentials at pH 7.2 and correspond to the group of polymers mediating the highest levels of uptake (Figure 5.3). In these experiments, a DNA/polymer weight ratio of 1:20 corresponds to a polymer nitrogen/DNA phosphate (N/P) ratio of roughly 20:1 for polymers containing only one amine per repeat unit (taken as an average for the entire library). The N/P ratio for polymers containing two amines per repeat unit (e.g., polymers containing amine monomers 10 and 13) is roughly 40:1 and helps explain the positive zeta potentials observed using these polymers. In general, the results above suggest that forming polymer/DNA complexes and screening at N/P ratios greater than 20:1 could increase the zeta potentials of these complexes, facilitate internalization, and lead to higher transfection efficiencies. The results of this current study address only those conditions used in initial transfection screening assays.

**Table 5.1.** Effective diameter (nm) of DNA/polymer complexes.

<table>
<thead>
<tr>
<th>Amines</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>E</th>
<th>G</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1029</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>175</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>350</td>
<td>561</td>
<td>150</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>302</td>
<td>301</td>
<td>328</td>
<td>352</td>
<td>363</td>
<td>238</td>
</tr>
<tr>
<td>6</td>
<td></td>
<td>193</td>
<td></td>
<td></td>
<td>269</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td></td>
<td>271</td>
<td>205</td>
<td></td>
<td></td>
<td>276</td>
</tr>
<tr>
<td>8</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>2471</td>
<td>1004</td>
<td>1583</td>
<td>1418</td>
<td>2033</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>209</td>
<td>217</td>
<td>195</td>
<td>318</td>
<td>259</td>
<td>300</td>
</tr>
<tr>
<td>11</td>
<td>268</td>
<td>317</td>
<td>269</td>
<td>1308</td>
<td>351</td>
<td>185</td>
</tr>
<tr>
<td>12</td>
<td>237</td>
<td>298</td>
<td>278</td>
<td>510</td>
<td>425</td>
<td>271</td>
</tr>
<tr>
<td>13</td>
<td>176</td>
<td>130</td>
<td>134</td>
<td>168</td>
<td>164</td>
<td>159</td>
</tr>
<tr>
<td>14</td>
<td>159</td>
<td>223</td>
<td>111</td>
<td>224</td>
<td>255</td>
<td>114</td>
</tr>
</tbody>
</table>

* Complexes were formed at DNA/polymer ratios of 1:20, with DNA added dropwise to the polymer while gently vortexing the mixture. Table entries omitted represent water-insoluble polymers or polymers unable to complex DNA. Measurements were made in 10 mM HEPES buffer (pH = 7.2).
Table 5.2. Zeta potential (mV) of polymer/DNA complexes.

<table>
<thead>
<tr>
<th>Amines</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>E</th>
<th>G</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td>-9.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td></td>
<td></td>
<td></td>
<td>-26.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>-17.0</td>
<td>-17.4</td>
<td>-41.6</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>-11.5</td>
<td>-19.3</td>
<td>-8.5</td>
<td>-14.0</td>
<td>-12.5</td>
<td>-3.5</td>
</tr>
<tr>
<td>6</td>
<td></td>
<td>-15.9</td>
<td></td>
<td></td>
<td>-17.2</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>-12.9</td>
<td>-20.6</td>
<td></td>
<td></td>
<td></td>
<td>-11.2</td>
</tr>
<tr>
<td>8</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>-6.0</td>
<td>-8.1</td>
<td>-4.4</td>
<td>-5.7</td>
<td>-7.9</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>6.9</td>
<td>6.0</td>
<td>6.6</td>
<td>2.2</td>
<td>0.1</td>
<td>2.9</td>
</tr>
<tr>
<td>11</td>
<td>-10.3</td>
<td>-17.8</td>
<td>-11.6</td>
<td>-12.5</td>
<td>-8.3</td>
<td>-11.5</td>
</tr>
<tr>
<td>12</td>
<td>-17.2</td>
<td>-11.8</td>
<td>-12.7</td>
<td>-10.7</td>
<td>-8.3</td>
<td>-8.2</td>
</tr>
<tr>
<td>13</td>
<td>19.0</td>
<td>23.6</td>
<td>19.5</td>
<td>15.6</td>
<td>16.5</td>
<td>15.8</td>
</tr>
<tr>
<td>14</td>
<td>5.0</td>
<td>1.8</td>
<td>24.8</td>
<td>-2.9</td>
<td>-13.3</td>
<td>22.8</td>
</tr>
</tbody>
</table>

* Complexes were formed at DNA/polymer ratios of 1:20, with DNA added dropwise to the polymer while gently vortexing the mixture. Table entries omitted represent water-insoluble polymers or polymers unable to complex DNA. Measurements were made in 10 mM HEPES buffer (pH = 7.2).

When the particle sizing and zeta potential measurements are taken together, a number of relationships emerge. For example, of the 10 polymer/DNA complexes with the highest levels of cellular uptake, all had measured effective diameters below 250 nm and all but one had positive zeta potentials (the notable exception being G5 with a zeta potential of -3.5 mV). Furthermore, the complexes combining the smallest effective diameters with the largest positive zeta potentials were C14 and G14, the two vectors resulting in the highest levels of cellular uptake. The unusually high level of uptake exhibited by polymer G5 is difficult to rationalize based solely on particle size and zeta potential measurements, and we continue to investigate the mechanistic aspects of transfection using this polymer.
5.3.5. Analysis of pH Environment

Because DNA is rapidly degraded by lysosomal enzymes, an efficient gene delivery system must be able to avoid the lysosomal trafficking pathway. Design elements generally incorporated into synthetic polymers to overcome this barrier include the incorporation of fusogenic peptides[17, 20, 21] and the use of pH-buffering “proton sponge” polymers[22] that are thought to promote the osmotic swelling and physical rupture of endosomes and the subsequent release of the vector into the cytoplasm. We,[15] and recently, another group[23] have employed the general flow cytometry-based approach outlined above to investigate the ability of different polymers to overcome the lysosomal barrier to efficient gene transfer. The details of this protocol have been previously described and are similar to the protocol used above for analysis of cellular uptake.[15] Briefly, the formation of polymer/DNA particles using plasmid DNA labeled with both fluorescein (pH sensitive) and Cy5 (pH insensitive) allows the local pH environment of transfected DNA to be determined as a linear function of pH (in the range of pH from 4 to 8), simply by measuring the emission ratios of these two fluorophores. The fluorescence emission ratios of NIH 3T3 cells incubated with labeled polymer/DNA complexes were measured using a flow cytometer and were used to calculate the average pH environment of the delivered DNA. The results of these experiments are displayed in Figure 5.4.
Figure 5.4. Measured average pH environment of DNA delivered via polymer/DNA complexes formed from members of polymer library (DNA/polymer = 1:20) and the controls PEI (DNA/polymer = 1:0.75) and PLL (DNA/polymer = 1:1). Measurements were performed in duplicate (sample discrepancies were within 0.2 pH units).

In this assay, particles unable to avoid or buffer lysosomes typically yield average pH measurements less than 5.0, while vectors able to escape the lysosomal pathway yield higher average pH measurements. For example, the local pH environment of DNA delivered using PEI, the prototypical “proton sponge” polymer, was measured to be 6.0, while that of DNA delivered using polylysine (PLL), a polymer with no known means for escaping lysosomes, was measured to be 4.4. While not a direct measure of compartmental location, the average pH experienced by delivered DNA is a reasonable indicator of the extent to which vectors have avoided or escaped acidic lysosomes (typical pH = 4.0–5.0).

Measurements of pH environment were made for the 10 complexes with the highest levels of cellular uptake. Of those complexes, those with the highest levels of transfection efficiency (G5, B14, A14, and G10) all had measured average pH values above 6.5, suggesting that they were able to successfully avoid trafficking to acidic lysosomes (Figure 5.4). The results surpassed that of even PEI, used in this experiment
as a positive control (measured pH value after internalization = 6.0). We[2, 24] and others[25] have previously incorporated imidazoles into polymers as pH-buffering moieties to aid in the escape of vectors from the lysosomal trafficking pathway. The high measured pH values for vectors containing monomer 14 support the hypothesis that the imidazole group (pKₐ ∼ 6.2) acts as a "proton sponge" in these polymers. It should be noted that the pH values measured for C14 and G14 (pH = 7.2 for both) could also be due to membrane permeabilization and subsequent pH equilibration with surrounding media resulting from the associated cytotoxicities of these materials. The near-neutral pH environment experienced by DNA complexed to polymer G5 is surprising in this context, as this material does not incorporate an obvious means of facilitating endosomal escape. Measured pH values for DNA delivered via complexes C10 (pH = 5.1) and D13 (pH = 4.0) were consistent with lysosomal pH, suggesting that these vectors were unable to avoid trafficking to lysosomes. Polylysine, a polymer with no known means for escaping the lysosomal trafficking pathway, was used as a negative control in the experiment and resulted in a pH measurement of 4.4.

5.3.6. Analysis of Cytotoxicity

We have previously assessed the degradability and cytotoxicity of 3 poly(β-amino esters).[13] For all three poly(β-amino esters), cells remained 100% viable relative to control cells even at concentrations as high as 100 µg/mL. In contrast, for cells treated with PEI, less than 30% were viable at a polymer concentration of 25 µg/mL and less than 10% were viable at a polymer concentration of 100 µg/mL. While this is a measure of the toxicity of a component of the gene delivery system, we felt it appropriate to analyze the cytotoxicity of the complete vector (DNA/polymer complex), as formulated for transfection experiments. Even if the polymers themselves are relatively non-toxic, the resulting DNA/polymer complexes may be more toxic due to enhanced cellular uptake of complex-associated polymer. The cytotoxicities of the 10 complexes with the highest levels of cellular uptake were determined using the MTT/thiazolyl blue dye reduction assay. Cells were incubated with complexes for 1 hr, after which time complexes were removed and fresh growth media was added. MTT assay was performed 24 hr later.
Figure 5.5. Relative viability of cells treated with DNA/polymer complexes formed from members of polymer library (DNA/polymer = 1:20), PEI (DNA/polymer = 1:0.75), and PLL (DNA/polymer = 1:1). Viabilities were determined relative to untreated control cells. Measurements were performed in triplicate (error bars represent standard deviation).

The results shown in Figure 5.5 suggest that complexes prepared with polymers synthesized using monomers C and G are most cytotoxic to cells. The most notable example is G5, which seems to kill nearly all cells under the conditions employed. By contrast, complexes prepared with polymers synthesized using A, B, and D monomers appear to be far less toxic. The control polymers PEI and PLL were not toxic under the conditions used in this experiment. These results are not necessarily inconsistent with the polymer cytotoxicity data reported in our prior work.13 First, we may expect polymer cytotoxicity to differ from DNA/polymer complex cytotoxicity. Second, due to the DNA/polymer weight ratios employed in the above experiments, a much higher concentration of poly(β-amino ester) is used, 20X and 27X relative to PLL and PEI, respectively. Although both polymers G5 and B14 resulted in high levels of transfection, based on their greatly differing levels of toxicity, B14 appears to be the more promising polymer for gene delivery.
5.4. Summary

We have described the parallel synthesis and biophysical characterization of a 140-member degradable poly(β-amino ester) screening library. *In vitro* transfection screening of this library led to the identification of two polymers, **B14** and **G5**, with transfection levels 4–8 times higher than that of PEI and levels rivaling those of a leading commercially available lipid-based reagent. In addition to providing a platform for the discovery of novel gene transfer vectors, a library of structurally diverse polymers affords the opportunity to correlate changes in polymer structure with differences in polymer function. The relatively small size of the current proof-of-concept library, combined with the diverse range of polymer molecular weights produced, currently prevents the assignment of definitive structure/function relationships. However, we have identified several structural features that correlate with improved physical and gene transfer properties. From the standpoint of DNA complexation, for example, we have found that polymers containing structural elements in which an oxygen atom is two carbons removed from an amine (e.g., polymers containing amine monomers 4, 7, or 8) are generally unable to form intimate electrostatic complexes with DNA. Characterization of the effective diameters and zeta potentials of polymer/DNA complexes suggests that, in general, small particle sizes and positive surface charges lead to higher levels of cellular uptake *in vitro*, and that polymers with multiple amines per repeat unit tend to yield complexes with smaller particle sizes and larger zeta potential. Evaluation of the pH environment of delivered DNA suggested that polymers incorporating an imidazole group or two amines in close proximity could successfully avoid low pH lysosomes. Finally, measurement of complex cytotoxicity implies that polymers synthesized using **C** and **G** monomers may be more toxic relative to there less hydrophobic counterparts **A**, **B**, and **D**.

The further elucidation of structure/function relationships will likely require the production of larger, more diverse libraries as well as greater control over polymer molecular weight. We recently synthesized a larger library consisting of several thousand unique structures, and initial transfection screening of this library has led to the identification of additional relationships, including the observation that the efficacy of polymer **G5** in this study appears to extend to several other polymers having different
alcohol-terminated side chains.[26] More work will be required to understand the role of specific functional elements with respect to the gene transfer properties of these and other cationic polymers. We believe that this parallel approach to the synthesis, discovery, and evaluation of libraries of cationic polymers and related materials will lead to an accelerated and improved understanding of the factors that currently limit non-viral gene delivery.
5.5. References

6. Synthesis of Poly(β-amino esters) Optimized for Highly Effective Gene Delivery

6.1. Introduction

To further enhance the efficacy of cationic polymer vectors, various groups have incorporated functional elements into polymers that improve their capacity to overcome specific barriers to gene delivery.[1, 2] Polyethylenimine (PEI),[3-5] a polymer generally considered to be the standard for polymer-based gene delivery, is an example of such a functional material. The buffering capacity of PEI is thought to lead to the osmotic swelling and rupture of endosomes, resulting in the escape of polymer-DNA complexes from the degradative lysosomal trafficking pathway.[3, 6] The discovery of functional polymers can be accelerated by generating and screening large, structurally diverse polymer libraries. Our group has previously reported the parallel synthesis and biophysical characterization of a 140-member library of unique, biodegradable poly(β-amino esters),[7, 8] and we have recently extended this approach to generate and screen a library containing thousands of such polymers.[9] As a class of materials, these polymers have shown promise for use as non-viral gene delivery vectors.

In addition to polymer structure, polymer molecular weight and polymer/DNA ratio are known to have a significant impact on transfection efficiency.[10-13] The number of polymer cation-DNA anion interactions, and thus polymer molecular weight, affects the affinity between the polymer chain and the DNA strand. Polymer chains that are too short do not effectively and stably condense DNA, while polymer chains that are too long retard the “un-packing” of DNA required for transcription and translation.[13] Tuning polymer chain length to result in an optimal polymer-DNA affinity may result in
improved efficacy for a given polymer-based delivery system. Polymer/DNA ratio controls the ratio of charges, affecting the complexation of polymer with DNA, which in turn impacts a number of important transfection properties, such as the stability, cellular uptake level, and cytotoxicity of the resulting complex. Although the importance of polymer molecular weight and polymer/DNA ratio have been demonstrated, to the best of our knowledge, the role of polymer chain end-groups has not yet been explored. Specifically, poly(β-amino esters) can have either amine- or acrylate-terminated chains. These polymers may have different transfection properties, and therefore, controlling end-groups may be important for improving the efficacy of poly(β-amino ester)-based gene delivery systems.

Several families of synthetic polymers, including degradable poly(β-amino esters), have been previously shown to effectively mediate gene transfer. However, the combined impact of numerous factors—such as polymer molecular weight, polymer chain end-group, and polymer/DNA ratio—on different gene transfer properties has not yet been systematically investigated. The elucidation of these relationships is likely to lead to the design of non-viral vectors with significantly enhanced gene transfer efficiencies. Here we examine the effect of molecular weight, polymer/DNA ratio, and chain end-group on the transfection properties of two unique poly(β-amino ester) structures. We demonstrate that these factors can have a dramatic effect of gene delivery function, and using high throughput screening methods, we have discovered poly(β-amino esters) that transflect better than PEI and Lipofectamine 2000, two of the best commercially available transfection reagents.

6.2. Materials and Methods

6.2.1. Polymer Synthesis

Poly-1 and Poly-2 polymers were synthesized by adding 1,4-butanediol diacrylate (99+%) and 1,6-hexanediol diacrylate (99%), respectively, to 1-aminobutanol (98%). These monomers were purchased from Alfa Aesar (Ward Hill, MA). Twelve versions each of Poly-1 and Poly-2 were generated by varying the amine/diacrylate stoichiometric ratio. To synthesize each of the 24 unique polymers, 400 mg of 1-aminobutanol was
weighed into an 8 mL sample vial with Telfon-lined screw cap. Next, the appropriate amount of diacrylate was added to the vial to yield a stoichiometric ratio between 1.4 and 0.6. A small Teflon-coated stir bar was then put in each vial. The vials were capped tightly and placed on a multi-position magnetic stir-plate residing in an oven maintained at 100 °C. After a reaction time of 5 hr, the vials were removed from the oven and stored at 4 °C. All polymers were analyzed by GPC.

6.2.2. Gel Permeation Chromatography (GPC)

GPC was performed using a Hewlett Packard 1100 Series isocratic pump, a Rheodyne Model 7125 injector with a 100-µL injection loop, and a Phenogel MXL column (5µ mixed, 300 x 7.5 mm, Phenomenex, Torrance, CA). 70% THF/30% DMSO (v/v) + 0.1 M piperidine was used as the eluent at a flow rate of 1.0 mL/min. Data was collected using an Optilab DSP interferometric refractometer (Wyatt Technology, Santa Barbara, CA) and processed using the TriSEC GPC software package (Viscotek Corporation, Houston, TX). The molecular weights and polydispersities of the polymers were determined relative to monodisperse polystyrene standards.

6.2.3. Luciferase Transfection Assays

COS-7 cells (ATCC, Manassas, VA) were seeded (14,000 cells/well) into each well of an opaque white 96-well plate (Corning-Costar, Kennebunk, ME) and allowed to attach overnight in growth medium. Growth medium was composed of 90% phenol red-free DMEM, 10% fetal bovine serum, 100 units/mL penicillin, 100 µg/mL streptomycin (Invitrogen, Carlsbad, CA). To facilitate handling, polymers stock solutions (100 mg/mL) were prepared in DMSO solvent. Note: we have demonstrated that the small residual amount of DMSO in the transfection mixture does not affect transfection efficiency and does not result in any observable cytotoxicity. Working dilutions of each polymer were prepared (at concentrations necessary to yield the different polymer/DNA weight ratios) in 25 mM sodium acetate buffer (pH 5). 25 µl of the diluted polymer was added to 25 µl of 60µg/ml pCMV-Luc DNA (Elim Biopharmaceuticals, South San Francisco, CA) in a well of a 96-well plate. The mixtures were incubated for 10 minutes to allow for complex formation, and then 30 µl of the each of the polymer-DNA solutions
were added to 200 µl of Opti-MEM with sodium bicarbonate (Invitrogen) in 96-well polystyrene plates. The growth medium was removed from the cells using a 12-channel aspirating wand (V&P Scientific, San Diego, CA) after which 150 µl of the Opti-MEM-polymer-DNA solution was immediately added. Complexes were incubated with the cells for 1 hr and then removed using the 12-channel wand and replaced with 105 µl of growth medium. Cells were allowed to grow for three days at 37°C, 5% CO₂ and were then analyzed for luciferase expression. Control experiments were also performed with PEI (MW = 25,000, Sigma-Aldrich) and Lipofectamine 2000 (Invitrogen). PEI transfections were performed as described above, but using polymer:DNA weight ratios of 1:1. Lipofectamine 2000 transfections were performed as described by the vendor, except that complexes were removed after 1 hour.

Luciferase expression was analyzed using Bright-Glo assay kits (Promega). Briefly, 100 µl of Bright-Glo solution was added to each well of the 96-well plate containing media and cells. Luminescence was measured using a Mithras Luminometer (Berthold, Oak Ridge, TN). A 1% neutral density filter (Chroma, Brattleboro, VT) was used to prevent saturation of the luminometer. A standard curve for Luciferase was generated by titration of Luciferase enzyme (Promega) into growth media in an opaque white 96-well plate.

6.2.4. Measurement of Cytotoxicity

Cytotoxicity assays were performed in the same manner as the luciferase transfection experiments with the following exception. Instead of assaying for luciferase expression after 3 days, cells were assayed for metabolic activity using the MTT Cell Proliferation Assay kit (ATCC) after 1 day. 10 µL of MTT Reagent was added to each well. After 2 hr incubation at 37 °C, 100 µL of Detergent Reagent was added to each well. The plate was then left in the dark at room temperature for 4 hr. Optical absorbance was measured at 570 nm using a SpectaMax 190 microplate reader (Molecular Devices, Sunnyvale, CA) and converted to % viability relative to control (untreated) cells.
6.2.5. Cellular Uptake Experiments

Uptake experiments were done as previously described,[8] with the exception that a 12-well plate format was used instead of a 6-well plate format. COS-7 cells were seeded at a concentration of $1.5 \times 10^5$ cells/well and grown for 24 hours prior to performing the uptake experiments. Preparation of polymer/DNA complexes was done in the same manner as in the luciferase transfection experiments, the only differences being an increase in scale (2.5 μg DNA per well of 12-well plate as opposed to 600 ng DNA per well of 96-well plate) and the use of Cy5-labeled plasmid[14] instead of pCMV-Luc. As in the transfection experiments, complexes were incubated with cells for 1 hr to allow for cellular uptake by endocytosis. The relative level of cellular uptake was quantified using a flow cytometer to measure the fluorescence of cells loaded with Cy5-labeled plasmid.

6.2.6. GFP Transfections

GFP transfections were carried in COS-7 (green monkey kidney), NIH 3T3 (murine fibroblast), HepG2 (human hepatocarcinoma), and CHO (Chinese Hamster Ovary) cell lines. All cell lines were obtained from ATCC (Manassas, VA) and maintained in DMEM containing 10% fetal bovine serum, 100 units/mL penicillin, 100 μg/mL streptomycin at 37 °C in 5% CO₂ atmosphere. Cells were seeded on 6-well plates and grown to roughly 80-90% confluence prior to performing the transfection experiments. Polymer/DNA complexes were prepared as described above using the pEGFP-N1 plasmid (Clontech, Palo Alto, CA) (5 μg/well). Complexes were diluted in 1 mL Opti-MEM and added to the wells for 1 hr. The complexes were then removed and fresh growth media was added to the wells. After 2 days, cells were harvested and analyzed for GFP expression by flow cytometry. Propidium iodide staining was used to exclude dead cells from the analysis.

6.2.7. Flow Cytometry

Flow cytometry was performed with a FACSCalibur (Becton Dickinson) equipped with an argon ion laser capable of exciting GFP (488 nm excitation) and a red
diode laser capable of exciting Cy5 (635 nm excitation). Distilled water was used as the sheath fluid. The emission of GFP was filtered using a 530 nm band pass filter and the emission of Cy5 was filtered using a 650 long pass filter. The cells were appropriately gated by forward and side scatter and 30,000 events per sample were collected.

6.3. Results and Discussion

6.3.1. Polymer Synthesis

As previously described,[15] the synthesis of poly(β-amino esters) proceeds via the conjugate addition of amines to acrylate groups. Because the reaction is a step polymerization, a broad, statistical distribution of chain lengths is obtained, with average molecular weight and chain end-groups controlled by monomer stoichiometry.[16, 17] Molecular weight increases as the ratio of monomers nears stoichiometric equivalence, and an excess of amine or diacrylate monomer results in amine- or acrylate-terminated chains, respectively. For this class of polymers, precise control of stoichiometry is essential for controlling polymer molecular weight. While monomer stoichiometry is the most important factor affecting chain length, consideration should also be given to competing side reactions that can impact the molecular weight and structure of polymer products. In particular, intramolecular cyclization reactions, where an amine on one end of the growing polymer chain reacts with an acrylate on the other end, can limit obtained molecular weights.[17] These cyclic chains may also have properties that differ from those of their linear counterparts.

In this work, we have modified the previously reported polymerization procedure[7] in order to better control monomer stoichiometry and to minimize cyclization reactions. First, the scale of synthesis was increased from roughly 0.5 g to 1 g to allow for control of stoichiometry within 1%. Further improvement in accuracy is limited by the purity (98-99%) of the commercially available monomers used. Second, all monomers were weighed into vials instead of being dispensed volumetrically. Discrepancies between actual and reported monomer densities were found to be non-negligible in some cases, leading to inaccuracies in dispensed mass. Third, polymerizations were performed in the absence of solvent to maximize monomer
concentration, thus favoring the intermolecular addition reaction over the intramolecular cyclization reaction. Eliminating the solvent also provides the added benefits of increasing the reaction rate and obviating the solvent removal step. Finally, since the previously employed methylene chloride solvent was not used, the reaction temperature could be increased from 45 °C to 100 °C. Increasing temperature resulted in an increased reaction rate and a decrease in the viscosity of the reacting mixture, helping to offset the higher viscosity of the solvent-free system. The combined effect of increased monomer concentration and reaction temperature resulted in a decrease in reaction time from roughly 5 days to 5 hours.

We synthesized polymers Poly-1 and Poly-2 by adding 1,4-butanediol diacrylate and 1,6-hexanediol diacrylate, respectively, to 1-amino butanol. Twelve unique versions of each polymer were synthesized by varying amine/diacrylate mole ratios between 0.6 and 1.4.

For both sets of polymers (Poly-1 and Poly-2), 7 of the 12 had amine/diacrylate ratios greater than 1, resulting in amine-terminated polymers, and 5 of the 12 had amine/diacrylate ratios less than 1, resulting in acrylate-terminated polymers. After 5 hr reaction at 100 °C, polymers were obtained as clear, slightly yellowish, viscous liquids. The polymers had observable differences in viscosity, corresponding to differences in molecular weight. Polymers were analyzed by organic phase gel permeation chromatography (GPC) employing 70% THF/30% DMSO (v/v) + 0.1 M piperidine eluent. Polymer molecular weights (M_n) ranged from 3350 (Poly-1, amine/diacrylate = 1.38) to 18,000 (Poly-1, amine/diacrylate = 0.95), relative to polystyrene standards.
(Figure 6.1). Molecular weight distributions were monomodal with polydispersity indices (PDIs) ranging from 1.55 to 2.20.

![Graph showing molecular weight vs. amine/diacrylate ratio for Poly-1 and Poly-2.](image)

**Figure 6.1.** Control of polymer molecular weight and chain end-group by varying amine/diacrylate ratio. Molecular weights ($M_w$) (relative to polystyrene standards) were determined by organic phase GPC. Polymers synthesized with amine/diacrylate > 1 have amine end-groups, while polymers synthesized with amine/diacrylate < 1 have acrylate end-groups.

### 6.3.2. Luciferase Transfection Results

Transfection experiments were performed with all 24 synthesized polymers (12 each of Poly-1 and Poly-2) at 9 different polymer/DNA ratios to determine the impact of molecular weight, polymer/DNA ratio, and chain end-group on transfection efficiency (Figures 6.2 and 6.3). As a model system, we used the COS-7 cell line and a plasmid coding for the firefly luciferase reporter gene (pCMV-Luc) (600 ng/well). To facilitate performance of the nearly 1000 transfections (data obtained in quadruplicate), experiments were done in 96-well plate format. Reporter protein expression levels were determined using a commercially available luciferase assay kit and a 96-well luminescence plate reader.
Figure 6.2. Luciferase transfection results for Poly-I as a function of polymer molecular weight, polymer/DNA ratio (w/w), and polymer end-group. (A) amine-terminated chains; (B) acrylate-terminated chains. (n = 4, error bars not shown)
Figure 6.3. Luciferase transfection results for Poly-2 as a function of polymer molecular weight, polymer/DNA ratio (w/w), and polymer end-group. (A) amine-terminated chains; (B) acrylate-terminated chains. (n = 4, error bars not shown)
The data displayed in Figures 6.2 and 6.3 demonstrate that polymer molecular weight, polymer/DNA ratio, and chain end-group impact the transfection properties of both Poly-1 and Poly-2 polymers. One striking, and somewhat unexpected, result was that none of the acrylate-terminated polymers mediated appreciable levels of transfection under any of the evaluated conditions. This result may be more broadly applicable for poly(β-amino esters), as we have yet to synthesize a polymer, using an excess of diacrylate monomer, that mediates appreciable reporter gene expression at any of the polymer/DNA ratios we have employed. These findings suggest that perhaps only amine-terminated poly(β-amino esters) are suitable for use as gene delivery vehicles. In contrast, there were distinct regions of transfection activity in the MW–polymer/DNA space for amine-terminated versions of both Poly-1 and Poly-2 (Figures 6.2-A and 6.3-A). Maximal reporter gene expression levels of 60 ng luc/well and 26 ng luc/well were achieved using Poly-1 ($M_w = 13,100$) and Poly-2 ($M_w = 13,400$), respectively. These results compare quite favorably with PEI (polymer/DNA = 1:1 w/w), which mediated an expression level of 6 ng luc/well (data not shown) under the same conditions.

While the highest levels of transfection occurred using the higher molecular weight versions of both polymer structures, the optimal polymer/DNA ratios for these polymers were markedly different (polymer/DNA = 150 for Poly-1, polymer/DNA = 30 for Poly-2). The transfection results we have obtained for Poly-1 and Poly-2 highlight the importance of optimizing polymer molecular weight and polymer/DNA ratio, and the importance of controlling chain end-groups. Further, the fact that two such similar polymer structures, differing by only two carbons in the repeat unit, have such different optimal transfection parameters emphasizes the need to perform these optimizations for each unique polymer structure. To improve our understanding of the obtained transfection results, we chose to study two important delivery characteristics that directly impact transgene expression, cytotoxicity and the ability to enter cells via endocytosis. [2]
6.3.3. Cytotoxicity

We evaluated the cytotoxicities of the various polymer/DNA complexes using a standard MTT/thiazolyl blue dye reduction assay. The experiments were performed exactly as the transfection experiments described above, except that instead of assaying for reporter gene expression on day 3, the MTT assay was performed after day 1 (see Materials and Methods). We initially hypothesized that the lack of transfection activity observed for acrylate-terminated polymers may have been due to the cytotoxicity of the acrylate end-groups. Figures 6.4-B and 6.5-B do indicate that high concentrations of acrylate are cytotoxic, as viability is seen to decrease with increasing polymer/DNA ratio and decreasing polymer $M_w$ (lower $M_w$ corresponds to a higher concentration of end-groups). However, cytotoxicity of acrylates does not sufficiently explain the lack of transfection activity at lower polymer/DNA ratios or higher molecular weights. Data shown in Figure 6.4-A demonstrates that cytotoxicity is not a limiting factor for Poly-1 vectors, since cells remain viable even at the highest polymer/DNA ratios. On the other hand, the data displayed in Figure 6.5-A suggests that cytotoxicity is a major factor limiting the transfection efficiency of Poly-2 vectors, especially for the lower molecular weight polymers. This result may explain why transfection activity is non-existent or decreasing for Poly-2 vectors at polymer/DNA > 30 (see Figure 6.3-A).
Figure 6.4. Cytotoxicity of Poly-1/DNA complexes as a function of polymer molecular weight, polymer/DNA ratio (w/w), and polymer end-group. (A) amine-terminated chains; (B) acrylate-terminated chains. (n = 4, error bars not shown)
Figure 6.5. Cytotoxicity of Poly-2/DNA complexes as a function of polymer molecular weight, polymer/DNA ratio (w/w), and polymer end-group. (A) amine-terminated chains; (B) acrylate-terminated chains. (n = 4, error bars not shown)
6.3.4. Cellular Uptake

The ability of polymer/DNA complexes to be taken up by cells was evaluated using a previously described flow cytometry-based technique[8] to measure the fluorescence of vector-delivered DNA. Briefly, polymer/DNA complexes were prepared using plasmid DNA covalently labeled with the fluorescent dye Cy5. To allow for comparison of the cellular uptake data with the gene expression data outlined above, complexes were formed at the same polymer/DNA ratios and in the same manner as in the transfection experiments. Labeled complexes were incubated with COS-7 cells for 1 hr at 37 °C to allow for uptake. The relative level of particle uptake was then quantified by measuring the fluorescence of cells loaded with Cy5-labeled DNA. The results of these uptake experiments are summarized in Figures 6.6 and 6.7. Data shown in Figure 6.6-B and 6.7-B suggest that the lack of transfection activity for the acrylate-terminated polymers is not due to cytotoxicity, as initially thought, but rather to an inability to enter the cell. Similarly, Poly-1 complexes are severely uptake-limited at all but the highest polymer/DNA ratios (Figure 6.6-A). While this data doesn't correlate exactly with the transfection results obtained for Poly-1, it is consistent with the fact that transfection activity is not observed until very high polymer/DNA ratios are employed. Poly-2 complexes show no appreciable cellular uptake at polymer/DNA ratios < 30 and increasing levels of uptake as polymer/DNA ratios increase beyond 30 (Figure 6.7-A). This result, combined with the above cytotoxicity results, helps to explain the transfection activity of Poly-2 complexes. At polymer/DNA ratios less than 30, complexes do not effectively enter the cell, but as polymer/DNA ratios increase much beyond 30, cytotoxicity begins to limit transfection efficiency, resulting in optimal transfection activity near polymer/DNA = 30.
Figure 6.6. Relative cellular uptake level of Poly-1/DNA complexes as a function of polymer molecular weight, polymer/DNA ratio (w/w), and polymer end-group. (A) amine-terminated chains; (B) acrylate-terminated chains. (n = 4, error bars not shown)
Figure 6.7. Relative cellular uptake level of Poly-2/DNA complexes as a function of polymer molecular weight, polymer/DNA ratio (w/w), and polymer end-group. (A) amine-terminated chains. Note: blank squares represent conditions where cytotoxicity of the complexes prevented a reliable measurement of cellular uptake; (B) acrylate-terminated chains. (n = 4, error bars not shown)
Where endocytosis is the main route of cellular entry, the effective formation of nanometer-scale polymer/DNA complexes is one requirement for achieving high levels of cellular uptake.[12, 18] We hypothesized that the poor uptake levels observed for many of the polymer/DNA complexes may have been attributable to the unsuccessful formation of stable, nanoscale complexes. Unfortunately, the poor neutral pH solubility of the polymers prevented us from making dynamic light scattering measurements of complex size using the transfection medium (Opti-MEM reduced serum media, pH 7.2) as the diluent. We noticed that the obtained readings were attributable to polymer precipitates, which were in some cases visible as turbidity in solutions of polymer in Opti-MEM. However, we were able to measure the effective diameters of complexes using 25 mM sodium acetate (pH 5) as the sample diluent. While this data cannot shed light on the size or stability of complexes in the transfection medium, it can indicate whether complexes were successfully formed prior to addition to the transfection medium. Specifically, we found that the lower molecular weight versions ($M_w < 10,700$) of Poly-1 were unable to form nanoscale complexes, even in acetate buffer. In all other cases we found that nanometer-sized polymer/DNA complexes were formed. While these results may explain the poor uptake levels associated with low molecular weight versions of Poly-1, they do not satisfactorily explain the low uptake activity of the acrylate-terminated polymers or the dependence of polymer/DNA ratio on cellular uptake. Although particle size and stability are important factors impacting cellular uptake, it is likely that other, yet unidentified, factors must also be considered in order to provide a more complete explanation of the obtained cellular uptake results.
6.3.5. Enhancement of Transfection Using a Co-Complexing Agent

Both Poly-1 and Poly-2 require relatively high polymer/DNA weight ratios to achieve high levels of gene transfer. One explanation may be that, compared to other polymers often used to compact DNA (e.g., polylysine (PLL) and PEI), these polymers have relatively low nitrogen densities. Poly-1 has a molecular weight per nitrogen atom (MW/N) of 301, and Poly-2 has a MW/N of 329. By comparison, for PLL and PEI, these figures are roughly 65 and 43, respectively. We reasoned that it might be possible to reduce the amount of Poly-1 or Poly-2 necessary to achieve high levels of transfection by incorporating a small amount of co-complexing agent. We felt this approach could be especially beneficial for Poly-2 vectors, since cytotoxicity appears to be an important limitation for these vectors. To test this hypothesis, PLL and PEI were used as model co-complexing agents. We focused our attention on the most promising member in each of the Poly-1 (amine-terminated, $M_w = 13,100$) and Poly-2 (amine-terminated, $M_w = 13,400$) sets of polymers. The data displayed in Figures 6.8 and 6.9 indicate that a significant reduction in polymer could be achieved, while maintaining high levels of transfection efficiency, through the use of PLL or PEI as co-complexing agents. In some cases, significant enhancement of transfection activity was realized. As expected, this co-complexation approach was particularly beneficial for Poly-2 vectors. This work, and prior work,[19-22] demonstrates that the blending of polymers with complementary gene transfer characteristics can in some cases produce more effective gene delivery reagents.
Figure 6.8. Enhancement of transfection activity of Poly-1 (amine-terminated chains, $M_w = 13,100$) based delivery vectors through the use of co-complexing agents. (A) PLL; (B) PEI. ($n = 4$, error bars not shown)
Figure 6.9. Enhancement of transfection activity of Poly-2 (amine-terminated chains, $M_w = 13,400$) based delivery vectors through the use of co-complexing agents. (A) PLL; (B) PEI. ($n = 4$, error bars not shown)
6.3.6. GFP Transfections

To further evaluate the transfection properties of the Poly-1/PLL and Poly-2/PLL blended reagents, we performed transfection experiments using a reporter plasmid coding for green fluorescent protein (pCMV-EGFP). Though the luciferase and GFP transfection assay systems both measure transfection activity, they provide different types of information. The luciferase system quantifies the total amount of exogenous luciferase protein produced by all the cells in a given well, providing a measure of cumulative transfection activity. In contrast, the GFP system can be used to quantify the percentage of cells that have been transfected, providing a cell-by-cell measure of transfection activity. Both systems are useful and offer complementary information regarding the transfection properties of a given gene delivery system.

GFP transfections were performed in a similar manner as the luciferase experiments, but were scaled up to 6-well plate format (5 μg plasmid/well). COS-7 cells were transfected using Poly-1/PLL (Poly-1:PLL:DNA = 60:0:1:1 w/w/w) and Poly-2/PLL (Poly-2:PLL:DNA = 15:0:4:1 w/w/w). Lipofectamine 2000 (μL reagent: μg DNA = 1:1), PEI (PEI:DNA = 1:1 w/w, N/P ~ 8), and naked DNA were used as controls in the experiment. After 1 hr incubation with cells, vectors were removed and fresh growth media was added. Two days later GFP expression was assayed using a flow cytometer. Nearly all cells transfected with Poly-1/PLL were positive for GFP expression (Figures 6.10 and 6.11). Experiments indicated that Poly-2/PLL vectors were less effective, resulting in roughly 25% positive cells. Positive controls Lipofectamine 2000 and PEI were also able to mediate effective transfection of COS-7 cells under the conditions employed. Although Lipofectamine 2000 and PEI transfections resulted in nearly the same percentage of GFP-positive cells as Poly-1/PLL, the fluorescence level of GFP-positive cells was higher for Poly-1/PLL (mean fluorescence = 6033) than that of both Lipofectamine 2000 (mean fluorescence = 5453) and PEI (mean fluorescence = 2882). Multiplying the percentage of positive cells by the mean fluorescence level of positive cells provides a measure of aggregate expression for the sample and should, in theory, better correlate with the results of luciferase gene expression experiments. Quantifying total GFP expression in this manner indicates that the highest expression level is achieved
by Poly-1/PLL, followed by Lipofectamine 2000 and PEI. This result is in general agreement with the luciferase expression results.

![Bar chart showing % positive cells for different treatments.]

**Figure 6.10.** GFP gene transfer into COS-7 cells using Poly-1/PLL (Poly-1:PLL:DNA = 60:0.1:1 w/w/w), Poly-2/PLL (Poly-2:PLL:DNA = 15:0.4:1 w/w/w), Lipofectamine 2000 (μL reagent: μg DNA = 1:1), PEI (PEI:DNA = 1:1 w/w, N/P ~ 8), and naked DNA. Cells were seeded on 6-well plates and grown to near confluence. Cells were incubated with complexes (5 μg DNA/well) for 1 hr, after which time complexes were removed and fresh growth media was added. Two days later GFP expression was assayed by flow cytometry. (n = 3, error bars indicate standard deviation)

![Image of GFP expression in COS-7 cells.]

**Figure 6.11.** GFP expression by COS-7 cells transfected using Poly-1/PLL.
Experiments have shown that Poly-1/PLL (Poly-1:PLL:DNA = 60:0.1:1 w/w/w) is a highly effective vector for transfecting COS-7 cells. The ability of this vector to mediate transfection in three other commonly used cell lines (CHO, NIH 3T3, and HepG2) was also investigated. It is very likely that each of these cell lines have optimal transfection conditions that differ from those used to transfect COS-7 cells; however, as a preliminary evaluation of the ability to transfect multiple cell lines, transfections were performed in the same manner and under the same conditions as the COS-7 transfections. Results indicate that Poly-1/PLL (Poly-1:PLL:DNA = 60:0.1:1 w/w/w) is able to successfully transfect CHO, NIH 3T3, and HepG2 cells, though not as effectively as COS-7 cells (Figure 6.12). This is not too surprising since the vector used was optimized by screening for gene transfer in COS-7 cells. We expect that the optimization of vector composition and transfection conditions specific for each cell type will result in even higher transfection levels.

Figure 6.12. GFP gene transfer into different cell lines using Poly-1/PLL (Poly-1:PLL:DNA = 60:0.1:1 w/w/w). Cells were seeded on 6-well plates and grown to near confluence. Cells were incubated with complexes (5 µg DNA/well) for 1 hr, after which time complexes were removed and fresh growth media was added. Two days later GFP expression was assayed by flow cytometry. (n = 5, error bars indicate standard deviation)
6.4. Summary

In this work, we have systematically investigated the role of polymer molecular weight, polymer chain end-group, and polymer/DNA ratio on a number of important gene transfer properties. All three factors were found to have a significant impact on gene transfer, highlighting the benefit of carefully controlling and optimizing these parameters. In addition, we found that the incorporation of a small amount of PLL, used to aid complexation, could further enhance gene transfer. Through these approaches we have generated degradable polymer-based vectors that rival some of the best available non-viral vectors for in vitro gene transfer. Through these approaches we have generated degradable polymer-based vectors that rival some of the best available non-viral vectors for in vitro gene transfer. We are currently optimizing polymer structure and formulation in the presence of serum in preparation for in vivo testing.
6.5. References


7. Conclusions

Gene therapy represents a revolutionary approach for treating human disease and is expected to have a tremendous impact on the field of medicine. For this potential to be realized, safe and effective gene delivery vectors need to be developed. Although both viral and non-viral approaches will likely be employed, non-viral vectors hold the most promise for the widespread application of gene therapy technology. Unfortunately, current non-viral vectors lack the functional sophistication required to overcome the numerous barriers to efficient gene transfer. As a result, they have had poor transfection efficiencies, limiting their clinical utility. Attempts to rationally design the necessary functionality into non-viral vectors have resulted in only modest successes, exposing major limitations in our current understanding of both cellular processes and the impact of vector structure on function. This thesis was driven by the hypothesis that an improved understanding of both the non-viral gene delivery process and the impact of vector structure on function would lead to the generation of more effective non-viral vectors.

As part of this approach, we developed the first quantitative assay designed to investigate the lysosomal barrier to gene transfer. Quantitative information regarding the cellular uptake of vectors is also provided by this assay. Using these tools, we investigated the mechanism of PEI mediated gene transfer and provided, arguably, the most complete validation of the proton sponge hypothesis to date. We showed that the relatively high transfection efficiency of PEI is due to its proton sponge character, resulting in its ability to escape the endosome and avoid trafficking to acidic lysosomes. We then performed a detailed study of the gene transfer properties of a 140-member
library of degradable polymers in order to improve our understanding of vector functionality. Through this study, a number of structural features correlating with improved physical and gene transfer properties were identified. Finally, we performed the first systematic investigation of the combined impact of numerous factors—such as polymer molecular weight, polymer chain end-group, and polymer/DNA ratio—on different gene transfer properties. Through this work we were able to formulate polymers that mediated in vitro gene transfer at levels that surpassed both PEI and Lipofectamine 2000, two of the best commercially available non-viral transfection reagents.
8. Future Work

The work described in this thesis can be extended in three major areas. First, although certain structure/function relationships have been elucidated, there is still much that is unknown about how polymer structure impacts specific steps in the gene transfer process. Second, the polymer vectors developed in this thesis can be further functionalized to enhance gene transfer properties. Third, vectors developed in this thesis can be investigated in vivo.

Though we have successfully correlated certain polymer structural features with specific gene transfer functions, we are a long ways away from the a priori rational design of non-viral vectors. Currently, our ability to make significant progress in this area is somewhat limited by our inability to adequately characterize polymer-DNA complexes. Since it is the complex that participates in the gene transfer process, we must improve our understanding of how polymer structure impacts complex structure, and subsequently, how complex structure impacts gene transfer function. Effective diameter and surface charge of complexes can be measured, but the nature of complex at a molecular level is not well understood. The main reason for this is that the tools to investigate the polymer-DNA complex at the molecular level have not yet been developed. What is needed is method of determining complex structure analogous to crystal structure determination for proteins. Development of such a tool would be a major advance toward understanding polymer-DNA complexation and would help elucidate the interactions of the complex in both intracellular and extracellular environments.
The vectors developed in this thesis, specifically Poly-1 and Poly-2, show great promise as non-viral vectors. Additional functionality could be incorporated into these vectors to produce even more effective vectors. One area of effort could be the use of PEG or surfactants to improve the serum stability of these vectors. Ligand moieties could easily be added to enhance cellular uptake (e.g. transferrin) or to provide a means for targeting specific cell types (e.g. galactose or mannose). Nuclear localization signals could also be incorporated into the vectors, either by conjugation to the polymer or the plasmid. We have also demonstrated that blending Poly-1 and Poly-2 with different polymers, for example PLL or PEI, can result in enhanced gene transfer properties. This approach could easily be expanded in a parallel, high-throughput manner to evaluate other co-complexing materials.

Many vector systems, with in vitro transfection efficiencies less than that of either Poly-1 or Poly-2, have been successfully used for in vivo gene transfer. Therefore, it is reasonable to expect that Poly-1 and Poly-2 could be useful for in vivo, as well as in vitro, gene delivery. Biodistribution experiments could be performed, with different routes of administration investigated. The results of these studies would help determine the general feasibility of using these vectors in vivo and would help identify the most appropriate disease targets.
Appendix: Measuring Buffering Capacity and Molecular Weight Effects

1. Introduction

Earlier work has suggested that the buffering capacity of PEI is responsible for its ability to avoid low pH cellular compartments. We decided to examine the role of both buffering capacity and polymer molecular weight on the ability of polymeric vectors to escape trafficking to acidic lysosomes. Measurements of the pH environment of DNA delivered using different molecular weight PEIs were made. In addition, measurements were made using various polylysine-\textit{graft}-imidazole acetic acid polymers.[1] These polymers had three different molecular weight polylysine backbones and varying degrees of imidazole grafted to the backbones (Figure A1). Varying the amount of imidazole groups (pKa ~ 6.2) in the polymer provides a means for varying the buffering capacity of the polymer.

\[ \text{Figure A1. Synthesis of polylysine-\textit{graft}-imidazole acetic acid polymers.} \]
2. Materials and Methods

All PEIs were purchased from Sigma-Aldrich (St. Louis, MO) or Polysciences (Warrington, PA). Polylysine-\textit{graft}-imidazole acetic acid polymers were prepared as previously described\cite{1} and generously provided by Dr. David Putnam (School of Chemical and Biomolecular Engineering, Cornell University).

The pH measurements were performed as described in Chapter 3.

3. Results and Discussion

The data displayed in Figure A2 indicate that the measured average pH of DNA delivered using PEI rises from 4.0-4.5 at a molecular weight of 2000 Da and levels off at pH 5.5-6.0 at molecular weights greater than 10,000 Da. This suggests low molecular weight PEIs ($< 10,000$ Da) are unable prevent the trafficking of DNA to acidic lysosomes. This is most likely due to the inability of low molecular weight PEIs to bind the plasmid tightly, leading to premature vector unpacking. As a result, the plasmid may not have the benefits of PEI’s proton sponge mechanism for escaping endosomes. Therefore, it seems that molecular weight does not impact the buffering capacity (or proton sponge nature) of PEI directly, but instead impacts the binding interaction between the DNA and the polymer.

![Graph showing pH changes with molecular weight](image)

\textbf{Figure A2.} Measured average pH environment of DNA delivered using different molecular weight PEIs at 2 hr time point. Complexes prepared at DNA:polymer = 1:0.75 (w/w). (\(n = 3\), error bars indicate standard deviation)
The results displayed in Figure A3 indicate that until 60% imidazole substitution, polylysine-graft-imidazole acetic acid polymers are not able to prevent the trafficking of DNA to lysosomes. As the imidazole substitution increases beyond 60% the average pH environment of delivered DNA increases, indicating that the additional polymer buffering capacity alters the lysosomal trafficking of plasmid DNA.

![Graph showing pH as a function of % imidazole substitution.](image)

**Figure A3.** pH measured for polyplexes prepared with various polylysine-graft-imidazole acetic acid polymers at 2 hr time point. Complexes were formed at DNA:polymer = 1:1 (w/w). Measurements were performed in duplicate, all points shown.

4. References