POLYMERIC NANOPARTICLE ENGINEERING FOR DELIVERY OF NUCLEIC ACIDS

by

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Abstract:

Nucleic acid delivery to human cells holds huge potential in treating disease by directly manipulating gene expression via the delivery of coding nucleic acids that lead to protein production (DNA, mRNA) or non-coding RNAs that lead to gene downregulation (siRNA, miRNA). The structure of nucleic acids makes them particularly difficult to functionally deliver to the cytosol of living cells, as nucleic acids are high molecular weight, strongly negatively charged due to their phosphate backbone and susceptible to many nucleases present in the extracellular fluid that lead to enzyme mediated degradation. These challenges can be overcome via the use of delivery vectors that complex nucleic acids into condensed nanoparticle formulations that facilitate cellular uptake, endosomal escape and cytosolic release of nucleic acids to target cells. To date, however, chemical delivery methods for nucleic acids have suffered from low efficacy preventing clinical translation. This thesis sought to engineer materials to yield more effective delivery of nucleic acids to mammalian cells as well as design assays to better understand the barriers to effective delivery and how those barriers might be overcome.

Chapter one provides an overview of the guiding aims of the thesis and summary of academic contributions towards its completion. In chapter two, I first provide an overview of the state of gene delivery via polymeric vectors followed by a review of the state of gene delivery for therapeutic cancer applications. Chapter three details my efforts to engineer a more effective fluorescent ratio based sensor of pH for investigation of endosomal pH following nanoparticle internalization. In chapter four, I describe efforts to adapt hydrodynamic flow focusing to form poly(beta-amino ester) (PBAE) nanoparticles and improve lyophilization techniques. Chapter five details optimization of assembly parameters of PBAE nanoparticles including mixing ratio, assembly time and buffer system. Chapter six is an interesting study where I worked with Donald Zack’s lab to deliver plasmid DNA to pluripotent stem cell derived post-mitotic retinal pigment epithelial cells by creating a large library of PBAE structures. Chapter seven details the creation of branched PBAE structures and their assessment relative to their linear counterparts using a tri-acrylate molecule. Chapter eight details the utilization of a canonical linear, end-capped PBAE to improve cytosolic delivery of STING agonist cyclic dinucleotides for improved cancer therapy at low therapeutic doses. Finally, chapter 9 provides a brief perspective on where I believe the field is headed and what are the most exciting advances I have seen in the past few years.
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I am most grateful to all the mentors I have had throughout my life. In particular, my parents served as a great inspiration for pursuing a degree in science as they encouraged me to be curious from a young age. As my mom likes to tell people, my favorite word as a young child was “why” and in the days before easy internet access I can imagine that would become irritating to any parent after the umpteenth time. In my undergraduate studies I cannot give enough thanks to my direct research mentor and academic advisor Becky Bader. Her training and guidance made it possible for me to make the leap to graduate school to pursue a PhD. Within graduate school, I am extremely grateful for the constant support and mentorship from my advisor Jordan J. Green who gave me more academic freedom than I would have ever expected. This PhD likewise would not have been possible without the support of my thesis committee of Dr. Hai-Quan Mao, Dr. Jamie Spangler and Dr. Donald Zack as well as the other faculty within the Translational Tissue Engineering Center (TTEC) faculty including Dr. Jennifer Elisseeff, Dr. Josh Doloff and Dr. Warren Grayson. All have provided advice and mentorship at different occasions during my PhD.

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Arman, who worked with me for four semesters, is currently a medical student at University of California, San Francisco after having completed a distinguished B.S. in materials science and the CBID masters program. Denis, who worked with me for six semesters, entered the MD/PhD program at Johns Hopkins University in Fall 2019 and graduating from Hopkins with a with a B.S. in BME. Rahul Upadhya, who worked with me for one summer, is currently pursuing a PhD in biomedical engineering at Rutgers University. Mark Suprenant, who worked with me for four semesters and graduated from Hopkins with a B.S. in BME, is currently pursuing a PhD in biomedical engineering at Boston University. Kamran Siddiq, who worked with me for four semesters and graduated from Hopkins with a B.S. in BME is working as a biotech consultant. Mahita Varanasi worked with me for four semesters and will be graduating Hopkins with a B.S. in BME in 2020 is considering graduate school. Shanelle Mendes, who worked with me for four semesters and will be graduating Hopkins with a B.S. in BME in 2020 is applying to MD/PhD programs. Ellen, who worked with me for three semesters and will graduate from Hopkins with a B.S. in BME in 2021 aims to go to medical school. Deepti, who worked with me for two semesters and will graduate
from Hopkins with a B.S. in BME in 2022 aims to go to medical school. Finally, Marranne, who worked with me one summer as an INBT REU student will graduate with a B.S. in Biology from Berea college and might have been convinced to pursue a PhD in biomedical engineering.

Finally I must thank my greatest champion and partner in life, Yuan Rui. Meeting her and eventually getting married was the highlight of my PhD years and I wouldn’t be where I am today without her loving support. Yuan is one of the best and most innovative scientists I know and being her partner through both the life events and science of a PhD was a tremendous privilege. In conclusion, my acknowledgements would be incomplete without also thanking my spectacular cat-son Mavid Suprenant Rui-Wilson and cat-daughter Dorkin Winnifred Rui-Wilson, who have brightened my life more than I would have imagined and provided countless hours of love and entertainment.
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Chapter 1. Introduction to the Thesis

1.1 Objectives

The primary objectives associated with this thesis were to advance the study of non-viral delivery technologies toward clinical utilization for gene therapies. The work in this thesis was accomplished broadly via the high level aims and sub-aims as follows:

1. Develop polymeric materials that enable nuclear delivery of nucleic acids to mammalian cells at high levels of efficacy.
   a. Develop assays to explore intracellular trafficking and endosomal escape of polymeric nanoparticles.
   b. Engineer polymer chemistries that explore influence of polymer structure on transfection efficacy
   c. Develop high throughput methods enabling semi-automated synthesis and testing of large polymeric nanoparticle libraries to identify trends in transfection efficacy and enable rapid screening in hard-to-transfect cell populations.

2. Evaluate efficacy of polymeric nanoparticles in vitro to clinically relevant cell populations and design mechanisms for clinical level scale up of polymeric nanoparticles.
   a. Develop methods for PBAE nanoparticles scale-up, clinical utilization and standardization of transfection
   b. Apply high-throughput synthesis and screening methods to identify polymeric nanoparticles formulations enabling delivery of nucleic acids in vitro

3. Test the efficacy of developed polymeric nanoparticle formulations to deliver nucleic acids in vivo for functional gene therapies.
   a. Assess ability of PBAE nanoparticles to deliver cyclic dinucleotides as adjuvants for cancer immunotherapy to immune cells in vitro and in vivo leading to robust prevention of tumor growth
   b. Apply PBAE nanoparticles for delivery of nucleic acids in vivo to therapeutically transfect cancer cells

1.2 Summary of Contributions

Chapter 2: A background in polymeric gene delivery


Chapter 3: A Triple-Fluorophore Labeled Nucleic Acid pH Nanosensor to Investigate Non-Viral Gene Delivery


Chapter 4: Continuous Microfluidic Assembly of Biodegradable Poly(beta-amino ester)/DNA Nanoparticles for Enhanced Gene Delivery


Chapter 5: The Role of Assembly Parameters on Polyplex Poly(Beta-Amino Ester) Nanoparticle Transfections

Chapter 6: A Combinatorial Library of Biodegradable Polyesters Enables Non-viral Gene Delivery to Post-mitotic Human Stem Cell-derived Polarized RPE Monolayers


Chapter 7: Differentially Branched Ester Amine Quadpolymers with Amphiphilic and pH Sensitive Properties for Efficient Plasmid DNA Delivery


Chapter 8: Biodegradable STING agonist nanoparticles for enhanced cancer immunotherapy


Other contributions not included in written thesis:


Chapter 2.a Biodegradable Polymeric Nanoparticles for Gene Delivery

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*These authors contributed equally to this work.

Foreword: I wrote the following book chapter for the textbook Perspectives in Micro- and Nanotechnology for Biomedical Applications with fellow graduate students Jayoung Kim and Kristen Kozielski.


Abstract

Increasingly scientists and clinicians are discovering the genetic basis of diseases, ranging from monogenic disorders to multigenic diseases such as various cancers. Moreover, human diseases are increasingly being understood on the molecular level, rather than as a phenotype, which is moving treatments towards stratified and personalized medicine. Gene therapy holds the promise of a technology that could address this growing need for genetic medicine as it can potentially tune individual cell gene expression on or off in a targeted and precise manner. This technology also could, theoretically, be applied to almost any human disease. The central challenge is that in practice, safe and effective delivery of desired nucleic acids to targeted human cells is very difficult. This chapter outlines these challenges in gene delivery and then discusses state-of-the-art approaches at overcoming these obstacles and obtaining successful gene delivery in in vitro and in vivo systems through the use of biodegradable polymers. Biodegradable gene delivery polymers, or plastics that are designed to safely deliver a biological cargo inside cells and then degrade, have certain advantages over other materials as well as over viruses for the delivery of genes. This chapter elucidates the diverse types of biodegradable polymers used for gene delivery, the related nanoparticulate systems they form with nucleic acids, and the structural properties that increase their efficacy and safety.
Introduction

The delivery of nucleic acids to manipulate gene regulation can be both a therapeutic and a scientific tool. Diseases caused by missing or defective genes could potentially be cured by replacing these genes, such as upregulating tumor suppressor genes in cancer.\textsuperscript{1-3} The immune system can be modulated by the introduction of DNA-based vaccines,\textsuperscript{4,5} or by introducing genes that would allow the immune system to better recognize or fight cancer.\textsuperscript{6,7} Additionally, suicide genes can be introduced to kill cancer cells.\textsuperscript{8} Ex \textit{vivo}, gene therapy can be used to manipulate stem cells for targeted differentiation,\textsuperscript{9} or to reprogram induced pluripotent stem cells from differentiated cells.\textsuperscript{10} Turning off or down regulating genes could treat diseases caused by gene overexpression.\textsuperscript{11,12} Technology to selectively turn genes off is also a valuable biological tool to elucidate the function of genes within a cell and in the context of a disease.\textsuperscript{13}

Viral gene delivery vectors, although effective, come with risks such as tumorigenicity and immunogenicity.\textsuperscript{14} Adenovirus-mediated gene delivery studies have found that dosage repeatability and concentrations can be limited by toxicity and humoral immune response.\textsuperscript{15} Although non-viral nucleic acid delivery can avoid these issues, it is typically less effective.\textsuperscript{16}

Lipid-based and inorganic delivery vehicles have previously been examined for their potential to deliver nucleic acids. Lipid-based delivery is well-characterized,\textsuperscript{17-19} and commercially-available lipid-based delivery vehicles are available for \textit{in vitro} delivery of DNA\textsuperscript{20,21} and siRNA.\textsuperscript{22} Lipid-based nanoparticles can potentially generate off-target and immunogenic effects,\textsuperscript{20} but there are strategies to attenuate these unwanted interactions such as the introduction of poly(ethylene glycol) (PEG) shielding.\textsuperscript{23,24} Calcium phosphate crystals,\textsuperscript{25-27} gold nanoparticles, quantum-dots and other inorganic materials have also been employed for non-viral gene delivery. Gold is advantageous because it’s biocompatible, easy to functionalize, and has malleable physical properties.\textsuperscript{28-32} Quantum dots are useful for fluorescent imaging as they are brighter and less prone to photobleaching than typical fluorophores.\textsuperscript{33,34} Several lipid-based and inorganic nanoparticle systems have also been combined with polymeric materials for enhanced gene delivery, particularly through the incorporation of PEG coatings\textsuperscript{24,35,36} for nanoparticulate shielding or polyamines for improved interaction with DNA and intracellular delivery.\textsuperscript{37,38}

Biodegradable polymeric gene delivery systems are a relatively newer class of materials for non-viral gene therapy. They are promising due to key features such as safety mediated by their biodegradability, design flexibility due to their tunable structure, large cargo capacity, and relative ease in manufacture. This chapter will focus on biodegradable polymeric nanoparticles for gene delivery. They will be discussed in the context of systemic and intracellular barriers to gene delivery and how polymer design can be utilized to overcome these barriers. New developments in the field of biodegradable polymeric gene delivery and an outlook for the future will be highlighted.
Obstacles to Gene Delivery

The central limitation of non-viral vectors for gene delivery is inefficient gene transfection arising from the natural mechanisms of the human body to protect itself against foreign substances. These barriers to biomaterial-mediated gene transfection span a spectrum from the systemic level to the cellular level (Figure 2-1). Different biomaterial properties and modifications to gene carriers are important for each step of the delivery process leading to successful expression of exogenous delivered nucleic acid. We will discuss seven major biological barriers to gene transfer using non-viral vectors and strategies to overcome each of these barriers. It is also important to note that the design properties seemingly optimal for one of the delivery obstacles could pose a challenge to other obstacles; hence further effort is needed to globally optimize polymeric nanoparticles to balance these potential trade-offs.
Nucleic acid binding / encapsulation

An efficient gene delivery vector must condense or encapsulate the nucleic acid to prevent enzymatic degradation and facilitate its cellular entry. As DNA is a strongly negatively charged material, early work in

Figure 2. Major barriers to nucleic acid delivery using nanoparticles include stable particle formation, systemic circulation, tissue and cell targeting, cellular uptake, endosomal escape, and release of nucleic acid. Reprinted by permission from Macmillan Publishers Ltd: Nature reviews. Genetics 2014, 15, 541-55, copyright 2014.
the field of non-viral gene delivery focused on the use of naturally occurring biological materials with
significant positive charge that could electrostatically bind to the DNA. In this manner, DNA could be
condensed into a smaller size, be made more resistant to potential enzymatic degradation, and have improved
ability to enter cells. Polycation poly(L-lysine) (PLL) was observed to bind to nucleohistones,\textsuperscript{42,43} and was
later investigated as one of the earliest polymers to form nanocomplexes with DNA.\textsuperscript{44-46} PLL is capable of
complexing with DNA to form nanoparticles that successfully undergo cellular uptake but fail to escape from
the endosome.\textsuperscript{47} To overcome this challenge, PLL has been used in combination with other materials that aid
in endosomal escape including other peptide molecules and pH sensitive moieties that make use of the
proton sponge effect.\textsuperscript{48-50} To improve delivery in comparison to PLL, alternative gene delivery materials
needed to be discovered. An off-the-shelf commercially produced polymer with very high charge density,
polyethylenimine (PEI), was first reported for use in transfection in 1995 by Boussif \textit{et al.}, who attributed its
high transfection efficiency to its ability to undergo endosomal escape via the proton sponge effect.\textsuperscript{51}
Unfortunately, while transfection efficacy is correlated with the molecular weight of PEI, cytotoxicity is
similarly correlated, making unmodified high MW PEI largely unsuitable for \textit{in vivo} applications. PEI is a non-
biodegradable polycation that requires excess polymer to effectively transfect cells and can lead to
accumulation upon repeated administration.\textsuperscript{52,53} To overcome this first obstacle to non-viral gene delivery,
other biomaterials can encapsulate nucleic acids into particulates. Amphiphilic lipids, such as N-1-(2,3-
dioleyloxy)propyl-N,N,N-trimethylammonium chloride (DOTMA), and relatively hydrophobic polymers,
including poly(lactide-co-glycolide) polymer (PLG), form DNA-encapsulated nanoparticles by either phase
separation alone or in combination with electrostatic interaction.\textsuperscript{54,55}

Polymeric vectors are able to deliver different types of nucleic acids. For DNA, standard double-
stranded plasmids as well as minicircles, which are plasmids that have had prokaryote sequences such as the
CpG islands removed, are widely used to introduce exogenous genes that encode for proteins of interest.\textsuperscript{56}
Because of the large size of DNA molecules, they are able to bind to cationic polymers such as PEI and
poly(\(\beta\)-amino ester)s (PBAE) and form stable nanoparticles.\textsuperscript{57,58} Small interfering RNA (siRNA), through the
RNA interference pathway, causes mRNA to be broken down and inhibits translation.\textsuperscript{59} As an siRNA
molecule is dramatically shorter (~20 bp vs. > 1,000 bp) and stiffer than a plasmid DNA molecule, stable
particle formulation through electrostatic interactions with cationic polymers is more difficult.\textsuperscript{60,61} Therefore,
siRNA-delivering nanoparticles can require more complex engineering solutions to form effective particles,
such as making siRNA more multivalent by introducing short complementary overhangs\textsuperscript{62} or multimerizing
siRNA molecules with cleavable disulfide linkages.\textsuperscript{63} More recently, an enzymatic RNA polymerization
technique has been used to condense RNA structures into self-assembled RNAi-microsponges.\textsuperscript{64} While
siRNA binding and encapsulation can be challenging, siRNA delivery overall is not necessarily more difficult
than DNA delivery, as unlike DNA, siRNA does not require nuclear import to function.\textsuperscript{65}
**Systemic circulation**

Both viral-based and non-viral based vectors face the problem of rapid clearance from the systemic circulation on the order of minutes. While viruses often suffer from specific antibody-mediated immune response, non-viral platforms are quickly cleared by several non-specific mechanisms. Most polymeric gene delivery materials are positively charged as electrostatic interaction is the prevalent driving force in forming many types of nanoparticles for gene delivery. The resulting positive surface charge of nanoparticles provides colloidal stability in aqueous solutions and facilitates interaction with cellular membrane. However, it also attracts anionic counter ions in physiological salt and serum proteins that cause opsonization and aggregation, leading to increased clearance by the mononuclear phagocyte system (MPS) and the reticuloendothelial system (RES).

Several strategies have been employed to minimize clearance from the systemic circulation, including charge shielding and shape control. One common “stealth” technology involves coating nanoparticles with PEG, which provides a relatively inert surface due to its neutral and hydrophilic structure. Conjugation of PEG or “PEGylation” to gene delivery particles composed of cationic polymers, lipids, dextran-spermine, and other materials have been reported demonstrating beneficial effects. An alternative approach is the neutralization of excess positive surface charge of particles through coatings with anionic biomacromolecules such as negatively charged polypeptides. More recently, engineering of particle shape has also been shown to be an important parameter to extend circulation time. Nanoparticles for drug delivery with higher aspect ratios had longer circulation half-lives than spherical particles, and this strategy has begun to be utilized for gene delivery nanoparticles.

**Tissue and cell targeting**

Tissue targeting can be accomplished through design of a targeted systemically administered nanoparticle, through a tissue-specific promoter, or through a local injection. Nanoparticles can be injected into anatomically accessible sites to enhance delivery in the local region of interest while reducing non-specific transfection at other sites. For example, intracerebroventricular delivery grants direct access to the brain, retrograde intrabiliary infusion to the liver, and intratumoral injection to tumors.

Nanoparticles that are administered intravenously must have mechanisms to exit the circulation at the target tissue. The application of nanoparticles to solid tumors is often benefited by passive targeting. The formation of new blood vessels near rapidly growing solid tumors allows nanoparticles with diameters of 400 nm or less to passively leak out of the neovasculature and distribute to the tumor tissue. Therefore,
prolonging systemic circulation by the aforementioned strategies, such as PEGylation, can increase the accumulation of nanoparticles at a tumor site, and this enhanced permeability and retention (EPR) effect has become an important tool in nanoparticle-mediated gene delivery for cancer therapy. On the other hand, molecular ligands and chemical moieties that bind specifically to overexpressed receptors on the vascular endothelial cells’ surface near a solid tumor have been conjugated to various nanoparticles as an active targeting mechanism. For example, arginine-glycine-aspartic acid (RGD) peptide sequence and other chemical antagonists to various integrin isoforms have been covalently conjugated or electrostatically bound via negatively charged polypeptides to biomaterials target tumor vasculature. Another emerging method of conferring tissue-specificity to nanoparticles utilizes aptamers as targeting ligands.

Nanoparticles with targeting ligands can be used to target many additional cell types as well. This is important as, when cell-specific RNA interference or therapeutic exogenous DNA expression is critical, nucleic acid delivery nanoparticles with cell-specific targeting can increase efficacy and reduce potential off-target side effects. Overexpressed receptors on the surface of specific cell types of interest are good candidates to target with ligands. The gene delivery and nanomedicine literature show that modification of nanoparticles ligands of many different types can be effective including: galactosylated PEI targeting hepatocytes, antibodies specific to the insulin receptor to target cancer cells in brain, synthetic peptides that bind to integrin α5β1 on neuroblastoma cells, small molecules targeting CD40 on ovarian cancer cells, and leukocyte function-associated antigen-1 to bind to melanoma cells.

Other methods for cell-specific gene delivery are also possible. Following a polymer library approach, a recent study by Guerrero-Cázares et al. showed that the specific chemical structure of a polymer that comprises a polymer/DNA gene delivery nanoparticle can confer cell specificity to one type of cell over another such as primary brain tumor initiating cells over healthy neural progenitor cells. Similar results have also been shown for polymeric nanoparticles that can target liver cancer cells and endothelial cells. A final approach is including a cell type-specific promoter in the plasmid to promote targeting of specific cells such as cancer cells. In this manner, even if some of the polymeric nanoparticles are delivered to off-target cells, there is only successful expression of the exogenous gene in the targeted cells where the specific promoter is active.

**Cellular uptake**

Once nanoparticles reach the cells of interest, they must overcome several barriers at the cellular level before successful transfection is achieved. First, gene carriers need to cross the cellular membrane, for which exist both non-specific and specific mechanisms. Macropinocytosis is a non-specific cellular uptake mechanism, where cells engulf extracellular fluid through actin-driven evagination. However, gene vectors
Entered via macropinocytosis result in poor transfection efficacy due to high rate of recycling. Also, the positive surface charge on nanoparticles formulated with cationic polymers or lipids promotes electrostatic interaction with the negatively charged cell surface, which in turn triggers another non-specific pathway, adsorptive endocytosis. It should be noted that positive surface charge is shielded by approaches designed to increase systemic circulation time such as PEGylation. These coatings minimize electrostatic interactions between nanoparticles and cellular membranes.

On the other hand, specific uptake mechanisms are mediated by receptors on the cell membrane, which can recognize various molecular ligands as well as chemical moieties of a nanoparticle. In the case of PEGylated nanoparticles, ligands can be conjugated to the terminal ends of the PEG chain, and chemical moieties can be exposed upon environment-stimulated PEG cleavage. There are two major specific uptake routes for gene carriers. Clathrin-mediated endocytosis is initiated with clathrin-coated pits of approximately 100 – 150 nm in size that pinch off from the plasma membrane to form endosomes. Nanoparticles modified with MC1SP-peptide and transferrin that target the melanocortin receptor-1 and the transferrin receptor respectively, and unmodified lipoplexes and liposomes are found to be endocytosed via this pathway. In comparison, caveolae-mediated endocytosis is characterized by flask-shaped invaginations of about 50 – 100 nm in diameter. Folic acid ligands that bind with folate receptors as well as unmodified polymeric nanoparticles can be directed to caveolae-mediated uptake.

Different uptake pathways lead to different intracellular fate, which underscores the importance of the cellular uptake on successful transfection. For example, the major route of uptake for PBAE nanoparticles does not necessarily lead to high transfection efficiency. The surfaces of nanoparticles can be modified to direct their uptake pathway, improve the intracellular fate of the plasmids that they contain, and increase subsequent transfection efficiency.
**Endosomal escape**

Once endocytosed, nanoparticles must escape the endosomal compartment and reach the cytoplasm. One mechanism of endosomal escape for polymeric nanoparticles is through the proton sponge effect. \(^{114}\) Reversibly protonated biomaterial vector can act as a buffer as the endosome gradually become acidic, thereby protecting the cargo. Subsequently, chloride ions enter the endosomes to neutralize the charge, creating osmotic pressure that eventually leads to endosomal burst and cargo release. PLL, owing to its primary amines that are easily protonated at pH 7, is unable to provide strong buffering capacity at endosomal pH. In order to neutralize the acidic pH, researchers have either co-delivered PLL with amphipathic amines, such as chloroquine, or substituted its lysine residues with histidine or arginine residues that have titratable amines. \(^{48,115}\) PEI and PBAE, on the other hand, have weakly basic tertiary amines in their structure that allows for the proton sponge effect. \(^{51,116}\) Although the proton sponge hypothesis is a widely believed mechanism of endosomal escape, it has been challenged and remains to be clearly elucidated. \(^{117}\)

Nanoparticles can also escape the endosome by destabilizing endosomal membranes. For example, amphipathic, fusogenic peptides, such as GALA (repeats of Glu-Ala-Leu-Ala) and KALA (repeats of Lys-Ala-Leu-Ala), have been utilized as the primary component of non-viral vectors and have also been associated non-covalently with nanoparticles. \(^{118,119}\) These fusogenic peptides are able to form alpha-helical structures at endosomal pH that can destabilize the endosomal membranes. Other amphipathic lipids, such as dioleoylphosphatidylethanolamine (DOPE), that assume non-bilayer structure, can also facilitate endosomal membrane destabilization when associated with liposomes. \(^{120,121}\)

**Release of nucleic acid and nuclear transport of DNA**

Although strong binding or encapsulation of nucleic acid is necessary to form stable particles, nucleic acids must be able to be released from the particles once the particles have entered the cytoplasm. This release typically occurs due to thermodynamics-driven disassociation of the anionic DNA from cationic polymers or due to degradation of the particle. Release is necessary in order to enable the nucleic acid to have a biological effect, as DNA that isn’t released from its carrier is unable to be transcribed as efficiently. \(^{122,123}\) This was illustrated by a recent study showing that the binding constant between polycations and DNA is biphasic with the transfection efficiency. \(^{124}\) Additionally, polymer degradability imparts decreased cytotoxicity, as polymer molecular weight has been shown to positively correlate with toxicity. \(^{125}\) Lack of degradability contributes to the toxicity of conventional polymers such as 25 kDa branched PEI. \(^{126,127}\) Below, we will discuss methods that allow for the chemical modification of conventional polymers in order to enable
them to degrade (Figure 2-2), in addition to the design of polymers with degradable moieties inherent in their chemical structure.

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Figure 2-2. Chemical moieties that can enable the degradation of polymeric nanoparticles. Chemicals listed above the reaction scheme arrow indicate those necessary for the reaction, while chemicals listed below indicate reaction catalysts.

**Hydrolysis**

The release of nanoparticle cargo can be achieved through polymer hydrolysis via the cleavage of ester, urethane, imine, and orthoester linkages. Poly[alpha-(4 aminobutyl)-L-glycolic acid] (PAGA) is a hydrolysable analog of PLL in which the amides linking conventional PLL monomers are replaced with ester linkages. PAGA based DNA delivery has been shown to lead to higher transfection efficacy and lower toxicity versus conventional PLL.\(^{128}\) Hydrolytically cleavable PEI can be synthesized by linking shorter PEI polymers together with ester-containing crosslinkers. Diacrylate monomers used to crosslink 800 Da PEI were shown to create nanoparticles with the same size, shape, charge, and DNA binding versus 25 kDa PEI.
while achieving 16-fold enhancement to transfection efficacy with no measureable toxicity.\textsuperscript{129} PBAE polymers are formed via Michael addition of amine-containing monomers with diacrylate monomers and therefore contain esters within the polymer backbone.\textsuperscript{130} Combining various amine and acrylate monomers enables the creation of libraries of PBAEs with various chemical properties,\textsuperscript{131} and whose binding constants are affected by polymer molecular weight.\textsuperscript{124} Other poly(amino ester)s can be synthesized to have a similar chemical structure and transfection efficacy as 25 kDa PEI but with reduced toxicity (Figure 2-4).\textsuperscript{132} Poly(lactic-co-glycolic) acid (PLGA)-based nanoparticles can encapsulate nucleic acids, particularly using a double emulsion method and polyamines, and successfully deliver nucleic acids.\textsuperscript{133} PLGA chemically modified with amine-containing molecules grafted onto their polymer backbone has also been shown to effectively deliver DNA and siRNA.\textsuperscript{134,135} Other hydrolytically cleavable polymer linkages have been explored for biodegradable polymer design. Amine containing polyurethanes can be designed to deliver DNA.\textsuperscript{136} Polyimines, which specifically allow for acid-labile hydrolysis, can be used to link short chain PEI.\textsuperscript{137} Additionally, polyorthoester polymers can form stable nanoparticles at neutral pH, but are acid-labile and release DNA at pH 5.\textsuperscript{138,139}

Reduction

Polymer bioreducibility via the inclusion of disulfide bonds enables cargo release targeted to the cytoplasm. Cytosolic reduction is due to the presence of reducing agents such as glutathione, a molecule present in concentrations roughly 1000 times higher in the cytosol versus extracellular space.\textsuperscript{140} This cellular compartment-specific degradation and release makes bioreducible polymers particularly useful for delivery of siRNA, mRNA, and miRNA whose site of action is within the cytosol. In contrast, DNA delivery via disulfide-containing polymers has sometimes been found to be less-effective.\textsuperscript{141} As with hydrolytic linkages, reducible linkages can improve delivery efficacy and reduce cytotoxicity of conventional polymers. PLL linked with disulfides has shown improved nucleic acid delivery.\textsuperscript{142} Methods to crosslink PLL with disulfides, either by incorporating cysteines into the polypeptide backbone,\textsuperscript{143} or by chemically modifying the lysine side chains to contain thiols,\textsuperscript{144,145} has led to improved siRNA and DNA delivery. Linear PEI linked with disulfides also showed improved siRNA delivery with lower toxicity than 25 kDa PEI.\textsuperscript{146} The KALA fusogenic peptide has also been modified with cysteines to allow for crosslinking.\textsuperscript{147}

Polymer bioreducibility can be imparted by synthesizing polymers from disulfide-containing monomers. Poly(amido amine)s (PAAs) are synthesized with diacrylamide and amine-containing monomers, and are therefore not biodegradable. However, disulfide-containing diacrylamides can also form PAAs and impart targeted cargo release to the cytoplasm. Disulfide-containing PAAs have been extensively studied for both DNA and siRNA delivery.\textsuperscript{146,148-151} PBAEs can also be bioreducible, either by end-capping with disulfide-containing monomers,\textsuperscript{9,152} or using diacrylate monomers with disulfides to incorporate them into the
polymer backbone. Bioreducible PBAEs have been shown to be successful as siRNA delivery vehicles, achieving near-complete gene knockdown with little toxicity, including at low siRNA doses.

**Triggered Release**

Other modes of degradability can enable targeted release of polymeric nanoparticles through triggered release. This release can be tissue or environment-specific. As an example, enzyme-cleavable linkages, specifically matrix-metalloprotease (MMP)-cleavable groups, can allow for release within tumor space. Polymers can be degraded by enzymes or polymeric nanoparticles can become deshielded in the presence of specific enzymes. Nanoparticle degradation and nucleic acid release can also be triggered by an external source. For example, light-induced degradation could allow for user-controlled release and spatially controlled cargo release.

**Nuclear transport**

Lastly, the final potential delivery step following delivery of nucleic acids to the cytoplasm is their transport into a specific organelle, such as the nucleus. Unlike siRNA, DNA plasmids have to be transported to the nuclear membrane and enter the nucleus for their biological effects to occur. Following nuclear import, exogenous DNA expression requires transcription and translation. The simian virus 40 large T antigen nuclear localization signal (NLS), which is a peptide sequence rich of lysine amino acid, is known to facilitate nuclear transport. Many vectors, including cationic peptides and lipids, as well as DNA plasmids, have been modified with variant forms of an NLS or NLS-binding motif in order to enhance transfection.
Biomaterials used for polymeric gene delivery

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Figure 2.3. Characteristic chemical structures of polymers used for non-viral gene delivery

**Peptides**

*Poly(L-lysine)*

While poly(L-lysine) (PLL), shown in Figure 2.3A, generally has low efficacy for gene delivery by itself, multicomponent nanoparticles that incorporate PLL as a polycation to bind nucleic acids have been more successful. In one example, PEG-PLL-DMMAn-Mel nanoparticles have shown potential improvement for PLL-based siRNA delivery. In this system, Meyer et al created PEG-modified PLL nanoparticles that utilized the lytic peptide melittin (Mel) shielded by pH cleavable dimethylmaleic anhydride (DMMAn) to only expose the lytic peptides for endosomal escape once a pH of 5. In addition to the pH sensitive lytic peptide exposure, siRNA release was achieved via disulfide cleavage between the siRNA and polymer. These PEG-PLL-DMMAn-Mel nanoparticles were shown to achieve 90% knockdown *in vitro*, with a caveat being potential cytotoxicity (70% metabolic activity as measured by an MTT assay). While results of this nanoparticle formulation were promising for siRNA delivery *in vitro*, *in vivo* testing revealed a high level of toxicity in healthy mice and tumor bearing mice alike requiring sacrifice of the animals shortly after application. This study highlights the potential role of biomaterial-induced toxicity in synthetic gene delivery systems.

Other uses of PLL for nucleic acid delivery have included pH cleavable PEGylated PLL-cholic acid nanoparticles shown to have a nine-fold reduction in gene expression *in vitro* with cell viability over 90%. *In vivo* results of PEGylated PLL-cholic acid nanoparticle delivery of VEGF siRNA achieved a tumor size reduction of 41% with a measured 70% qPCR knockdown of VEGF mRNA without a significant weight reduction in treated mice. Another PLL nanoparticle formulation utilizing dendritic PLL for the knockdown of Apolipoprotein B to reduce serum low-density lipoprotein levels was shown to achieve significant knockdown *in vivo* leading to a 40% reduction in serum LDL. PLL has been shown to be an effective polycation when modified to enable endosomal escape and cellular targeting, but its current use is limited as it lacks the versatility of many other polycations for the delivery of nucleic acids.

**Cell-penetrating peptides**

Peptides have been incorporated in many other nanoparticle designs as both the backbone structure and as surface molecules. Amphipathic endosomal escape peptides such as GALA and KALA, as mentioned previously, are well documented for improving transfection among various cell types and with different nanoparticle formulations. Other CPPs have been used in the creation of nanoparticles for the delivery of siRNA with lysine residues often being used to increase the cationic nature of the peptides. Peptides have also been incorporated into nanoparticles for the purposes of endosomal release. For example, sHGP, a 15 amino acid oligopeptide from HIV gp41, has been shown to improve endosomal release. Peptide sequences from influenza, specifically Inf7, have also been utilized in the design of nanoparticles to aid in
endosomal escape and has been shown to improve efficacy of transfection with siRNA.\textsuperscript{166} Control over the enzymatic degradation rate of peptide-based nanoparticles has also been achieved by Chu \textit{et al}. who designed nanoparticles utilizing both \textit{D} and \textit{L} amino acids for controlled cleavage by Cathepsin B.\textsuperscript{167} In this way, a stereospecific enzymatic degradation strategy was shown to offer excellent stability extracellularly with a controlled rate of intracellular degradation to release nucleic acids.\textsuperscript{167}

CPP based nanoparticles termed PF6 for the delivery of siRNA were created by Andaloussi \textit{et al}. and demonstrated to be able to knockdown a reporter gene up to 90\% in serum containing media with minimal cytotoxicity and inflammatory effects.\textsuperscript{164} Importantly, PF6 nanoparticles were shown to be stable over a span of weeks in water as well as being stable over a short term in serum containing media. These nanoparticles has a diameter between 125-200 nm and a zeta potential of approximately -10 mV.\textsuperscript{164} Intravenous administration of PF6 with luc-siRNA to transgenic mice with bioluminescent liver cells showed effective knockdown peaking on day 5 at a 75\% reduction.\textsuperscript{164} PF6 knockdown of the functional protein HPRT1 was observed to be greatest in the liver and in addition there was silencing of greater than 60\% in the kidneys.\textsuperscript{164}

\textit{Synthetic polymers}

\textit{Polyethylenimine}

High molecular weight 25 kDa Polyethylenimine (PEI), shown in Figure 2-3B, has previously been shown to condense DNA to form nanoparticles, undergo endosomal escape, and successfully deliver DNA.\textsuperscript{51,168} Unfortunately while transfection efficacy is correlated with the molecular weight of PEI, cytotoxicity is likewise correlated making unmodified 25 kDa PEI largely unsuitable for \textit{in vivo} applications.\textsuperscript{168} Related to issues of immediate cytotoxicity, PEI is a non-biodegradable polycation that requires excess polymer to effectively transfect cells that can lead to accumulation upon repeated administration.\textsuperscript{52,53} PEI of approximately 25 kDa molecular weight was shown to have higher transfection efficiency than higher molecular weight versions, such as 50 kDa and 800 kDa, but still suffered from cytotoxicity \textit{in vivo} in mice.\textsuperscript{51,169} To further minimize the cytotoxicity of non-biodegradable PEI, lower molecular weight versions of linear and branched PEI have been investigated, as have partially biodegradable cross-linked PEI and hyperbranched oligoethyleneimine.\textsuperscript{170-173} Biodegradable linkages between low molecular weight PEI segments have primarily included bioreducible disulfides and hydrolysable esters, both of which have been shown to improve transfection efficacy as well as decrease cytotoxicity compared to 25 kDa PEI.\textsuperscript{129,172} In 2003, Forrest \textit{et al}. created hydrolysable PEI polymers from 800 Da PEI and diol-diacrylate monomers that improved transfection efficiency and reduced cytotoxicity.\textsuperscript{129} Using disulfide cross-linked 1.8 kDa low molecular weight PEI, Liu \textit{et al}. were able to achieve greater than 60\% transfection with 90\% cell viability in serum containing media in 2010.\textsuperscript{174}

These improvements to polymer structure have made PEI much less toxic \textit{in vitro} but do not fully avoid the problems of polycation accumulation \textit{in vivo} that challenge non-biodegradable polymeric delivery.
PEI-based nanoparticle formulations have been investigated in human clinical trials. One example is a Phase I clinical trial for the delivery of a plasmid encoding interleukin-12, which was administered to thirteen patients with recurrent ovarian cancer and indicated favorable safety results. The nanoparticle was composed of a lipopolymer, PEG-PEI-cholesterol, and was administered in a series of four increasing doses every 4 weeks intrapleurally.

Poly(beta-amino ester)s

The development of poly(beta-amino ester)s (PBAEs), shown in Figure 2-3C, as a material for transfection has been greatly advanced by high throughput screening of PBAE polymer libraries in which monomers that make up the backbones, side chains, and end capping groups have been systematically varied. This rapid screening technique has allowed for a large variety of PBAEs to be tested and patterns in transfection to be determined. Transfection by PBAEs has been shown in some cases to be cell type specific, with cellular uptake and transfection differences between healthy cells and tumor cells due to variation in polymer structure. Using polymer libraries and high throughput screening, PBAE nanoparticle formulations for the delivery of siRNA and DNA to human glioblastoma cells have been achieved up to 85% and 90% respectively in vitro, significantly greater than Lipofectamine 2000 and other commercial transfection reagents (Figure 2-4). While most PBAE nanoparticles for the delivery of nucleic acids have used linear PBAEs for rapid intracellular degradation and release of DNA polyplexes, cross-linked PBAEs formed by Michael addition using triacrylate monomers and N,N-dimethylethylenediamine have also been created to reduce the rate of degradation and DNA release.

In vivo results of PBAE nanoparticle-mediated delivery of DNA in mice have demonstrated functional transfection for treating diseases such as ovarian cancer. Transfection of brain tumor-initiating cells (BTICs) in 3D oncospheres with pDNA has been accomplished with PBAE nanoparticles at up to 76% transfection in vitro. In this work, PBAE nanoparticle specificity of transfection for BTICs over fetal neural progenitor cells (fNPCs) has been demonstrated both in vitro and in vivo, supporting the notion that in vitro monolayer culture screening of PBAE nanoparticles has relevance for in vivo efficacy. Transfection of ovarian tumor bearing mice via intratumoral injection of PBAE nanoparticles containing a plasmid encoding diphtheria toxin showed a mean tumor load reduction greater than that of administration with dual chemotherapeutics. The intrapleural injection route used in this study mirrors the current injection route of chemotherapeutics for advanced ovarian cancer, supporting the clinical relevance of the work. Of importance to clinical relevance, PBAE/DNA nanoparticles, although readily hydrolysable, have been demonstrated to be stable when stored at -20°C for up to two years upon lyophilization with sucrose as a cryoprotectant.
PBAEs have also been used as a cationic polymer to supplement other materials in the creation of nanoparticles for the \textit{in vivo} delivery of siRNA. Cohen \textit{et al.} have created acetalated-dextran nanoparticles with 10 wt\% PBAE that showed pH sensitive degradation and release of DNA.\textsuperscript{182} PBAE has been used as a cationic polymer for binding DNA in conjunction with PLGA to form microspheres capable of transfecting macrophages to express a tumor specific antigen and induce an adaptive immune response in mice.\textsuperscript{183}

For localized delivery of DNA and siRNA amenable to tissue engineering applications, PBAEs have been used in the development of multilayer polyelectrolyte films shown to enable contact dependent transfection.\textsuperscript{184} Multilayered films, such as those developed in the lab of David Lynn, rely on charge association between layers of polycations, in this case cationic PBAEs and anionic DNA, to respond in a pH and temperature dependent manner for localized transfection.\textsuperscript{185} DNA release was shown to be largely dependent on multilayer film degradation and released DNA in a relaxed conformation compared to the typical supercoiled conformation resulting from nanoparticle delivery.\textsuperscript{184} Multilayered polyelectrolyte films have been further developed for localized delivery of siRNA with release due in large part to diffusion out of the film rather than film degradation\textsuperscript{186} Notably, the multilayer film design allowed for sustained release of DNA for 30 hours, while siRNA was released in a burst manner.\textsuperscript{184,186} The Hammond and Irvine groups have developed PBAE-based layer-by-layer coatings of microneedles that can be used for DNA vaccination and delivery of immunostimulatory RNA through the skin.\textsuperscript{187} Using this approach, the authors found potent cellular and humoral immunity \textit{in vivo} in mice and enhanced gene delivery \textit{ex vivo} in non-human primate skin.\textsuperscript{187}
Figure 2-4. Poly(beta-amino ester)s (PBAE)s for gene delivery to brain cancer. (A) Libraries of PBAEs can be synthesized by reacting different acrylate and amine-containing monomers. (B) DNA-containing nanoparticles can be lyophilized and stored prior to in vivo administration. (C) Intracranially administered PBAE/DNA nanoparticles selectively transfect (red) human brain cancer cells (green) while avoiding healthy
Poly(amido amine)s

Dendrimers are symmetrically branched polymer structures that have been used as base units to encapsulate and deliver various materials through charge interactions or conjugation. Many dendrimers, including poly(amidoamine) (PAA or PAMAM) shown in Figure 2-3D, are synthesized by a series of Michael addition reactions, allowing for great specificity of size and nitrogen content for complexation with nucleic acids by a fine-tuned N:P ratio. The exterior surface of dendrimer molecules can also be modified with hydrophilic groups to allow for improved solubility in water or with targeting ligands for attempted improved active cellular uptake. The most frequently used dendrimer for nucleic acid delivery to date has been PAMAM, although peptide dendrimers have also been utilized with some success. Bioreducible PAMAM nanoparticles for the delivery of DNA have been created with very high cell viability and transfection efficiency up to 200 times that of branched PEI. The degree to which the structure of these hyperbranched PAMAM particles were able to be reduced was able to be finely tuned by the changing the monomer molar ratios used in the Michael addition reactions used to create the polymer. Beyond PAMAM, Barnard et al. developed an ester hydrolysable dendrimer with surface amine groups capable transfection of in vitro up to 10 times more efficiently than PEI.

Poly(lactide-co-glycolide) and poly(caprolactone)

PLGA microparticles containing 25 wt% PBAE were used to transfect macrophages with reporter gene DNA both in vitro and in vivo. In vivo results showed that these microparticles containing a plasmid for the expression of a tumor antigen were able to induce rejection of the transplanted tumor matching the antigen. The resulting adaptive immune response was sufficient to cause a reduction in measured tumor growth by day 11 following transfection. In another nanoparticle formulation, copolymer hybrid poly(ester amine) nanoparticle formulations of polycaprolactone and PEI have been formulated with improved transfection over 25 kDa PEI for a number of cell lines. Thus, biodegradable polymer blends are an appealing approach for nucleic acid delivery.

Polysaccharides

Chitosan

Chitosan, a natural linear polysaccharide derived from chitin and shown in Figure 2-3E, has been used in the delivery of pDNA and siRNA. Chitosan varies by the degree of deacetylation from chitin expressed as a ratio of β-(1–4)-linked D-Glucosamine to N- acetylated-D-glucosamine. Highly deacetylated
chitosan has been used more frequently for nucleic acid delivery due to its greater cationic nature and corresponding ability to complex with the negatively charged backbone of DNA or RNA. Mao et al. created PEGylated chitosan DNA nanoparticles with the targeting molecule transferrin capable of transfection.\textsuperscript{194} Chitosan-DNA nanoparticles were also found to transfect intestinal epithelium \textit{in vivo}, generate immunologic protection, and reduce allergen-induced anaphylaxis when administered orally to mice.\textsuperscript{195} For siRNA delivery, chitosan thiamine pyrophosphate nanoparticles have been shown to achieve knockdown up to 70\% with cell viability above 90\% \textit{in vitro} for hepatocarcinoma cells, notably greater than Lipofectamine.\textsuperscript{196} Trimethyl chitosan has also been used in conjunction with the polysaccharide polysialic acid (PSA) for the delivery of transcription factor decoy oligonucleotides resulting in a reduction of inflammation measured by excreted cytokines \textit{in vitro}.\textsuperscript{197}

\textit{Hyaluronic acid}

Hyaluronic acid (HA), shown in Figure 2-3F, has been utilized in nanoparticles for the delivery of nucleic acids as well as a targeting molecule for the CD44 cell receptor often overexpressed on the surface of tumor cells.\textsuperscript{198, 199} HA chitosan-PEG nanoparticles synthesized for the delivery of pDNA and siRNA have been shown to have transfection efficiency equivalent to that of Lipofectamine 2000 \textit{in vitro}.\textsuperscript{198} Nanoparticles for siRNA delivery composed of HA-spermine and HA-PEI have achieved above 90\% knockdown \textit{in vitro} with specificity for the CD44 receptor.\textsuperscript{199} When the HA-PEI particles were tested \textit{in vivo} for targeting of a metastatic lung cancer model implanted subcutaneously in mice, knockdown measured by qPCR of up to 55\% was observed.\textsuperscript{199} Improving serum stability of nanoparticles is another area in which HA has been utilized in combination with polycations such as PEI, functioning in much the same way as glycosylation of proteins \textit{in vivo}.\textsuperscript{200} HA has also been utilized in the creation of hydrogels capable of delivering DNA at controlled rates for \textit{in vivo} tissue engineering applications.\textsuperscript{201}

\textit{Cyclodextrin}

β-Cyclodextrin, shown in Figure 2-3G, is a three-dimensionally stable oligomer of glucose that forms cup like structures with a hydrophobic core. Chemical modification of β-cyclodextrin with acetyl groups enables the polymer structure to complex with nucleic acids as a cationic polymer. Cyclodextrin-based nanoparticles developed in the lab of Mark Davis for the intravenous delivery of siRNA have reached clinical trials. These nanoparticles, shown in Figure 2-5A, are formulated from β-cyclodextrin, adamantine-PEG, and the targeting ligand transferrin, and have been shown to have favorable characteristics for the delivery of siRNA including a small nanoparticle size between 60-80 nm, a zeta potential +10-20 mV, and the ability to protect siRNA from nuclease activity in the presence of serum for at least 4 hours.\textsuperscript{202} In 2009 following animal trials in monkeys, this nanoparticle formulation for the delivery of RRM2-siRNA was tested in FDA
Phase I clinical trials as a cancer therapeutic. The Phase I trial involving 24 patients has since concluded with favorable results for the nanoparticle safety, including evidence for the lack of a complement response. Additionally, RRM2 mRNA levels intratumorally were shown to have been reduced up to 77% and a 32% partial knockdown of RRM2 was measured for RRM2 protein levels in the tissue as shown in Figure 2-5B. While this level of knockdown may not be effective as a monotherapy for cancer, it provides early evidence that with this non-viral system siRNA can be targeted to tumor cells in human patients with measurable knockdown of a specific protein.

Figure 2-5. Cyclodextrins for siRNA delivery. (A) The three components that formulate CALAA-01, a cyclodextrin-containing nanoparticle for RRM2 RNAi tested in FDA phase I clinical trials. (B) mRNA and protein levels of RRM2 are both knocked down in the targeted tissue of one patient (C). RRM2 staining (red) of human tumor tissue before and after systemic administration of the cyclodextrin-containing nanoparticles. Reprinted by permission from Nature Publishing Group: Nature. 2010, 464, 1067-70, copyright 2010.

Dextran
Dextran, shown in Figure 2-3H, is a branched polysaccharide of repeating glucose units and has been used in the formation of nanoparticles for the delivery of siRNA and pDNA. Dextran is often acetalated to improve solubility in organic solvents and allow for pH dependent degradation. While the structure of unmodified dextran does not fit the requirements of an ideal biomaterial for the delivery of nucleic acids, its status as an easily modified biocompatible and biodegradable polymer allows for it to be utilized with other materials in the formulation of nanoparticles for nucleic acid delivery. Acetalated dextran has been used in conjunction with PBAE and spermine for the successful delivery of both siRNA and DNA. Ac-Dex/PBAE particles created for the delivery of DNA have been shown to undergo endosomal pH dependent degradation and have also been coated with cell penetrating peptides for improved endosomal escape.

Spermine

Spermine, shown in Figure 2-3I, is a natural oligoamine that has been used primarily as an oligomer grafted onto non-cationic polymers to enable the resulting polymer to have improved charge association with DNA. Biodegradable polysaccharide-based particles using spermine as a polycation have been explored with varying degrees of success. In 2002, a library of over 300 polysaccharide-oligoamine particles were created with some polysaccharide-spermine particles reaching transfection efficiency equal to that of Transfast cationic lipids. Since then, acetalated-dextran spermine nanoparticles have been created for siRNA as well and shown to cause up to 60% knockdown of GFP in HeLa cells.

Nucleic-acid based particles

Nanoparticles composed entirely of nucleic acids have been created for the delivery of siRNA. In this case, the natural biodegradable polymer is the nucleic acid itself, which functions as both the structure for the particle as well as the cargo of the particle. Lee et al. used six 30 bp segments of DNA with complementary overhanging siRNA segments to create self-assembling tetrahedral oligonucleotide nanoparticles by complementation between the DNA and overhanging siRNA as shown in Figure 2-3J. These particles, each carrying six siRNA molecules, were shown to have a circulation time four times longer than unprotected siRNA. Oligonucleotide nanoparticles improved siRNA delivery for the knockdown of luciferase both in vitro and in vivo with a 60% reduction in bioluminescence of luciferase expressing tumors in a rat model two days after treatment. These initial studies, particularly for improved in vivo delivery of siRNA have indicated that nucleic acid origami particles are a high capacity siRNA delivery method that deserves further study. Paula Hammond and colleagues have used RNA polymerase to form long strands of RNA that can self-assemble into nanostructures and microstructures. These structures, RNA microsponges, with or without PEI, were able to generate in vivo knockdown when administered.
intratumorally in mice. In an alternative approach, Chad Mirkin and colleagues have developed spherical nucleic acids, which are densely packed nucleic acids arranged in a spherical geometry, with or without a core. These materials are promising for nucleic acid delivery for varied applications, including cancer therapy. In one example, gold core siRNA-based spherical nucleic acid nanoparticles were found to cross the blood-brain barrier, knockdown Bcl2L12, and induce apoptosis of brain cancer cells, increasing in vivo survival.

Conclusion

Gene therapy holds great promise in treating various diseases of genetic origin by introducing exogenous nucleic acid to express desired proteins and by knocking down the expression of undesirable genes. A key challenge to gene therapy is effective delivery, and significant effort has been invested into developing biomaterials that can form nanoparticles to deliver genes to specific targets safely and efficiently. As highlighted in this chapter, a number of non-viral, biodegradable polymers have been developed to form biodegradable nanoparticles for gene delivery and are promising due to their ease of synthesis, low toxicity, and efficacy at transfection. Importantly, strategies for polymer modifications have been identified to overcome major biological barriers to gene delivery. While a polymeric nanoparticle system for human gene therapy has yet to be FDA-approved, numerous systems for polymeric DNA and siRNA delivery are in preclinical and clinical trials. These systems, or their future derivatives, may be able to achieve the promise of genetic medicine.

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Chapter 2.b Targeted Polymeric Nanoparticles for Cancer Gene Therapy

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Key terms
Gene delivery, Cancer therapy, Nanoparticles, Polymeric biomaterial, Targeting ligands, Promoter, Tissue-specificity, Cell-specificity

Foreword: I wrote the following review article with graduate students Jayoung Kim and Camila Zamboni covering non-viral gene delivery for therapeutic cancer applications.


Abstract

In this article, advances in designing polymeric nanoparticles for targeted cancer gene therapy are reviewed. Characterization and evaluation of biomaterials, targeting ligands, and transcriptional elements are each discussed. Advances in biomaterials have driven improvements to nanoparticle stability and tissue targeting, conjugation of ligands to the surface of polymeric nanoparticles enable binding to specific cancer cells, and the design of transcriptional elements has enabled selective DNA expression specific to cancer cells. Together, these features have improved the performance of polymeric nanoparticles as targeted non-viral gene delivery vectors to treat cancer. As polymeric nanoparticles can be designed to be biodegradable, non-toxic, and to have reduced immunogenicity and tumorigenicity compared to viral platforms, they have significant potential for clinical use. Results of polymeric gene therapy in clinical trials and future directions for the engineering of nanoparticle systems for targeted cancer gene therapy are also presented.

Introduction

Molecular origins of many human diseases, such as cancer, are increasing being elucidated and understood on a genetic level. Gene therapy is an emerging technology that could treat these disorders by permanently or transiently replacing genetic defects with exogenous nucleic acids.1,2 Nucleic acids can be used as new biological cancer therapeutics in multiple ways. For example, tumor suppressor genes can be upregulated
or mutated oncogenes downregulated in affected cells, suicide genes can signal cell death, and DNA vaccines can trigger the immune system to fight cancer.\textsuperscript{3-6}

The challenge in gene therapy is to develop safe vectors that can efficiently deliver nucleic acids. Viral vectors, such as adenovirus and lentivirus, are highly effective yet liable to immunogenicity and/or tumorigenicity from insertional mutagenesis.\textsuperscript{7} While non-viral gene delivery vectors are considered safe, this increased safety comes at the cost of lower efficiency. Polymeric vectors, both natural and synthetic, compose a class of non-viral vectors that show great promise as their chemical structure can be tuned to allow design flexibility for increased safety and efficacy.

A key component for cancer gene therapy success is delivery. While physical methods of delivery enhancement, such as using ultrasound, heat, light, and applied magnetic and electric fields, have been utilized to deliver gene carriers to target cancer sites, these methods rely on precise knowledge of the location of tumors. To reach both known and unknown locations of cancer cells, other research efforts have focused on the development of non-viral vectors, such as polymeric nanoparticles, as delivery systems to ensure site-specific accumulation and cancer-specific transfection through both passive and active targeting.\textsuperscript{2} As illustrated in Figure 2-6, small nanoparticles (or polyplexes) with neutral surface charge and steric hindrance can circulate for extended periods of time and accumulate near tumor sites through the enhanced permeation and retention (EPR) effect. In addition, polymeric biomaterials, with or without targeting ligands, can induce tissue-specific accumulation and cell-specific uptake. Finally, nucleic acid engineering can enable cell type-specific transcription and translation.

This review highlights advances to improve the targeted delivery of therapeutic nucleic acids through the utilization of polymeric nanoparticle vectors. The presented nanoparticles are able to specifically target and enhance efficient delivery to tumors.
Figure 2-6. Overall scheme of gene delivery to cancer cells using targeted polymeric nanoparticles. Three broad categories of targeting include biophysical targeting, ligand-mediated targeting, and transcriptional targeting.

Biophysical and Biomaterial-mediated Targeting
Intrinsic physicochemical characteristics of polymeric nanoparticles can facilitate tumor targeting. Characteristics such as particle size, surface charge, and chemical functional groups exposed on a biomaterial’s surface can either drive cellular uptake or block cellular interaction. In some cases, biomaterial composition can dictate tumor specificity in the absence of known ligands. Technology that enables passive targeting to cancer cells is an expanding area of research in the design of polymeric gene delivery vectors.

**Biophysical targeting**

One of the most widely utilized mechanisms of passive targeting is based on size. The enhanced permeation and retention (EPR) effect, or leaky vasculature and a lack of lymphatic drainage around a tumor mass, allows for nanoparticles of less than 500 nm to accumulate in the regions of tumor vasculature. Larger nanoparticles are usually eliminated from the body by the reticuloendothelial system (RES) or trapped in the lungs, while nanoparticles with hydrodynamic diameters smaller than 5 nm are rapidly excreted by the kidneys. To prevent particle aggregation and maintain the effective size for the EPR effect, nanoparticles are often coated with hydrophilic molecules, such as poly(ethylene glycol) (PEG) that provide neutral surface charge and steric hindrance.

Many polymeric nanoparticles for gene delivery are formed through electrostatic interactions between positively charged cationic polymers and negatively charged anionic DNA. The net balance of these charges determines a nanoparticle’s surface charge, which is one of its key biophysical properties. A positively charged nanoparticle surface interacts with negative charges on the cellular membrane and promotes cellular uptake. In addition, non-specific adsorption of anionic serum proteins on to positively charged surfaces of nanoparticles changes the surface properties of nanoparticles. In some cases, these interactions have the potential to induce receptor-mediated endocytosis. Other studies have shown that adsorbed serum proteins can reduce cellular uptake. Moreover, these non-specific protein interactions can induce particle aggregation and opsonization, thereby shortening systemic circulation and reducing the EPR effect and cancer targeting potential.

To prevent undesirable particle-serum interactions, various methods have been explored to shield the surface of polymeric nanoparticles with biomolecules. One common strategy is the utilization of neutrally charged PEG molecules as previously discussed. Researchers have also made stimuli-responsive versions of PEG coatings that aid in tumor targeting. For example, with a stimuli-responsive linker, such as metalloproteinase-cleavable linker, PEG can detach from a nanoparticle surface, restore a positive nanoparticle surface charge in close proximity to cancer cells, and better interact with these target cells. Other approaches for nanoparticle surface shielding have utilized other polyanions, such as poly(glutamic acid), carboxymethyl
poly(L-histidine), and hyaluronic acid (HA) to coat positively charged nanoparticle surfaces, promote colloidal stability, and facilitate gene delivery \textit{in vitro and in vivo}.

\textbf{Biomaterial-mediated targeting}

Many biomaterials have been investigated as polymeric vectors capable of delivering genetic cargo to cells of interest. Polymers used for gene delivery include polypeptides (i.e. poly(L-lysine)), natural polymers (i.e. chitosan, dextran, and HA), and synthetic polymers (i.e. polyethylenimine (PEI), polyamidoamine (PAMAM) and poly(β-amino ester) (PBAE)).\textsuperscript{26-41} Interestingly, some of these polymers have shown a bias for transfecting one cell type over another, indicating that they can enable tissue- or cell-specificity without an active targeting modification.

Tissue-targeting and biodistribution of polymeric nanoparticles is one of the key elements of pharmacokinetics in systemic delivery. Polymeric nanoparticles can demonstrate an intrinsic tendency to accumulate in particular organs or tissues. For example, dextran sulfate is able to bind to more receptors on liver sinusoidal endothelial cells than hyaluronic acid, and thus accumulates more in liver.\textsuperscript{42} Biodistribution of PEI nanoparticles varies depending on the molecular weight, the structure of the polymer, and the polymer amine to DNA phosphate (N/P) ratio.\textsuperscript{43} Polymer coatings can also generate tissue specificity as work by Harris \textit{et al.} showed that PBAE nanoparticles coated with anionic poly(glutamic acid)-based peptides demonstrated changes in biophysical properties and tissue-specificity to spleen and bone marrow (Fig. 2-7A).\textsuperscript{44} Such tissue specificity of biomaterials could potentially be utilized to target tumors as well.

Biomaterial-mediated targeting to specific cell-types has been demonstrated with PBAE polymeric nanoparticles. The physicochemical properties of PBAE nanoparticles can be tuned by the monomer composition used in the synthesis of the constituent polymers, and structure-function relationships have been extensively investigated.\textsuperscript{45-47} For example, specific PBAE structures have significant specificity of transfecting cancer cells over their healthy counterparts as shown in Fig. 2-7B/C. PBAE nanoparticles show an order of magnitude higher transfection efficacy at transfecting human primary brain tumor initiating cells (BTICs) over primary fetal neural progenitor cells (fNPCs) and similarly at transfecting hepatoma cells over hepatocytes.\textsuperscript{41, 48} The authors also showed that the total uptake of nanoparticles as well as the division rate of cells were similar between BTICs and fNPCs from multiple primary samples, indicating that other factors are the major contributors of the biomaterial-mediated specificity. This research highlights that differential biomaterial-cell interactions can occur in cancer cells as compared to healthy cells and can enable gene delivery specific to cancer cells. Further research is needed in this area of drug delivery to elucidate the mechanisms responsible for this nanoparticle targeting.
Figure 2-7. Biomaterial-mediated tissue- and cell-specificity of nanoparticles. (A) Accumulation of PBAE nanoparticles coated with poly(glutamic acid) chain terminating with additional cationic amino acid residues in spleen and bone marrow. (B) Selective PBAE transfection of brain tumor initiating cells over fetal neural stem cells in vivo (Delivery of dsRed to BTIC labeled with GFP; Only Nanoparticles is a control group with nanoparticles but no tumor) and of (C) hepatoma cells over hepatocytes in separate cultures (left) and in co-culture (right). Reprinted with permission from Elsevier: Biomaterials. 2010, 31, 998-1006, American Chemical Society: ACS Nano. 2014, 8(5), 5141-53, and John Wiley and Sons: Journal of Biomedical Materials Research. 2013, 101(7), 1837-45. 41, 44, 48

Ligand Targeting
The most well studied method to target cancer cells with polymeric nanoparticles is through the use of ligands conjugated to the surface of nanoparticles. Cancer results in the dysregulation of a large number of genes including many cell surface receptors, whose upregulation can be exploited for targeting via specific ligands. Transferrin, folate, epidermal growth factor, RGD and other peptides, hyaluronic acid and specific carbohydrates have been utilized as targeting moieties for selective polymeric nanoparticle delivery to tumor cells. Additionally, antibodies and antibody fragments have been utilized to target cancer specific antigens such as the HER2 receptor and prostate specific antigen. Many of the targeting moieties used for polymeric gene delivery to treat cancer have been previously utilized for the delivery of chemotherapeutics and in targeting of liposomes for gene delivery. Among factors affecting whether conjugation of targeting ligands improves transfection efficiency are ligand density and ligand binding affinity as well as effect on zeta potential and nanoparticle stability. Ligand density can increase cooperative binding and avidity to improve cellular uptake.

**Transferrin (Tf)**

Transferrin (Tf), an 80 kDa glycoprotein, has been conjugated to nanoparticles containing poly(L-lysine) (PLL), PEI, cyclodextrin, and PAMAM to improve targeting to tumor cells overexpressing the Tf-receptor. Conjugation of transferrin to the surface of PEI and PEI-PEG nanoparticles for plasmid delivery has been shown to clearly improve tumor targeting and reduce off-target transfection as shown in Figure 2-8. Importantly, conjugation of Tf to the PEI-PEG nanoparticles was shown to improve tumor cell transfection at 48 hours, thus Tf was thought to improve the number of plasmids that reached each transfected cell. These Tf coated PEG-PEI nanoparticles were shown to transfect distant tumor cells in vivo 10-100 times greater compared to non-tumor cells following intravenous injection. Transferrin has continued to be used in many nanoparticle formulations, notably as a component of cyclodextrin-PEG-Tf nanoparticles (CALAA-01) having undergone FDA Phase I clinical trials for the delivery of short interfering RNA (siRNA) to solid tumors.
Figure 2-8. Conjugation of transferrin to PEI and PEI-PEG for xenograft tumor targeting was shown to clearly improve tumor transfection at 48 hours and reduce off-target transfection in mice following tail vein injection using the reporter gene luciferase and bioluminescent imaging. Reprinted by permission from Nature Publishing Group: *Gene Therapy*. 2003, 10, 758-764, copyright 2003.51

**Folic Acid (FA)**

Receptors for folate or folic acid (FA) are upregulated in rapidly dividing cell types, including tumor cells, and were proposed as an early target for drug delivery to cancer cells.72 For gene delivery, folate has been used to target bPEI-PEG nanoparticles for improved uptake and tranfection with a reporter gene by folate receptor expressing cells both *in vitro* and *in vivo*.57 Folate targeting for these nanoparticles was shown to improve transfection with minicircle DNA by approximately 3.6 fold *in vivo* although broad transfection was seen as folate receptors are expressed on the surface of most proliferating cells.57 In another study, folic acid was notably shown to be the most effective targeting ligand to cancer cells among 30 (primarily peptides) ligand tested for delivery by oligonucleotide-based nanoparticles.56 These oligonucleotide-based nanoparticles were
shown to be effective for ~60% knockdown of a reporter luciferase gene in a xenograft tumor model *in vivo* following IV administration.\(^58\)

**Epidermal growth factor (EGF)**

The epidermal growth factor receptor (EGFR) is a common molecular target for cancer therapy overexpressed in approximately 30% of solid tumors in addition to being a predictor of poor treatment outcome.\(^73\) EGF was conjugated to PEI-PEG as early as 2001 for improved transfection measured to be between 10-100 times greater than untargeted nanoparticle *in vitro*.\(^59\) Since then, synthetic peptides designed to target the EGFR, including GE11, have been conjugated to linear PEI-PEG nanoparticles for the delivery of plasmids coding for a sodium iodine symporter gene for treatment of liver cancer.\(^60\) These EGFR targeted polymeric nanoparticles were shown to improve tumor specific transfection compared to non-targeted nanoparticles and increased survival demonstrating the potential efficacy of targeting ligands.\(^60\)

**RGD peptide**

The RGD peptide (arginine, glycine, aspartic acid) and other oligopeptide variations have been shown to strongly target the \(\alpha_v\beta_3\) integrin receptor that is selectively expressed in tumor vasculature, with weaker binding to many other integrin receptors for improved cellular uptake.\(^70\) Conjugation of RGD peptide and cyclic-RGD peptide to nanoparticles for targeted delivery of plasmids to tumor cells has also been shown to improve transfection *in vivo* one day following IV administration in mice, although there were notably near equal increases in transfection of cells in the lungs and liver.\(^62\) The bPEI based nanoparticles for plasmid delivery Sakae *et al* designed utilized an anionic version of PEG having conjugated succinic acid residues, PEG-suc, and RGD peptide, both of which increased transfection efficiency.\(^62\) RGD peptide has also been electrostatically attached to PBAE nanoparticles for the delivery of DNA and shown to improve transfection of cells overexpressing integrin receptors but was not specifically applied to targeting tumor cells.\(^61\)

**Hyaluronic acid (HA)**

The CD44 cell receptor for hyaluronic acid is often overexpressed on tumor cells and is under active investigation for its putative role in cancer initiation/stem cells.\(^74\) As a targeting moiety, HA can greatly affect zeta potential of the nanoparticles created depending on the amount used due to its negative charge at physiological pH.\(^63\) Zwitterionic nanoparticles composed only of hyaluronic acid-bPEI in varying ratios have been explored for targeted transfection.\(^63\) While these nanoparticles were not applied for targeting tumor cells

54
specifically, they could be adapted for this purpose based on the use of HA in targeted delivery of chemotherapeutics to cancer cells.\textsuperscript{53, 64}

\textit{Antibodies}

Antibodies can be used as efficient and specific targeting moieties for tumors expressing a tumor specific antigen such as HER\textsubscript{2}\textsuperscript{65} or prostate specific antigen.\textsuperscript{66} The conjugation of HER2 antibodies to PEI for the delivery of DNA was shown to improve transfection of HER2+ breast cancer cell lines \textit{in vitro} 20-fold.\textsuperscript{65} Antibodies for prostate specific membrane antigen have also been used to target PEI/DNA nanoparticles to prostate cancer cells \textit{in vivo} with 20-fold improvement over non-targeted nanoparticles.\textsuperscript{66} Antibody fragments have also been used to target cancer cells; specifically, a fusion protein composed of HER2 single-chain fragmented antibodies and a cationic polypeptide for the delivery of siRNA to HER2+ breast cancer cells was shown to improve tumor targeting and reduce metastasis \textit{in vivo} following multiple weekly tail vein injection.\textsuperscript{67} Although antibodies can bind with high affinity, the use of monoclonal antibodies and antibody fragments as targeting ligands can be significantly more expensive than other cell receptor ligands and are not necessarily more effective. Antibody use for targeting of therapeutics has increased dramatically yet with high associated costs may not be the best strategy for targeted polymeric gene delivery.\textsuperscript{75}

\textit{Transcriptional-level Targeting}

For the case of DNA delivery, biological targeting of cancer cells can also be achieved following intracellular nanoparticle delivery. Although polymer, nanoparticle, and ligand properties are key to bind to target cells and lead to internalization by target cells, delivered DNA can be engineered to impart further cancer targeting on the intracellular level. To further constrain therapeutic transgene expression to just targeted cancer cells, and obviate undesirable effects associated with expression in healthy cells, transcriptional targeting can be utilized. Transcriptional targeting can be achieved by restricting the expression of transgenes to only tumors or particular tissues through the use of specific promoters.\textsuperscript{76}

\textit{Transcriptional Control of Gene Expression}

In eukaryotes, the transcription of protein-coding genes involves the interaction between RNA polymerase II, cis-regulatory DNA elements, transcription factors, co-factors and chromatin structure.\textsuperscript{77, 78} Cis-regulatory DNA elements refer to promoters and distal regulatory sequences that contain recognition sites for transcription factors (trans-acting elements).\textsuperscript{78} Promoters are located upstream from the transcriptional start
site of a gene and comprise a core promoter and proximal regulatory elements. The binding of RNA polymerase II to the core promoter, which initiates transcription, requires general (basal) transcription factors. Together, RNA polymerase II and general transcription factors form the preinitiation complex (PIC), which provides only basal levels of transcription. This basal machinery activity can be increased by the binding of other set of proteins, site-specific transcription factors, to the proximal and distal regulatory elements. In contrast to the ubiquitous expression of general transcription factors, site-specific transcription factors are expressed in a spatiotemporal and/or condition-specific manner. This tight regulation is critical in determining where and to what extent a given gene is expressed. Accordingly, a gene of interest will be expressed only in cells containing sufficient levels of the transcription factors involved in activating transcription from the promoter located upstream from this gene.

The regulation provided by the coordinated interaction between transcription factors is the primary mean by which cells orchestrate gene expression in response to various stimuli. Thus, the cis-regulatory elements that recruit these factors can be used and tailored to drive transcription of an exogenously delivered therapeutic gene in a tissue- or tumor-specific manner.

It is important to highlight that the transcriptional targeting strategy for gene therapy relies on the accurate delivery of DNA to the cells of interest, i.e., the gene under control of a specific promoter must reach the cells containing the right transcriptional factors; otherwise no transcription occurs. Thus, the efficiency of the control at the transcriptional level is highly dependent on other levels of targeting, i.e. delivery to specific cells. Moreover, cell surface binding, endosomal escape, translocation to the nucleus, and nucleic acid release also represent barriers that have to be overcome in order to a gene reaches the transcription machinery.

**Promoters for transcriptional targeting**

**Tissue-specific promoters**

The application of tissue-specific promoters in transcriptional targeting exploits genes that are specifically expressed in a tissue of interest. If a particular promoter is active in certain healthy tissues in addition to tumor cells, then the ability for cancer targeting is reduced. One of the first tissue-specific promoters evaluated for transcriptional targeting in cancer gene therapy was the promoter for the tyrosinase gene, which is preferentially active in melanocytes. In 1993, Vile et al. showed that this promoter can drive expression of a reporter gene to both human and murine melanoma cells and melanocytes, but not to a range of other cell types. Since then, the tyrosinase promoter has been extensively applied in the research of gene therapy for melanoma. Similarly, in the past two decades, candidates of prostate specificity, such as prostate-specific antigen (PSA), prostate-specific membrane antigen (PSMA), and probasin, have been widely explored.
Tissue-specific promoters have also been applied to drive siRNA expression in target cells. Song et al. demonstrated that the siRNA expression driven by the prostate-specific antigen (PSA) promoter can lead to gene silencing specifically in the androgen-responsive prostate cancer cell line, in an androgen-dependent and tissue-specific manner.96

**Tumor-specific promoters**

Tumor-specific promoters are involved in the transcription of genes that are overexpressed in tumor cells but have limited or no activity in normal cells.86 Cancer-specific promoters can be specific to a particular cancer type85 or general to many types of cancer without a particular tissue specificity.88, 97 The progression-elevated gene-3 (PEG-3)98, for example, was identified as a mutant gene overexpressed during tumorigenesis of different cancer types that presents tumor progression promoting properties. The PEG-3 promoter was shown to drive cancer-specific gene expression and cell killing, while sparing normal cells.98, 99 Applying in vivo jetPEI (Polyplus transfection) as a vector for intravenous administration, Hyo-eun et al. successfully demonstrated PEG-3 promoter-driven firefly luciferase expression (PEG-Luc) specifically to small metastatic deposits of human melanoma and breast cancer in mouse models (Figure 2-9).100 Other relevant cancer-specific promoters include the human telomerase reverse transcriptase (hTERT)101, survivin102, 103, and astrocyte elevated gene-1.
Figure 2-9. Transcriptionally mediated targeting of gene expression. (A) Bioluminescence imaging showing cancer-specific expression of firefly luciferase gene in a representative of the metastatic melanoma group (Mel-3) and no expression in a healthy control mouse (Ctrl-2). (B) Computed tomography imaging and gross anatomical views of the lung from a healthy control mouse (Ctrl-2) and one representative of the metastatic melanoma group (Mel-2). Black arrows are pointing toward the metastatic nodules in the lung. Scale bars: 5 mm. (C) Quantification of bioluminescence imaging signal intensity in the control group (Ctrl) and metastatic melanoma group at 24 and 48 h following i.v. administration of the pPEG-Luc–PEI polyplex. Quantified values are shown in total flux. ***P < 0.0001. Reprinted with permission from Nature Publishing Group: Nat Med, 2011, 17(1), 123-9.
Tumor-selective promoters can take advantage of fetal genes that become re-expressed in certain malignancies\textsuperscript{88, 97}, such as the promoters of the oncofetal \(\alpha\)-fetoprotein (AFP) and carcinoembryonal antigen (CEA) genes, reactivated in hepatocellular carcinomas (HCC) and adenocarcinomas, respectively.\textsuperscript{86} Hu \textit{et al.} used a construct with the fragment EA4D of the AFP promoter and a novel polymeric delivery system to induce cell killing mediated by tBid overexpression only in AFP-producing HCC.\textsuperscript{108} Other tumor-selective promoters are also able to induce gene expression only in particular cancer types. Huang \textit{et al.} tested PBAE-mediated delivery of a construct containing the diphtheria toxin suicide protein (DT-A) gene under control of two promoters of genes that are highly active in ovarian tumors, MSLN and HE4.\textsuperscript{109} The authors observed suppression of ovarian tumor growth following intraperitoneal (i.p.) injection of particles twice a week, while minimal nonspecific toxicity was observed in other tissues. Other groups have also explored promoters of genes whose activity is related to additional cancer hallmarks\textsuperscript{85, 86, 88, 97}, such as the hypoxic tumor environment\textsuperscript{110}, the proliferating endothelium of tumor blood vessels\textsuperscript{111}, the altered signaling pathways\textsuperscript{112}, and the cell-cycle abnormalities and hyperproliferation.\textsuperscript{113}

\textbf{Inducible promoters}

In contrast to tissue/tumor-specific promoters, which usually induce constitutive transgene expression in a target tissue\textsuperscript{86}, inducible promoters can be applied to achieve temporal, spatial and dose-dependent regulation.\textsuperscript{114, 115} These promoters can be regulated by agents such as drugs, radiation and heat.\textsuperscript{97} An example of such a promoter is the early growth response gene-1 (Egr-1) promoter, which is radiation-induced and has been used to optimize the control of gene expression in different tissues.\textsuperscript{116-120} Inducible promoters essentially drive ubiquitous transgene expression\textsuperscript{121}, however, tissue/tumor-specific and inducible promoters can be combined to enable the control of transgene expression in a tissue/tumor specific and inducible manner.\textsuperscript{121, 122} Recently, Xiong \textit{et al.}\textsuperscript{118} successfully combined the hTERT promoter to CArG elements, which is known to be the radiation-responsive motifs within the \textit{Egr1} promoter\textsuperscript{123-125}, to drive radiation-inducible and cancer-specific gene expression. The synthetic promoter containing 6 repeating CArG units (C6) showed the best radiation inducibility.
**Design of stronger promoters**

As shown in some of the examples described above, a composite promoter can be engineered by incorporating different functional elements from natural promoters. These strategies aim to optimize the natural promoter systems and to overcome the generally weak transcriptional activity of eukaryotic promoters, while maintaining specificity.

**Post-transcriptional targeting**

Controlled expression of factors encoded by exogenously delivered DNA can also be targeted to cancer cells by post-transcriptional targeting. The expression of transgenes can be regulated post-transcriptionally by controlling RNA splicing, RNA stability, and translation initiation.

**RNA splicing**

RNA splicing consists of the removal of introns and coupling of exons from the pre-mRNA transcripts to form mRNA before its transport to the cytoplasm. The variation of the splicing sites within a transcript, called alternative RNA splicing, generates multiple mRNA isoforms in different cell types, tissues, or stages of development. Changes in alternative RNA splicing are commonly observed in malignancies. The alternative RNA splicing events involved on the expression of the CD44 gene, for example, generate mRNA isoforms called CD44R1, which exists on activated and/or malignant cell types. Only CD44R1-positive cells contain the necessary machinery to accurately splice a particular intron within the CD44 pre-mRNA. Hayes et al. observed that constructs in which the expression of a transgene is dependent upon removal of this intron from the sequence can be used to specifically target CD44R1-positive cells.

**RNA stability**

The stability of mRNA molecules and their decay rates are determined by regulatory elements within the mRNA sequence and proteins or small non-coding RNAs that bind these elements. The post-transcriptional regulation mechanism by which small non-coding RNAs interact with specific mRNAs and induce selective gene silencing is called RNA interference (RNAi). siRNAs perform RNAi by degrading mRNAs containing fully complementary sequences and by translation repression. siRNA-mediated silencing has been applied to target genes associated with various cancer-relevant pathways, such as apoptosis.
(e.g. Bax\textsuperscript{135} and Bcl-2\textsuperscript{136}) and cell signaling (e.g. K-Ras\textsuperscript{137}), and to knockdown viral oncogenes (e.g. HPV E6\textsuperscript{138}). Recently, Urban-Klein \textit{et al.} demonstrated significant growth inhibition following siRNA-mediated silencing of the HER-2 proto-oncogene\textsuperscript{139}, which is overexpressed in a variety of human cancers and related with unfavorable prognosis.\textsuperscript{140} Micro RNA (miRNA) delivery for tumor targeting can cause inhibition of oncogenic nucleic acids.\textsuperscript{141} miR-34 and let-7 are tumor-suppressor miRNAs\textsuperscript{141} with reduced expression levels in many types of cancer cells.\textsuperscript{142, 143} Replacement therapy with miR-34 has been shown to successfully induce apoptosis in cancer cell lines\textsuperscript{144} while let-7 replacement can inhibit tumor growth.\textsuperscript{145} On the other hand, the knockdown of miR-155, overexpressed in many solid malignancies, has demonstrated to inhibit tumor growth, migration and invasion.\textsuperscript{146} Short hairpin RNAs (shRNA), which are transcribed in the nucleus, can be effective to target cancer cells when under control of efficient promoters.\textsuperscript{147} Gao \textit{et al.} demonstrated silencing of an oncogene serine/threonine kinase specifically in hepatoma cells by shRNA under control of AFP promoter/hypoxia-inducible enhancer.\textsuperscript{148}

\textit{RNA translation}

The translation initiation process consists of the recruitment and assembling of the initiator tRNA and the 40S and 60S ribosomal subunits at the start codon of the mRNA molecule.\textsuperscript{149} Excessive secondary structures in the 5′ untranslated region (UTR) of the mRNA are normally inefficiently translated.\textsuperscript{149-153} However, overexpression of the eIF4E factor, a rate-limiting component of translation initiation, results in a specific increase in the translation of highly structured 5′ UTR mRNAs.\textsuperscript{149, 154, 155} eIF4E is commonly overexpressed in solid tumors\textsuperscript{155} and many of the mRNAs with excessive secondary structures encode products that stimulate cell growth and angiogenesis\textsuperscript{155} (e.g.: fibroblast growth factor-2; FGF-2\textsuperscript{155, 156}). DeFatta \textit{et al.} found that the 5′ UTR of FGF-2 mRNA can be placed in front of the herpes simplex virus type 1 thymidine kinase (HTK) gene to restrict the translation of HTK mRNA and, therefore, the susceptibility of the prodrug ganciclovir, to cancer cells.\textsuperscript{155}

\textbf{Other targeting strategies that can be utilized in polymeric nanoparticle-mediated cancer gene therapy}

The choice of the gene product to be expressed itself can introduce another level of tumor specificity for cancer gene therapy. Polymeric gene therapy can be used to restore function of tumor suppressor genes that have been inactivated during tumorigenesis, such as p53, by reintroducing the wild type gene into tumor cells.\textsuperscript{157, 158} The delivery of constructs encoding the TNF-related apoptosis-inducing ligand (TRAIL) gene represents another strategy of cancer targeting. TRAIL has the unique capacity of inducing apoptosis specific
This cancer specificity involves enhanced activity of pro-apoptotic receptors (DR4 and DR5) in tumor cells and apoptotic priming of cancer cells by oncogenes.

An alternative method of active tumor targeting can be achieved by the capacity of mesenchymal stem cells (MSCs), such as the human adipose-derived MSCs (hAMSCs), to migrate towards tumors. MSCs can be used as a cell-based therapeutic delivery vehicle following gene delivery with DNA-encoded anti-cancer molecules. For example, MSCs can be engineered with genes that encode differentiation agents such as bone morphogenetic protein-4 (BMP-4), which promotes the terminal differentiation of putative cancer stem cells (CSCs) and facilitates tumor eradication. This MSC plus gene therapy approach has proven to be useful in treating glioblastomas to induce brain tumor-initiating cell (BTIC) differentiation and prolong survival in mice.

**Clinical Trials**

The translation of polymeric gene delivery nanoparticles for cancer treatment has proved challenging and few polymeric nanoparticle formulations have yet made it to clinical trials. Table 1-1 lists selected polymeric nanoparticles used in clinical trials. Phase I/II clinical trials listed in Table 1-1 were designed to evaluate safety, dose-response pharmacodynamics, dose-dependent accumulation of nanoparticles in tumor tissue, and show a signal of efficacy. The cyclodextrin-PEG-Tf nanoparticle formulation, termed CALAA-01, for the delivery of anti-RRM2 siRNA was notable for the first evidence of RNA interference in humans following systemic delivery in a Phase I trial. Multiple clinical trials have been undertaken using the PEI delivery system for diphtheria-toxin A termed BC-819 and developed by BioCancell Ltd. Other PEI based nanoparticle formulations have utilized glycosylated JetPEI and PEG-PEI-cholesterol. The genes chosen for delivery included DCK::UMK to increase chemosensitivity of tumor cells to gemcitabine, SSTR2 to inhibit tumor cell proliferation as well as IL-12 to induce anticaner activity from the patient’s immune system. PEI has also been evaluated in a clinical trial for the dual delivery of siRNA and plasmid DNA for treating a number of hematopoietic cancers. These phase I/II trials are notable for demonstrating RNAi and delivery of plasmid DNA using polymeric vectors, as well as preliminary clinical evidence of gene therapy efficacy in preventing tumor growth in patients.

<table>
<thead>
<tr>
<th>NP Formulation</th>
<th>Cargo Target</th>
<th>Status</th>
<th>Phase</th>
<th>Clinicaltrials.gov Identifier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cyclodextrin adamantane-PEG-Tf (CALAA-01)</td>
<td>siRNA anti-MMR2</td>
<td>Solid tumors</td>
<td>Terminated</td>
<td>I</td>
</tr>
<tr>
<td>PEI DNA Diphtheria-toxin A</td>
<td>DNA Ovarian cancer</td>
<td>Completed</td>
<td>I/II</td>
<td>NCT0071997168</td>
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<tr>
<td>PEI DNA Diphtheria-toxin A</td>
<td>DNA Pancreatic cancer</td>
<td>Completed</td>
<td>I/II</td>
<td>NCT00826150</td>
</tr>
<tr>
<td>PEI DNA Diphtheria-toxin A</td>
<td>DNA Transitional cell carcinoma of bladder</td>
<td>Recruiting</td>
<td>I</td>
<td>NCT01878188</td>
</tr>
<tr>
<td>PEI DNA Diphtheria-toxin A</td>
<td>DNA Bladder cancer</td>
<td>Active</td>
<td>II</td>
<td>NCT00595088</td>
</tr>
<tr>
<td>JetPEI DNA Diphtheria-toxin A</td>
<td>DNA SSTR2 DCK::UMK Metastatic pancreatic ductal adenocarcinoma</td>
<td>Completed</td>
<td>I</td>
<td>NCT01274455169</td>
</tr>
<tr>
<td>PEG-PEI Cholesterol DNA IL-12</td>
<td>DNA Persistent or recurrent ovarian epithelial cancer</td>
<td>Completed</td>
<td>I</td>
<td>NCT01489371170</td>
</tr>
<tr>
<td>PEG-PEI Cholesterol DNA IL-12</td>
<td>DNA fallopian tube cancer, primary peritoneal cancer</td>
<td>Active</td>
<td>II</td>
<td>NCT01118052</td>
</tr>
<tr>
<td>PEG-PEI Cholesterol DNA IL-12</td>
<td>DNA Colorectal cancer</td>
<td>Recruiting</td>
<td>I/II</td>
<td>NCT01300858</td>
</tr>
<tr>
<td>PEI DNA, siRNA eIF5A&lt;sup&gt;S50R&lt;/sup&gt;-anti-eIF5A</td>
<td>DNA Multiple myeloma, mantle cell lymphoma, B cell lymphoma, plasma cell leukemia</td>
<td>Active</td>
<td>I/II</td>
<td>NCT01435720171</td>
</tr>
</tbody>
</table>
Conclusion and Future Prospects

Targeted polymeric gene delivery for cancer therapy has made advances through rational engineering of biomaterials, utilization of targeting ligands, and engineering of nucleic acids for transcriptional and translational regulation. In some ways, targeted gene therapy has followed similar improvements to delivery as nanoparticle-based chemotherapeutic formulations. Cancer is a disease well-suited for nanoparticle-based gene delivery strategies due to the multiple levels of targeting that can specifically treat cancer cells over healthy cells. These include opportunities for passive targeting via the EPR effect and active ligand targeting due to the changes in expression patterns of many cancer cell receptors. Polymeric nanoparticles can be readily engineered with active targeting moieties on their surfaces and produced on the scales required for pharmaceutical applications. Selection of biomaterials through broad polymer library screens and rational polymer engineering design have both been utilized to improve tissue and tumor cell specificity as well improve transfection efficacy. Furthermore, polymer structures can be readily modified with PEG and other biomolecules for charge shielding and to improve tumor cell specificity and transfection in vivo. Compared to nanoparticle chemotherapeutic formulations, gene therapy possesses the additional level of targeting afforded in transcriptional and translational control for even greater reduction in off-target effects and potential reduction of side effects during treatment. Systemic delivery and tissue targeting improvements through charge shielding and targeting moieties will continue improve efficacy for intravenous administration. Improvements to nanoparticle stability in blood serum and reduction of alternative complement pathway activation for increased circulation time are also important aspects for improved systemic polymeric gene delivery. Although PEG is often used as a moiety to improve the stealth nature of nanoparticles, depending on how it is utilized, it may not be devoid of complement activation and can lead to hypersensitivity reactions. Targeting at the cellular level can be improved by identification of receptors and ligands that lead to improved uptake, delivery to the cytoplasm, and intracellular trafficking leading to efficient transfection. Increased understanding of endocytosis and resulting endosome trafficking pathways will lead to more rational design of targeting moieties for improved intracellular delivery. Additionally, determination of differences in transfection between tumor cells and healthy cells following equal levels of nanoparticle uptake will allow improved design of nanoparticles.

Tissue- and tumor-specific promoters for transcriptional targeting and RNAi for gene knockdown have become key elements of non-viral gene therapy for cancer. Ongoing research will improve understanding of cancer cell-selective gene activity and enable the recognition of more potent regulatory components to drive cancer cell-specific gene expression and silencing. For the most effective cancer cell targeting, these orthogonal systems for cancer specificity of polymeric gene delivery nanoparticles can be combined together.
Polymeric nanoparticle based cancer gene therapy is still in its infancy at the clinical level but has great potential for the future. Compared to gene therapies utilizing viral vectors, polymeric nanoparticle based approaches to gene therapy have lagged in transfection efficacy but have improved safety, lower risks of immunogenicity and tumorigenesis, improved manufacturing and quality control, improved targeting capabilities, and much greater nucleic acid carrying capacity. With advances in transfection efficacy and tumor specificity through multiple targeting strategies, polymeric nanoparticle-based gene therapy has a bright future.

Acknowledgement

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Declaration of Interest

The authors report no declarations of interest.
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149. Meric F, Hunt KK. Translation Initiation in Cancer: A Novel Target for Therapy 1 FM is supported by The University of Texas MD Anderson Cancer Center Physician-Scientist Program and by NIH Grant 1KO8-CA 91895-01. KKH is supported by Department of Defense Award DAMD-17-97-1-7162. 1. Molecular Cancer Therapeutics. 2002;1(11):971-9.


<table>
<thead>
<tr>
<th>Polymeric Vector</th>
<th>Nucleic Acid</th>
<th>in vivo Animal Model</th>
<th>Method of Targeting</th>
<th>Results / Efficacy</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Biophysical (EPR)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PEI</td>
<td>DNA</td>
<td>A/J mice with s.c. Neuro 2A tumor</td>
<td>PEGylation</td>
<td>Reduced plasma protein binding, prolonged circulation, tumor accumulation</td>
<td>1999&lt;sup&gt;12&lt;/sup&gt;</td>
</tr>
<tr>
<td>PEI</td>
<td>DNA (GM-CSF)</td>
<td>ddY mice with s.c. B16 tumor</td>
<td>Hyaluronic Acid Coating</td>
<td>Prevented aggregation, more concentrated particles via lyophilization/rehydration, 50% tumor suppression</td>
<td>2010&lt;sup&gt;25&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>Biomaterial-mediated</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PEI</td>
<td>DNA (IL-2)</td>
<td>ICR mice</td>
<td>Molecular weight and N/P ratio of PEI</td>
<td>Longest half-life with bPEI 25kDa (10:1), different tissue accumulations depending on MW and N/P ratio</td>
<td>2007&lt;sup&gt;43&lt;/sup&gt;</td>
</tr>
<tr>
<td>PBAE</td>
<td>DNA</td>
<td>Athymic mice with JHGBM-276 cells</td>
<td>PBAE Structure</td>
<td>PBAE structure 447 transfected in cell-specific manner: brain tumor initiating cells over fetal neural stem cells</td>
<td>2014&lt;sup&gt;41&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>Ligand-mediated</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PEI-PEG-Tf</td>
<td>DNA (Luc)</td>
<td>A/J mice with human hepatoma</td>
<td>Transferrin</td>
<td>Transferrin ligand conjugation improved tumor targeting following and transfection following IV injection</td>
<td>2003&lt;sup&gt;51, 71&lt;/sup&gt;</td>
</tr>
<tr>
<td>Cyclodextrin-PEG-Tf (CALAA-01)</td>
<td>siRNA (anti-RRM2)</td>
<td>CD-1 nude mice with human hepatocellular carcinoma xenograft.</td>
<td>GE11 (EGF analog)</td>
<td>First evidence of RNAi knockdown in humans for cancer treatment</td>
<td>2009&lt;sup&gt;55, 56&lt;/sup&gt;</td>
</tr>
<tr>
<td>LPEI-PEG-GE11</td>
<td>DNA (Sodium iodine symporter)</td>
<td>BALB/c nude mice with Luc-KB carcinoma</td>
<td>Folic Acid</td>
<td>EGFR-specific peptide GE11 targeted delivery of NIS with systemic iodine administration significantly improved survival</td>
<td>2011&lt;sup&gt;60&lt;/sup&gt;</td>
</tr>
<tr>
<td>Oligonucleotide</td>
<td>siRNA (anti-Luc)</td>
<td>ddY mice with subcutaneous B16 tumors</td>
<td>RGD Peptide</td>
<td>FA conjugated oligonucleotide origami nanoparticles for reporter gene knockdown</td>
<td>2012&lt;sup&gt;58&lt;/sup&gt;</td>
</tr>
<tr>
<td>PEI/Suc-PEG-RGD</td>
<td>DNA (Luc, GFP)</td>
<td>Athymic BALB/c-nu mice bearing HER2+ xenograft</td>
<td>HER2 antibody fragment</td>
<td>RGD ligand improved delivery to mouse melanoma tumor with following IV administration</td>
<td>2008&lt;sup&gt;62&lt;/sup&gt;</td>
</tr>
<tr>
<td>Cationic peptide – Ab fragment</td>
<td>siRNA (anti-PLK-1)</td>
<td>Athymic BALB/c-nu mice bearing HER2+ xenograft</td>
<td>HER2 antibody fragment</td>
<td>Reduction of tumor metastasis for HER2+ breast cancer xenograft following weekly IV administration</td>
<td>2012&lt;sup&gt;67&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>Transcription-level</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>in vivo-jetPEI*</td>
<td>DNA (PEG-Luc)</td>
<td>Human melanoma or breast cancer metastasis</td>
<td>Cancer-specific promoter</td>
<td>PEG-3 promoter drive expression of Luc reporter gene specifically to micrometastatic deposits after i.v. delivery</td>
<td>2011&lt;sup&gt;100&lt;/sup&gt;</td>
</tr>
</tbody>
</table>
Table 2-2. Examples of targeted polymeric nanoparticles with different targeting mechanism

| Nanopolymer folic acid-grafted PEI600-CyD (H1) | DNA (EA4D-tBid) | DNA (MSLN or HE4/DT-A) | model in NCR nu/nu mice BALB/C athymic mice with s.c. AFP-producing or non-producing HCC | Tumor-selective promoter | Tumor-selective promoter | Cell killing and tumor growth inhibition only in AFP-producing HCCs following i.t. injection | Suppressed tumor growth in ovarian carcinoma while little nonspecific toxicity occurs in other tissues after i.p. injection | 2014 108

| PBAE | DNA | DNA (MSLN or HE4/DT-A) | Cell killing and tumor growth inhibition only in AFP-producing HCCs following i.t. injection | Tumor-selective promoter | Tumor-selective promoter | Suppressed tumor growth in ovarian carcinoma while little nonspecific toxicity occurs in other tissues after i.p. injection | 2009 109

| Post-transcription
| PEI (anti-HER-2) | siRNA | Athymic nude (nu/nu) mice with s.c. ovarian carcinoma | Regulation at the RNA stability level | Significant growth inhibition following intraperitoneal injection of PEI-complexed anti-HER-2 siRNA | 2005 139
**Chapter 3: A Triple-Fluorophore Labeled Nucleic Acid pH Nanosensor to Investigate Non-Viral Gene Delivery**

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**Foreword:** The following article was my first 1st-author accepted research article in graduate school and while it didn’t pave the way for any incredible discoveries I remain proud of this work as a demonstration of persistence in finding a way to make things work. I am glad to have published in Molecular Therapy as well, as it is the society journal of the American Society for Gene and Cell Therapy (ASGCT). This work was also presented at the ASGCT National Conference as an oral presentation.


**Abstract:** There is a need for new tools to better quantify intracellular delivery barriers in high-throughput and high-content ways. Here, we synthesized a triple-fluorophore labeled nucleic acid pH nanosensor for measuring intracellular pH of exogenous DNA at specific time-points in a high-throughput manner by flow cytometry following non-viral transfection. By including two pH-sensitive fluorophores and one pH-insensitive fluorophore in the nanosensor, detection of pH was possible over the full physiological range. We further assessed possible correlation between intracellular pH of delivered DNA, cellular uptake of DNA, and DNA reporter gene expression at 24-hours post-transfection for poly-L-lysine and branched polyethyleneimine polyplex nanoparticles. While successful transfection was shown to clearly depend on median cellular pH of delivered DNA at the cell population level, surprisingly, on an individual cell basis, there was no significant correlation between intracellular pH and transfection efficacy. To our knowledge, this is the first reported instance of high throughput single-cell analysis between cellular uptake of DNA, intracellular pH of delivered DNA, and gene expression of the delivered DNA. Using the nanosensor, we
demonstrate that the ability of polymeric nanoparticles to avoid an acidic environment is necessary, but not sufficient for successful transfection.

**Keywords:** polymeric nanoparticles, non-viral gene delivery, pH sensor, proton sponge

**Introduction:**

Non-viral gene delivery has great promise in clinical applications such as for cancer therapy where transient expression can be sufficient to result in clinical efficacy.\(^1\) Compared to DNA based viral gene therapy, non-viral delivery methods are more amenable to repeat administration due to reduced risk of immunogenicity and insertional mutagenesis, but remain less efficient than viral delivery methods.\(^1,2\) Despite extensive progress in the previous decade, many challenges in engineering successful non-viral gene delivery platforms remain.\(^1,3\) Polymeric gene delivery in particular has made extensive progress towards increased performance through rational engineering of polymer structures as well as screening of broad libraries of polymer structures, but new quantitative bioassays are required to fully understand the mechanisms by which existing nanoparticles achieve transfection.\(^4,11\)

Barriers to successful polymeric gene delivery at the level of individual cells include cellular internalization, endosomal escape, nucleic acid unpacking, and nuclear transport.\(^12,13\) Of these barriers, overcoming endosomal escape has been specifically identified as a critical rate-limiting step in polycation nanoparticle mediated transfection as generally only a minor fraction of endocytosed polypelexes manage to escape to the cytoplasm.\(^14\) Non-endolysosomal trafficking that does not require endosomal escape, but enables nuclear entry through the endoplasmic reticulum, has been shown to occur for certain nanoparticles, such as specific histone targeted nanoparticles, but has not been demonstrated for the majority of nanoparticle formulations.\(^15,16\) The presence of intracellularly delivered nucleic acid at an acidic pH indicates that the nucleic acid is not in the cytoplasm and is not in the environment required for successful gene expression. Failure to escape the endosome to the cytoplasm can result in nucleic acid degradation when the early endosome transitions to a late endosome/lysosome, typically within an hour following uptake.\(^17\) The shift from early endocytic vesicle to late endosome and eventually lysosome results from fusion of the early endosome with other vesicles. The latter contain hydrogen pump V-ATPases and digestive enzymes which result in acidification and degradation of the nucleic acid contents of polymeric nanoparticles.\(^17,18\)

To escape the endosome and avoid lysosomal degradation, polymeric nanoparticles have been designed specifically either with moieties that facilitate membrane pore formation or amine groups designed to enable them to buffer vesicle acidification and consequently escape the endosome via the hypothesized proton sponge mechanism.\(^18\) Beginning with branched polyethyleneimine (bPEI), many polymeric nanoparticles have
been engineered to take advantage of endosome acidification as a means to protect their nucleic acid cargo and enable endosomal escape. Despite extensive study in the two decades since the first use of bPEI, the mechanism of endosomal escape for cationic nanoparticles is still not universally agreed upon. The ability of the cationic polymers containing tertiary amines to effectively buffer in the physiological pH range between 5-7 has been demonstrated in multiple settings, yet improved buffering capacity at low, physiologically relevant pH has been shown to not always result in more effective transfection. As a means of studying this mechanism universally among cell types and polymer systems, we sought to create an intracellular pH sensor for probing the mean compartmental pH environment of exogenously delivered DNA in individual cells and to design this sensor to be readable in a high throughput manner by flow cytometry.

Approaches to single cell and single compartment pH measurements have previously utilized fluorescence. A ratiometric, fluorophore-based assay utilizing a pH sensitive fluorophore and a pH insensitive reference fluorophore was reported by Murphy et al. using FITC and RITC to measure endosomal pH over time following insulin internalization. Similar assays have been utilized to measure endosomal pH by fluorophore labeling polymeric gene delivery vectors or plasmid DNA. More sensitive ratiometric pH probes have recently been developed for non-gene delivery applications using fluorescent polymers and have shown the importance of using multiple pH-sensitive fluorophores to effectively probe the lysosomal pH range.

Specifically for gene delivery applications, we aimed to investigate the local pH of the exogenously delivered DNA, not the local pH of delivery polymers that may dissociate from the DNA. Here we report a triple fluorophore-labeled plasmid DNA based pH nanosensor with improved sensitivity at lysosomal pH used for probing cellular pH of exogenously delivered DNA to individual cells and cell populations following polymeric gene delivery. This ratiometric pH nanosensor enabled an investigation of endosomal buffering following polymeric gene delivery in a high throughput manner by flow cytometry. Average pH of the nucleic acid pH nanosensor was monitored at specific time points within individual cells to quantify trends between cellular uptake of DNA, local pH environment of delivered DNA, and successful expression of the DNA in both easy-to-transfect and hard-to-transfect cell lines using bPEI and poly-L-lysine (PLL).

Methods:

Materials. Branched polyethylenimine 25 kDa (bPEI) (408727), Poly-L-Lysine sodium chloride 15-30 kDa (PLL) (P2658), buffer salts and organic solvents were purchased from Sigma Aldrich (St. Louis, MO). Succinimidyl-[4-(psoralen-8-yloxy)]-butyrate (NHS-psoralen) (23013), Oregon green cadaverine (O10465) and fluorescein cadaverine (A10466) were purchased from Thermofisher (Halethorpe, MD). Cy5-amine (230C0) was purchased from Lumiprobe (Hallandale Beach, FL). All fluorophores were dissolved in DMSO at a concentration of 10 µg/µL and stored at -20°C in small aliquots. Plasmid pDsRed-Max-N1 (Addgene 21718,
Cambridge, MA) was used for construction of the pH nanosensor, while pEGFP-N1 (Addgene 2491) was used for initial transfection efficacy screens.

**Nucleic Acid pH Nanosensor Synthesis.** DsRed plasmid DNA at a concentration of 1 µg/µL was mixed in a mass ratio of 16:1 with NHS-psoralen in DMSO at a concentration 1 µg/µL. The solution was distributed to a 96-well round bottom plate with 50 µg DNA/well and placed on ice. Psoralen was then crosslinked into the DNA by 25 minutes of UV exposure using a 0.16 amp 365nm lamp placed directly over the plate. For each well, 8 µL of 10x PBS, 17 µL DMSO, 25 µg fluorescein cadaverine, 25 µg Oregon green cadaverine and 2.5 µg Cy5-amine were added, well mixed and then incubated at room temperature for 1h while protected from light to prevent photobleaching. It was necessary to fine tune DMSO volume percent of the solution to 30% to facilitate reaction of the more hydrophobic fluorophores. Labeled DNA was then ethanol precipitated two times to remove excess reactants and purify the DNA. The purified pH nanosensor was then resuspended in ultrapure water at 75% of the original volume. The concentration and labeling efficacy were determined using a Nanodrop spectrophotometer (Thermofisher Scientific) and the volume was increased to make the pH nanosensor concentration 1 µg/µL. DNA was then divided into aliquots and stored at -20°C protected from light. Additional DNA was labeled using only fluorescein or Oregon green and Cy5 for comparison of the triple-labeled pH nanosensor to more commonly utilized dual labeling techniques with only one pH sensitive fluorophore. Fluorescence over the pH range from 3.0 to 9 for the different versions of the pH nanosensor was assessed using a Synergy 2 multiplate reader (Biotek, Winooski, VT) with four replicates.

**Cell Culture.** Human glioblastoma astrocytes (GB319) were grown as adherent cells as previously described on tissue culture flasks in DMEM/F12 (11330057, Thermofisher) with 10% heat inactivated fetal bovine serum (16140071, Thermofisher) and 1% antimycotic/antibiotic (15240062, Thermofisher). HEK293T were purchased from ATCC (Manassas, VA) and cultured in DMEM (11965092, Thermofisher) with 10% fetal bovine serum and 1% penicillin/streptomycin.

**pH Standard Curves.** The pH nanosensor was introduced to cells for creation of the standard curve relating fluorescence ratio to pH by electroporation using the Neon Transfection System (Thermofisher). In detail, cells were passaged to individual tubes with 200,000 cells/tube then fully aspirated and kept on ice for 20 minutes. The cells were then resuspended in 10 µL of the Neon System supplied R-buffer containing 1 µg of 100% labeled DNA and electroporated with 1 pulse at 1300V for 20ms. For the flow cytometry based pH curve the electroporated cells were transferred to a solution of PBS with 2% FBS, centrifuged and aspirated to remove R-buffer. Cells were then resuspended in PBS containing 4% formalin for 15 minutes for simultaneous fixation and permeabilization, centrifuged and aspirated to remove formalin, then resuspended
in known pH point buffers prior to flow cytometry. Known pH point 150 mM buffer solutions were made for pH values between 4.0-9.0 with a final concentration of 120 mM NaCl and 30 mM citrate phosphate buffer with 2% v/v FBS. Precise pH was measured using a SevenEasy pH Meter (Mettler Toledo, Columbus, OH) following addition of FBS. A BD Accuri C6 (BD Biosciences, San Jose, CA) flow cytometer with two lasers (488 and 633 nm) with four channels corresponding to green, yellow, red, and far-red fluorescence (FL1 at 530±15 nm, FL2 at 565±10 nm, FL3 at 610±10 nm, and FL4 at 675±12.5 nm respectively) was used for all flow cytometry experiments in combination with a HyperCyt autosampler (Intellicyt, Albuquerque, NM). For plate reader experiments, pH nanosensor was diluted to 0.01 µg/µL in known pH buffer solution in 96 well plates with four replicates. Fluorescence measurements were taken with a Synergy 2 plate reader (Biotek, Winooski, VT).

Transfection. For transfection efficacy experiments, cells were plated in CytoOne 96-well tissue culture plates (USA Scientific, Ocala, FL) plates 24h prior to transfection with 15,000 cells/well in 100 µL media. Nanoparticles were formed by mixing eGFP-N1 DNA and polymer solutions at 1:1 v/v ratio followed by 10 minutes of incubation to allow for particle formation. PLL and bPEI from stock aqueous concentrations of 10 µg/µL were diluted to their necessary concentrations in 10 mM HEPES or 150 mM NaCl respectively before being mixed 1:1 with DNA diluted to 0.06 µg/µL in the equivalent buffer. For notation, w/w 1 or w/w 2 for bPEI and PLL denotes a 1:1 or 2:1 weight-weight ratio with plasmid DNA respectively. Lipofectamine® 2000 was used according to manufacturer instructions, prepared in Opti-mem at w/w 2 and w/w 1 ratios. Twenty microliters of the nanoparticle solution were added to each well of cells containing 100 µL of complete media and allowed to incubate for two hours, at which point the media was replaced. Transfection efficacy was assessed for percent-transfected cells and geometric mean expression approximately 48h following transfection. Cell viability was assessed using MTS Celltiter 96 Aqueous One (Promegra, Madison, WI) cell proliferation assay approximately 24h following transfection.

Cellular Uptake and pH Measurements. Cells were again plated at a density of 15,000 cells/well in 96 well plates in 100 µL media and allowed to adhere for 24h. Nanoparticles were formed identically to the procedure for determination of transfection efficacy other than the use of 20% plasmid pH nanosensor pre-mixed with 80% unlabeled DsRed DNA. Following two hours of incubation, media was aspirated and the cells were washed two times with 50 µg/µL heparin in 150 mM PBS with five triturations followed by a single PBS wash to remove surface-bound polyplexes. PBS was then aspirated and replaced with fresh complete media. At specified time points, cells were lifted and resuspended in 150 mM PBS containing 2% v/v FBS before being run through a BD Accuri C6 flow cytometer with HyperCyt CFlow autosampler as previously detailed. Flow cytometry data was gated using FlowJo (Ashland, OR) as shown in Supplementary Fig. 3-S4 to determine
cellular uptake and exclude cells with fluorescence in either channel of the pH nanosensor below background autofluorescence levels. Cellular uptake was quantified using Cy5 from the pH nanosensor for the percent of cells having uptaken polyplexes and geometric mean fluorescence. Data from the cells above background level for both FL1H and FL4H were exported and analyzed in Matlab. The ratio of FL1H/FL4H for pH sensitive/pH insensitive fluorescence (FL1H/FL4H) was calculated for each cell. Then the median FL1H/FL4H ratio for each well was determined and used to calculate the average pH for that well using the linear standard pH curve (Figure 3-2c).

Confocal Microscopy. GB319 cells were plated on Nunc Lab-Tek chambered borosilicate coverglass well plates (155411, Thermofisher) at 37,500 cells per well for the same seeding density as transfections in 96 well plates. Cells were incubated for two hours with nanoparticles formed from 1500 ng of the plasmid pH sensor and the respective polymer. Cells were then washed three times with 50 µg/mL heparin (379059, Sigma Aldrich) in 150 mM PBS. At 24 hours post-transfection, cells were stained for 10 minutes with Hoechst 33342 (H3570, Thermofisher) for nuclei visualization as well as Cell Navigator Lysosome Staining dye (AAT Bioquest, Sunnyvale, CA) then washed three times with PBS and imaged in live cell imaging solution (A14291DJ, ThermoFisher) at 37°C in 5% CO₂. Images were acquired using a Zeiss LSM 780 microscope with Zen software and 63x oil immersion lens. Specific laser channels used were 405 nm diode, 488 nm argon, 561 nm solid-state and 639 nm diode lasers. Laser intensity and detector gain settings were optimized for cells transfected with PEI w/w 2 and maintained for all imaging experiments.

Nanoparticle Characterization. Three samples were independently prepared for each nanoparticle formulation at the same concentrations as outlined in the transfection methods section. Nanoparticles for pH nanosensor measurements were formed from 100% labeled DNA. Nanoparticle hydrodynamic diameters were determined by dynamic light scattering (DLS) in disposable micro-cuvettes using a Malvern Zetasizer NanoZS (Malvern Instruments, Marlvern, UK) with a detection angle of 173°. Each sample was then diluted in 10 mM NaCl by a dilution factor of 5 and zeta potential was measured by electrophoretic light scattering in disposable zeta cuvettes at 25°C using the same Malvern Zetasizer NanoZS (Malvern Instruments, Marlvern, UK).

Heparin binding competition assay between polyplex nanoparticles and DNA was run similarly to previously published. Briefly, polyplex nanoparticles were formed between unlabeled or 100% labeled plasmid DNA and either bPEI or PLL at a w/w ratio of 2 and concentration of 0.03 ug/uL in 10 mM NaCl. The nanoparticles were then added to separate solutions of heparin sodium salt (Sigma H3393) diluted in 10 mM NaCl to give the specified amounts of heparin per well in nanograms. The solutions of polyplex
nanoparticles with heparin were then diluted with 30% glycerol to give a 5% glycerol solution, after which 15 μL were loaded into a 1% agarose gel.

Yo-Pro-1 iodide binding assays were run similarly to previously published results, where DNA and Yo-Pro-1 iodide (Thermofisher) were both diluted to a concentration of 1 μM in 10 mM NaCl. For pH nanosensor trials, 100% Cy5 only labeled DNA was used to avoid fluorescence from the green channel fluorophores. The solution of Yo-Pro-1 and DNA was then mixed in a 1:1 ratio with a polymer solution to give the specified polymer concentration per well. Green channel fluorescence was then measured using a plate reader (Biotek Synergy 2).

Data Analysis, Statistics and Figures. Prism 6 (Graphpad, La Jolla, CA) was used for all statistical analyses and curve plotting. Unless otherwise specified, statistical tests were performed with a global alpha value of 0.05. ChemDraw (Perkin Elmer, Waltham, MA) was used for chemical structures and schematics. Unless otherwise stated, absence of statistical significance markings where a test was stated to have been performed signified no statistical significance. Figures were structured such that red lines, bars or dots specifically denote DsRed positive populations, while DsRed negative populations were denoted with black (HEK293T) or grey (GB319).

Results

Nucleic Acid pH Nanosensor Synthesis and Validation
The triple-fluorophore labeled plasmid pH nanosensor was synthesized in a batch process using the reaction scheme shown in Figure 3-1a using NHS-psoralen UV crosslinked into DsRed plasmid DNA prior to reaction with fluorescein cadaverine (FL), Oregon green cadaverine (OG) and cyanine-5-amine (Cy5). It was designed to have a green channel to far-red channel fluorescence ratio that would increase with increasing pH as shown in Figure 3-1b,c. The reaction ensured that each plasmid statistically had all three fluorophores with approximately 50 green and 50 far-red fluorophores per 4700 base-pair plasmid as determined by spectrophotometry. The labeling efficacy was not particularly sensitive to the amount of NHS-psoralen used, but required sufficient DMSO (~30%) in the reaction solution to allow the more hydrophobic fluorophores to react effectively.
Figure 3-1. pH Nanosensor Synthesis. (a) Synthesis of the triple-labeled pH sensor was achieved via a two-step process of UV photocrosslinking NHS-psoralen to plasmid DNA followed by reaction with three primary amine fluorophores (fluorescein, Oregon green and Cy5). (b) Far red channel fluorescence from Cy5 is pH-insensitive over the physiological pH range of interest, whereas Oregon Green and fluorescein exhibit pH-sensitive quenching relative to their pKa values of 4.6 and 6.4. (c) The combination of these fluorophores results in a pH sensitive ratiometric relationship for cells electroporated, fixed and run through flow cytometry in known pH solutions.

The pH nanosensor sensitivity over the entire pH range of interest was verified to be improved with the inclusion of two pH sensitive fluorophores and one pH insensitive fluorophore in comparison to plasmids labeled with only one pH sensitive fluorophore and one pH insensitive fluorophore (Figure 3-2a). This nanosensor was observed to have an approximately linear relationship between pH and fluorescence ratio over the physiological pH range of interest (Figure 3-2b). For flow cytometry experiments, a calibration curve relating fluorescence ratio to pH for the nanosensor was created by electroporating the pH nanosensor into human embryonic kidney cells (HEK293T) and human primary grade IV glioblastoma cells (GB319)\textsuperscript{5}, fixing the cells to permeabilize them to free flow of ions, and running them through the flow cytometer following resuspension in known pH buffer solutions (Figure 3-2c). The pH nanosensor was further confirmed to be effective in a third cell line and was stable when stored at -20°C over six months (Supplementary Figure 3-S1).
Figure 3-2. Fluorescence ratio pH calibration curve. (a) Plate reader normalized fluorescence curves fit with a logarithmic function show that two pH sensitive fluorophores were necessary for pH sensitivity over the range of interest (all curves also contain Cy5 for pH-insensitive fluorescence on the FL4 channel; OG = Oregon green; FLC = fluorescein). The triple labeled pH nanosensor gave an approximately linear relationship over the pH range of interest measured using (b) a fluorescence plate reader and (c) flow cytometry following electroporation into HEK293T cells. Values shown are mean ± SEM of four wells for (a,b) and median ratio of >10,000 cells for (c).

Additionally, fluorophore labeling of plasmid DNA to form the pH nanosensor was confirmed not to affect nanoparticle physicochemical properties in terms of hydrodynamic diameter or zeta potential when combined with cationic polymers to form polyplex nanoparticles (Figure 3-3a,b). Overall, PLL nanoparticles were approximately 50 nm in size with a zeta potential of +20 mV and PEI nanoparticles were approximately 250 nm in size with a zeta potential of +25 mV. To test the influence of DNA labeling for the pH nanosensor on polymer/DNA binding strength, a heparin competition binding assay using gel electrophoresis and a Yo-Pro-1 iodide competition binding assay were performed that showed that polymer/DNA interaction was minimally affected by covalent labeling (Figure 3-3c,d).

Intracellular trafficking of covalently modified plasmid DNA has likewise been a concern in previous studies, which we sought to address by utilizing only 20% pH nanosensor DNA in studies involving cellular uptake and expression. While it is true that the fluorescent labeling method does prevent expression from labeled plasmids, this reduction in reporter gene expression was due to UV exposure that likely results in nicks in the DNA during the labeling process (Supplementary Figure 3-S2) and not necessarily fluorophore covalent conjugation. Recent studies have shown that bPEI polyplexes have on the order of 10 plasmids per nanoparticle, meaning that with 20% pH nanosensor DNA used to form polyplexes in uptake and expression experiments each polyplex would statistically contain unmodified plasmids.
Figure 3-3. Biophysical properties of polymeric gene delivery nanoparticles were not affected by pH nanosensor labeling. Polymeric gene delivery nanoparticles were formed by self-assembly of cationic polymers using the pH nanosensor or unlabeled plasmid DNA and assessed using dynamic light scattering for (a) hydrodynamic diameter and (b) zeta potential, where no statistical differences were observed. Values shown are mean ± SEM of three independently prepared samples. Differences between nanoparticles formulations were assessed using multiple t-tests with multiple comparisons corrected for using the Holm-Sidak method. Polymer binding strength with labeled pH nanosensor or unlabeled plasmid DNA was compared using a (c) Yo-Pro-1 binding assay and (d) heparin binding assay, where only minor differences were observed in polymer/DNA interaction. Values on the heparin binding assay show the weight-weight ratio of heparin to the mass of polycation per well. A possible increase in binding interaction between pH nanosensor DNA and cationic polymer may be accountable by the presence of carboxylic acid groups in two of the fluorophores used.

Cellular Uptake and Transfection

Polymeric nanoparticles have been demonstrated previously in HEK293 cells to traffic via an endolysosomal pathway and the cell line is particularly amenable as a well-established cell type for non-viral transfection experiments. Human GB319 cells in contrast are a difficult to transfect primary cell line, which show relatively low reporter gene expression for a wide variety of transfection reagents. Initial screens of the nanoparticle formulations were done to identify optimal ratios between transfection reagent and DNA that
were effective for transfection without being highly cytotoxic (Supplementary Figure 3-S3c,d), where polymer:DNA weight-weight (w/w) ratios of 1:1 and 2:1 were selected. These ratios between polymer and DNA were in line with polymer doses used previously\(^\text{20}\), and can be converted from w/w to N/P ratio by multiplying by 7.6 or 2.2 respectively for 25 kDa bPEI and PLL.

For cellular uptake studies, including for pH measurements, cells were washed with the polyanion heparin prior to flow cytometry to remove any surface bound but non-internalized nanoparticles (Supplementary Figure 3-S4).\(^\text{35}\) Cellular uptake efficacy was determined via flow cytometry by the fluorescence of the pH insensitive fluorophore (Cy5) at two hours after adding nanoparticles to the cells (Figure 3-4a,b) as gated in Supplementary Figure 3-S5b. All nanoparticle formulations tested demonstrated similar levels of particle uptake within each cell type in terms of percent of uptake positive cells as well as the normalized geometric mean uptake, which correlates to the average number of nanoparticles taken up by each cell. Due to the high labeling efficacy using NHS-psoralen, and the sensitivity of the nanosensor, the fraction of fluorophore labeled DNA for pH determination and cellular uptake experiments was able to be reduced to 20% of the total DNA used to form polyplex nanoparticles. This was particularly important, as exposure of the plasmid DNA to UV to promote covalent bond formation in the pH nanosensor synthesis protocol resulted in DNA nicks that eliminated expression from the labeled plasmid DNA (Supplementary Figure 3-S2).

Transfection efficacy was assessed via flow cytometry (Figure 3-4c,d) and fluorescence microscopy (Supplementary Figure 3-S3a,b) in HEK293T and GB319 cells, respectively. Despite the high levels of cellular uptake of PLL/DNA nanoparticles, transfection via PLL polyplexes at either w/w ratio was very ineffective in both HEK293T and GB319, as anticipated. bPEI/DNA nanoparticles were more effective in both cell lines and did so in a polymer dose specific fashion with bPEI w/w 2 being superior to bPEI w/w 1 (Figure 3-4c,d).
Figure 3-4. Cellular uptake and reporter gene transfection in two cell lines. (a) HEK293T and (b) GB319 cellular uptake as percent of all cells and normalized geometric mean fluorescence of Cy5 labeled DNA were similar between PLL and bPEI. Transfection efficacy in (c) HEK293T and (d) GB319 cells as percent of all cells and normalized geometric mean expression showed that bPEI was much more effective than PLL in transfecting cells. Lipofectamine® 2000 showed similar levels of cellular uptake and transfection to bPEI with the conditions tested. Bars show mean ± SEM of four wells.

Calculated pH Measurements

HEK293T and GB319 cells were transfected with the nanoparticle formulations using 20% pH nanosensor plasmid DNA and average pH of DNA was calculated at specified time points following the initial addition of nanoparticles (Figure 3-5a,e). The fluorescence on the FL1H pH sensitive and FL4H pH insensitive channels for individual cells as gated in the third panel of Supplementary Figure 3-S5a was imported to Matlab for processing. The ratio between the FL1H and FL4H from individual cells was computed and input into the linear standard curve relating fluorescence ratio to pH (Figure 3-2c) for individual cells and the median calculated cell pH per well was determined. For all polyplex nanoparticles studied, acidification rapidly occurred within the first 2 hours of transfection. pH of the nanosensor then remained effectively constant over the subsequent day for all nanoparticles except PLL in HEK293T, where acidification continued to occur at a slower rate until 8 hours post transfection. PLL/DNA polyplexes at both w/w 1 and w/w 2 acidified to an average pH of approximately 4.5 for HEK293T and 5.5 for GB319. bPEI/DNA polyplexes in contrast avoided dramatic acidification in both cell lines with HEK293T displaying an average
pH of 6.0 at both polymer doses. bPEI nanoparticles in GB319s appeared to reduce acidification in a polymer dose dependent fashion with w/w 1 having an approximate median pH of 6.1 and w/w 2 having an approximate median pH of 6.6.

At the 24-hour time point, DsRed expression was assessed as shown in flow cytometry gating (Supplementary Figure 3-S5c) to determine characteristics of cells having been successfully transfected compared to those cells that were not successfully transfected. Fluorescence from the pH sensitive (green), pH insensitive (far-red) and DsRed (red) channels could also be clearly seen for each nanoparticle formulation using fluorescence microscopy at 24 hours post-transfection (Supplementary Figure 3-S6).

Overall, bPEI and PLL both showed statistically significant differences at the population level for median pH of the DsRed positive and negative populations (Figure 3-5b,f) but the pH distributions (Figure 3-5c,d,gh) were not qualitatively different except in the case for w/w 1 bPEI in GB319 cells. For transfections with bPEI w/w 1 in GB319 cells, acidic pH (5.0-6.0) correlated with a decreased frequency of successful transfection (DsRed- > DsRed+) whereas neutral pH (~7.0) weakly correlated with a higher frequency of successful transfection (DsRed+ > DsRed-). This trend was not noted in HEK293T cells however, despite the similar trend in increased transfection efficacy with bPEI w/w 2 particles. For PLL, in both cell types the DsRed negative population had a narrow, acidic pH distribution indicative of lysosomal fate (Figure 3-5d,h). For HEK293T cells positively transfected with PLL nanoparticles, the DsRed positive population was shifted to more neutral pH.

Figure 3-5. Calculated population pH and relationship to transfection at 24 hours. Median pH of intracellular DNA calculated at various time points following transfection for the entire cell populations showed PLL failing to avoid a lysosomal fate, while bPEI showed a pH closer to neutral in both (a).
HEK293T and (e) GB319 cells. (b) Comparison via Holm-Sidak corrected multiple t-tests of the median pH values from the DsRed positively transfected populations and negative untransfected populations at 24 hours showed statistically significant differences between the populations for bPEI w/w 1, bPEI w/w 2, PLL w/w 1, and PLL w/w 2 for HEK293T cells. (f) In GB319 cells, statistically significant differences in median pH values between the successfully transfected populations and untransfected populations for for bPEI w/w 1, bPEI w/w 2, and PLL w/w 1 were observed. (c) For HEK293T cells transfected with bPEI w/w 2 and w/w 1, less acidic pH of the delivered DNA in individual cells (>5.5) was slightly correlated with an increase in fraction of cells transfected compared to more acidic pH of the delivered DNA in the individual cells (<5.5). (d) Interestingly, HEK293T cells transfected with PLL showed the same behavior to a greater extent. (g) For GB319 cells transfected with bPEI, transfection resulted in a wider distribution of calculated pH than with PLL particles. Following transfection with bPEI, acidic pH (5.0-6.0) correlated with a decreased frequency of successful transfection (DsRed- > DsRed+) whereas neutral pH (~7.0) correlated with a higher frequency of successful transfection (DsRed+ > DsRed-). (h) For GB319 cells transfected with PLL, there were only minor differences between the DsRed positive and negative populations. All errors bars show the mean ± SEM of four wells. Histograms show the binned flow cytometry data from four wells.

**Individual cell relationships between calculated pH, cellular uptake, and exogenous gene expression**

An advantage of the fluorescence-based nanosensor developed is the capability for high-throughput measurements of cellular uptake of exogenous DNA, measurement of local pH of the exogenous DNA, and expression of the exogenous DNA. Assessment of DsRed reporter gene expression at 24 hours post-transfection as compared to pH nanosensor measurements and cellular uptake measurements on the pH-insensitive channel of the nanosensor enabled the analysis of trends at the single cell level. To clearly elucidate the DsRed positive population of cells from possible increased autofluorescence, the ratio of FL3H fluorescence to FL1H fluorescence was calculated for each cell, which showed a clear difference for DsRed positive cells (see gating of Supplementary Figure 3-S6c). Plotting the DsRed expression in terms of FL3H/FL1H versus calculated pH on an individual cell level basis did not show a strong apparent correlation for bPEI or PLL at either w/w ratio or cell line tested (Figures 3-6,a-h). There did, however, seem to be a weak correlation with bPEI but only at w/w 1 ratio in GB319 cells (Figure 3-6f). Quantification of the correlation between variables specifically for the DsRed expressing cell populations using Pearson’s correlation coefficient (PCC) in Matlab showed that there was quantitatively minimal correlation between most of the variables (Figures 3-6i-n). Weak positive correlation (0.2<PCC<0.4) between DsRed expression and pH was observed only for bPEI in either cell line (Figure 3-6i,l), while cells transfected with PLL showed no significant correlation between the variables. This result was surprising, given the cases of strong statistical significance between the median pH values of the DsRed positive and negative populations for
both bPEI and PLL. (Figure 3-5b,f). For other relationships, nanoparticle uptake and cell pH showed weak correlation only for bPEI w/w 2 in GB319 cells (Figure 3-6m) and nanoparticle uptake and gene expression showed weak correlation only for bPEI w/w 2 and PLL w/w 2 in GB319 cells (Figure 3-6n).

Figure 3-6. Relationships between individual cell pH, uptake and gene expression at 24 hours.
Representative scatter plots of DsRed positive (red) and negative (black/grey) populations of cells transfected with (a,e) bPEI w/w 2, (b,f) bPEI w/w 1, (c,g) PLL w/w 2 and (d,h) PLL w/w 1 qualitatively showed minimal correlation between gene expression and calculated pH. To quantify correlation between variables, Pearson’s correlation coefficient (PCC) was calculated for each well for variables in the case of HEK293T (i-k) and GB319 (l-n). PCC between (i,l) pH and gene expression showed weak (0.2<PCC<0.4) correlation only in the case of HEK293T cells transfected with bPEI at w/w 1 and w/w 2 as well as GB319 cells with bPEI w/w 1. (j,m) pH and nanoparticle uptake showed weakly positive correlation only for GB319 cells transfected with bPEI w/w 2. (k,n) Nanoparticle uptake and gene expression showed weak correlation only for GB319 cells transfected with bPEI w/w 2 and PLL w/w 2. All errors bars show the mean ± SEM of four wells for PCC calculated for the cells in that well.

Microscopy verification and lysosome colocalization
The pH nanosensor was verified to function visually using confocal microscopy in the harder to transfect GB319 cell line, where it was noted that bPEI was prone to having high variability in the fluorescence ratio.
between individual endosomes, spanning both neutral and acidic pH ranges (Figure 3-7a). Following transfection with bPEI/DNA nanoparticles, within individual cells, some endosomes had a high fluorescence ratio indicating a near neutral pH, while other endosomes had the pH sensitive fluorophores effectively quenched, thus indicating their presence in lysosomes. PLL in contrast had low pH sensitive fluorescence and low fluorescence ratios with only the pH-insensitive fluorescence strongly observed. These results were confirmed by staining for lysosomes with a pH sensitive dye (see methods) and analyzing colocalization of the pH insensitive fluorophore with lysosomes (Figure 3-7a,b). The fraction of DNA colocalized with lysosomes was above 75% for PLL at both 3 hours and 24 hours post-transfection while for bPEI the fraction of DNA colocalized with lysosomes was below 40% at 3 hours and increased between the 3 and 24 hour time points (Figure 7c).

![Figure 3-7. Confocal microscopy qualitative assessment of pH nanosensor function and lysosome colocalization.](image)

(a) Endosomes 3 hours post-transfection containing bPEI w/w 2 or PLL w/w 2 nanoparticles formed with the pH nanosensor show qualitative functioning of the nanosensor for live cell
microscopy using GB319 cells, whereby the pH insensitive fluorophore intensity was not dependent on pH while the pH sensitive fluorophores were quenched when DNA was present in lysosomes. Images were thresholded for analysis of colocalization of DNA via the pH insensitive channel with lysosomal tracking dye. Compartments containing DNA non-colocalized with lysosomes are shown in pink, while lysosomes not containing DNA are shown in red, and compartments containing DNA colocalized with lysosomes are shown in yellow. Scale bar 20 µm. (b) Scattergrams show the analysis of the representative microscopy images with distribution of pixels with DNA fluorescence intensity shown on the x-axis, lysosome fluorescence intensity shown on the y-axis and region 3 showing thresholded colocalized pixels. (c) Quantification of Pearson’s correlation coefficient M1 for colocalization of DNA and lysosomes showed PLL nanoparticles colocalized with lysosomes at both 3 hours and 24 hours post-transfection while bPEI nanoparticles partially avoided an initial lysosomal fate at 3 hours but not at 24 hours. Quantified results were tested using multiple t-tests corrected for multiple comparisons using the Holm-Sidak method. Errors bars show mean ± SEM of 5 images taken with the same microscope settings.

Application to non-polymeric non-viral nanoparticles:

We additionally evaluated the pH nanosensor using the liposomal transfection reagent Lipofectamine® 2000 to test its efficacy with a non-polymeric non-viral transfection agent. (Figure 3-8). Lipofectamine® 2000 at a w/w 1 ratio showed a median pH of DNA of approximately 6.0 demonstrating that some plasmids fail to fully escape a late endosome or lysosomal fate. The mechanism of endosomal escape of lipid reagents like Lipofectamine® 2000 is not fully established, but recent reports have shown that the vast majority of lipid nanoparticles still reach lysosomes and escape is a relatively rare event.39, 40 Overall, we detected only minimal differences in the calculated pH between DsRed positive and negative populations of cells transfected with Lipofectamine® 2000 and did not find any correlation between nanoparticle uptake and transfection or nanoparticle uptake and pH. That being said, acidic pH (4.0-6.0) correlated with a slightly decreased frequency of successful transfection (DsRed- > DsRed+) whereas higher pH (7.0-8.0) weakly correlated with a higher frequency of successful transfection (DsRed+ > DsRed-). However, as high transfection was seen with Lipofectamine® 2000 across the whole range of pH (4.0-8.0), significant trafficking of the delivered DNA to acidic compartments was not a significant barrier to Lipofectamine® 2000 as a non-viral transfection agent, unlike with PLL.
Figure 3-8. Lipid nanoparticle pH nanosensor measurements. (a) pH of DNA in Lipofectamine® 2000 nanoparticles introduced to HEK293T cells was determined to average approximately 6.0 at all time points following transfection. There was only a minor statistical difference between the (b) the median pH of cells that were dsRed positive and negative at 24 hours post-transfection, which was only slightly evident when looking at a (c) representative distribution of the populations. (d) Likewise to bPEI and PLL there was no highly significant correlation between DsRed expression and measured pH for the cell at 24 hours post-transfection. (e) Cell pH and DsRed gene expression showed weak (0.2<PCC<0.4) correlation using PCC and no significant correlation was observed between the other relationships.

Discussion

In this work, we have created a nucleic acid based pH nanosensor for investigating the local pH of plasmid DNA following transfection with non-viral carriers. Notably, the pH nanosensor synthesized here utilized two pH sensitive fluorophores, fluorescein and Oregon green, with respective pKa values of 4.6 and 6.4 for improved sensitivity at lower pH ranges of interest (Figure 3-2a), which was required to probe lysosomal pH levels.22 Using the ratio of the fluorescence of the pH sensitive fluorophores to the pH insensitive fluorophore, a standard curve was calibrated for intracellular pH readings (Figure 3-2c) that could be quantified in a high throughput manner through flow cytometry.
Previous studies have focused on labeling polymers directly as a pH probe while less research has
been done constructing pH sensors from nucleic acids to specifically investigate vesicles containing DNA.20, 24, 30, 31 Labeling of polymers directly has been shown to influence the size and properties of the particles studied.30 In this study, nanoparticles formed from cationic polymers complexed with 100% pH nanosensor DNA were not statistically different in size, zeta potential or polymer binding strength from nanoparticles formed with unlabeled DNA (Figure 3-3). Additionally, given that free polymer has been shown to play a large role in transfection and that some polymers may form particles in the absence of DNA, studying the trafficking of a fluorescently labeled polymer is a less direct method to assess the behavior of those particles and endosomes specifically containing plasmids.20, 41 Labeling plasmids directly with fluorophores also enabled a single ratiometric curve to be generated for the specific batch of labeled plasmid as nanosensors, eliminating the need to label and characterize multiple polymers individually when using the nanosensor to probe biomaterial structure/function relationships.

PLL and bPEI were optimal polymers to use as a proof of concept, as they are established gene
delivery materials and were shown to have similar degree of nanoparticle uptake (Figure 3-4a,b). As expected from previously published results in other cell types, bPEI was much more effective than PLL for overall transfection in both cell lines tested (Figure 3-4c,d). Using the pH nanosensor, we observed that plasmid DNA delivered with bPEI maintained a near neutral pH while DNA delivered with PLL was trafficked to acidic compartments (Figure 3-5a,e).24, 31 Interestingly, the dose-dependent difference in median pH of the GB319 cell population for bPEI was not observed in the HEK 293T cells and disappeared when examining only the DsRed positive population (Figure 3-5f). Examining differences between DsRed positive and negative populations at 24 hours post-transfection showed significant differences in the median pH of the populations for both bPEI and PLL, although the significance was greatest for bPEI at a w/w 1 dose (Figure 3-5b,f). Analysis of the DsRed positive and negative populations (Figure 3-5) showed that for both polymers in both cell lines, when the average pH was close to neutrality, the relative frequency of successful transfection (DsRed+/DsRed-) increased. Intriguingly, when moving from a population perspective to a single cell perspective, the strong positive correlation between increased intracellular pH and increased transfection is no longer observed (Fig. 3-6). These results are in agreement with previous claims that only a small fraction of polyplexes ever manage to escape endolysosomes to the cytosol.20 Thus, the many polyplexes that do not escape the endolysosomes and the many cells that do not become successfully transfected obscure the potential correlation of those that do. Nonetheless, with the right polyplex material, these non-escaping polyplexes and these non-transfected cells can still all exhibit an increase in pH sensed by the nanosensor that correlates to a transfection advantage for the cell population overall.

To further investigate functioning of the pH nanosensor following transfection and to evaluate polyplex activity in GB319 human glioblastoma cells in the context of previous reports that have observed
the vast majority of polyplexes taken up by a cell fail to escape the endosomes, we performed confocal microscopy at 24 hours post-transfection (Figure 3-7). The pH sensor was confirmed to function as expected, further validating results from flow cytometry, with the pH sensitive channel showing much greater fluorescence in proportion to the pH insensitive channel for bPEI mediated transfection than for PLL. Interestingly, endosomes containing the pH nanosensor and bPEI appeared to exhibit a wide variance in pH, which was apparent from confocal images and was further confirmed by assessing the specific fraction of DNA in lysosomes via colocalization analysis (Figure 3-7b,c). For nucleic acid containing nanoparticles that manage to escape the endosome, they will no longer appear as punctate points in microscopy images and imaging would be hindered by the much brighter concentrated pixels of nanoparticles in endolysosomes; these same nanoparticles that have successfully escaped the endosomes would, however, be accounted for in total fluorescence with flow cytometry. This could explain in part the possible discrepancies between flow cytometry and confocal microscopy analysis, whereby nucleic acids delivered with bPEI have been reported in other cell types to maintain a median pH above five when assessed with flow cytometry, but below five with confocal microscopy.20, 24, 31

Our studies set out to investigate to what extent certain non-viral gene delivery nanoparticles reach a state of acidification in endolysosomes or else manage to avoid that fate through the use of a DNA-based nanobiosensor. An important aspect to note is that there are a variety of means for a nanoparticle to avoid acidification. While preventing acidification through endosomal buffering and titratable amine groups has been demonstrated in part by some biomaterials such as PEI, this is not the only method by which particles escape a lysosomal fate.22 An alternative possibility is avoidance of these acidic pathways all-together through uptake into non-acidifying vesicles13, 16 or direct entry to the cytoplasm through induced nanopores.42 A related aspect is that the type of endocytosis, such as clathrin-mediated vs. caveolae-mediated, can also make a significant difference to the efficiency of transfection, and that this can occur in a polymer structure-specific manner.43 None-the-less, it is clear that sequestration of delivered DNA into acidic late-endosomes and lysosomes, means that those DNA molecules are not available to be transcribed in the pH-neutral nucleus, the target of the gene delivery. Thus, this nanobiosensor can play an important role in probing this barrier in a high-throughput, single cell, and dynamic fashion.

Even given successful and canonical endocytosis of a nanoparticle, there are additional intracellular barriers beyond endosomal escape to successful transfection including DNA unpacking, nuclear import and nuclear retention of DNA that must be considered for a delivery approach, but the endosomal barrier remains in many cases the rate limiting step in expression following nanoparticle uptake.14 In recent studies, the rarity of endosomal escape of lipid nanoparticles as well as polymeric nanoparticles have been likewise well documented.39, 40 These studies in particular have noted the difficulty in using fluorescence based imaging for tracking labeled molecules or particles that have successfully escaped the endosome, as the fraction of
total fluorescence detectable in the cytosol is very low. With this in mind, we have chosen to focus on the specific barrier to transfection of endosomal escape in our creation of a new tool.

The proton sponge hypothesis posits that due to endosomal buffering by a protonatable base, such as PEI (the “proton sponge”), there will be a concomitant flux of anions into the endosome to maintain electroneutrality and consequent buildup of osmotic pressure that can lead to endosomal rupture. The results of this study do not confirm or deny the existence of the proton sponge mechanism as it was first proposed by Boussif et al but do argue against a simple buffering limited endosomal escape path for polymeric vectors. If transfection of cells were to be limited by the buffering capacity of the polymeric vectors used, including bPEI, buffering capacity of nanoparticles in the pH 5-7 range would be expected to show strong direct correlation with transfection efficacy, which is not what we observe when we look at the cells on a single cell level in our study (Figure 3-6).

Theoretical approaches to assessing the feasibility of the proton sponge mechanism are inconclusive. Purely theoretical assessment of the feasibility of the proton sponge mechanism based on the combination of osmotic pressure and pressure from the charged polymer has been used to argue that buffering of endosomal pH with enough free (uncomplexed) cationic polymer was sufficient for rupturing endosomes. Assessment of the feasibility of the proton sponge mechanism via quantification of the amount of fluorescently labeled bPEI that accumulated in endosomes in combination with the theoretical buffering capacity and estimated critical lysosomal membrane tension of 10 mJ/m² has been used to argue that osmotic pressure could not lead to endosomal escape on its own. Likewise, the maximum endosomal osmotic pressure possible based on the buffering capacity of bPEI has been estimated to be below the level necessary for full endosomal membrane disruption.

Perhaps buffering capacity of polymers for gene delivery may make endosomal escape more likely without directly causing endosomal escape via the hypothesized proton sponge mechanism on its own. Polymer buffering capacity may cause an increase in osmotic pressure across the membrane in such a way as to make it more vulnerable to disruption by other attributes of the polymer, such as its increasingly high charge, similarly to how an overly inflated balloon is easier to pop with a pin than a partially inflated balloon. This hypothesis is supported by experimental results from multiple groups demonstrating interaction of polymer directly with cellular membranes, as charged cationic polyplexes have been demonstrated to lead to membrane disruption directly. Further, the spacing of cationic charges in polymer structure has been demonstrated to affect both transfection efficacy and cellular viability, as observed in the odd-even effect with polyaspartamides. Coupled with results from groups demonstrating efficacy of amphiphilic polymers for gene delivery that have relatively low charge densities, this balance between ability to possibly generate osmotic pressure due to buffering capability and ability to disrupt membranes by physical means may be critical for polymeric vector cytosolic delivery.
In conclusion, we have demonstrated that a triple-fluorophore labeled nucleic acid can function as a sensitive pH nanosensor to probe the environment of exogenous nucleic acid delivered by polymeric and liposomal non-viral carriers through the whole range of physiological pH. bPEI in comparison to PLL, exhibited a higher average intracellular pH as well as a higher variance in intracellular pH. Population average measurements of pH were found to correlate well to successful transfection of human cells, whereas single cell measurements were found to be only weakly correlative. These results suggest limitations to the proton sponge hypothesis and also that the ability of polymeric nanoparticles to avoid an acidic environment is necessary, but not sufficient for successful transfection. To our knowledge, this is the first reported instance of high throughput single cell analysis between cellular uptake of DNA, intracellular pH of delivered DNA, and gene expression of the delivered DNA. This nanosensor technology can be of benefit to increase fundamental quantitative understanding of how biomaterial properties affect intracellular delivery and to glean high-content and high-throughput information on the local environment of DNA as it transports through the cell.

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References:


**Supplementary:**

Supplementary Figure 3-1: pH nanosensor flow cytometry standard curve relating pH to fluorescence ratio in two cell types

Supplementary Figure 3-2. UV effect on plasmid DNA expression

Supplementary Figure 3-3. Transfection efficacy in HEK293T and GB319 cells

Supplementary Figure 3-4. Effectiveness of heparin washing.

Supplementary Figure 3-5. Flow cytometry population gating

Supplementary Figure 3-6. HEK293T cells 24-hours post-transfection with 20% pH nanosensor DNA polyplexes show clear reporter gene expression.

Supplementary Methods: Processing of flow cytometry data
Supplementary Figure 3-S1. **pH nanosensor flow cytometry standard curve relating pH to fluorescence ratio in two cell types.** The pH nanosensor gave consistent readings following electroporation into different cell types (GB319 and F54) and at time points six months apart.

Supplementary Figure 3-S2. **UV effect on plasmid DNA expression.** UV exposure resulted in decreased expression of plasmid DNA, making the labeled plasmid unsuitable by itself to induce expression. HEK293T cells were transfected with bPEI 2 w/w nanoparticles with DNA post-exposure to UV. Bars show the mean ± SEM of four wells. Transfection efficacy was assessed using flow cytometry and fluorescence intensity.
shows geometric mean fluorescence on the red channel normalized to that of cells transfected with unexposed plasmid. Error bars show the mean ± SEM of four wells. Scale bars are 500 µm for 5x images.

Supplementary Figure 3-S3. Transfection efficacy in HEK293T and GB319 cells. bPEI, PLL and Lipofectamine 2000 were complexed DNA and added at a dose of 600 ng of eGFP-N1 plasmid DNA per well to determine optimal reagent dose for (a) HEK293T and (b) GB319 cells. Images were captured with a 10x fluorescence microscope with equal exposure time two days following transfection. Scale bar 200 µm. Cell viability following transfection for (c) HEK293T and (d) GB319 was assessed using MTT cell titer and was normalized to the untreated wells absorbance values. Error bars show the mean ± SEM of four wells.
Supplementary Figure 3-S4. Effectiveness of heparin washing. The protocol for washing cells was confirmed to be sufficient to remove surface bound but non-internalized polyplex nanoparticles. Cells were incubated with nanoparticles for one hour at 4 °C to inhibit endocytosis then washed two times with polyanion 50 µg/mL heparin sulfate in 150 mM PBS followed by a single PBS rinse. Cells incubated at 4 °C and washed (orange) were shown to have fluorescence on the pH insensitive channel FL4H for Cy5 reduced to that of the untreated control (red) compared to cells incubated at 4°C and unwashed (blue). Cells incubated with nanoparticles under standard conditions of a two hour transfection at 37 °C (green) had over two orders of magnitude higher fluorescence than washed cells demonstrating that washing was effective to remove surface bound nanoparticles.
Supplementary Figure 3-S5. Flow cytometry population gating. Flow cytometry data was gating according to the following plots. (a) Singlet cells were gated from all detected particles using FSC-H vs SSC-H followed by FSC-H vs FSC-A. Gates for cells with fluorescein (FL) and Cy5 fluorescence greater than the untreated population of cells were selected as shown. (b) Cells were gated as being positive for uptake of DNA using the Cy5 channel (FL4-H) compared to the untreated population of cells. More than 90% of cells had detectable DNA uptake for both bPEI and PLL. Cells in the Double+ region with fluorescence greater
than cell autofluorescence on both channels were used for purposes of flow cytometry pH measurements. (c) From the cells in the Double+ region, cells were strictly gated in FL3-H vs FL1-H to determine those cells positively expressing the reporter protein dsRed at 24 hours post-transfection.

Supplementary Figure 3-S6. HEK293T cells 24-hours post-transfection with 20% pH nanosensor DNA polyplexes show clear reporter gene expression. Microscope images were acquired with a 40x lens. Scale bar 40 μm.

**Supplementary Methods:**

**Processing of flow cytometry data.** Flow cytometry data acquired with an Accuri C6 flow cytometer and attached HyperCyt CFlow Automator were exported to FCS files for each well. The FCS files were imported to FlowJo and analyzed as shown (Supplementary Figure 3-S5) to identify singlet cells as well as DsRed positive and negative cell populations at 24 hours post-transfection. The individual cell data was then exported to .csv files from FlowJo and imported into Matlab for quantitative analysis with the following scripts. The `plateTF` script reformats vector matrices to a 96 well block plate format, while the `import_flow_data` script allows for pH calculation of individual cells as well as calculation of Pearson’s correlation coefficient (PCC).
Chapter 4: Continuous Microfluidic Assembly of Biodegradable Poly(beta-amino ester)/DNA Nanoparticles for Enhanced Gene Delivery

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Foreword: This article came out effective concurrently with my pH Nanosensor article and was my first introduction to microfabrication practices and a frankly cool demonstration of using nanoparticle tracking analysis to specifically track particles containing fluorescently labeled plasmid DNA. Published in the Society for Biomaterials (SFB) society journal – Journal of Biomedical Materials Research Part A, the article has been well received by the field, having already been cited 26 times from 2017-2019.


ABSTRACT:
Translation of biomaterial-based nanoparticle formulations to the clinic faces significant challenges including efficacy, safety, consistency and scale-up of manufacturing, and stability during long-term storage. Continuous microfluidic fabrication of polymeric nanoparticles has the potential to alleviate the challenges associated with manufacture, while offering a scalable solution for clinical level production. Poly(beta-amino esters) (PBAE)s are a class of biodegradable cationic polymers that self-assemble with anionic plasmid DNA to form polyplex nanoparticles that have been shown to be effective for transfecting cancer cells specifically in vitro and in vivo. Here we demonstrate the use of a microfluidic device for the continuous and scalable production of PBAE/DNA nanoparticles followed by lyophilization and long term storage that results in improved in vitro efficacy in multiple cancer cell lines compared to nanoparticles produced by bulk mixing as well as in
comparison to widely used commercially available transfection reagents polyethylenimine and Lipofectamine® 2000. We further characterized the nanoparticles using nanoparticle tracking analysis (NTA) to show that microfluidic mixing resulted in fewer DNA-free polymeric nanoparticles compared to those produced by bulk mixing.

Keywords: gene delivery, microfluidic, polymer, nanoparticle, hydrodynamic focusing, nanoparticle tracking analysis

Introduction:

Non-viral gene delivery has great promise in clinical applications where transient expression can be sufficient to result in clinical efficacy. Compared to viral gene therapy, non-viral delivery of plasmid DNA is more amenable to repeated administration due to the reduced risk of immunogenicity and insertional mutagenesis, as well as also being generally less expensive to manufacture than its viral counterparts. Non-viral gene delivery has traditionally been much less effective than viral delivery methods and presents many challenges in clinical scale production. Improvements in non-viral delivery methods for plasmid DNA and RNA via polyplex or lipoplex formulations have resulted in efficacy both in vitro and in vivo in preclinical models leading to improved therapeutic outcomes. With many studies demonstrating preclinical efficacy in animal models, there are now multiple clinical trials based on polyplex nanoparticles in the US and abroad for the treatment of a variety of diseases. Poly(beta-amino ester)s (PBAEs) in particular are a promising class of cationic polymers capable of transfecting a diverse variety of cells both in vitro and in vivo, typically more effectively than PEI and similar reagents as well as commercial transfection reagents such as Lipofectamine® 2000. PBAEs offer design flexibility that allows for the synthesis of polymer libraries to screen for the ability to self-assemble with DNA and transfect different cell types. Beyond allowing chemical diversity in structure, PBAEs are biodegradable in water, making them less cytotoxic than non-biodegradable cationic polymers such as polyethylenimine.

Production of non-viral gene therapeutics at clinically relevant scales of production is challenging, as nanoparticles prepared by batch processes have been shown to suffer from batch-to-batch and intra-batch variability and nanoparticle batch manufacturing processes can lead to nanoparticles with different properties when scaled-up to larger batch sizes. In an approach to overcome the aforementioned challenges, microfluidic mixing has been used to form nanoparticles from many materials including hydrophobic polymers such as PLGA to form hard nanoparticles as well as soft nanoparticles composed of lipoplexes and polyplexes for gene delivery. Microfluidic methods have been demonstrated to form polyplex nanoparticles in particular with smaller diameters and improved nanoparticle stability as well as more favorable cellular viability and increased transfection. Bulk mixing of solutions via pipetting results in time scales on the order of seconds to reach homogeneity, while microfluidic platforms allow mixing of
solutions to occur in time scales of microseconds to milliseconds that have been documented to result in smaller and more monodisperse nanoparticles.

Polyplex nanoparticles that form as a result of electrostatic interactions are particularly vulnerable to heterogeneity resulting from bulk mixing via pipet or vortex. Microfluidic approaches have thus been applied to the formation of polyethylenimine (PEI) and similar DNA containing polyplex nanoparticles with promising results. Of the available approaches to microfluidic mixing for polyplex nanoparticle formation, three dimensional hydrodynamic flow focusing relies on diffusive mixing via inertial flow focusing for rapid formation of nanoparticle in a continuous flow process which is amenable to the scale-up with the possibility of parallelization. To analyze the effect of microfluidic mixing on nanoparticle properties, many of these studies have assessed nanoparticle stability on the time-span of hours in aqueous solution following formation, but few have addressed the challenge of storage of nanoparticle formulations once created over multiple months. This is especially a concern for a biodegradable product where point of care fabrication prior to administration is not feasible. Biomanufacturing processes must overcome multiple challenges during the synthesis of biodegradable nanoparticles, such as PBAE/DNA self-assembled polyplex nanoparticles, to be readily translatable from the lab bench to the clinic. These challenges include robustness and scalability in the fabrication process and stability of the final nanomedicine product.

In this report, we demonstrate microfluidic flow focusing for the rapid formation of PBAE and plasmid DNA nanoparticles followed by rapid freezing and lyophilization for long term storage. Following continuous microfluidic assembly, the lyophilized nanoparticles are shown to be more effective overall than bulk mixed nanoparticles at transfection of three cancer cell lines and are shown to retain equivalent efficacy in transfection at three months following production when stored at -20°C. We further characterize these nanoparticles with a novel assay using nanoparticle tracking analysis (NTA) to determine the properties and concentration of the nanoparticles specifically containing plasmid DNA, the effect of lyophilization on nanoparticle properties, and the ability of the nanoparticles to mediate co-expression of exogenously delivered DNA.

**Methods:**

**Materials**

Small molecule monomers for PBAE synthesis, 4-Amino-1-butanol (S4) and 4-butanediol-diacylate (B4) were purchased from Alfa Aesar; 2-(3-Aminopropylamino)ethanol (E6) was purchased from Fluka and Monomer-Polymer. Succinimidyl-[4-[(psoralen-8-yloxy)]-butyrate (NHS-psoralen) was purchase from Thermofisher. Cy3-NH2 was purchased from Lumiprobe (Hallandale Beach, FL). Plasmid pDsRed-Max-N1 (Addgene 21718, Cambridge, MA) and peGFP-N1 (Addgene 2491) were used for transfection efficacy screenings.

**Polymer Synthesis and Characterization**
PBAE 446 was synthesized in a Michael addition reaction as previously described (Figure 4-1) to create acrylate terminated polymer base structures, then dissolved in THF and reacted with small molecules to endcap the polymer. Specifically, monomers B4 (4-butanediol-diacrylate) and S4 (4-Amino-1-butanol) were mixed at a ratio of 1.1:1 to form 1g of base polymer and stirred for 24 hour at 90°C. The base polymer was then dissolved in THF at a concentration of 167 µg/µL and mixed in a 3:2 ratio with a 0.5M solution of the end capping molecule E6 (2-(3-aminopropylamino)ethanol) for 1 hour at room temperature. The end-capped polymer was then ether purified by precipitating in diethyl ether two times and placed in a vacuum chamber for 3 days to remove residual ether. The purified PBAE 446 polymer was then dissolved in anhydrous DMSO at 100 µg/µL and stored at -20°C in small aliquots. The molecular weight was determined using gel permeation chromatography (GPC; Waters, Milford, MA) as previously described in comparison to polystyrene standards and nuclear magnetic resonance using a 500 MHz Bruker NMR.

Microfluidic Chip Design and Simulation
AutoCAD (Autodesk, Rafael, CA) was used for creating the design of the photolithography mask, which was printed by CAD/Art Services, Inc (Bandon, OR). The flow resistance due to channel diameters were designed to allow a volumetric flow ratio of 3:4:1:1 for the DNA, polymer primary inlet and polymer pinch channel inlets respectively. The mixing domain of the masks were extruded in AutoCAD to a depth of 100 µm and exported as STL files to be imported to COMSOL. Multiphysics 4.4 (COMSOL, Inc., Burlington, MA). COMSOL was used to simulate laminar, incompressible flow with convection and diffusion of dilute species of polymer and plasmid DNA. eGFP Plasmid DNA of 4700 bp was modeled with a diffusivity coefficient of 5.14*10⁻⁸ cm²/s. PBAE polymer in aqueous solution was modeled with a diffusivity of 5.0*10⁻⁸ cm²/s based on results from NTA of polymer only nanoparticles.

Nucleic Acid Fluorescent Labeling
Plasmid DNA at a concentration of 1 µg/µL in water was mixed in a mass ratio of 16:1 with NHS-psoralen in DMSO at a concentration 1 µg/µL. The solution was distributed to a 96-well round bottom plate with 50 µg DNA/well and placed on ice. Psoralen was then crosslinked into the DNA by 25 minutes of UV exposure using a 0.16 amp 365 nm lamp placed directly over the plate. For each well, 8 µL of 10x PBS, 17 µL DMSO, 35 µg Cy3 were added, well mixed and then incubated at room temperature for 1h. Labeled DNA was then ethanol precipitated two times to remove excess reactants and purify the DNA. The labeled plasmid was resuspended in ultrapure water at 75% of the original volume after which, the concentration and labeling efficacy were determined using a Nanodrop spectrophotometer (Thermfischer Scientific) and the volume was increased to concentration 1 µg/µL. Labeled DNA was divided into aliquots and stored in the dark at -20°C.

Gel Electrophoresis
Plasmid DNA was run in 1% agarose gels for DNA binding experiments as previously described.39 Similarly, for shear experiments, DNA at working concentration in buffer was run through the microfluidic chip at various flow rates, then diluted and run in a 1% agarose gel for 40 minutes.

**Microfluidic Chip Fabrication**

The master wafer was prepared using silicon wafers <100> (University Wafer, Boston, MA) 100 mm in diameter, which were dehydrated on a hot plate and plasma etched with oxygen for four minutes at 100 W and pressure between 0.3-0.5 Torr. The silicon wafer was then patterned with SU-8 2075 negative photoresist (Microchem, Woburn, MA) according to manufacturer instructions. Briefly, the plasma etched silicon wafer was spin coated with approximately 1 mL of SU-8 2075 photoresist by spinning at 2100 rpm for 30 seconds. The coated wafer was then soft baked for 5 minutes at 65°C followed by 15 minutes at 95°C. The wafer was exposed to UV light using a mask aligner for 240 mJ/cm² followed by post exposure bakes at 65°C for 5 minutes and 95°C for 12 minutes. The baked wafer was then washed in a bath of SU-8 developer for 10 minutes to remove uncrosslinked photoresist followed by a rinse with isopropyl alcohol.

Sylgard 184 (Dow Corning, Auburn, MI) polydimethylsiloxane (PDMS) was prepared at a monomer to crosslinker ratio of 10:1 and poured over the patterned wafer. The wafer and PDMS were baked for one hour at 80°C and the PDMS was carefully separated from the wafer. A biopsy punch (Fisher, 0.1 cm diameter) was used to create inlet and outlet holes for 20 gauge PTFE tubing (Cole Parmer, EW-06417-31, Court Vernon Hills, IL). The patterned PDMS and glass slides were oxygen plasma etched for 1 minute at 30W and 0.4 Torr and then pressed together to bond the PDMS to the glass. Devices were then hard baked for 90 minutes at 85°C. Tubing was inserted into the PDMS without any adapter to connect devices to two syringe pumps.

**Nanoparticle Formation and Lyophilization**

Bulk mixed nanoparticles were formed by mixing plasmid DNA (0.06 mg/mL) and polymer solutions at 1:1 v/v ratio in 25 mM sodium acetate (NaAc, pH=5.0) to give the stated weight-weight ratios. Ten minutes of incubation were then allowed for particle formation before analysis or addition to cells. Bulk lyophilized nanoparticles were formed as described above, then supplemented with sucrose to a concentration of 30 mg/mL and frozen at -80°C. Bulk drip nanoparticles were formed as described above with the DNA solution supplemented with sucrose before being mixed with the polymer solution. Bulk drip nanoparticles were then dripped via pipet into a tube of liquid nitrogen to allow for near instantaneous freezing, mirroring nanoparticles formed in the microfluidic process. Lyophilization was achieved for all samples using a Labconco (Kansas City, MO) lyophilizer set to -45°C maintaining a pressure of approximately 14 Torr.

Microfluidic mixed nanoparticles were formed with the assembly shown in Figure 4-2A using the microfabricated PDMS/glass chip and two syringe pumps to drive rapid chip-based mixing of aqueous polymer and DNA solutions. For all microfluidic experiments, the first syringe pump for controlling polymer
solution flow rate was set to be two times the flow rate of the second syringe pump for controlling DNA flow rate. For all microfluidic prepared nanoparticles, DNA was diluted in 25 mM NaAc buffer to 0.09 mg/mL and mixed in a 1:2 ratio with polymer solution to give a final DNA concentration of 0.03 mg/mL. For lyophilized nanoparticles, the DNA solution was additionally supplemented with sucrose to give a post-mixing sucrose concentration of 30 mg/mL.

**Nanoparticle Tracking Analysis**

Nanoparticle hydrodynamic diameters and concentrations were determined by nanoparticle tracking analysis using a Nanosight NS500 with a 532 nm laser at 25°C (Malvern, Westborough, MA) running software version 3.1. A minimum of three samples were independently prepared for each nanoparticle formulation using PBAE 446 and Cy3 labeled plasmid DNA at concentrations as outlined in the transfection methods section. Ten minutes were allowed for nanoparticle self-assembly in 25 mM sodium acetate (NaAc, pH=5.0), after which each sample was diluted in 150 mM PBS (pH = 7.4) between 500-2000 fold using a two or three step dilution to give a Nanosight particle concentration of 20-80 particles per frame. Each sample was then loaded into a disposable 1 mL plastic syringe and perfused into the Nanosight chamber. A fluorescent 565 nm long pass filter was inserted and three 60 second captures were made at the fluorescent stage position. Immediately after capture, analysis was postponed and another three 60 second captures were made with the fluorescent filter removed and the stage at the scatter position. For purposes of determining average number of plasmids per nanoparticle, the number of plasmids/mL was calculated to be 5.824*10^{12} plasmids/mL at a concentration of 0.03 mg/mL given a plasmid size of 4700 base-pairs and approximate molecular weight of 3,102 kDa. This value was then used in relation to Nanosight concentration measurements and number or volume weighted diameter distributions to estimate the number of plasmids per nanoparticle.

**Cell Culture**

Patient derived human glioblastoma cells (GB319) were grown as adherent cells on tissue culture flasks in DMEM/F12 (Thermofisher) with 10% heat inactivated fetal bovine serum (Thermofisher) and 1% antimitotic/antibiotic (Thermofisher). Human triple-negative breast cancer cells (MDA-MB-231) and murine melanoma cells (B16-F10) from ATCC were grown as adherent cells on tissue culture flasks in DMEM (Thermofisher) supplemented with 10% heat inactivated bovine serum and 1% penicillin/streptomycin. All cells were incubated at 37°C in a 5% CO₂ environment.

**Transfection and Viability Assessment**

For transfection efficacy experiments, cells were plated in CytoOne 96-well tissue culture plates (USA Scientific) plates 24h prior to transfection with 15,000 cells/well in 100 µL media. PBAE nanoparticles were formed as described in the previous section detailing nanoparticle formation and lyophilization.
Lipofectamine® 2000 (Thermofisher) and 25 kDa branched polyethylenimine (PEI) were used as positive controls for transfection. Lipofectamine® 2000 was prepared in Opti-MEM 1 with GFP plasmid DNA according to manufacturer instructions at a ratio of 1x. PEI was prepared in 150 mM sodium chloride at weight/weight ratios of 1, 2, 4 and 8 to GFP plasmid DNA. Twenty microliters of the nanoparticle solution were added to each well of cells containing 100 µL of complete media and allowed to incubate for two hours, at which point the media was replaced. Transfection efficacy was assessed for percent-transfected cells and geometric mean expression approximately 48 h following transfection using an Accuri C6 flow cytometer and Hypercyt autosampler. Cell viability was assessed using MTS Celltiter 96 Aqueous One (Promega, Madison, WI) cell proliferation assay at 24 hours post transfection.

Data Analysis, Statistics and Figures
Results are reported as mean ± standard error of the mean (SEM) unless otherwise specifically stated. Matlab (Mathworks, Natick, MA) was used for quantitative analysis of Nanosight data. FlowJo (Ashland, OR) was used for flow cytometry gating as shown in Supplementary Figure 4-S1. ImageJ with FigureJ were used for image analysis and figure assembly. ChemBioDraw was used for schematics. Prism 6 (Graphpad, La Jolla, CA) was used for all statistical analyses and curve plotting. To assess statistical significance between differences in transfection efficacy, one-way ANOVA was performed using Dunnett’s method for multiple comparisons. To assess statistical significance between differences in nanoparticle concentrations, multiple t-tests were performed using the Holm-Sidak method to correct for multiple comparisons. Statistical significance notation: *(p<0.05); **(p<0.01); ****(p<0.001); *****(p<0.0001). The absence of statistical significance notation on figures where a statistical test was stated to have been performed indicates that no statistical significance was obtained.

Results

Polymer synthesis
Following the naming scheme for PBAEs established in previous publications, PBAE polymer 446 was synthesized in a ratio of 1.1:1 for the acrylate to amine small molecule monomers as shown in Figure 4-1. PBAE 446 was selected for this study as it has been shown to be effective in a wide variety of cell types and demonstrated minimal autofluorescence, which was necessary for use in nanoparticle tracking analysis assays. PBAEs containing ring structures in the backbone or endcap were observed to have sufficient autofluorescence to interfere with fluorescent nanoparticle tracking analysis measurements using the 532 nm laser and 565 nm long-pass filter of the Nanosight NS500. GPC was used to determine the $M_N$ and $M_W$ of PBAE 446 to be 7.4 kDa and 14.0 kDa respectively with a PDI of 1.89 relative to polystyrene standards. $^1$H NMR of the endcapped polymer, shown in Supplementary Figure 2, was used to estimate the $M_N$ of PBAE 446 to be 6.2 kDa with a degree of polymerization of 21.
Figure 4-1. PBAE synthesis from small molecule monomers. PBAE base polymer (B4S4) was synthesized by step growth polymerization via Michael addition reactions from monomers B4 and S4. The base polymer was then dissolved into THF and endcapped with small molecule E6 to yield PBAE 446.

Microfluidic chip fabrication and evidence against DNA shearing

A microfluidic chip design shown in Figure 4-2 was designed based on previously reported chips in the literature for hydrodynamic flow focusing devices capable of rapid, transition state mixing of aqueous solutions of cationic polymer and anionic plasmid DNA.23,43,44 COMSOL Multiphysics simulations of laminar, incompressible flow with convection and diffusion of dilute species were performed for microfluidic chip designs with minor variations in the chip dimensions and design. The chip was purposefully designed to confine uncomplexed DNA to the center of the channel via flow focusing in both the vertical and horizontal axes via the hairpin turn and pinch channels respectively. At the primarily utilized flow rate of 45 mL/h, simulation of plasmid DNA and polymer only particle diffusion resulted in equilibrium being reached less than 10 milliseconds following the point at which the two aqueous solutions come into contact.
Figure 4-2. Schematic of microfluidic device for 3D-HF for polyplex formation. (A) The chip was designed to be run with two syringe pumps flowing aqueous solutions of PBAE polymer and plasmid DNA. Lyophilized particles from the microfluidic device were frozen by allowing the particles to drip directly into liquid nitrogen. (B) The negative mask designed in Autocad for the microfluidic device with single inlets for aqueous polymer and DNA solutions. The mixing domain is outlined in red. (C) The schematic shows the dimensions of the microfluidic chip mixing domain with 100 µm feature height. (D) The COMSOL multiphysics simulation of laminar flow with transport of diluted species showed rapid mixing of DNA, shown here as concentration isosurfaces as a function of the final DNA concentration. Simulation showed focusing of the DNA in the vertical z-dimension to occur via inertial focusing around the hairpin turn followed by focusing in the y-dimension via 90° pinch channels. At flow rates used for in vitro experiments, the simulation showed complete mixing in less than 10 ms.

For the microfluidic channels, flow was calculated to remain in the transition domain between
laminar and turbulent flow with a maximum Reynolds number up to 125 for the primarily utilized flow rate of 45 mL/h. High shear forces in microfluidic devices can result in nicks to the DNA backbone that cause relaxation of plasmid DNA from the more effective supercoiled state to the relaxed open circular form.\textsuperscript{45,46} Three dimensional hydrodynamic flow focusing devices have been shown not to result in DNA shear previously\textsuperscript{23}, which we confirmed for this device via simulation (Supplementary Fig. 4-S3) and gel electrophoresis (Fig. 3A). Shear was shown via simulation to occur as anticipated near the top, bottom and sides of the channel with maximum shear occurring immediately after the horizontal pinch channels. As shown in Supporting Information Fig. 4-3, uncomplexed DNA was confined to the center of the channel and minimal if any DNA shear was anticipated. To confirm no evidence of DNA shear, we ran plasmid DNA through the MF chip at various flow rates up to 240 mL/h and then separated the supercoiled and relaxed circular DNA using gel electrophoresis (Fig. 4-3A).

![Figure 4-3. Gel electrophoresis of plasmid DNA.](image)

(A) At total flow rates up to 240 mL/h, plasmid DNA was not sheared as evidenced by the lack of a DNA smear by gel electrophoresis. (B) PBAE 446 was 100% effective at binding plasmid DNA at 10, 30, 60 and 90 w/w ratios of polymer to DNA.

**Nanoparticle characterization via nanoparticle tracking analysis**

Nanoparticle tracking analysis (NTA) was used to characterize PBAE 446 polyplex nanoparticles formed at different w/w ratios with plasmid DNA via bulk or MF mixing. Certain PBAE nanoparticles have been documented previously to form polymer only micellar nanoparticles due to their amphiphilic structure even in the absence of DNA.\textsuperscript{7} To assess the characteristics of nanoparticles specifically containing plasmid DNA and differentiate those nanoparticle properties from the properties of the particles only containing polymer,
plasmid DNA was labeled with the fluorophore Cyanine-3 (Cy3). Cy3 labeling enabled those nanoparticles containing plasmid DNA to be detectable by fluorescence using NTA while non-fluorescent nanoparticles containing no plasmid DNA would be filtered out and not tracked. To validate that DNA was fully bound to polymer at all weight-weight ratios tested, gel electrophoresis was used to show retardation (Fig. 4-3B). Using these methods, the total nanoparticle and DNA containing nanoparticle diameter distributions of bulk prepared PBAE nanoparticles were compared at different w/w ratio as seen in Figure 4-4. Interestingly, for the total nanoparticle size distribution, as w/w ratios increased, the size distribution approached that of PBAE only (DNA-free) nanoparticles (Fig. 4-4A). In contrast, nanoparticles containing plasmid DNA maintained approximately the same size distribution across the tested w/w ratios (Fig 4-4B).

![Graphs](image)

**Figure 4-4.** Bulk prepared nanoparticle properties assessed using NTA. (A) As w/w ratio increased from 10 w/w, the diameter distribution of all nanoparticles approached that of PBAE only nanoparticles formed in
the absence of DNA. (B) In contrast, nanoparticles containing plasmid DNA as assessed by fluorescent NTA showed only minimal differences in the diameter distribution between the tested w/w ratios. (C) The concentration of all nanoparticles and those nanoparticles specifically containing plasmid DNA were analyzed by fluorescent and non-fluorescent NTA. An increase in w/w ratio was shown to increase the total nanoparticle concentration assessed using multiple t-tests performed using the Holm-Sidak method to correct for multiple comparisons while the concentration of nanoparticles containing plasmids did not statistically change when assessed using one-way ANOVA. Bars show ± SEM with n ≥ 4 independently prepared samples. (D) The average number of plasmids per nanoparticle was assessed at the different w/w ratios using the volume-weighted and number-weighted diameter distributions. Bars show ± SEM of n ≥ 4 independently prepared samples.

NTA also enabled determination of total nanoparticle concentration and DNA containing nanoparticle concentrations for the samples (Fig. 4-4C). Total nanoparticle concentration was shown to increase with increasing w/w ratio between polymer/DNA, but DNA containing nanoparticle concentration did not statistically change with increasing w/w ratio when assessed using multiple t-tests performed using the Holm-Sidak method to correct for multiple comparisons. Using the known plasmid DNA concentration, knowledge that all of the DNA was fully encapsulated in the nanoparticles (Fig. 4-3B), and the measured particle size distributions, the number of plasmids per nanoparticle was then estimated (Fig.4-4D). The number-weighted average of plasmids per particle was between 8-12 plasmids, whereas the volume-weighted average of plasmids per particle was 14-20. The number-weighted average gives an estimate of the number of plasmids in a randomly chosen nanoparticle, whereas the volume-weighted average gives an estimate of the number of plasmids in the same nanoparticle as a randomly chosen plasmid. Most nanoparticles containing DNA were estimated to contain fewer than 20 plasmids as can be seen in from the volume and plasmid per particle distributions in Supplementary Figure S-S4. In comparison to our previously reported plasmids per particle findings with related PBAE/DNA nanoparticles composed of differently structured polymers, our volume-weighted plasmid per particle findings with polymer 446 are smaller than what was reported with polymers 551, 537, and 447. This difference may be due to the differences in the polymer structures and/or may be attributable in part to the previous estimate having been made using an older version of the Nanosight hardware (LM10) and software.

To determine any effect MF mixing may have on nanoparticle characteristics, MF mixed nanoparticles were prepared equivalently at a 30 w/w concentration and total flow rate of 45 mL/h. For freshly prepared PBAE nanoparticles, microfluidic mixing had no effect on the particle size distribution of all nanoparticles or DNA containing nanoparticles (Fig. 4-5A). For a therapeutic application, these hydrolytically degradable nanoparticles must be capable of being stably stored dry for long periods of time. To evaluate
the feasibility of long-term dry storage, bulk and MF mixed nanoparticles were lyophilized and analyzed by NTA following resuspension in water. For both bulk and MF mixed nanoparticles, the nanoparticle size distribution was not strongly affected by lyophilization (Fig. 4-5B). However, the concentration of total nanoparticles before and after lyophilization was shown to be dependent on the mixing method (Fig. 4-5C), as the total nanoparticle concentration of bulk prepared nanoparticles significantly decreased (P<0.001 when multiple t-tests were performed with Holm-Sidak method) with lyophilization while the DNA containing nanoparticle concentration decreased to a smaller degree following lyophilization as well. Presumably, this indicates a loss of the polymer-only nanoparticles during freezing and lyophilization as well as a partial decrease in DNA-containing nanoparticles too. In contrast, the nanoparticles formed by microfluidic mixing contain relatively few polymer-only (DNA-free) nanoparticles, and the concentrations of both total and DNA containing nanoparticles formed by microfluidic mixing was not observed to change significantly with lyophilization (Fig. 4-5C).

Figure 4-5. NTA diameter distribution and concentration of MF and bulk mixed 30 w/w PBAE 446 nanoparticles. (A) For 30 w/w nanoparticles, there was no significant difference in the nanoparticle diameter distribution with the different mixing methods or between all nanoparticles and those nanoparticles containing plasmid DNA. (B) The diameter distribution also did not change following lyophilization of the nanoparticles. (C) NTA concentration measurements comparing fresh and lyophilized nanoparticles did show a difference in particle concentration upon lyophilization for the total nanoparticle concentration of bulk mixed nanoparticles only. Bars show ± SEM with n ≥ 4 independently prepared samples.

Transfection efficacy of three cancer cell lines
Efficacy of the PBAE nanoparticles formed via either MF or bulk mixing was assessed by reporter gene expression two days following transfection in three cancerous cell lines of variable transfection difficulty. The primary, patient derived human glioblastoma cells (GB319) used have been shown to be well-transfected by
PBAE nanoparticles, with over 70% transfection efficacy previously reported. Murine melanoma cells (B16) were selected as a more difficult to transf ect cell line and human triple negative breast cancer (MDA-MB-231) cells were selected as a very difficult to transf ect cell line. In vitro transfection efficacy of the three cell lines with PBAE 446/DNA nanoparticles at three weight/weight ratios of polymer:DNA was assessed using flow cytometry in terms of percent of cells transfected (Fig. 4-6A) and geometric mean fluorescence (Fig. 4-6B) from GFP expression. For freshly made PBAE nanoparticles formed via MF mixing, no difference in transfection efficacy was detectable when compared to fresh PBAE nanoparticles formed via bulk mixing, as was evident from both flow cytometry data and fluorescence microscopy images (Fig. 4-7) from treated cells. Additionally, MF mixing did not statistically affect cytotoxicity for freshly prepared nanoparticles when compared against the fresh bulk mixed nanoparticles at the same dose (Fig. 4-6C).

Positive transfection controls Lipofectamine® 2000 and branched PEI were prepared in parallel and used to transf ect the same three cell lines with three different doses tested for each material (Supplementary Fig. 4-S5). PEI was minimally effective even with evident cytotoxicity, while Lipofectamine® 2000 was moderately effective for transf ection in some of the cells at a 1:1 w/w ratio. However, PBAE 446 was more effective for transf ection while maintaining high cell viability in all three cell lines compared to either PEI or Lipofectamine® 2000.
Figure 4-6. Flow cytometry assessment of transfection efficacy of in three cell lines using PBAE 446 nanoparticles prepared either fresh and lyophilized at three w/w ratios. Lipofectamine® 2000 and 25 kDa PEI were included as transfection controls. (A) The percent of cells transfected ranged from 2% to 92%. Conditions with significantly lower transfection efficacy than the fresh bulk mixed nanoparticles are denoted by α, while conditions with significantly lower transfection than MF lyophilized nanoparticles are denoted with β. Statistical tests were performed as one-way ANOVA using Dunnet’s method for multiple
comparisons of each treatment against either bulk fresh or MF lyophilized nanoparticles. (B) Geometric mean expression, presented on a logarithmic scale showed clear differences in efficacy of transfection between MF and bulk or BD lyophilized nanoparticles. Symbols α and β again corresponded to significant differences in geometric mean expression compared to the bulk fresh and MF lyophilized nanoparticles respectively. (C) Cellular viability assessed by MTT metabolic activity assay 24 hours post-transfection showed minimal cytotoxicity under nearly all conditions. Only GB319 cells treated with 60 w/w and 90 w/w nanoparticles showed a drop in cellular viability. Lipofectamine® 2000 showed significant toxicity in all cell lines. Bars show ± SEM with n=4 wells.

Figure 4-7. Transfection efficacy using freshly prepared PBAE nanoparticles with GFP plasmid DNA. No differences in transfection efficacy between freshly made bulk and MF mixed PBAE 446 nanoparticles were observed in the three cell lines. Images show brightfield with GFP overlay and GFP fluorescence on the bottom with a fixed exposure time of 100 ms. Scale bar 200 µm.
Lyophilized nanoparticle efficacy

To prepare nanoparticles for storage in a lyophilized form, sucrose was added as a stabilizer to a final concentration of 30 mg/mL for all samples prior to freezing. Bulk prepared and lyophilized nanoparticles were frozen at -80°C, while nanoparticles coming out of the microfluidic chip effluent tube were allowed to drip directly into a container of liquid nitrogen as shown in Figure 4-2A to promote near instantaneous freezing. To determine if the method of freezing influences nanoparticle efficacy, control nanoparticles termed bulk drip (BD) were prepared by dripping the bulk mixed nanoparticles prepared with sucrose into a tube containing liquid nitrogen. For GB319 cells, transfection efficacy as measured by both percent of cells transfected and geometric mean expression was generally not affected by lyophilization (Fig. 4-6A and Fig. 4-6B). Lyophilization of the bulk prepared nanoparticles did however significantly reduce transfection efficacy in the more difficult to transfect B16 melanoma and MDA-MB-231 human breast cancer cell lines when compared against the bulk fresh prepared nanoparticles when assessed using one-way ANOVA (Fig. 4-6A and Fig. 4-6B, p<0.05 using Dunnett’s method for multiple comparisons). The manner of freezing for the bulk mixed nanoparticles prior to lyophilization was shown to have no significant impact on transfection efficacy, as the BD prepared nanoparticles rarely performed differently than the bulk lyophilized nanoparticles. Nanoparticles prepared by microfluidic mixing followed by drip freezing and lyophilization did perform statistically better than both bulk lyophilized and BD lyophilized nanoparticles (Fig. 6A and Fig. 6B, p<0.05 using Dunnett’s method for multiple comparisons). Transfection when using the lyophilized MF prepared nanoparticles was greater than lyophilized bulk prepared nanoparticles in multiple instances, particularly for 60 and 90 w/w formulations in the harder to transfect B16 and MDA-MB-231 cells (Fig. 4-8). To evaluate whether MF produced PBAE/DNA nanoparticles could be stably stored over multiple months, microfluidic prepared and lyophilized nanoparticles were stored for three months at -20°C and tested for transfection efficacy in the same three cell lines with no significant decrease in efficacy (Fig. 4-9). Specific statistical results are detailed in Supplementary Tables 1-4.
Figure 4-8. Transfection efficacy of cells with lyophilized PBAE 446 nanoparticles that were prepared by bulk, bulk drip (BD) or MF methods. No differences in transfection efficacy were observable for GB319 cells, while transfection was visibly greater when using the MF prepared lyophilized nanoparticles in harder to transfect B16 and MDA-MB-231 cells. Images show brightfield with GFP overlay and GFP fluorescence on the bottom with a fixed exposure time of 100 ms. Scale bar 200 µm.
Figure 4-9. PBAE nanoparticles prepared by microfluidic mixing retained efficacy for at least three months following lyophilization and storage at -20°C. Bars show ± SEM with n=4 wells.

Multiple plasmid delivery

To confirm the results from NTA indicating the ability of the PBAE nanoparticles to contain multiple plasmids within one nanoparticle transfections were performed transfections using plasmid DNA premixed with two reporter genes on separate plasmids. In the three different cell lines MF produced PBAE nanoparticles enabled successful delivery of multiple types of DNA and co-expression (Fig. 4-10). Co-expression followed the same trends as delivery of a single gene and MF fabricated nanoparticles had the same levels of co-expression as bulk prepared nanoparticles.
Figure 4-10. Dual reporter gene delivery in PBAE nanoparticles. (A) An example micrograph of cells transfected with either GFP, dsRed or both plasmids pre-mixed with co-expression shown in yellow. Scale bar 200 µm. Flow cytometry analysis of cells transfected under the different conditions showed expression of both GFP and dsRed in a majority of cells for (B) GB319 cells while for harder to transfect (C) B16 and (D) MDA-MB-231 cells, a larger fraction of transfected cells were expressing only one reporter gene. No
significant differences between nanoparticles prepared by bulk or MF mixing methods were observed in the reporter gene expression. Bars show ± SEM with n=4 wells.

Discussion

Polymeric gene delivery has the potential to address a wide variety of maladies through the delivery of exogenous DNA, siRNA or miRNA but has been slow to translate from the benchtop to the clinic. Our group has demonstrated improved tumor survival following polymeric nanoparticle delivery of plasmid DNA specifically in an orthotopic brain cancer rat model via suicide gene delivery and through transfection of cancer-honing mesenchymal stem cells that lead to a reduction in stem-characteristics of tumor initiating cells. Other groups have demonstrated similar approaches for polymeric nanoparticle delivery of plasmid DNA in the lungs for treatment of cystic fibrosis, demonstrating the potential for clinical translation of polymeric particles for delivery of DNA. A related challenge is the robust and scalable manufacture of such nanobiotechnology for use in the clinic, a challenge that the developed microfluidic system for gene therapy aims to address. To date, no non-viral gene delivery formulations have successfully passed through clinical trials in the US.

Translation of nanoparticle formulations to the clinic requires reproducible, large-scale production to be an economically viable approach. Here we have demonstrated continuous flow assembly via microfluidic mixing of PBAE/DNA nanoparticles effective for delivery of plasmid DNA to a variety of cancerous cell lines. Continuous processes are advantageous for manufacturing compared to batch processes as they can be run in an uninterrupted fashion, easily scaled up without changing the properties of the product, and can effectively eliminate batch-to-batch variability. To this end, many nanoparticle formulations prepared by microfluidic means have recently been demonstrated with equivalent or improved efficacy from freshly made nanoparticles. Unlike some microfluidic platforms for nanoparticle formation, our device does not require two immiscible phases or removal of organic solvents. Additionally, the lack of turbulent mixing and chip design for uncomplexed DNA confinement to the center of the channel, where shear forces were greatly reduced, was shown to prevent DNA shearing that could lead to DNA relaxation and lower rates of transfection.

While continuous flow formation of nanoparticles is arguably more translatable than batch processes for clinical scale nanoparticle synthesis, the rate of nanoparticle production from a microfluidic process has been a concern in many studies. In this study, nanoparticles were produced at a rate of approximately 100 mg/h from a single device operating at a relatively fast flow rate of 45 mL/h. At this flow rate and the concentrations used in the study, an estimated clinically relevant size dose (100 mg) could be produced in one hour based on scale-up from nanoparticle doses shown to be effective in rat brain cancer. Larger doses could
be manufactured by increasing the DNA concentration, running the device for longer periods of time, and/or running multiple devices in parallel.\(^3\)

Another important feature of nanoparticle manufacturing is the evaluation of the product stability following continuous microfluidic assembly and long-term storage. Storage of polyplex nanoparticles is particularly challenging, as they exist in a partial dynamic equilibrium while in aqueous solution that can lead to aggregation over time.\(^23,49\) PBAEs in particular are not amenable to long-term storage in aqueous solutions as they possess hydrolysable ester linkages in the backbone of the polymer and are subject to degradation with a bond half-life on the time span of hours depending on the pH of the solution.\(^20\) To overcome this challenge, our group has demonstrated long-term storage of lyophilized, bulk mixed, sucrose stabilized PBAE nanoparticles that maintained comparable transfection efficacy in terms of percent of cells transfected for some cell types through two years when stored at -20°C.\(^36,49\) Geometric mean expression was noted to be variable depending on sucrose concentration and length of time stored to -20°C.\(^36\) Here, utilizing bulk nanoparticle fabrication methods, we likewise observe no decrease in transfection efficacy of glioblastoma cells (GB319) following lyophilization of bulk nanoparticles but did observe modest decreases in transfection of melanoma (B16) and breast cancer (MDA-MB-231) cells. Microfluidic produced PBAE nanoparticles, in contrast, retained efficacy in all cell lines following lyophilization, including after storage for three months. This finding is highly significant as it ensures robustness, scalability, and stability of these MF fabricated nanoparticles over time.

We developed a new assay to evaluate plasmid-containing polyplex nanoparticles utilizing new NTA hardware and software on the NS500 with the capability of discerning fluorescent nanoparticles that contain plasmid DNA from non-fluorescent nanoparticles. The estimated number of plasmids per PBAE nanoparticle from this method agrees with those published for bPEI nanoparticles analyzed by single particle microfluidic analysis when scaled by particle volume, providing validation for our method.\(^55\) While NTA does require dilution of the sample for analysis, we hypothesize that the characteristics of the particles analyzed following dilution accurately represent the characteristics of the nanoparticles as they would exist following effective dilution for \textit{in vitro} or \textit{in vivo} use. In contrast to methods such as TEM\(^56\) that have previously been used to estimate the number of plasmids contained in a nanoparticle, relatively little perturbation of the particles occurs with our methods utilizing NTA. We showed that for PBAE 446/DNA nanoparticles formed at higher w/w ratios, there was a significant difference between the measured concentration of all nanoparticles and those nanoparticles specifically containing plasmids. Using fluorescent NTA, we studied the effect of polymer to DNA weight/weight ratio on the particle size distribution of all nanoparticles and the particle size distribution of those nanoparticles specifically containing plasmids. Interestingly, the number of nanoparticles containing plasmid DNA and the particle size distribution of those particles remained effectively constant from 30 w/w through 90 w/w.
Microfluidic mixing, particularly three-dimensional hydrodynamic flow focusing used here, has been shown to reduce nanoparticle sizes of some polyplex nanoparticles that only form when they electrostatically complex with DNA and have their high density of positive charges rapidly neutralized.\textsuperscript{23,32} In contrast to other types of polyplex nanoparticle formations, microfluidic mixing did not result in a statistically different PBAE/DNA nanoparticle size distribution when analyzed via NTA. We hypothesize that this result may be attributable to the fact that PBAE polymer is less charged and more amphiphilic than other cationic gene delivery polymers, such as PEI. Differences in freshly made nanoparticles formed via bulk or microfluidic mixing indicated a difference in the fraction of nanoparticles containing plasmid DNA, as there were fewer polymer only, or DNA-free nanoparticles measured when assembled via microfluidic mixing.

For PBAE/DNA nanoparticles lyophilized with sucrose, we find that microfluidic mixing enables enhanced stability and transfection efficacy compared to bulk prepared lyophilized nanoparticles. These readily scalable methods of producing stable and efficacious self-assembled biodegradable PBAE/DNA nanoparticles are promising for translational utilization.

**Conclusions**

We have demonstrated a continuous flow process for microfluidic mixing and lyophilization of highly effective PBAE/DNA nanoparticles for plasmid DNA delivery. The microfluidic assembled lyophilized nanoparticles were more effective than bulk assembled lyophilized nanoparticles in multiple cell lines and enabled co-delivery of multiple plasmids. As these microfluidic assembled, biodegradable nanoparticles were demonstrated to be efficacious, safe, robust, easily scalable, and stable following long-term storage, they may be promising biomaterials for therapeutic non-viral gene therapy.

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**Conflicts of interest.** The authors declare no conflicts of interest.

**References:**


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48. Sunshine JC, Peng DY, Green JJ. Uptake and transfection with polymeric nanoparticles are dependent on polymer end-group structure, but largely independent of nanoparticle physical and chemical properties. Mol Pharm 2012;9(11):3375-83.
Supporting Information:

Supplementary Figure 4-S1. Flow cytometry gating. (A) Singlet cells were gated as shown in frame one and two for each cell population. Cells expressing GFP were gated against the untreated population as shown in frame 3. Gates were adjusted to have an average of 0.1% false positive cells in the untreated control well. (B) Cells were gated into four regions for untransfected (Region 1), GFP expression (Region 2), dsRed expression (Region 3) or dual reporter gene expression (Region 4).
Supplementary Figure 4-S2. $^1$H NMR (500 MHz Bruker) of end-capped and diethyl ether purified PBAE 446 in CDCl$_3$. Shifts were integrated and estimated $M_N$ was determined to be 6.2 kDa with a mean repeat unit number of 21. Some diethyl ether with a shift at $\sim$1.4 ppm from ether precipitation to eliminate excess endcap monomer remained at the time of NMR but was removed by one more day of storage in a vacuum chamber.
**Supplementary Figure 4-S3.** COMSOL simulation of laminar flow and transport of dilute species. (A) The numbered cut lines show locations of cross sectional frames used to assess DNA mixing and shear rate. (B) Calculated shear rate was greatest near the walls immediately downstream of the pinch channels (frame 4) at the primarily utilized flow rate of 45 mL/h. (C) At the flow rates used for all *in vitro* experiments, DNA was shown to be fully mixed with the solution of polymer within 10 ms of initial contact. Cross sections of DNA concentration showed flow focusing in both the vertical and horizontal directions, preventing uncomplexed DNA interaction with the channel walls at sites of high shear rate.
Supplementary Figure 4-S4. Number-weighted (A) volume and (B) plasmid distributions were calculated along with volume-weighted (C) volume and (D) plasmid distributions of DNA containing bulk prepared PBAE nanoparticles. Distribution shapes demonstrate that the majority of DNA-containing nanoparticles contained 30 plasmids or fewer.
Supplementary Figure 4-S5. Positive controls Lipofectamine® 2000 and 25 kDa branched PEI were tested at three different weight/weight ratios in the three cell lines. (A) Microscopy images of brightfield with overlay and GFP with 100 ms exposure overlay showed substantial transfection only for B16 cells and substantial cytotoxicity in both GB319 and MDA-MB-231 cells. Scale bar 200 μm. (B) Transfection efficacy was quantitatively assessed using flow cytometry and cell viability was assessed by MTT cell metabolic assay. Lipofectamine® 2000 and PEI both showed substantial cytotoxicity in the three cell lines and only Lipofectamine® 2000 was able to transfect a substantial percent of cells. Bars show ± SEM with n=4 wells.
Table 4-S1. Statistical test of differences in transfection efficacy measured by percent of cells transfected. One-way ANOVA was performed using Dunnet’s method for multiple comparisons of each treatment to the control treatment of bulk, freshly prepared nanoparticles with P<0.05 noted as $\alpha$ in Figure 4-6a.

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Table 4-S2. Statistical test of differences in transfection efficacy measured by percent of cells transfected. One-way ANOVA was performed using Dunnet’s method for multiple comparisons of each treatment to the test treatment of MF lyophilized nanoparticles with P<0.05 noted as $\beta$ in Figure 4-6a.

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Table 4-S3. Statistical test of differences in geometric mean expression of all cells. One-way ANOVA was performed with using Dunnet’s method for multiple comparisons of each treatment to the test treatment of MF lyophilized nanoparticles with P<0.05 noted as β in Figure 4-6b.

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Table 4-S4. Statistical test of differences in geometric mean expression of GFP positive cells. One-way ANOVA was performed using Dunnet’s method for multiple comparisons of each treatment to the test treatment of bulk fresh nanoparticles with P<0.05 noted as α in Figure 4-6b.

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Chapter 5: The Role of Assembly Parameters on Polyplex Poly(Beta-Amino Ester) Nanoparticle Transfections

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Foreword: This work was completed with the extensive assistance of undergraduate student Mark Suprenant, after whom my first cat Mavid is named (a combination of D-avid and M-ark). The work in this manuscript published in Biotechnology and Bioengineering and clears up many of my concerns about using PBAEs for routine transfections in vitro and clears up many misconceptions about these materials regarding their susceptibility to freeze-thaw cycles, mixing ratios and other application notes. This will hopefully be informative to anyone using these materials for routine transfection experiments as the lab of Jamie Spangler is now doing with PBAE 446.

Abstract:
Intracellular delivery of nucleic acids to mammalian cells using polyplex nanoparticles remains a challenge both in vitro and in vivo, with transfections often suffering from variable efficacy. To improve reproducibility and efficacy of transfections in vitro using a next-generation polyplex transfection material poly(beta-amino ester)s (PBAEs), the influence of multiple variables in the preparation of these nanoparticles on their transfection efficacy was explored. The results indicate that the even though PBAE/pDNA polyplex nanoparticles are formed by self-assembly of polyelectrolytes, their transfection is not affected by the manner in which the components are mixed, facilitating self-assembly in a single step, but a timing for self-assembly of 5-20 min is optimal. In addition, even though the biomaterials are biodegradable in water, their efficacy is not affected by up to 8 freeze-thaw cycles of the polymer. It was found that there is greater stability of nucleic acid-complexed polymer as a polyplex nanoparticle compared to free polymer. Finally, by exploring multiple buffer systems, it was identified that utilization of divalent cation magnesium or calcium acetate buffers at pH 5.0 are optimal for transfection using these polymeric materials, boosting transfection several fold compared to monovalent cations. Together, these results can improve the reproducibility and efficacy of PBAE and
similar polyplex nanoparticle transfections and improve the robustness of using these biomaterials for bioengineering and biotechnology applications.

Keywords: Polyplex nanoparticle, gene delivery, poly(beta-amino ester), transfection

1. Introduction:

Non-viral gene delivery is a routinely used approach in the laboratory to introduce genetic materials to mammalian cells. Nanoparticles (NPs) are often used to mediate effective cellular uptake, endosomal escape, and delivery to either the cytosol or the nucleus. Cationic polymeric materials, including polyethyleminine (PEI) \(^1\) and poly(beta-amino ester)s (PBAEs) \(^2\)-\(^4\), have been used extensively for gene delivery both \textit{in vitro} and \textit{in vivo} with promising results but to date have been slow to advance to the clinic due largely to insufficient efficacy of the materials \(^5\).

In contrast to non-biodegradable PEI-based polymers, PBAEs are cationic polyesters that contain tertiary amines in the backbone of the polymer that facilitate rapid hydrolysis in aqueous solution, enabling PBAEs to be used with generally low cytotoxicity and effectively no risk of accumulation following repeat administration \textit{in vivo}. PBAE NPs have been demonstrated to yield higher transfection efficacy than many commercial reagents \textit{in vitro} and have been demonstrated to effectively deliver nucleic acids \textit{in vivo}, even leading to improved survival outcomes in multiple tumor models with delivery of various plasmid DNA cargoes \(^6\)-\(^7\). Many researchers performing simple \textit{in vitro} transfections may further benefit from the utilization of PBAEs for routine transfections for bioengineering and biotechnology applications due to their high efficacy, low cytotoxicity and rapid degradation rates that fully eliminate the polymers prior to peak gene expression approximately two days following transfection \(^8\)-\(^9\). The relative ease of synthesis of these materials and improvements in performance over many canonical transfection reagents makes PBAEs prime candidates for use as routine transfection reagents. Unlike many current commercial reagents, however, PBAEs are susceptible to degradation in aqueous solution, which may make them more sensitive to the manner in which these materials are stored and their NP derivatives are prepared.

To improve the reproducibility and efficacy of pre-clinical experiments using PBAE NPs and better enable high throughput methods for studying these materials, we performed assays to assess the influence of various experimental factors on transfection efficacy. To achieve this, we utilized two canonical, linear, end-capped PBAE polymers to transfect two well characterized cell lines with a reporter gene \textit{in vitro} and quantified expression via flow cytometry. Multiple experimental parameters were explored with a focus on factors that can affect polyplex self-assembly including order of polyelectrolyte addition during polyplex self-assembly, volume of polyelectrolyte components during self-assembly, environmental conditions during polyplex self-assembly, polyplex self-assembly incubation time, and influence of hydrolysis of polymer. This
approach enabled the discovery of parameters that produced optimized transfection as well as the determination of parameter ranges that ensure robust reproducibility.

2. **Experimental Section:**

2.1 **Materials**

Monomers for PBAE synthesis were purchased from vendors listed in Table S1. Stock solutions of 3 M sodium acetate (NaAc) (Sigma), 1 M HEPES (Quality biological), 1 M magnesium acetate (MgAc) (Boston Bioproducts), 1 M calcium acetate (CaAc) (VWR) were diluted to desired osmolarity and adjusted to set pH values. Buffer salts for 2-ethanesulfonic acid (MES), sodium citrate (Na2HCitr), sodium phosphate (Na2HPO4), magnesium citrate (MgHCitr) and calcium citrate (CaHCitr) were purchased from Sigma Aldrich. Branched PEI (BPEI, 25 kDa MW) was purchased from Sigma Aldrich. Plasmid eGFP-N1 (Addgene 2491) was used for assessment of transfection efficacy.

2.2 **Transfection Assessment**

All PBAE NPs were prepared in 25 mM NaAc, pH 5.0 at a 60 weight-weight (w/w) polymer/DNA ratio for all experiments and pipetting to mix as previously described unless otherwise noted. BPEI NPs were prepared in 150 mM NaCl by adding polymer to DNA in a 1:1 volume ratio at a 2 w/w ratio. Particles were added to HEK293T or MDA-MB-231 cells at DNA doses of 200 and 400 ng per well, respectively, in complete medium containing 10% serum and incubated for two hours, followed by a complete media change. Transfection efficacy was observed using a fluorescence microscope (Axiovert Observer A.1, Zeiss) and quantified using flow cytometry approximately 48 hours following transfection, using an Accuri C6 flow cytometer (BD Biosciences, Franklin Lakes, NJ) with Hypercyt high-throughput sampler and reader (Intelicyt Corp., Albuquerque, NM). Transfection efficacy is generally reported as percent transfection in MDA-MB-231 cells and as normalized geometric mean expression in HEK293T cells, as the transfection was typically too high across all PBAE transfection conditions (>90% cells positive) in this cell type to differentiate between conditions using percent transfection. Figure 5-S14 shows gating for flow cytometry analysis.

2.3 **Mixing Volume ratio**

PBAE NPs were prepared by pipet-mixing polymer and DNA at several volumetric ratios, with the polymer volume fractions varying from 0.15 to 0.95 in 25 mM NaAc buffer, pH 5.0. Follow-up experiments in which PBAE in DMSO was resuspended directly with dilute plasmid DNA were performed by aliquoting the PBAE in anhydrous DMSO (100 mg/mL) to tubes or round-bottom plates, then directly pipetting plasmid DNA pre-diluted in 25 mM NaAc, pH 5.0, to resuspend the PBAE for complexation into NPs.

2.4 **Pre-transfection incubation time**

To determine the influence of preparation time on transfection efficacy, NPs were prepared by pipet-mixing in a 1:1 volume ratio of dilute polymer and plasmid DNA at set times prior to addition of NPs to cells. For PBAE polymers, the additional step of dissolving polymer from anhydrous DMSO to aqueous
buffer was performed two minutes prior to mixing dilute polymer solution with dilute plasmid DNA. Additional experiments were conducted in which polymer was dissolved in 25 mM NaAc by vortexing and pre-incubated for set amounts of time prior to complexation with plasmid DNA; after mixing with DNA, NPs were further incubated 5 minutes then added to cells. For all presented experiments, NPs were then added to HEK293T and MDA-MB-231 cells at low doses (200 ng and 400 ng DNA, respectively) and incubated with cells for two hours, after which media was aspirated and replaced with 100 μL/well of fresh, complete medium.

2.5 Freeze-Thaw Cycles and Water Content

Individual PBAE aliquots at 100 mg/mL in anhydrous DMSO were subjected to repeated freeze-thaw cycles performed on subsequent days. For thaw only cycles, polymer aliquots were thawed at room temperature for one hour in a container with desiccant then refrozen at -20°C a set number of times. Under matched conditions for open-air thaw conditions, polymer aliquots were thawed at room temperature with desiccant for one hour, then opened to the laboratory atmosphere for 5 minutes, capped, and refrozen to simulate controlled multiple uses from a single polymer aliquot. After freeze-thaw cycles were completed, all polymer aliquots were stored at -20°C for one month then used for assessment of transfection efficacy as described above. For water fraction experiments, PBAE 4-4-6 in anhydrous DMSO at 100 μg/μL was diluted to 50 μg/μL with additional solvent so that the final aliquot contained between 0-50% water by volume. Aliquots were then frozen at -20°C for two months, thawed, and held at room temperature for set amounts of time prior to be used for transfection efficacy assessment as described above.

2.6 Polymer degradation:

Two replicates each of PBAE (free polymer) and PBAE/DNA (60 w/w NPs) were incubated in either PBS (pH 7.4), citrate buffer (pH 6), or sodium acetate buffer (pH 5.2) at 37°C. At each specified time point, samples were frozen and lyophilized to remove aqueous solvent. Polymer was then dissolved in THF, filtered, and analyzed by gel permeation chromatography (GPC) as previously described. Number average (Mn) and weight average (Mw) molecular weights were determined against linear polystyrene standards at each time point. One-phase decay plots were fit to degradation plots to determine ester bond half-life.

2.7 Buffer system

For the initial experiment, buffer solutions of sodium acetate (NaAc), 2-(N-morpholino)ethanesulfonic acid (MES), sodium phosphate (NaH₂PO₄), sodium citrate (Na₂HCitr), potassium acetate (KAc), and magnesium acetate (MgAc₂) were prepared as 1 M stocks in ultrapure water and sterile-filtered. Each stock buffer was split into four groups, diluted to 25 mM in ultrapure water, and adjusted to pH 4, 5, 6, or 7 assessed with a Mettler Toledo SevenEasy pH meter. PBAE NPs were formed in each buffer by resuspending PBAE polymer at 100 mg/mL in DMSO in a 96-well round-bottom plate, followed by mixing with dilute plasmid DNA using a multichannel pipette. NPs were then added to HEK293T and MDA-MB-
231 cells at low doses (200 ng and 400 ng DNA, respectively) and incubated with cells for two hours, after which media was aspirated and replaced with 100 μL/well of fresh, complete medium. Following results from the first experiment, additional buffers of calcium acetate (CaAc$_2$), magnesium citrate (MgHCitr), and calcium citrate (CaHCitr) were prepared at 25 mM concentrations and pH 5.0. Divalent cation citrate buffers were marginally soluble and used at their solubility limits, estimated to be approximately 4 mM. Composite buffers mimicking calcium phosphate transfection conditions were also utilized $^{11}$. Uptake experiments were performed with 20% of the plasmid DNA labeled with Cy5 as previously described $^{12}$.

2.8 Data analysis, statistics and Figures:

FlowJo (FlowJo, LLC) was used for flow cytometry analysis. Servier Medical Art (CC license) was used for illustrations. Prism 6 (Graphpad, La Jolla, CA) was used for all statistical analyses and curve plotting. For multiple comparisons between all tested conditions, ordinary one-way ANOVA with Tukey corrected multiple comparisons were performed. Similarly, for multiple comparisons tests against a specific control condition one-way ANOVA with Dunnett corrected multiple comparisons was performed. Following one-way ANOVA, a Brown-Forsythe test of equal variance was performed to assess if specific conditions possessed significantly different standard deviations. Unless otherwise specified, statistical tests were performed with a global alpha value of 0.05, and experiments were repeated at least twice with representative results shown. Unless otherwise stated, absence of statistical significance markings where a test was stated to have been performed signifies no statistical significance. Statistical significance is denoted as follows: *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001.

3. Results and Discussion

We sought to determine if incubation time influences transfection efficacy with these materials since PBAE polymers undergo hydrolysis following dissolution in aqueous solutions, with ester bond half-life usually between 2-6 hours $^{9, 13}$. The experiments revealed that the optimal aqueous incubation time for PBAE polymer prior to and after mixing with plasmid DNA are <10 minutes and between 5-20 minutes, respectively. Further exploring the influence of hydrolysis in stored aliquots of PBAE on NP efficacy, our results indicated that polymer aliquots stored at -20°C were stable over up to eight freeze-thaw cycles, making these materials promising for use as general-use transfection reagents where a single polymer aliquot can be used multiple times without concern of reduction in efficacy.

We further explored the influence of volumetric ratio of PBAE to DNA during NP mixing to show that, as long as NPs are sufficiently mixed, the volume of each solution prior to mixing made no difference in transfection efficacy. These results were further expanded to show that PBAE polymer in DMSO could be directly resuspended with dilute plasmid DNA in buffer to give equally effective NPs, which has implications for making NPs at high DNA concentrations where PBAE polymer solubility would otherwise limit the dose. Finally, we explored alternative buffer systems to the traditional pH 5 sodium acetate buffer used and
discovered that divalent cation magnesium and calcium acetate buffers improve transfection efficacy. Here, we explored the influence of assembly parameters on only two relatively hydrophilic PBAE structures which are representative of the most commonly used PBAE materials for gene delivery in the literature. PBAEs possessing more hydrophilic (such as PEG-diacrylate based) or hydrophobic (such as PBAE utilizing alkyl side chains) may perform differently.

**Polymer synthesis and characterization**

PBAE polymers were synthesized as previously described. PBAE 4-4-6 used in this study was characterized via GPC to have $M_n$ 5,170 Da, $M_w$ 10,220 Da, and dispersity (D) 1.98, while PBAE 4-5-39 was characterized to have $M_n$ 4,580 Da, $M_w$ 14,290 Da, and dispersity (D) 3.12. PBAE 4-5-39 is a new chemical entity using end-cap E39, which is similar to our previously reported E7 end-cap but is slightly more hydrophilic, possessing a secondary amine in the piperazine ring instead of a tertiary amine. Together, these two PBAE structures are representative of relatively hydrophilic PBAE structures (via the use of PBAE 4-4-6) and more hydrophobic structures similar to C32 (via 4-5-39).

**Pre-Transfection Incubation Time**

We performed experiments to test if the incubation time of PBAE NPs in buffer prior to adding to cells influenced transfection efficacy. Many transfection reagents recommend rapidly pipetting or vortexing aqueous solutions of transfection reagent and plasmid DNA followed by an undisturbed incubation period ranging from 5-30 minutes for DNA complexation. We hypothesize that the rapid degradation of PBAE polymers in aqueous solution might influence the optimal incubation time of these materials prior to adding them to cells. To explore this variable, we tested both the pre-incubation time effects of free PBAE polymer and the incubation time effects of complexed PBAE/DNA NPs (Fig. 5-1A). First, the pre-incubation time of PBAE polymer alone in aqueous solution was tested, showing decreases in efficacy following pre-incubation times greater than 10-20 minutes (Fig. 5-1B,C). This result was consistent across cell lines and with two PBAE polymers of varying hydrophobicity (Fig. 5-S2A,B). Following these results, we tested the effect of incubating PBAE/DNA NPs following NP formation to determine optimal incubation time to allow for effective polymer/DNA self-assembly and found that an incubation time of between 5-20 minutes was optimal (Fig. 5-1D,E and 5-S2C,D). While long incubation times showed decreases transfection efficacy presumably due to degradation, short incubation times of less than one minute following mixing of plasmid DNA and PBAE polymer also showed statistically lower transfection efficacy across polymers and cell types.
Figure 5-1. Effect of pre-incubation time of free PBAE polymer and of formed PBAE/DNA nanoparticles on transfection. A) Either PBAE 4-4-6 nanoparticles were formed and pre-incubated for set timepoints before adding to cells or PBAE 4-4-6 was pre-dissolved in 25 mM NaAc buffer and pre-incubated for set timepoints before forming nanoparticles that were then added to cells. PBAE polymer pre-incubated for 10 minutes or less prior to nanoparticle formation was most effective for transfection in (B) HEK293T and (C) MDA-MB-231 cells. Nanoparticles that were pre-incubated 5-20 minutes before adding to cells were most effective for transfection of (D) HEK293T and (E) MDA-MB-231 cells.

To identify why PBAE NPs were susceptible to optimal incubation times, we assessed the diameter of PBAE 4-4-6 free polymer and NPs over the time span of one hour in 25 mM NaAc buffer, pH 5. Supporting the transfection results, PBAE/DNA NPs were stable over one hour (Fig. 5-S3), indicating that PBAE/DNA NPs were effectively stable in acidic buffer over time. We also measured the degradation rate of free PBAE polymer and PBAE polymer/DNA complexes in aqueous buffer at different pH values (Fig. 5-S4). At pH 5.2 in NaAc buffer, the ester bond half-life of free PBAE polymer $M_N$ was only 1.6 hours, while PBAE/pDNA polyplexes was 3.5 hours (Table 5-S2). This difference in degradation rate for free PBAE polymer compared to PBAE/pDNA complexes was statistically significant as assessed by a one-phase decay model at pH 5.2 ($p<0.0001$ for both $M_N$ and $M_W$). Free PBAE polymer was further shown to degrade faster at pH 6 and pH 7.4 than pH 5.2, though there were no statistical differences between free PBAE polymer
and PBAE/pDNA polyplexes in degradation rate at higher pH values. This result is consistent with ours and others' previously published results showing that degradation of this class of polymers is more rapid at neutral pH $^{2,13,17}$ than at weakly acidic conditions. We hypothesize that when many of the amines in the PBAE backbone are not protonated (i.e., >pH 6), they behave as bases and can locally generate -OH species near the ester backbone. The -OH species is then free to act as a nucleophile and degrade the ester bonds. At lower pH values (<pH 6), when more amines in the PBAE backbone are protonated, the amines no longer generate as much hydroxide, and degradation of the polymer backbone is slowed, although this degradation is still significantly faster than the degradation of other polyesters such as PLGA, which degrade with a half-life on the order of weeks $^{18}$.

Together, these results support limiting the residence time of free PBAE polymer in aqueous solution to <20 minutes and keeping the PBAE polymer/DNA complex incubation time between 5-20 minutes for optimal transfection. In contrast, we performed a similar assay with BPEI/DNA and found that pre-incubation times between 10-60 minutes were not statistically different in transfection efficacy for this non-degradable cationic polymer (Fig. 5-S5). We hypothesize that the differences observed in the optimal pre-incubation time between PBAE NPs and BPEI NPs are primarily attributable to degradation associated with PBAEs.

**Mixing Volume Ratio**

Polyplex NPs of pDNA interacting with PBAEs and BPEI prepared by pipet mixing have traditionally been mixed in a 1:1 volume ratio of dilute nucleic acid to dilute polymer for simplicity $^{1,19}$. Other strategies for forming polyplex NPs, including microfluidic mixing $^{20}$ and flash nanoprecipitation $^{21}$, vary from this 1:1 mixing volume ratio only minimally, with nucleic acids accounting for at least one third of the total volume of solution. For many assays, including semi-automated high throughput screening situations, and for *in vivo* administration, however, the ability to vary the volume fraction of the polymer solution in forming polyplex NPs would be beneficial. For PBAEs, their amphiphilic nature makes them less water-soluble than many other polyplex NP systems with a solubility limit generally approximately 20 mg/mL in acidic buffer.

For this purpose, we sought to test if the volume fraction of mixing dilute PBAE polymer with dilute plasmid DNA influenced the transfection efficacy of the resulting NPs (Fig. 5-2A). Testing PBAE polymer volume fractions of 0.15-0.95 demonstrated that the mixing volume ratio did not have a statistically significant influence on the transfection efficacy in either cell line with PBAE 4-4-6 (Fig. 5-2B,C). These results were confirmed with PBAE 4-5-39 as well (Fig. 5-S6), where no statistically significant difference in transfection efficacy was noted among the different polymer volume fractions. The standard deviations of wells transfected with high polymer volume fractions were statistically higher as assessed by the Brown-Forsythe test of equal variance, but only for HEK293T cells transfected with PBAE 4-4-6.
Figure 5-2. Effect of polymer and plasmid mixing ratio. (A) PBAE NPs are not highly sensitive to the mixing volume ratio between dilute polymer and plasmid DNA. There were no statistical differences (one-way ANOVA with Tukey corrected multiple comparisons) between any transfected groups for (B) HEK293T or (C) MDA-MB-231 cells transfected with PBAE 4-4-6. The method of resuspending PBAE polymer in aqueous solution likewise did not influence the efficacy of the nanoparticles in (D) HEK293T or (E) MDA-MB-231 cells. Bars show mean ± SEM of four wells.

To facilitate high-throughput screening of polymeric NPs, we further sought to test if resuspending PBAE polymer by vortexing, resuspending by pipetting in a plate with buffer, or resuspending by pipetting in a plate with pre-diluted pDNA in buffer influenced the resulting transfection efficacy. The ability to resuspend PBAE polymer and form NPs in a single step simply by simply adding pre-diluted pDNA in buffer...
to a set volume of stock polymer at 100 mg/mL in DMSO would considerably facilitate rapid testing of these materials by skipping the polymer dilution step and enable parallelization in preparation of NPs. Surprisingly, we found no difference in transfection efficacy resulting from resuspending PBAE polymers in a round-bottom plate by forming the NPs in two steps or by direct resuspension to NPs in a single step by pipetting dilute pDNA in buffer to resuspend the polymer (Fig. 2D,E). Together, these results regarding methods of self-assembly are promising for using PBAEs in parallel semi-automated screening techniques and for their use as routine transfection reagents, robust to mixing technique variability among users.

**Freeze/Thaw Cycles**

PBAE polymers are notable for their rapid degradation in aqueous solution compared to traditional polyesters such as poly(lactide-co-glycolide) (PLGA) or polycaprolactone (PCL) \(^2,4\). The degradation half-life of the ester bond in the backbone of PBAE polymers has been measured to typically range from 2 to 6 hours depending on hydrophobicity \(^2,9\) and local pH \(^2,9,13,17\). To avoid the early degradation that would be expected to reduce efficacy of the polymers, PBAEs are traditionally stored in anhydrous DMSO at \(-20\)°C with silica desiccant, but the use of molecular sieves or other stronger desiccants is not needed. Utilization of PBAE polymers as routine in vitro transfection reagents or for clinical formulations requires sufficient stability during storage to ensure the polymers have the same efficacy after storage.

To address these concerns, we performed thawed PBAE aliquots in anhydrous DMSO repeatedly on subsequent days either under anhydrous conditions or under open-atmosphere laboratory conditions to simulate multiple opening-closing events for individual aliquots of polymer. After a set number of freeze-thaw cycles on subsequent days, polymer aliquots were stored at \(-20\)°C for one month, then thawed a final time and used for transfection. We hypothesized that the repeated freeze-thaw process or water absorption from the atmosphere would result in sufficient polymer degradation to reduce efficacy of the materials following multiple freeze-thaw cycles. DMSO is a highly hygroscopic solvent, and water absorption from the atmosphere can be a major limiting factor for high throughput compound library screening when compounds are stored as DMSO stocks \(^22\). As reported, open-air exposure of DMSO to typical laboratory air for one hour can result in absorption of 6% water by volume \(^22\).

Transfections were conducted on two cell lines with two PBAE structures (Fig. 5-3 and 5-S7) and surprisingly demonstrated no statistical change in transfection efficacy of these materials over eight freeze-thaw cycles for the PBAE aliquots exposed to anhydrous or open-atmosphere conditions compared to the control aliquot of polymer thawed only once. While the hygroscopic nature of DMSO was a concern, the amount of time the polymers spent at room temperature and open to laboratory air did not seem to appreciably influence the stability of the polymers stored at \(-20\)°C.
Figure 5-3. PBAE polymers are not prone to freeze-thaw induced reductions in efficacy. (A) Schematic of experimental outline for testing effect of freeze-thaw cycles on polymer efficacy. (B) HEK293T and (C) MDA-MB-231 cell transfection efficacy were not statistically different even with PBAE 4-4-6 having undergone eight freeze-thaw cycles (one-way ANOVA with multiple comparisons to the fresh polymer). Similarly, PBAE 4-4-6 aliquots pre-mixed to 50 mg/mL prior to storage with varying degrees of hydrated DMSO showed no loss in efficacy when stored at -20°C for two weeks and then used within an hour after thawing in (D) HEK293T or (E) MDA-MB-231 cells.

We next sought to determine if water absorption, specifically in anhydrous DMSO, and subsequent degradation when stored at -20°C has the potential to reduce efficacy of these materials over time. For this experiment, polymer aliquots were prepared with defined water content ranging from 0.25% to 50%, frozen at -20°C for two months, then thawed and used for transfection. Our results surprisingly showed that PBAE
4-4-6 lost no efficacy over two months when stored at -20°C and used immediately after thawing, even with 50% water solutions (Fig. 5-S8). The amount of time that polymer spent in hydrated DMSO at room temperature did, however, affect transfection efficacy. Aliquots stored with 25% and 50% water by volume became completely ineffective at three and five hours of room temperature incubation respectively, while aliquots with 10% water by volume maintained equal efficacy to anhydrous polymer aliquots even after five hours of room temperature incubation (Fig. 5-3D,E). These results were consistent between both HEK293T and MDA-MB-231 cells, which we hypothesize is attributable to appreciable polymer degradation in hydrated DMSO solutions. Given the water absorption rate of DMSO, we recommend limiting room temperature incubation of these polymers to less than five hours and open-air exposure of the DMSO stocks to less than an hour to keep absorbed water content below 10%. With these recommendations, PBAEs are unlikely to suffer degradation induced reductions in efficacy. These results are largely consistent with previous reports of storing lyophilized PBAE NPs at 4°C and -20°C, demonstrating that the minute amounts of water absorbed from the air are effectively inconsequential as long as the materials are stored at -20°C and the amount of time at room temperature is limited to during use for transfection.

**Buffer System**

PBAE NPs have traditionally been prepared in low salt (25 or 50 mM), acidic, pH 5 sodium acetate (NaAc) buffer, which ensures tertiary amines in the backbone of the polymer are charged, facilitating complexation with anionic DNA. Due to the amphiphilic and pH-sensitive nature of PBAE polymers, buffer pH as well as osmolarity and valence were all expected to influence NP formation conditions by affecting the cationicity and hydrophobicity of the PBAE polymer as well as potentially influencing DNA structure. At pH 5, most tertiary amines of linear PBAE structures are protonated, whereas, at neutral pH, tertiary amines in the polymer backbone remain largely unprotonated. This increase in cationicity likewise influences the solubility of PBAE structures, as more highly charged cationic structures are able to undergo hydrogen bonding at low pH but are less soluble above pH 6.0.

The sodium acetate buffer system is notably monovalent for both ions, which are expected to participate minimally in interactions between cationic polymer and anionic DNA. To explore the influence of buffer valence, we evaluated buffer systems with both divalent cations and anions, which were anticipated to participate in binding interactions with either the cationic polymer or anionic DNA. Among anions, citrate buffers have long been used when forming liposomes and lipid NPs, typically at pH 4. Similarly, the zwitterionic buffer HEPES has often been used to buffer BPEI in 150 mM NaCl, but due to its limited neutral buffering range, we substituted MES as a single-component zwitterion buffer. Phosphate buffers have likewise been used in transfection, although primarily as a means to precipitate calcium phosphate DNA particles for low-efficiency calcium phosphate-based transfection.
We initially tested six biological buffer systems at 25 mM concentration and pH values of 4, 5, 6 and 7 with PBAE 4-4-6/pDNA polyplex NPs. Comparing by pH across all buffer systems, PBAE 4-4-6 yielded statistically higher transfection efficacy in both cell lines when using acidic buffers of pH of 4 or 5 compared to pH 7 buffers (Fig. 5-4A,B), although this result was not universal across all buffer systems and was more pronounced in HEK293T cells. No buffers resulted in dramatic differences in measured cell viability (Fig. 5-4C,D), and all buffer systems yielded some level of transfection. Magnesium acetate and sodium citrate buffers at pH 5 repeatedly yielded the highest transfection efficacy and were further explored in subsequent transfection screens in which we also included a calcium acetate (CaAc₂) buffer (pH 5, 25 mM).

Figure 5-4. Buffer effect on PBAE polyplex NP transfection efficacy. Buffers from six salts were prepared at 25 mM osmolarity and pH values of 4, 5, 6 and 7 and used to make PBAE 4-4-6 nanoparticles compared against the historical buffer of pH 5 sodium acetate (NaAc) shown as the hatched bar. Several buffers showed promising increases in transfection efficacy in (A) HEK293T and (B) MDA-MB-231 cells with effectively no difference in cytotoxicity (C,D). To assess the influence of buffer pH on transfection, we performed a Tukey-corrected matched comparisons one-way ANOVA across all buffer conditions with respect to pH to
demonstrate that pH 4 and pH 5 buffers were statistically the most effective buffers among all groups with significantly greater transfection than pH 7 buffers. Bars show mean ± SEM of four wells.

Divalent cation MgAc$_2$ and CaAc$_2$ buffers at pH 5 and 25 mM concentrations were observed to statistically improve transfection efficacy in all tested cases with PBAE NPs 4-4-6 and 4-5-39 in both HEK293T and MDA-MB-231 cells (Fig. 5-5 and 5-S9). The improvement in transfection efficacy was notable in both cell types as assessed by either geometric mean expression, where calcium acetate yielded five-fold higher expression in either cell type, and percent transfection, where calcium acetate yielded 20% and 50% higher number of cells detectably expressing GFP in HEK293T and MDA-MB-231 cells respectively. To identify why divalent cation buffers improve transfection efficacy, we measured the uptake of PBAE 4-4-6 NPs formed in different acetate buffers but found no statistically significant differences in the level of NP uptake in HEK293T cells (Fig. 5-S10). MDA-MB-231 cell uptake was statistically but only modestly higher with divalent cation buffers (Fig. 5-S10,C,D). We also measured the particle diameter and zeta-potential of NPs formed in NaAc and MgAc$_2$ buffers, which were observed to have the no statistically significant difference between particles prepared in either buffer (Fig. 5-S11). These results indicated that the presence of divalent cations was not leading to aggregation of the PBAE NPs or a change in their diameters that would bias them towards greater uptake or transfection efficacy exclusively due to size differences.

Figure 5-5. Acetate anion buffers with divalent magnesium or calcium cations improve transfection efficacy over monovalent sodium acetate buffer. PBAEs 4-4-6 and 4-5-39 had statistically higher transfection efficacy with MgAc$_2$ and CaAc$_2$ buffers than NaAc in (A) HEK293T cells and (B) MDA-MB-231 cells, shown as
normalized to the level of transfection efficacy in pH 5, 25 mM NaAc buffer. (C) Microscopy showed increase in geometric mean expression of HEK293T cells using divalent cation acetate buffers. Results show multiple experiments with four replicates each, normalized to NaAc buffer transfection efficacy for that experiment. Scale bars 200 µm.

To further examine how divalent cation buffers might be affecting transfection efficacy, we measured the degree of pDNA binding with PBAE polymer using a variety of assays. Gel electrophoresis retention assays with a PBAE w/w titration and heparin sulfate competition binding assay both showed slightly reduced binding efficacy between PBAE polymer and pDNA in MgAc₂ buffer compared to NaAc buffers (Fig. 5-S12). Competition binding assays performed using the DNA-binding carbocyanine nucleic acid dyes YO-PRO-1 and YOYO-1 were further consistent with gel retention assay results, indicating slightly stronger binding of PBAE polymers to pDNA in NaAc buffer (Fig. 5-S12,F,G). The presence of magnesium ions notably affected the fluorescence of YOYO-1 and YO-PRO-1 even in the absence of PBAE polymer, indicating that the concentration of divalent cation salts used were likely influencing DNA structure (Fig. 5-S12,C-E). YOYO-1 and YO-PRO-1 are known to primarily interact with DNA via intercalation, resulting in approximately 1000-fold higher fluorescence upon binding double stranded nucleic acids. In the absence of PBAE polymer, we observed a dramatic increase of over 40% in the fluorescence of YOYO-1 binding to pDNA in MgAc₂ buffer compared to that in NaAc, whereas YO-PRO-1 fluorescence decreased by 85% in MgAc₂ buffer compared to that NaAc buffer, which is consistent with the magnesium ion's influencing pDNA structure via electrostatic interactions.

The presence of magnesium, calcium and other divalent cations in solution has long been known to affect DNA structure, as hexahydrated magnesium ions bind the major groove of double stranded DNA and are involved in stabilization of single-stranded RNAs. Magnesium or calcium ions are further required to facilitate the DNA binding activity of many enzymes and affect the melting temperature of oligonucleotides in predictable fashions. To determine if the presence of magnesium and calcium improved transfection by changing NP properties or by changing cell phenotype due to their presence in media during transfection, we added extra divalent cation salts to cell culture media directly either before or after transfection with PBAE NPs formed in NaAc buffer (Fig. 5-6A). Interestingly, the increase in transfection efficacy associated with the use of divalent cation buffers only occurred when PBAE NPs were prepared directly with divalent cation acetate buffers and not when divalent cation salts were simply added to media (Fig. 5-6B,C). Magnesium sulfate and calcium chloride are present at 0.8 and 1.8 mM concentrations in DMEM, respectively, meaning that the addition of NPs formed in 25 mM divalent cation acetate buffer increased the media concentration of magnesium or calcium only by 5.2 and 2.3 fold respectively. This short duration of exposure to higher divalent cation salt concentrations was not expected to change cell phenotype directly in vitro. Magnesium and calcium ions are largely considered biocompatible with the normal range of blood plasma levels of
magnesium and calcium between 0.6-1.1 and 2.1-2.6 mM respectively. The fact that divalent cation buffers improved transfection only when used to form PBAE NPs and not when simply added to cell culture medium indicates that these buffers may likewise improve in vivo transfection efficacy.

Figure 5-6. Divalent cation buffers only improve transfection when used directly to prepare NPs instead of simply by being added to media. (A) To identify if divalent cations were affecting NPs or cells directly, NaCl, MgCl₂ or CaCl₂ were added at concentrations to mimic presence when using MgAc₂ or CaAc₂ buffers and added to cells either prior to or after removal of NPs prepared in NaAc buffers only. In parallel, NPs formed with divalent cations were complexed in NaAc, MgAc₂ or CaAc₂ buffers and added to cells. Transfection efficacy with PBAE 4-4-6 was improved only for NPs prepared in divalent cation acetate buffers in both (B) HEK293T and (C) MDA-MB-231 cells and not by addition of divalent cation salts to media. Bars show mean ± SEM of four well replicates.

Inspired by historical transfection systems using divalent calcium and phosphate buffers to co-precipitate plasmid DNA, we also explored using composite buffer systems that, once mixed, resulted in insoluble or marginally soluble composites designed to purposefully precipitate NPs of polymer and plasmid DNA (Fig. 5-S13). While calcium phosphate transfection is no longer commonly used due to its inconsistency
and low transfection efficacy (usually <10% cells), we hypothesized that the ability to selectively enrich the media in direct contact with cells with NPs containing plasmid DNA may prove useful. Our results indicated that these buffer systems were either generally ineffective, unreliable in the distribution of transfected cells or highly cytotoxic at the doses explored. Divalent calcium and magnesium acetate buffers outperformed all other buffer combinations, with notably higher cell viability and consistency of transfection throughout each well.

4. Conclusions:

The transfection studies demonstrate the importance of multiple experimental variables involved in the reproducible utilization of polyplex poly(beta-amino ester)/pDNA NPs for transfection. Importantly, these results demonstrate that PBAE polymers are largely insensitive to changes in the ratio of mixing between polymer and pDNA and are able to be resuspended directly from DMSO stocks using dilute DNA solutions to self-assemble into nanoparticles in a single step. These polymers are further insensitive to freeze-thaw cycles and contamination by water as long as they are stored at -20°C and are not kept thawed at room temperature for extended times. While consistent when used within 20 min, the polyplex NPs formed with these polymers become sensitive to degradation in aqueous buffers beyond 20 min of complexation time. Finally, our exploration of buffer systems other than sodium acetate identified divalent cation acetate buffers, such as magnesium acetate and calcium acetate, as optimal for transfection efficacy in multiple PBAE structures and cell lines in vitro, boosting transfection by severalfold. Together, these results demonstrate PBAE polymers a promising general-purpose transfection reagent for in vitro transfections and high-throughput screening applications.

Acknowledgements

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References:


List of Supplemental Methods, Figures and Tables

Supplemental Methods
PBAE Synthesis
Cell Culture
Dynamic Light Scattering
DNA Binding Assays

Supplemental Figures
Figure 5-S1. Schematic of PBAE synthesis
Figure 5-S2. PBAE 4-5-39 pre-incubation time
Figure 5-S3. PBAE diameter time-course
Figure 5-S4. PBAE degradation rate
Figure 5-S5. BPEI NP pre-incubation time
Figure 5-S6. PBAE 4-5-39 mixing ratios
Figure 5-S7. PBAE 4-5-39 freeze-thaw cycles
Figure 5-S8. PBAE 4-4-6 water fraction
Figure 5-S9. PBAE nanoparticle acetate buffer transfections.
Poly(beta-amino ester)s (PBAEs) were synthesized following previously described methods shown in Fig. S1. Briefly, one diacrylate-terminated backbone monomer (B) was polymerized with one primary amine-containing sidechain monomer (S) in a neat solution by stirring for 24 hours at 90°C, forming the base polymer via Michael addition. This base polymer was dissolved in anhydrous tetrahydrofuran (THF) and mixed with one end-cap small molecule (E), then stirred at room temperature for 1 hr. The end-capped PBAE was then precipitated into diethyl ether, washed twice, and left under vacuum for 48 hours for complete removal of ether. The dry PBAE was dissolved in anhydrous DMSO at 100 mg/mL and stored at −20°C in small aliquots to minimize freeze-thaw cycles. The specific PBAEs used for the following studies were referred to as 4-4-6 or 4-5-39 for polymers composed of monomers B4-S4-E6 and B4-S5-E39 respectively.

**Cell Culture**

HEK293T human embryonic kidney cells and MDA-MB-231 human triple-negative breast cancer cells were maintained in high-glucose DMEM with 10% FBS and 1% penicillin/streptomycin. All cells were cultured at 37°C in a 5% CO₂ atmosphere. For transfection, cells were plated at a density of 12,000 cells/well in 100 µL media approximately 24 hours prior to transfection on CytoOne 96-well tissue culture plates (USA Scientific). Transfections were performed in complete medium containing 10% serum.

**Dynamic Light Scattering**

NPs were prepared independently in triplicate for each condition at 60 w/w mass ratio of polymer to DNA with DNA concentrations of 0.06 µg/µL. The NP Z-average hydrodynamic diameter was then determined by dynamic light scattering in disposable micro-cuvettes using a Malvern Zetasizer NanoZS (Malvern Instruments, Malvern, UK) with a detection angle of 173°. Samples were then diluted six-fold in complete medium containing 10% serum and measured again to determine NP hydrodynamic diameter.
following dispersion in media. For time-course sizing experiments, NP samples were prepared, and a measurement was acquired from undisturbed samples every ten minutes for one hour.

**DNA Binding Assays**

Gel retention assays were performed as previously described\(^{31}\) to assess binding affinity between PBAE polymer and DNA by titrating the PBAE:DNA w/w ratio or by competition binding with heparin sulfate. Quantitative DNA binding assays were performed using the DNA binding fluorophores YO-PRO-1 and YOYO-1 (Thermo Fisher) at a concentration of 1 µM and 1:1 molar ratio of fluorophore to plasmid DNA as previously described\(^{13}\). Fluorescence was measured using a plate reader (Biotek Synergy 2), and percent quenching of the fluorophore was calculated by normalizing to plasmid DNA and dye without polymer in the matched buffer system.

Figure 3. PBAE polymers are not prone to freeze-thaw induced reductions in efficacy. (A) Schematic of experimental outline for testing effect of freeze-thaw cycles on polymer efficacy. (B) HEK293T and (C) MDA-MB-231 cell transfection efficacy were not statistically different even with PBAE 4-4-6 having undergone eight freeze-thaw cycles (one-way ANOVA with multiple comparisons to the fresh polymer). Similarly, PBAE 4-4-6 aliquots pre-mixed to 50 mg/mL prior to storage with varying degrees of hydrated DMSO showed no loss in efficacy when stored at -20°C for two weeks and then used within an hour after thawing in (D) HEK293T or (E) MDA-MB-231 cells.
Figure 5-S1. Schematic of PBAE synthesis for polymer (A) 4-4-6 and (B) 4-5-39. Acrylate terminated base polymers were synthesized at a 1.1:1 ratio of acrylate to amine monomers followed by end-capping to yield linear, end-capped polymers.
Figure 5-S2. PBAE 4-5-39 pre-incubation time in HEK293T cells. Pre-incubation of dilute PBAE polymer in aqueous solution showed similar levels of transfection efficacy (A) and geometric mean expression (B) with no significant difference with incubation times between 1-30 minutes. Complexation time incubation of dilute PBAE polymer with dilute plasmid DNA showed reduced efficacy in percent of cells transfected (C) and geometric mean expression (D) for incubation times of <1 minute and >30 minutes. Differences analyzed by one-way ANOVA with Dunnett corrected multiple comparisons.
Figure 5-S3. PBAE 4-4-6 in 25 mM NaAc, pH 5 over time. DLS z-average measurements of PBAE polymer + DNA at a 60 w/w ratio showed no difference over the span of an hour in acidic buffer. PBAE polymer in the absence of DNA gave poor quality detectable measurements with DLS. Points show mean ± SEM of three individually prepared replicate samples.

Figure 5-S4. Effect of DNA interaction on PBAE degradation. Free PBAE 4-4-7 or PBAE 4-4-7/DNA nanoparticles at a 60 w/w ratio were dissolved in buffers at pH 5.2, 6.0 or 7.4 and incubated at 37°C. Fitted curves show one-phase decay models fit to the data.
Figure 5-S5. BPEI 25 kDa pre-incubation time. Dilute BPEI was mixed with dilute plasmid DNA and allowed to self-assemble to form polyplex nanoparticles for a set duration prior to adding the polyplex nanoparticles to HEK293T cells. A pre-incubation complexation time of 20 minutes was optimal for HEK293T (A) transfection efficacy and (B) geometric mean expression, although no statistically significant differences were detectable for incubation times between 10-60 minutes. Incubation time of 5 minutes or less resulted in statistically lower transfection efficacy. Differences analyzed by one-way ANOVA with Dunnett corrected multiple comparisons.
Figure 5-S6. PBAE 4-5-39 mixing ratio. Mixing aqueous solutions of dilute PBAE 4-5-39 and plasmid DNA resulted in no statistical differences in (A) transfection efficacy or (B) geometric mean expression in HEK293T cells for PBAE volume fractions between 0.15-0.95. Differences analyzed by one-way ANOVA with Dunnett corrected multiple comparisons. Geometric mean values did not demonstrate statistically significant variation in variance as assessed by Brown-Forsythe test (P = 0.8612). Bars show mean ± SEM of four wells.

Figure 5-S7. PBAE 4-5-39 freeze-thaw cycle influence. No significant difference was detectable in the level of transfection following up to 8 freeze-thaw cycles of the polymer aliquots in (A) HEK293T or (B) MDA-MB-
Figure 5-S8. The effect of water fraction content over time on PBAE 4-4-6 NP transfection efficacy over time. PBAE 4-4-6 aliquots were diluted to have specific water volume fractions, frozen at -20°C for two months and then thawed and held at room temperature for set amounts of time prior to transfection. Immediately after thawing (0 hours at room temperature) and at one hour of incubation at room temperature, no significant difference was detectable in the level of transfection in (A) HEK293T or (B) MDA-MB-231 cells. Efficacy of polymer aliquots containing 50% water fraction dropped to zero transfection by three hours of room temperature incubation, while efficacy of aliquots containing 25% water fraction Differences between the 0% water fraction aliquot and others were analyzed by one-way ANOVA with Dunnett corrected multiple comparisons with results statistically lower than the anhydrous DMSO (0%) polymer aliquot are shown in red. Bars show mean ± SEM of four wells.
Figure 5-S9. The role of buffer cation for PBAE nanoparticle transfections in acetate buffers. MgAc$_2$ and CaAc$_2$ resulted in statistically higher percent transfection efficacy as a percent of all cells transfected in (A) HEK293T and (B) cells. Results are shown normalized to 25 mM NaAc transfection efficacy results. (C) Microscopy images show high levels of viability with no difference from untreated cells for any of the acetate conditions.
buffer conditions of cells transfected with PBAE 4-5-39. Level of GFP expression visibly increases with MgAc$_2$ and CaAc$_2$ buffers. Scale bar 200 µm. Bars show mean ± SEM of four wells.

Figure 5-S10. PBAE 4-4-6 acetate buffer DNA uptake. MgAc$_2$ and CaAc$_2$ buffers marginally improved DNA uptake with PBAE 4-4-6 nanoparticles. Differences in geometric mean level of DNA uptake were insignificant in (A,B) HEK293T cells but statistically significant in (C,D) MDA-MB-231 cells. (E) No differences in level of uptake or appearance of internalized DNA were visually observable by microscopy of HEK293T cells. Scale bar 25 µm. Bars show mean ± SEM of four wells.
Figure 5-S11. PBAE 4-4-6 buffer diameter measurements. (A) PBAE 4-4-6 nanoparticles prepared in pH 5.0, 25 mM NaAc or MgAc\(_2\) were not statistically different in diameter either in the nanoparticle formation buffer or following 1:5 dilution into complete media containing 10% serum. (B) Zeta-potential of nanoparticles formed in pH 5.0, 25 mM NaAc or MgAc and dilute 1:6 in 10 mM NaCl had no statistically significant differences in nanoparticle zeta potential. Each value represents the z-average diameter measured by dynamic light scattering of individually prepared nanoparticles.
Figure 5-S12. Buffer influence on DNA binding. Nanoparticles prepared in MgAc$_2$ showed reduced DNA binding capacity as assessed by all assays. (A) Gel electrophoresis with PBAE 4-4-6 nanoparticle w/w titration showed a greater release of DNA at 7.5 and 3.8 w/w ratios in MgAc$_2$ compared to NaAc buffer. (B) Similarly, DNA binding in competition binding assay with heparin sulfate showed greater release of DNA at heparin w/w ratios 7.5 and 3.8. (C) MgAc$_2$ influenced fluorescence of YOYO-1 and Yo-Pro-1 in the absence of PBAE polymer making YOYO-1 bind 40% more effectively and (F) Yo-Pro-1 bind 85% less effectively in the absence of DNA. (D,E) In the presence of PBAE polymer these dyes were quenched due to competition of DNA binding from the PBAE polymer. Using quenching values normalized to either MgAc$_2$ or NaAc
specifically for (F) YOYO-1 and (G) Yo-Pro-1 showed reduced binding efficacy of PBAE polymer in MgAc2 compared to NaAc buffer. All points show mean ± SEM of four well replicates prepared independently.

Figure 5-S13. Composite buffer transfection. (A) Buffers systems in which plasmid DNA and PBAE polymer were diluted in separate buffer were explored. MgAc2 and CaAc2 buffers remained most effective. Minimally soluble buffer systems including MgHCitr and CaHCitr were also utilized and showed high levels of expression but high levels of cytotoxicity at the 200 ng DNA dose used (highlighted in red). (B) Microscopy images show high toxicity resulting from minimally soluble buffer systems including MgHCitr, CaHCitr and
calcium phosphate. Phosphate buffers in particular were notable for causing PBAE nanoparticles to crash out of solution as visibly cloudy particulates and no composite buffers showed greater transfection in HEK293T or MDA-MB-231 cells. Bars show mean ± SEM of four wells.

Figure 5-S14. Flow cytometry gating in FlowJo. Single cells were identified by gating forward-scatter height (FSC-H) against side-scatter height (SSC-H) followed by elimination of double cells by gating forward-scatter height against area (FSC-A). Cells were gated for eGFP expression in 2D plot of FL1H vs FL2H and for Cy5 labeled plasmid DNA uptake by FL4-H vs FL1-H.
Table 5-S1.

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Table 5-S2. PBAE 4-4-7 degradation as free polymer or polyplex nanoparticles with one-phase decay model

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0.408 Half Life (h) 3.51
Chapter 6: A combinatorial library of biodegradable polyesters enables non-viral gene delivery to post-mitotic human stem cell-derived polarized RPE monolayers

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Foreword: The following manuscript is the product of a multi-year collaboration between the Green lab and Don Zack’s lab. I am extremely appreciative of the opportunity, as the Zack lab’s utilization of more relevant stem cell derived culture models and access to a large amount of technology to support research has been transformative in my PhD.


ABSTRACT:
Safe and effective delivery of DNA to post-mitotic cells, especially highly differentiated cells, remains a challenge despite significant progress in the development of gene delivery tools. Non-viral and biodegradable polymeric nanoparticles (NPs) can offer an array of advantages for gene delivery over viral vectors due to improved safety, carrying capacity, ease of manufacture, and cell-type specificity. Here we demonstrate the use
of a high-throughput screening (HTS) platform to synthesize and screen a library of 148 biodegradable polymeric nanoparticles, successfully identifying structures that enable efficient transfection of human pluripotent stem cell differentiated human retinal pigment epithelial (RPE) cells with minimal toxicity. These NPs can deliver plasmid DNA (pDNA) to RPE monolayers more efficiently than leading commercially available transfection reagents. Novel synthetic polymers are described that enable high efficacy non-viral gene delivery to hard-to-transfect polarized human RPE monolayers, enabling gene loss- and gain-of-function studies of cell signaling, developmental, and disease-related pathways. One new synthetic polymer in particular, 3,3′-iminobis(N,N-dimethylpropylamine)-end terminated poly(1,5-pentanediol diacrylate-co-3 amino-1-propanol) (5-3-J12), was found to form self-assembled nanoparticles when mixed with plasmid DNA that transfect a majority of these human post-mitotic cells with minimal cytotoxicity. The platform described here can be utilized as an enabling technology for gene transfer to human cells that are primary, fragile, and resistant to conventional approaches of gene transfer.

Key Words:
Retinal pigment epithelial cell, human stem cell, nanoparticles, non-viral gene therapy, poly(beta-amino ester), polymer, ophthalmology

Lay Summary:
Many retinal diseases are attributable to dysregulation in gene expression or lack of expression of specific genes, allowing for the possibility of prevention or cure of these diseases by effective delivery of nucleic acids coding for the necessary gene to the retina. Delivery of nucleic acids to cells of the retina is challenging due to the non-dividing nature of most retinal cells, preventing DNA from reaching the nucleus. To overcome this barrier, we engineered and tested a library of nanoparticle formulations to identify polymers that enabled safe and effective delivery of nucleic acid cargoes to retinal pigment epithelial cells. The nanoparticle technology explored here has the potential to be utilized for therapeutic delivery of nucleic acids to retinal cells, possibly enabling treatment for otherwise untreatable retinal diseases for which a specific genetic deficit is known but no drugs are available.

1. INTRODUCTION:

Gene therapy holds promise for treating many acquired and inherited blinding disorders [1]. Gene therapy for long-term expression, particularly in vivo, has traditionally utilized viral vectors to deliver double stranded DNA. For retinal gene therapy, adeno-associated virus (AAV) in particular has been successfully utilized for effective delivery to various cells of the retina [2]. However, utilization of AAV vectors for gene therapy does have a number of drawbacks, including limited cargo capacity of the AAV capsid [3-5], risk of
insertional mutagenesis,[6] the challenge of large scale production of clinical grade vector for human therapy,[7] difficulty in transducing some cell types, and as pre-existing patient immunity to specific AAV serotypes [2, 8]. To overcome these challenges and to develop a potentially safer approach, an alternative strategy that is receiving increased attention is the formulation and development of biodegradable non-viral vectors to facilitate delivery of the gene of interest to the target site of interest. Non-viral vectors, although they have their own challenges such as relatively low efficiency, have the potential to overcome many of the drawbacks of AAV and other viral-based gene delivery methods[9].

With this goal of developing safe and efficient non-viral methods for gene delivery, a wide variety of non-viral nanoparticles (NPs) have been engineered and tested [10-16]. NPs have been developed that can effectively complex with nucleic acids, mediate cell uptake, achieve endosomal escape, and result in cellular gene expression. However, despite these significant successes, DNA transducing NPs that have been developed to date have tended to suffer from low efficacy [17]. Efficacy of non-viral transfection reagents in post-mitotic cell types that have exited the cell cycle is particularly low, as nuclear uptake of plasmid DNA remains a major hurdle to effectively mediating expression [18, 19]. Poly(beta-amino ester)s (PBAEs) are a promising class of synthetic, cationic polymers with large structural diversity that have been demonstrated to effectively transfect a wide variety of cells including embryonic stem cells [20], as well as immortalized human RPE cell lines in vitro and mouse RPE cells in vivo [21].

The RPE, which is essential for retinal function, is composed of a monolayer of pigmented bipolar epithelial cells at the backside of the retina. Compromise of the cellular environment of the RPE is associated with many hereditary and acquired retinal diseases, including age-related macular degeneration (AMD) and retinitis pigmentosa (RP) [22]. Mutations of genes expressed in the RPE are associated with a number of retina diseases, and viral-based gene therapies are actively being pursued for several of these diseases [23]. In fact, the first FDA approved gene therapy for an inherited disease (voretigene neparvovec-ryzl) is for an RPE-related disease, using an AAV-based vector system. Building upon this success, a number of academic and industry groups are pursuing gene therapies for a number of other RPE-related genetic diseases. A challenge in this work is that a number of the diseases of interest involve genes that exceed the limited carrying capacity of AAV. One approach to address this challenge involves clever efforts to artificially increase AAV’s carrying capacity [24]. A second approach involves efforts to develop other classes of viral gene vectors for retinal disease [25, 26]. A third approach, as noted above, involves development of non-viral approaches. However, despite the testing of many different non-viral strategies to deliver DNA to RPE, the success of the technologies that have been tested to date has been limited [27-36]. To address this challenge, we first established a high throughput-assay platform to screen potential PBAE nanoparticles for their ability to efficiently transfect iPSC-derived human RPE monolayers in vitro. Additionally, we hypothesized that the efficiency of cationic PBAE-pDNA NPs to transduce RPE monolayers could be significantly increased by
tuning the hydrophilicity and end group chemistry of the constituent polymers. To test this hypothesis, we synthesized 4 PBAE base polymers with different backbones and then end-capped the linear polymers in a parallel plate-based format to yield a library of 148 polymer structures. This library of polymers, that included novel amine-containing small molecules as end-groups to serve as putative transfection enhancers, was then screened using the high throughput human iPSC RPE monolayer assay. Here we describe identification of several promising NPs for RPE transfection.

2. MATERIALS AND METHODS:

2.1 Polymer synthesis and characterization

Monomers for base polymer synthesis were purchased from vendors listed in Table S2, while end-cap monomers used were purchased from vendors listed in Table S3 and S4. Acrylate monomers were stored with desiccant at 4°C, while amine monomers were stored with desiccant at room temperature. PBAE polymers were synthesized neat at 1.1:1 B:S monomer ratios for polymers 3-5-Ac, 4-4-Ac and 4-5-Ac and 1:1.05 monomer ratio for 5-3-Ac for 24 hours at 90°C. Following synthesis, neat polymers were dissolved at 200 mg/mL in anhydrous DMF then precipitated in diethyl ether twice at a solvent ratio of 1:10 by vortexing the solvents and centrifuging at 3000 rcf. Polymers were allowed to dry under vacuum for 24 hours, at which point they were massed and dissolved at 200 mg/mL in anhydrous DMSO and allowed to remain under vacuum to remove additional diethyl ether for another 24 hours. Finally, acrylate terminated polymers were aliquoted and stored at -20°C until use in end capping reactions.

For polymer characterization, samples of the initial neat polymer and neat polymer following diethyl ether removal were set aside for characterization via 1H NMR and gel permeation chromatography (GPC). GPC was performed on polymer samples both before and after double precipitation in diethyl ether using a Waters system with auto sampler, styragel column and refractive index detector to determine Mn, Mw and PDI relative to linear polystyrene standards. GPC measurements were performed as previously described with minor changes consisting of a modified flow rate (0.5 mL/min) and an increase in sample run time to 75 minutes per sample[37]. Analysis of polymers via 1H NMR (Bruker 500 MHz) following diethyl ether precipitation and drying was performed to confirm the presence of acrylate peaks. For NMR, neat polymer was dissolved in CDCl3 containing 0.05% v/v tetramethysilane (TMS) as an internal standard.

2.2 Polymer library preparation

PBAE polymers were prepared for transfection screening experiments by high-throughput, semi-automated synthesis techniques using a ViaFlo 384 (Figure 6-2B). For end capping reactions, 25 µL of endcap molecules in anhydrous DMSO at a concentration of 0.2 M were distributed to source wells of a deep-well 384 well plate, then distributed to corresponding replicate wells in groups shown in multiple colors of the end capping reaction 384-well deep plate (240 µL volume). Acrylate terminated base polymers at 200 mg/mL in anhydrous DMSO were thawed and distributed to wells containing 36 different endcap molecules and a single
well containing DMSO only for the acrylate terminated polymer control. End capping reactions were allowed to proceed for two hours at room temperature on a gentle shaker, after which endcapped PBAE polymers were diluted to 50 mg/mL in anhydrous DMSO and aliquoted to 5 µL per well on the left side of 384-well nanoparticle source plates. Nanoparticle source plates were sealed and stored at -20°C with desiccant until needed for transfection. Following large-scale screening of the PBAE library in 384-well plates, larger batches of top PBAE structures were synthesized from frozen base polymer using the same protocol described above. Endcapped polymers were then aliquoted to individual tubes and stored at -20°C with desiccant.

For end capping, reaction volumes of 50 µL at 100 mg/mL polymer concentration and 0.1 M amine monomer End-cap concentration were selected as sufficient to enable effective reactivity over a two-hour time period. For initial studies, endcap molecule E1 was titrated between 0.2 and 0.0625 M in reactions with base polymer PBAE 4-5-Ac at 100 mg/mL over two hours. Reacted polymers were then precipitated twice in diethyl ether to remove excess endcap monomer, dried and assessed using 1H NMR to determine efficacy of the end capping reaction by the disappearance of acrylate moiety peaks between 5.5-6.5 ppm. These results demonstrated effective end capping down to a concentration of 0.05 M for endcap molecule E1. To allow for varying levels of reactivity between endcap molecules, an endcap molecule concentration of 0.1 M was used for parallel large-scale end capping reactions.

2.3 Nanoparticle characterization

The hydrodynamic diameter of top PBAE structure 3,3′-iminobis(N,N-dimethylpropylamine)-end terminated poly(1,5-pentanediol diacrylate-co-3 amino-1-propanol) (5-3-J12) was characterized at three different w/w ratios to assess the influence of w/w ratio on nanoparticle characteristics. For dynamic light scatter (DLS) measurements, nanoparticles were initially formed in 25 mM MgAc₂, pH 5.0 then diluted 1:6 into 10% FBS in PBS dynamics and analyzed in disposable micro-cuvettes using a Malvern Zetasizer NanoZS (Malvern Instruments, Malvern, UK) with a detection angle of 173°. For zeta potential, nanoparticles were prepared and diluted as for DLS, but were analyzed by electrophoretic light scattering was in disposable zeta cuvettes at 25°C using the same Malvern Zetasizer NanoZS. For nanoparticle tracking analysis, nanoparticles were formed in 25 mM MgAc₂, pH 5, then diluted 1:500 in 150 mM PBS as previously described using a Nanosight NS300 [38].

A gel retention assay to assess PBAE: DNA binding strength was performed as previously described [39] using a 1% agarose gel. Acrylate terminated PBAE 5-3-Ac was compared against top PBAE structure 5-3-J12 at w/w ratios from 0 to 50 to demonstrate improved binding of endcapped PBAE structures.

Transmission electron microscopy (TEM) images were acquired using a Philips CM120 (Philips Research, Briarcliffs Manor, New York) on 400 square mesh carbon coated TEM grids. Samples were prepared at a DNA concentration of 0.045 µg/µL and polymer 90 w/w ratio in 25 mM MgAc₃, pH 5.0 after which 30
µL were allowed to coat TEM grids for 20 minutes. Grids were then dipped briefly in ultrapure water, wicked dry and allowed to fully dry before imaging.

2.4 Plasmid Design

For the *in vitro* transfection, a plasmid coding for the mCherry open reading frame was created by PCR amplification of the mCherry-N1 plasmid (catalog no. 632523; Clontech). Since this plasmid has no start site, an ATG initiation codon was added to the forward primer. After PCR amplification, mCherry was inserted into the directional pENTR-D-TOPO gateway entry vector (catalog no. K240020; Invitrogen). Positive colonies were selected by PCR and confirmed by sequencing. 100 ng of purified entry plasmid was mixed with pCAGG-DV destination vector, created by incorporating a gateway cassette containing attR recombination sites flanking a ccdB gene into the pCAGEN vector (Addgene #11160), in the presence of LR clonase II (catalog no. 11791019). After recombination clones were selected and sequenced and deposited (Addgene 108685). For experiments using co-delivery of two plasmids, iRFP670-N1 (Addgene 45457) [40] was used.

2.5 Differentiation and Culture of RPE From hPSCs

RPE monolayers were differentiated as described previously [41, 42] from the EP1-GFP human iPS cell line that constitutively expresses H2B-nuclear-GFP. In brief, iPS cells to be differentiated were plated at 60,000 cells per cm\(^2\) on Matrigel-coated 384-well plates and allowed to grow for 25 days in RPE medium consisting of 70% DMEM (catalog no. 11965092; ThermoFisher Scientific), 30% Ham’s F-12[43] Nutrient Mix (catalog no. 11765-054; Invitrogen), serum free B27 supplement (catalog no. 17504044; ThermoFisher Scientific), and antibiotic-antimycotic (catalog no. 15240062; ThermoFisher Scientific). Coating of plates with Matrigel (25 µL per well), seeding of cells (50 µL per well), and media change every other day (replaced with fresh 25 µL per well) were accomplished using a high throughput Viaflo microplate dispenser (catalog no. 6031; Intergra). Cells were confirmed to possess an RPE monolayer phenotype at day 25 following plating.

2.6 Imaging and Analysis using HCS studio 2.0 Software

Images were acquired on an ArrayScan VTI HCA Reader (Thermo Fisher Scientific) using a 20x objective. For analysis, the Thermo Scientific AdvancedTargetValidationV4.1 application was used with the assay described in Figure 6-S4.

2.7 *In vitro* nanoparticle mediated gene delivery

On the day of transfection, the old media was discarded and replaced with 40 µL of fresh RPE media. To form PBAE/DNA nanoparticles, pDNA was diluted in 25 mM magnesium acetate buffer (MgAc\(_2\), pH 5) and aliquoted to individual wells on the right half of the 384-nanoparticle-source plate. End capped PBAEs from the left half of the 384 well round bottom source well plate (Figure 6-2D) were then resuspended in parallel in 25 mM MgAc\(_2\) using a Viaflo microplate dispenser. After a brief centrifugation (1000 RCF for 1 minute) the solutions of unique PBAE structures were then transferred to the right half of the 384 well round
bottom source well place containing pDNA (Figure 6-2D) in a 3:1 (vol/vol) ratio, resulting in a defined weight-weight (w/w) ratio between 20-100 of PBAE:DNA. The nanoparticle source plate containing the PBAE/DNA mixtures was then briefly centrifuged (1000 RCF for 1 minute). To dispense nanoparticles to cells, 5 µL volumes of the NPs in each well were then added to the RPE monolayer (Figure 6-2E) and incubated with cells for 2 hours inside the 37°C incubator; all nanoparticles and media were then replaced with 50 µL of fresh RPE media. After 48 hours to allow for reporter gene expression, images were acquired using an automated fluorescence-based imaging system (Cellomics ArrayScan ; Thermo Fisher Scientific) for nuclear-GFP and mCherry. Transfected cells were identified as those expressing both the endogenous nuclear GFP and exogenous mCherry reporters, and the percent of transfected cells, as well as cell viability, was determined for each NP and condition.

2.8 Immunostaining and Confocal Microscopy

iPS EP1 cells, without a nuclear-GFP reporter, were differentiated by plating them at 25,000 cells per cm² on Matrigel-coated borosilicate sterile 8-well chambered cover glasses (catalog no. 155409; Lab-Tek II; ) and allowed to grow for 25 days in RPE medium. On the day of transfection, the old media was discarded and replaced with 300 µL of fresh RPE media. The PBAE 5-3-J12 was then mixed with CAG-mCherry pDNA in a 3:1 (vol/vol) ratio, resulting in a defined weight-weight (w/w) ratio of 90:1 of PBAE:DNA. To dispense nanoparticles to cells, 50 µL volumes of the NPs containing 1500 ng DNA were then added to the RPE monolayer and incubated with cells for 2 hours inside the 37°C incubator; all nanoparticles and media were then replaced with 300 µL of fresh RPE media. After 48 hours, to allow for reporter gene expression, the cells were fixed with 4% paraformaldehyde in PBS, cells were blocked and permeabilized for 30 min in 5% goat serum, 0.25% Triton X-100 in PBS, and then incubated for 1 h at room temperature with polyclonal mouse anti–ZO-1 (1/500; catalog no. 40-2200; Invitrogen) monoclonal rat anti–mCherry (1/1000; catalog no. M-11217; Molecular Probes). Cells were then incubated for 1 h at room temperature with the corresponding secondary antibody conjugated to Alexa 488 or Alexa 568 (Invitrogen), and counterstained with Hoechst 33342 (Invitrogen). Images were captured with a confocal microscope (Zeiss LSM 710).

2.9 Co-expression Assay

To assess the ability of top PBAE nanoparticles to co-deliver two plasmids, plasmids CAG-mCherry (Addgene 108685) and CMV-iRFP670 (Addgene 45457) [40] were diluted together in 25 mM MgAc₂ and used to form PBAE 5-3-J12 nanoparticles at a 90 w/w ratio. These nanoparticles were used to transfect and DNA dose of 200 ng/well in 384 well plates. For the co-delivered condition, plasmids in 25 mM MgAc₂, were pre-mixed prior to complexation with PBAE and added to RPE monolayers together in the same nanoparticles. Transfection efficacy for iRFP and mCherry was then assessed 72 hours following transfection using the HCS assay described below.

2.10 Statistical analysis
Graph pad prism software (v.7.0) was used for data analysis. One-way ANOVA test was used for comparison of the results. For finding the differences between groups, data was analyzed by post-Hoc, Dunnett’s multiple comparisons test. P values of ****p<.0001; ***p<.001; **p<.01; *p<.05 were considered as statistically significant.

3. RESULTS
3.1 Polymer Synthesis and Characterization

Four acrylate-terminated PBAEs that were shown previously to be effective for gene delivery in various cell types were synthesized as previously described [44] as neat polymers from small molecule diacrylate and amino monomers (Figure 6-1). The acrylate-terminated polymers were also washed twice with diethyl ether and characterized via gel permeation chromatography and $^1$H NMR to assess number average molecular weight ($M_n$) and to ensure that the majority of polymer molecules were acrylate terminated to allow for effective end-capping (Figure 6-S1). The results indicated that all synthesized polymers, were acrylate terminated with number average molecular weights between 6.2 to 7.8 kDa (Figure 6-S2). Precipitation in diethyl ether has previously been utilized to remove excess end-cap monomers reacted with base-polymers to avoid free monomer induced cytotoxicity [45]. We hypothesized that precipitation via diethyl ether even in the absence of end-cap monomers to be removed would increase molecular weight and reduce polydispersity of synthesized acrylate terminated polymers by removing oligomers that have been shown to be ineffective for gene delivery purposes [46, 47]. This effect was confirmed by GPC analysis of polymer before and after precipitation, whereby $M_n$ increased by an average of 41 ± 9\% (mean ± SD) and PDI decreased by an average of 16 ± 9\% (mean ± SD). The molecular weights of the base polymers are all within the optimal range for transfection efficacy previously reported for similar polymer structures [46].

Utilization of end-cap monomers in linear PBAEs has been demonstrated to greatly improve transfection efficacy compared to side-chain monomer terminated polymers (i.e. C32) [48-52]. Furthermore, polymer end-capping groups have been shown to significantly increase the efficacy of acrylate terminated base polymers that are almost entirely ineffective for both uptake and transfection [53]. With this rationale, we selected potential end-cap monomers from those previously published and available potential primary amine monomers from chemical supply companies. Polymer 4-4-Ac was endcapped with each potential monomer initially and pre-screened for transfection efficacy in HEK293T cells to separate out wholly ineffective end-cap monomers (Figure 6-S3 and Table S6-4). From this pre-screen, we selected 36 end-cap monomers (Figure 6-1) to use for polymeric library preparation, having eliminated 24 of the structures for RPE screening tests.

The polymer nomenclature “B$_{n}$-S$_{n}$-XY” in the synthesized polymer library denotes base monomer (B) carbon number between acrylate moieties, and side-chain monomer (S) number denotes carbon number
between hydroxyl as previously described.[44] Due to the large number of amino end-cap monomers, we utilized a new naming scheme whereby end-cap monomers were given a letter (J-P) for specific structural category (denoted X above) followed by a number for specific monomer in the category (denoted Y above). Structural categories of end-cap monomers included amino alkanes (J), amino piperidines (K), amino pyrrolidines (L), amino alcohols (M), amino piperazines (N), diamino-ethers (O) and amino morpholinos (P). By this nomenclature, 3,3′-iminobis(N,N-dimethylpropylamine)-end terminated poly(1,5-pentanediol diacrylate-co-3 amino-1-propanol) (PBAE 5-3-J12) was synthesized from monomers B5 and S3 to yield an acrylate terminated PBAE followed by endcapping with monomer J12 to yield a linear, end-capped polymer.

**Figure 6-1. Sequential poly(beta-amino ester)s (PBAEs) library construction and synthesis scheme (A)**

Synthesis scheme of linear PBAEs from diacrylate and primary amine small monomers to yield acrylate terminated polymers, followed by end-capping to yield linear end-capped PBAEs. **(B)** Representative PBAE 5-3-J12 formed from monomers B5, S3 and end-cap J12. **(C)** Three diacrylate monomers and **(D)** three side-chain amino alcohols utilized in library synthesis. **(E)** 36 end-cap monomers identified as effective for transfection.

### 2.2 Polymer Library Preparation

The polymer library described above was prepared in a semi-automated, high-throughput manner to identify polymer formulations effective for transfection of iPSC derived RPE cells in a highly parallel manner as shown in **Figure 6-2**. For high-throughput polymer end-capping reactions, end-cap monomers in DMSO were distributed to deep-well 384-well plates, after which acrylate terminated polymers were distributed likewise.
in parallel and allowed to react for two hours at room temperature with gentle shaking. End-capped polymers in the master reaction plate were diluted further in DMSO and a set volume was distributed in parallel to individual nanoparticle source plates (384 well round-bottom plates). Nanoparticle source plates were then sealed and stored at -20°C with desiccant until the time of transfection. This method allowed many nanoparticle source plates to be prepared at one occasion to ensure reproducibility between transfections on different days.

![Diagram of combinatorial PBAE library construction](image)

**Figure 6-2. Schematic of combinatorial PBAE library construction (A)** Linear base polymer PBAEs were synthesized in vials to be acrylate terminated, then characterized via 1H NMR and GPC (B) Synthesized polymers were dispensed into a 384 well round bottom plate using a Viaflo 96/384 microplate dispenser and end-capped with each base polymer. A total of 4 different base polymers as shown in different color scheme were end-capped per master plate containing 36 end-cap monomers each. (C) Source plates were then replicated from one master plate and stored them at -80°C for future use. (D) End capped linear polymers (left 12 columns of the plate) were mixed with plasmid DNA (right 12 columns of the plate) to formulate NPs. (E) The RPE monolayers were transfected using an automated Viaflo microplate dispenser and incubated for 2 hours with NPs. (F) Images were captured using Cellomics.

### 2.3 High Throughput Semi-Automated NP Transfection to RPE Monolayers

To access the transfection efficacy of the PBAE nanoparticles in mature RPE monolayers at day 25 following seeding of differentiated RPE cells (Figure 6-S5), we conducted a high throughput-screening assay...
with the prepared library of 148 PBAE structures. For high-throughput transfections, a nanoparticle source plate with polymers in one-half of the wells was thawed and DNA diluted in pH 5 magnesium acetate (MgAc₂) buffer was distributed to the right half of the plate. For initial screening assays we choose to use a plasmid in which expression of the fluorescent reporter mCherry is driven by the chicken β-actin with a CMV enhancer (CAG) promoter because the CAG promoter was previously shown to be highly active in human iPSC-derived RPE cells [54]. Each polymer was resuspended in pH 5 MgAc₂ buffer in parallel and mixed with the dilute DNA to form polyplex nanoparticles by electrostatic self-assembly. Nanoparticles were then distributed to plates of cells in parallel to screen for transfection efficacy using an image-based High Content Screening assay (Figure 6-S4). This setup enabled 148 polymer structures per nanoparticle source plate to be tested with two replicates for each polymer per plate of cells.

Our library of polymers was first screened at a 60 w/w ratio of polymer to plasmid DNA to assess transfection efficacy (Figure 6-3A) and viability (Figure 6-3B) relative to untreated RPE monolayers. As previously observed, acrylate terminated polymers (5-3-Ac, etc) lacking any end-cap monomer failed to yield detectable transfection [55]. Heat map arrays of transfection efficacy demonstrated that more hydrophobic polymer structures (base polymers 5-3- and 4-5-) generally yielded greater transfection and the end-cap played an important role in the efficacy of polymers overall. Several leading PBAE structures, 5-3-J12, 5-3-F3 and 5-3-F4, resulted in 42%, 37% and, 34% positively transfected cells, respectively while maintaining high cell viability (90%, 97% and, 98%, respectively). Among all polymers evaluated, cell viability was not directly proportional to a polymer’s ability to transfect RPE monolayers, as some other PBAEs demonstrated extremely low transfection efficacy despite high cell viability. Interestingly, the results of screening this library of polymers on mature RPE monolayers at day 25 post-seeding differed from transfection efficacy screening in mitotic RPE cells on day 3 post-seeding (Figure 6-S6), where more hydrophilic polymer structures demonstrated the highest efficacy and greater cytotoxicity was notable among all polymers. These results thus confirm our prior experience that polymer transfection efficiency can be highly cell type/state dependent and highlight the importance of optimizing polymers on the specific cell type and cell state of interest.
Figure 6-3. High throughput screening of PBAE nanoparticles in confluent D25 RPE monolayers. (A) Heat maps showing the percentage transfected RPE cells and (B) percentage survival rate following the introduction of a combination of 148 different nanoparticles to confluent RPE monolayers at day 25 post seeding using a 60 w/w ratio of polymer to CAG-mCherry plasmid DNA. The color scale bar refers to the percentage transfection efficiency and percentage survival that was calculated based on the number of mCherry positive cells detected from the total cell population.
2.4 Validation of 5-3-J12 Nanoparticle Transfection Efficacy

In order to validate and optimize the top polymer structure identified in our screen, 5-3-J12, polymer to DNA w/w ratio and overall polymer dose per well were varied to identify optimal transfection conditions (Figure 6-S7). With this optimization, 90 w/w 5-3-J12 nanoparticles were demonstrated to yield the greatest transfection efficacy (up to 60% transfection) compared to 30 w/w and 60 w/w nanoparticles of the same polymer structure at an equal polymer dose per well of cells. Assessment of a wide range of leading commercially available transfection reagents at multiple ratios of reagent to DNA and multiple DNA doses further demonstrated the enhanced efficacy of this new chemical compound in transfection of RPE monolayers (Figure 6-S8). In direct comparisons, PBAE 5-3-J12 yielded statistically significantly higher transfection efficacy (Figure 6-4A) than all tested commercial transfection reagents as well as our previously developed top PBAE polymer for transfection of RPE cells (PBAE 557) {Sunshine, 2012 #43}. Viability of RPE monolayers with nanoparticle 5-3-J12 was not statistically significantly different from untreated cells, in contrast to most commercial reagents, which induced significant cytotoxicity to achieve lower transfection efficacy (Figure 6-4B). PBAE 5-3-J12 further yielded a greater degree of transgene expression than the top commercial reagent ViaFect, as shown by both flow cytometry (Figure 6-4C) and by microscopy (Figure 6-4D). Overall transfection efficacy for all transfection reagents varied together across independently prepared iPSC-derivations of mature RPE monolayers over a 9-month time-span, but the top polymer structure, 5-3-J12, always yielded transfection efficiencies (20% to 60%) that were statistically higher than the top commercial reagent, ViaFect (Figure 6-S9).
Figure 6-4. Transfection of RPE monolayers with top PBAE nanoparticles and with commercially available reagents. (A) Transfection efficacy and (B) relative cell count of optimized nanoparticle formulation compared to commercial reagents. (One-way ANOVA with Dunnett corrected multiple comparisons) (C) Relative expression level of cells transfected with 5-3-J12 and ViaFect demonstrating a greater number of cells expressing at all levels of expression. (D) Representative microscope images of RPE cells expressing nuclear GFP (green) and transfected with mCherry (red). Scale bar is 100 μm. Bars show mean ± SEM of four wells.

2.5 Biophysical Characterization of 5-3-J12 Nanoparticle

To further investigate the biophysical properties of 5-3-J12, we characterized these nanoparticles at multiple w/w ratios via dynamic light scattering (DLS), electrophoretic light scattering for zeta-potential, and nanoparticle tracking analysis (NTA). 5-3-J12 nanoparticles at w/w ratios of 30, 60 and 90 polymer to DNA varied significantly in diameter assessed by DLS and NTA with 90 w/w nanoparticles forming significantly smaller particles (Figure 6-5A,B). This result is consistent with our previously published findings that forming PBAE nanoparticles at high w/w ratios with excess polymer has a tendency to yield a smaller overall population of nanoparticles [56, 57]. Zeta-potential of 5-3-J12 did not vary with w/w ratio, with all 5-3-J12 nanoparticles measured to have a surface charge of between 25-30 mV (Figure 6-5C). The addition of the J12 end-cap moiety to base polymer 5-3-Ac was demonstrated to improve DNA binding capacity via a gel retention assay (Figure 6-5D), likely due to increased positive charge from the amine-containing J12 end-group. The diameter of PBAE 5-3-J12 nanoparticles assessed by TEM was similarly consistent with hydrodynamic diameter sizing measurements, showing dried particles of approximately 100 nm in diameter (Figure 6-5E).
Figure 6-5. PBAE 5-3-J12/DNA Nanoparticle Characterization (A) Nanoparticle hydrodynamic diameter measurements assessed via DLS z-average and (B) NTA showed that average diameter decreased as polymer:DNA w/w ratio increased. DLS z-average measurements were statistically lower for 90 w/w nanoparticles, compared to 30 w/w nanoparticles. (C) Nanoparticle zeta-potential did not statistically differ between the nanoparticles at different w/w ratios. (D) End-capping with monomer J12 improved DNA binding compared to acrylate-terminated polymers. PBAE 5-3-J12 fully retarded DNA at w/w ratios down to 5 w/w, in contrast to the acrylate terminated polymer, which was only effective down to a 10 w/w ratio. (E) TEM showed 5-3-J12/DNA nanoparticles have a spherical morphology and size of approximately 100 nm. Graphs show the mean of three independently prepared samples. *p < 0.01, **p < 0.001, based on one-way ANOVA with Tukey's post hoc test.

2.6 Co-transfection and Properties of RPE Monolayer Transfected Cells

For in vitro mechanistic studies as well as potentially for in vivo ocular gene therapy applications, it could be advantageous to transfrect cells simultaneously with multiple genes. We therefore tested whether our non-viral delivery system could provide efficient co-expression of multiple genes. Transfection of mature RPE monolayers with 5-3-J12 90 w/w nanoparticles prepared with CAG-mCherry and CMV-iRFP670 plasmids
yielded up to 22% of cells showing co-expression of both genes (Figure 6-6A). Among cells detectably expressing either fluorescent protein, most showed expression of both genes (53.5 ± 1.4%), rather than expressing just one gene (15.0 ± 2.6% and 31.4 ± 2.7% for CAG-mCherry and CMV-iRFP670 respectively). In all transfection experiments, among the transfected cells we observed a wide range of expression levels. We also observed that there seemed to be a relationship between expression level and cell viability. With the strong synthetic CAG promoter, with both PBAE 5-3-J12 as well as ViaFect-mediated transfection, the cells that expressed the exogenous reporter genes at the highest levels were more likely to undergo cell death (see Supplementary Video that demonstrate time-lapse microscopy of highly expressing cells undergoing cell death). However, the induction of cell death was determined not to be directly attributable to the polymers themselves as nanoparticles prepared with non-coding Cy5-labeled plasmid DNA [58] did not induce a significant level of cell death, whereas a reduction in the total number of nuclear-GFP positive cells per well for RPE monolayers transfected with the same nanoparticles prepared with the CAG-mCherry plasmid was observed (Figure S10). Finally, confocal microscopy assessment of RPE monolayers transfected and stained for tight junctions (ZO-1) demonstrated the integrity of tight junctions following transfection with 5-3-J12 (Figure 6-7)
Figure 6-6. Co-transfection of two reporter plasmids with PBAE 5-3-J12/DNA nanoparticles. (A) Nanoparticles formed with pre-mixed plasmids encoding CAG-mCherry and CMV-iRFP enable RPE monolayers to co-express two exogenous genes. The majority of the cell population detectable as expressing either fluorescent protein, expressed both fluorescent proteins. (B) Representative RPE monolayers co-transfected with both CAG-mCherry (red) CMV-iRFP670 (blue) reporter constructs. A purple color indicates co-expression of the two exogenous genes. Scale bar is 100 µm. Bars show mean ± SEM of four wells.
Figure 6-7. Transfected RPE monolayer tight junction expression. (A) Maximum intensity projection confocal microscope image of RPE monolayer transfected with 5-3-J12/DNA nanoparticles at 90 w/w ratio demonstrates the presence of tight junctions stained with ZO-1. Scale bar is 100 µm.

DISCUSSION

Safe and effective non-viral gene delivery of plasmid DNA to post-mitotic cells remains challenging in part due to the lack of nuclear membrane breakdown, which is a major barrier to gene delivery. Delivery of plasmid DNA to post-mitotic cells is of great utility for the study of retinal biology as well as for the development of non-viral therapeutic materials for retinal gene therapy. Here, we designed and utilized a semi-automated high throughput system to generate and identify candidate biodegradable polymer structures that mediate effective delivery to post-mitotic, mature RPE monolayers derived from induced pluripotent stem cells [41, 42]. While prior studies have utilized immortalized RPE cell lines such as ARPE-19 or hTERT-RPE1 to study gene delivery to RPE monolayers \textit{in vitro}, these cell lines are provide only a limited model of the cell behavior of RPE cells \textit{in vivo} both in terms of cellular phenotype and gene expression profile. The ES-derived RPE monolayers used here have been previously validated to better mimic native RPE phenotype and gene
expression of RPE cells in the human retina, enabling them to serve as a useful model for the study RPE biology in high throughput formats [42].

The approach for high throughput PBAE library generation described here is highly adaptable for rapid library generation and screening techniques. In contrast to prior automated and semi-automated synthesis libraries that have used pipetting robots such as Tecan [59], our simplified semi-automated technique facilitates parallel testing of polymer structures that can be implemented with any parallel pipetting framework, including in 96 well plates with a simple multichannel pipet. Our approach also demonstrates the importance of end-cap monomer structure for determining the efficacy of linear PBAEs for transfection, an approach which is highly amenable to simple parallelization [52].

With these validated RPE monolayers and semi-automated polymer library preparation and screening technique, we identified multiple candidate polymer structures for transfection of post-mitotic RPE monolayers with the most promising candidate, 5-3-J12, yielding up to 60% transfection efficacy with minimal cytotoxicity. This polymer structure was further shown to enable co-delivery of two separate plamids coding for two fluorescent proteins, and we demonstrated that tight junctions stained with anti-ZO-1 remained largely intact following transfection. The utilization of this screening approach is notable given that our prior candidate for transfection of RPE cells (PBAE 557), identified by screening in ARPE-19 monolayers [21], yields <15% transfection efficacy in our current assay with mature RPE monolayers. Similarly, widely-used commercial transfection reagents such as Lipofectamine 3000 and JetPRIME polyethylenimine were minimally effective at transfecting difficult-to-transfect post-mitotic RPE monolayers. The commercial reagent ViaFect was the most effective commercially available reagent tested, but it yielded less than half the level of transfection of our top identified polymer, PBAE 5-3-J12.

Our top identified polymer structure, a new composition that utilizes 3,3′-iminobis(N,N-dimethylpropylamine) as an end-group, was characterized as a nanoparticle and shown to possess biophysical properties very similar to other leading non-viral gene delivery particles (~100 nm in size with a positive surface charge). Given the relative ease of synthesis of PBAEs and their overall low cytotoxicity and permanence due to their rapid hydrolytic degradation rate [55], they represent a promising biological technology for routine in vitro transfections in the laboratory to further research into retinal biology and genetics.

Despite the promise of PBAEs for transfection of difficult to transfect cells, we do also want to mention a note of caution. Previous work has demonstrated that high levels of transgene expression, including high levels of fluorescent reporter protein expression, can induce apoptosis and other undesirable cellular changes in transfected cells, which may be a challenge for the use of high efficiency non-viral vectors such as those described in this manuscript [60]. Presumably related to this phenomenon, we did observe cellular changes and cell death in experiments in which RPE cells were induced to express high levels of mCherry here, a phenomenon that was not observed when using non-coding plasmid DNA. Potential approaches to limit the
negatives effects of over-expression include utilization of self-limiting expression cassettes and use of weaker or regulatable promoters, as well as developing methods to directly reduce the cellular toxicity of over-expressed proteins.

CONCLUSIONS

In summary, here we report the high-throughput screening and development of PBAE-based, biodegradable nanoparticles as efficient vehicles for delivering pDNA to human iPSC-RPE monolayers using a combinatorial chemistry approach. By screening a total of 140 synthesized PBAEs with varying chemical structures, we identified lead PBAE structures that resulted in markedly increased pDNA delivery efficiency in vitro. Our results suggest that PBAE can effectively complex pDNA into nanoparticles, and protect the pDNA from being degraded by environmental nucleases and deliver pDNA effectively to RPE monolayers. Furthermore, our results support our hypothesis that PBAE-mediated pDNA delivery efficiency can be modulated by tuning PBAE end group chemistry. Using human iPSC-RPE monolayers as model cell types, we identified several PBAE polymers that allow efficient pDNA delivery at levels that are double that of leading commercial transfection reagents, while maintaining high cell viability. The top synthetic polymer, 3,3′-iminobis(N,N-dimethylpropylamine)-end terminated poly(1,5-pentanediol diacrylate-co-3 amino-1-propanol) (5-3-J12), formed ~100 nm nanoparticles when mixed with plasmid DNA, could co-deliver multiple plasmids to human iPSC-RPE monolayers, and was capable of transfecting a majority of iPSC-RPE cells with minimal cytotoxicity. Together, our results highlight the promise of PBAE-based nanoparticles as novel non-viral gene carriers for pDNA delivery into hard-to-transfect cells such as RPE monolayers.

ACKNOWLEDGEMENTS

We are grateful to Baranda S. Hansen for technical assistance. The authors thank the Wilmer Microscopy and Imaging Core Facility (EY001765) at Johns Hopkins for use of their confocal microscopy.

CONFLICT OF INTEREST STATEMENT

DJZ is on the scientific advisory board of Spark Therapeutics, which is interested in developing optimized approaches for retinal gene delivery.

FUNDING

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**AUTHOR CONTRIBUTION**


**REFERENCES:**


Supporting Methods

1.1 Commercial transfection reagent testing

1. Supporting Figures

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Figure 6-S2. Gel permeation chromatograph characterization
Figure 6-S3. Ineffective endcap monomers
Figure 6-S4. Cell identification using Cellomics Arrayscan software
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3. Supporting Tables

Table S1. Commercial reagents
Table S2. Base polymer monomers
Table S3. End-cap monomers
Table S4. Minimally effective end-cap monomers
Supporting Methods:

1.1 Commercial Transfection Reagent Preparation

All commercial reagents were prepared according to manufacturer recommendations with CAG-mCherry at the ratios listed in the table below. After 15-20 minutes for particle formation, nanoparticles were added to day 25 differentiated RPE monolayer cells in 384 well plates at the specified DNA doses by varying dispensing volume. After either 4 or 24 hours, media was entirely replaced with fresh medium and cells were cultured for two additional days, at which point transfection efficacy was assessed by image analysis with Cellomics. Minimal transfection (<1%) was generally noted for the tested 4-hour incubation times, so only 24 hour incubation time data is presented.

Table S6-1. Commercial reagents

<table>
<thead>
<tr>
<th>Reagent Name</th>
<th>Company</th>
<th>Product #</th>
<th>Ratios tested (µL/µg DNA)</th>
<th>Incubation Time (h)</th>
</tr>
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<tbody>
<tr>
<td>ViaFect</td>
<td>Promega</td>
<td>E4981</td>
<td>2, 4</td>
<td>24</td>
</tr>
<tr>
<td>Dreamfect Stem</td>
<td>OZ Biosciences</td>
<td>ST30500</td>
<td>1.5, 3</td>
<td>24</td>
</tr>
<tr>
<td>Dreamfect Gold</td>
<td>OZ Biosciences</td>
<td>DG80500</td>
<td>2, 4</td>
<td>24</td>
</tr>
<tr>
<td>FuGENE HD</td>
<td>Promega</td>
<td>E2311</td>
<td>1, 3</td>
<td>24</td>
</tr>
<tr>
<td>FuGENE 6</td>
<td>Promega</td>
<td>E2691</td>
<td>2, 6</td>
<td>24</td>
</tr>
<tr>
<td>JetPRIME</td>
<td>Polyplus</td>
<td>114-07</td>
<td>0.5, 1</td>
<td>24</td>
</tr>
<tr>
<td>DNA-IN Stem</td>
<td>MTI-Global Stem</td>
<td>73750</td>
<td>0.5, 1</td>
<td>24</td>
</tr>
<tr>
<td>Lipofectamine 3000</td>
<td>Thermofisher</td>
<td>L3000001</td>
<td>0.5, 1, 2</td>
<td>24</td>
</tr>
</tbody>
</table>
Figure 6-S1. Base polymer PBAEs $^1$H NMR. Following synthesis, polymers were washed with diethyl ether twice and characterized by $^1$H NMR (500 Mhz) to verify that base polymer structures were acrylate terminated. The ratio of integrated acrylate peak area to s-monomer carbon area was used to determine
molecular weight $M_N$ of base polymers. Calibration and contamination peaks include CDCl$_3$ 7.26; DMSO 2.62; diethyl ether 3.48, 1.20; tetramethyl silane (TMS) 0.

Figure 6-S2. Gel permeation chromatograph polymer size characterization. PBAEs were characterized via gel permeation chromatography to assess molecular weight against linear polystyrene standards following synthesis and after dissolved in DMSO and washed with diethyl ether twice. Washing with diethyl ether was shown to remove unreacted monomers units and small oligomers, (A) increasing polymer number average weight $M_N$ and (B) reducing the polydispersity index (PDI).
Figure 6-S3. Ineffective endcap monomers. Endcap structures shown were tested and confirmed to effectively react with acrylate terminated PBAE polymer 4-4-Ac but the resulting polymers were wholly ineffective for delivery of plasmid DNA to HEK293T cells. These E-monomers were excluded from large library end-capping for transfection efficacy studies in harder-to-transfect RPE monolayers.
Figure 6-S4. Cell identification using Cellomics Arrayscan software. 20x images acquired using Cellomics Arrayscan were computationally analyzed to identify cell nuclei from H2B-GFP (nucGFP) expression (485 +/- 10 nm excitation), followed by characterization of efficacy transfection from mCherry fluorescence (549 +/- 7.5 nm excitation) above background. Cells with nucGFP expression below a cutoff threshold, nuclei size below a cutoff threshold or overlapping nuclei with nuclei area above cutoff threshold were discarded (orange in nuclei identification). Cells marked by a red “1” denote positive transfection events. Scale bar is 50 µm.
Figure 6-S5. Post-mitotic status of differentiated RPE monolayers. Human iPS cells seeded in 384 plates were allowed to differentiate over 25 days in culture in 384 well plates. (A) Cell number per well increases from day 2 to day 10, at which point cell number peaked and cells began to differentiate. (B) Cells were visibly more dense at day 25 post-seeding compared to day 3 post-seeding with smaller nuclei. RPE monolayer at day 25 additionally possessed textured appearance. Bars show mean ± SEM of eight wells for each condition. Scale bar is 100 µm for 20x images.
Figure 6-S6. *In vitro* high throughput screening of PBAE nanoparticles in sub-confluent D3 RPE monolayer. 

(A) Representative images showing mCherry transfected RPE cells. Heat maps showing the (B) percentage transfected RPE cells and (C) percentage survival rate following the introduction of a combinations of 140
different nanoparticles to confluent RPE monolayer at day 3 post seeding. The color scale bar refers to the percentage transfection efficiency and percentage survival that was calculated based on the number of mCherry positive cells detected from total number of cell population. Scale bar 100 µm.

Figure 6-S7: PBAE nanoparticle 5-3-J12 dose and w/w ratio optimization. (A) RPE monolayers (D25) receiving equivalent DNA doses between 50-125 ng/well demonstrate a w/w ratio of 90 w/w and 75 ng DNA dose as optimal for transfection efficacy and (B) relative viability from nucGFP count per well. (C) Holding polymer dose constant and varying DNA dose to vary w/w ratio demonstrated that transfection efficacy was dependent on the ratio between polymer to DNA and not simply dependent on the amount of polymer dosed per well. Nanoparticles formed at 30 w/w were largely ineffective, even when the total polymer amount was equivalent to that used for 90 w/w nanoparticles. (D) Relative cell viability of RPE monolayer cells treated with equal polymer dose at specified w/w ratios.
Figure 6-S8. Commercial reagent transfection efficacy optimization. (A) A broad range of commercial transfection reagents as well as prior polymer PBAE 557 were tested in transfection of RPE monolayers at a range of ratios between polymer to plasmid DNA. (B) Each grouping for transfection and viability show multiple DNA doses tested organized from high to low. (C) Relative cell count to untreated wells following transfection. (D) Representative microscope images of cells transfected with specific commercial reagents and PBAE 557. Bars show mean ± SEM of four wells for each condition. Scale bar is 100 µm for 20x images.
Figure 6-S9. Transfection efficacy over multiple preparations of RPE monolayers. RPE monolayers were differentiated from human pluripotent stem cells and transfected on four separate occasions to account for variability between cell preparations and transfection efficacy. (A) PBAE 5-3-J12/DNA NPs at a 90 w/w ratio and dose of 75 ng/well was always more statistically effective for transfection than ViaFect at a 4x ratio to plasmid DNA and dose of 200 ng/well (multiple t-tests with Holm-Sidak corrected multiple comparisons).

(B) Cell viability following treatment was similar between each transfection occasion. (C) Microscope images of cells transfected on four different occasions show variation in the level of transfection. (D) Microscope image montage of nine fields demonstrates the level of consistency in transfection across the entire well area.
Figure 6-S10. Live cell nuclei count over time and expression time-course. (A) Representative microscopy images of nuclear GFP expression of RPE monolayers on days following transfection. Only cells induced to express CAG-mCherry show difference in phenotype of nuclei. (B) Reduction in live cell number per well only occurs for cells induced to express CAG-mCherry and not cells receiving the same nanoparticle.
treatment with non-coding Cy5 labeled plasmid DNA. (C) mCherry gene expression peaked on day four following transfection. Error bars show mean ± SEM of four wells for each condition. Scale bar 100 µm.

Table 6-S2. Base polymer monomers. Monomers used for PBAE library synthesis for screening RPE cells. Acrylate terminated polymers were synthesized from small molecule diacrylate and primary amine monomers.

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Monomer Name</th>
<th>MW</th>
<th>CAS number</th>
<th>Supplier</th>
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</thead>
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<td><strong>Base-Monomers (B)</strong></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1,3-propanediol diacrylate</td>
<td>B3</td>
<td>184.19</td>
<td>24493-53-6</td>
<td>Monomer-Polymer and Dajac Labs</td>
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<tr>
<td>1,4-Butanediol diacrylate</td>
<td>B4</td>
<td>198.22</td>
<td>1070-70-8</td>
<td>Alfa Aesar</td>
</tr>
<tr>
<td>1,5-Pentanediol diacrylate</td>
<td>B5</td>
<td>212.24</td>
<td>36840-85-4</td>
<td>Monomer-Polymer and Dajac Labs</td>
</tr>
<tr>
<td><strong>Side-chain-Monomers (S)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3-amino-1-propanol</td>
<td>S3</td>
<td>75.11</td>
<td>156-87-6</td>
<td>Alfa Aesar</td>
</tr>
<tr>
<td>4-amino-1-butanol</td>
<td>S4</td>
<td>89.14</td>
<td>13325-10-05</td>
<td>Fisher Scientific</td>
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<tr>
<td>5-amino-1-pentanol</td>
<td>S5</td>
<td>103.16</td>
<td>2508-29-4</td>
<td>Alfa Aesar</td>
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Table 6-S3. End-cap monomer library. Acrylate terminated base polymers were end-capped in a high-throughput manner with the 36 small molecule amine monomers below to yield end-capped linear polymers.

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Monomer Name</th>
<th>MW</th>
<th>CAS number</th>
<th>Supplier</th>
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</thead>
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<td><strong>Endcap Monomers</strong></td>
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<td></td>
<td></td>
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<tr>
<td>1,3-diaminopropane</td>
<td>J1</td>
<td>74.12</td>
<td>109-76-2</td>
<td>Sigma Aldrich</td>
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<tr>
<td>2,2-dimethyl-1,3-propanediamine</td>
<td>J2</td>
<td>102.18</td>
<td>7328-91-8</td>
<td>Sigma Aldrich</td>
</tr>
<tr>
<td>1,3-diminoopentane</td>
<td>J3</td>
<td>102.18</td>
<td>589-37-7</td>
<td>TCI America</td>
</tr>
<tr>
<td>2-methyl-1,5-diminoopentane</td>
<td>J4</td>
<td>116.2</td>
<td>15520-10-02</td>
<td>TCI America</td>
</tr>
<tr>
<td>Diethylenetriamine</td>
<td>J5</td>
<td>103.17</td>
<td>111-40-0</td>
<td>EMD Millipore</td>
</tr>
<tr>
<td>Triethylenetetramine</td>
<td>J6</td>
<td>146.23</td>
<td>112-24-3</td>
<td>Sigma Aldrich</td>
</tr>
<tr>
<td>Tetraethylenepentamine</td>
<td>J7</td>
<td>189.3</td>
<td>1112-57-2</td>
<td>Sigma Aldrich</td>
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<tr>
<td>Pentaethylenhexamine</td>
<td>J8</td>
<td>232.44</td>
<td>4067-16-7</td>
<td>Santa Cruz</td>
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<tr>
<td>N,N-Dimethylpropylenetriamine</td>
<td>J9</td>
<td>159.27</td>
<td>10563298</td>
<td>Sigma Aldrich</td>
</tr>
<tr>
<td>3,3'-Diamo-N-methylpiperazine</td>
<td>J10</td>
<td>145.25</td>
<td>105-83-9</td>
<td>MP Biomedicals</td>
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<tr>
<td>N,N-Diethyldiethylene ether diamine</td>
<td>J11</td>
<td>159.27</td>
<td>24426-16-2</td>
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<td>3,3'-Iminobis(N,N-dimethylpropyamine)</td>
<td>J12</td>
<td>187.33</td>
<td>6711484</td>
<td>Santa cruz</td>
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<tr>
<td>Tris(2-aminoethyl)amine</td>
<td>J13</td>
<td>146.23</td>
<td>4097-89-6</td>
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</tr>
<tr>
<td>Tris(2-methylnamino)ethylamine</td>
<td>J14</td>
<td>188.31</td>
<td>65604-89-9</td>
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<tr>
<td>1-(2-aminopropyl)piperidine</td>
<td>K1</td>
<td>128.22</td>
<td>27578-60-5</td>
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<tr>
<td>N-(3-Aminopropyl)piperidine</td>
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<td>128.22</td>
<td>3529-08-6</td>
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<tr>
<td>2-(Aminomethyl)piperidine</td>
<td>K3</td>
<td>114.19</td>
<td>22990-77-8</td>
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<td>4-(Aminomethyl)piperidine</td>
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<td>1-(3-Aminopropyl)pyrrolidine</td>
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<td>N-(3-Hydroxypropyl)ethylenediamine</td>
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<td>118.18</td>
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<td>N-(2-Hydroxyethyl)ethylenediamine</td>
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<td>104.15</td>
<td>111-41-1</td>
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<td>N,N'-Bis(2-hydroxyethyl)ethylenediamine</td>
<td>M4</td>
<td>148.2</td>
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<td>TCI America</td>
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<td>2-(2-Aminoethoxy)ethanol</td>
<td>M5</td>
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<td>1-(2-Aminoethyl)piperazine</td>
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<td>1-(3-Aminopropyl)-4-methylpiperazine</td>
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<td>2,2'-Oxybis(ethylenimine)</td>
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<td>1,11-dimino-3,6,9-trioxaundecane</td>
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<td>TCI America</td>
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<td>4246-51-9</td>
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<tr>
<td>3-Morpholinopropylamine</td>
<td>P1</td>
<td>144.21</td>
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<td>Sigma Aldrich</td>
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<tr>
<td>4-(2-Aminoethyl)morpholine</td>
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<td>130.19</td>
<td>2038-031</td>
<td>Sigma Aldrich</td>
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Table 6-S4. Minimally effective end-cap monomers. Base polymer 4-4-Ae end-capped with the 24 monomers below showed minimal (<3%) or non-existent expression following transfection in HEK293T cells.

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<thead>
<tr>
<th>Chemical</th>
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<th>MW</th>
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<th>Supplier</th>
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<td>4-Aminophenyl disulfide</td>
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<td>51-45-6</td>
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<td>E14</td>
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<td>351-50-8</td>
<td>Sigma Aldrich</td>
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<td>E15</td>
<td>155.15</td>
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<td>2,4,6-Trimethyl-phenylenediamine</td>
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<td>101-80-4</td>
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<tr>
<td>4-Diaminobenzenilide</td>
<td>E29</td>
<td>227.26</td>
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<td>N,N-Dimethyl-4,4'-azodianiline</td>
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Chapter 7: Differentially Branched Ester Amine Quadpolymers with Amphiphilic and pH Sensitive Properties for Efficient Plasmid DNA Delivery

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TOC/Graphical Abstract:

Foreword: The idea of using branched polymers for nucleic acid delivery is not new, but I felt that no one had yet fairly and definitively tested the efficacy of branched poly(beta-amine ester)s against their linear counterparts for delivery of plasmid DNA. The resulting paper published in the ACS journal Molecular Pharmaceutics is a deep dive into the structural effect of polymer branching on cellular scale delivery of plasmids.


Abstract:

Development of highly effective non-viral gene delivery vectors for transfection of diverse cell populations remains a challenge despite utilization of both rational and combinatorial driven approaches to nanoparticle engineering. In this work, multifunctional polyesters are synthesized with well-defined branching
structures via $A_2 + B_2/B_3 + C_1$ Michael addition reactions from small molecule acrylate and amine monomers and then end-capped with amine-containing small molecules to assess the influence of polymer branching structure on transfection. These Branched poly(Ester Amine) Quadpolymers (BEAQs) are highly effective for delivery of plasmid DNA to retinal pigment epithelial cells and demonstrate multiple improvements over previously reported leading linear poly(beta-amino ester)s, particularly for volume-limited applications where improved efficiency is required. BEAQs with moderate degrees of branching are demonstrated to be optimal for delivery under high serum conditions and low nanoparticle doses further relevant for therapeutic gene delivery applications. Defined structural properties of each polymer in the series, including tertiary amine content, correlated with cellular transfection efficacy and viability. Trends that can be applied to the rational design of future generations of biodegradable polymers are elucidated.

Keywords: non-viral, gene delivery; polymeric nanoparticle; transfection; branched polymer, plasmid DNA

1. Introduction

Safe and effective gene delivery to specific cell populations has the potential to revolutionize medicine by enabling gene expression to be turned on or off precisely with the delivery of DNA or RNA. While viral vectors, particularly adeno-associated virus (AAV), have shown gains in the therapeutic delivery of DNA in some diseases, clinical level production of AAV remains an enormous challenge, a nucleic acid carrying capacity is limited, and patient existing immunity can limit eligible patient populations.\textsuperscript{3-4} In contrast, non-viral nanoparticle based gene delivery methods have the potential to be both less expensive to produce, less immunogenic, and enable greater nucleic acid cargo capacity than AAV. However, non-viral gene delivery systems have suffered from low delivery efficacy to many cell types due to both systemic and intracellular delivery inefficiencies, which prevent translation to the clinic.\textsuperscript{5} While non-viral vectors have been demonstrated capable for effective delivery \textit{in vivo}, there remains a need to develop enhanced nanoparticles that are more efficient, particularly for applications in which the administration route limits the dose.

Polyesters are a class of polymers that have been utilized for non-viral gene delivery with high efficacy both \textit{in vitro} and \textit{in vivo} to a variety of cell types.\textsuperscript{6-9} Synthesis of poly(beta-amino ester)s (PBAEs) in particular via Michael addition reactions is relatively easy to achieve and vast libraries of linear polymers have been synthesized to explore the solution space of possible polymer structures for purposes of gene delivery.\textsuperscript{10-12} Until recently, however, only linear PBAEs have been explored for their ability to deliver nucleic acids to mammalian cells, despite the demonstration that branching polymers are often more effective than their linear counterparts for delivery of plasmid DNA in a variety of polymer systems such as polyethylenimine (PEI),\textsuperscript{13} and poly(2-dimethylaminoethyl methacrylate) (PDMAEMA).\textsuperscript{14, 15} Recent advances in the use of triacrylate monomers to synthesize branched polymers by Michael addition reaction have yielded
polymers highly effective for delivery of nucleic acids to a variety of cell types, including cancer cells\textsuperscript{14,15}, skin cells\textsuperscript{16}, neural cells\textsuperscript{17} and mesenchymal stem cells.\textsuperscript{17} Much of this prior work in the synthesis of branched PBAEs has either failed to assess the efficacy of branched polymers against linear polymers across the entire range of possible w/w ratios or has only utilized linear polymer structures of insufficiently high molecular weight and cationicity to achieve effective gene delivery.\textsuperscript{16,19}

Polymers with beta-amino groups are rapidly biodegradable and finely tunable for properties such as hydrophobicity, molecular weight and cationic charge by selection of constituent monomers. These features enable certain structures to be highly effective for gene delivery but often require large empirical screens to identify effective structures. The biodegradability of PBAEs in aqueous solution is uncharacteristically short for polyesters with typical bond half-lives of 4-6h for the backbone ester bonds,\textsuperscript{18} enabling the polymers to degrade to non-toxic, hydrophilic oligomers within 24 hours. Hydrophobicity can be modulated for transfection of different cell types\textsuperscript{19} and molecular weight can be modulated by tuning the overall vinyl to amine ratio.\textsuperscript{11,20} Linear acrylate-terminated PBAE polymers can also be end-capped with a variety of small molecule primary amines that increase the cationic charge of the polymer by adding secondary as well as primary amines to the polymer.\textsuperscript{21}

Whereas with polyethylenimine (PEI), branching structure changes the cationic character of the polymer (linear polymers contain mostly secondary amines while branched polymers contain a tertiary amine at each branch point and a primary amine at each new terminal group), branching in a PBAE synthesis scheme does not dramatically change tertiary amines present in polymer structures of the same molecular weight. On the other hand, for PBAEs, branching structure can increase the density of end-capping functional groups, and these molecules have been shown previously to greatly enhance the transfection efficacy of linear polymers.\textsuperscript{18,21} Branching in other polymeric systems has been further hypothesized to enhance the “needle effect” of endosomal escape mediated by polymer swelling, which could help explain this increase in efficacy.\textsuperscript{22,24}

Here we present the synthesis and characterization of a new polymer series, Branched poly(Ester Amine) Quadpolymers (BEAQs). They are composed of four constituent monomers in ratios that influence the cationic character and hydrophobicity of the polymer species in a predictable manner. This work builds on the successes of poly(ester amine) materials such as linear PBAEs,\textsuperscript{12} poly(amine-co-ester) (PACE) terpolymers,\textsuperscript{25} and poly(alkylene maleate mercaptamines) (PAMA)s\textsuperscript{26} that have demonstrated the utility of amines to bind nucleic acids, ester linkages to facilitate nucleic acid release and reduce toxicity as well as the ability to modulate cation density and hydrophobicity. We utilized A2+B2/B3 Michael addition reactions to synthesize primarily acrylate terminated polymers with well-defined degrees of branching that were then end-capped with a C monomer to explore the influence of branching structure on transfection efficacy and nanoparticle properties. This further enabled us to incorporate fine control of small amine-containing molecule end-groups for engineering of polymer and nanoparticle surface properties and hypothesized cell-
specific delivery.\textsuperscript{18, 27-29} Thus, the four components of the quadpolymers control degradability, hydrophobicity, branching, and cationicity which have large effects on delivery efficacy and cytotoxicity.\textsuperscript{30} We assessed each polymer quantitatively for plasmid DNA binding under various conditions to demonstrate that increased DNA binding is attributable to increased cationicity resulting from multiple end-caps as well as branching structure. Branching was further shown to improve DNA binding and transfection efficacy under conditions that normally destabilize polyplex nanoparticles.

2. Experimental

2.1 Materials: Trimethylolpropane triacrylate (TMPTA/B8, CAS 15625895), Bisphenol A glycerolate (1 glycerol/phenol) diacrylate (BGDA/B7, CAS 4687-94-9) and 2-(3-Aminopropylamino)ethanol (E6, CAS 4461-39-6) were purchased from Sigma Aldrich and used without further purification. 4-amino-1-butanol (S4, CAS 13325-10-05) was purchased from Alfa Aesar. Acrylate monomers were stored with desiccant at 4°C, while amine monomers were stored with desiccant at room temperature. Plasmid peGFP-N1 (Addgene 2491) was used for transfection efficacy screens. Cy5-amine (230C0) was purchased from Lumiprobe (Hallandale Beach, FL), dissolved in DMSO at a concentration of 10 µg/µL and stored at -20°C in small aliquots. Plasmid DNA (eGFP-N1) was labeled as previously described using NHS-Psoralen with the fluorophore Cy5-amine at a density of approximately 1 fluorophore/50 base pairs DNA.\textsuperscript{31}

2.2 Polymer synthesis: BEAQs were synthesized according to the ratios in Table S1 at an overall vinyl:amine ratio of 2.2:1 and monomer concentration of 200 mg/mL in anhydrous DMF. The diacrylate monomer (B7) was first weighed out to a 20 mL scintillation vial, after which triacrylate monomer (B8) was added. Anhydrous DMF was added to the vial and monomers were fully vortexed into solution and heated to 90°C before adding primary amine monomer S4. Monomer purity was accounted for in synthesis calculations based on the vendor characterization of each lot. Monomer B7 was assumed to be 90% pure in the absence of any reported purity information. Monomer solutions were then stirred at 90°C for 24h, after which polymers were removed from the oven and mixed with a solution of monomer E6 (2-(3-Aminopropylamino)ethanol) in anhydrous DMF (final concentration 0.2 M) in the dark at room temperature for 1h. End-capped polymer solutions were then precipitated twice in diethyl ether (10x volume followed by 5x volume) and dried under vacuum for three days. Polymers were finally re-dissolved in anhydrous DMSO at 100 mg/mL and stored at -20°C in small volume aliquots. Polymers were named according to the triacrylate mole fraction; thus B8-50% corresponds to the 50% triacrylate mole fraction polymer formed between the diacrylate (B7), triacrylate (B8), amino (S4) and diamino (E6) monomers with the triacrylate (B8) monomer accounting for 50% of the vinyl moieties in the initial monomer mixture.
2.3 Polymer characterization: Acrylate terminated polymers were sampled from reaction vials prior to end-capping reactions and precipitated twice in 10x volumes of diethyl ether to recover neat polymer. Acrylate terminated polymers were then dried under vacuum for 2h and analyzed via \(^1\)H NMR in CDCl\(_3\) (Bruker 500 MHz) to confirm the presence of acrylate peaks and quantify degree of branching. End-capped polymer likewise was characterized via \(^1\)H NMR in CDCl\(_3\) to confirm complete reaction of end-cap monomer with acrylate terminated polymers. End-capped polymer was also characterized via gel permeation chromatography (GPC) using a Waters system with autosampler, styragel column and refractive index detector to determine \(M_N\), \(M_W\) and polydispersity index (PDI) relative to linear polystyrene standards. GPC measurements were performed as previously described with minor changes of flow rate (0.5 mL/min) and increase in sample run time to 75 minutes per sample.\(^{32}\)

2.4 Polymer buffering capacity: End-capped polymer buffering capacity as a function of polymer structure was assessed by titrating 10 mg (100 µL at 100 mg/mL) of polymer dissolved in 10 mL of acidified, 100 mM NaCl from pH 3.0 to pH 11.\(^{18}\) For titrations, pH was determined using a SevenEasy pH Meter (Mettler Toledo) with pH assessed after stepwise addition of 100 mM sodium hydroxide.

2.5 Polymer solubility limit: Polymers were dissolved in pH 7.4, 150 mM PBS or pH 5.0, 25 mM NaAc at the specified maximum concentration and aliquoted (50 µL) to a round bottom 96 well plate (n=3 wells). Polymers were then diluted stepwise in their respective buffers and absorbance measurements were acquired with a plate reader (Biotek Synergy 2) at 600 nm (for opacity indicative of solubility limit). Absorbance measurements of 0.5 were defined as the maximum solubility point for purposes of plotting polymer solubility (Figure 7-S2).

2.6 DNA binding assays: Yo-Pro-1 iodide binding assays were run similarly to previously published results,\(^{33}\) where DNA and Yo-Pro-1 iodide (Thermo Fisher) were both diluted to a concentration of 1 µM (3.1 µg/mL plasmid) in either 25 mM NaAc, pH 5.0 or 150 mM PBS, pH 7.4 then mixed with polymer to give a 100 µL well volume in opaque black well plates. Green channel fluorescence was then measured using a plate reader after 30 minutes of incubation (Biotek Synergy 2). Gel electrophoresis binding experiments were run as previously described\(^9\) with nanoparticles prepared in either 25 mM NaAc buffer, pH 5.0 or 150 mM PBS, pH 7.4, diluted with 30% glycerol for loading into a 1% agarose gel.

2.7 Nanoparticle characterization: Three samples were independently prepared for each nanoparticle formulation at the same concentrations as outlined in the transfection methods section. Nanoparticle hydrodynamic diameters in 25 mM NaAc, pH 5.0 were then determined by dynamic light scattering (DLS) in disposable
micro-cuvettes using a Malvern Zetasizer NanoZS (Malvern Instruments, Marlvern, UK) with a detection angle of 173°. Samples were then diluted in 150 mM PBS at a dilution factor of 6 and measured again to determine nanoparticle hydrodynamic diameter in neutral, isotonic buffer followed by determination of zeta potential by electrophoretic light scattering in disposable zeta cuvettes at 25°C using the same Malvern Zetasizer NanoZS.

Transmission electron microscopy (TEM) images were acquired using a Philips CM120 (Philips Research, Briarcliffs Manor, New York) on 400 square mesh carbon coated TEM grids. Samples were prepared at a DNA concentration of 0.045 µg/µL and polymer 40 w/w ratio in 25 mM NaAc, pH 5.0 after which 30 µL were allowed to coat TEM grids for 20 minutes. Grids were then dipped briefly in ultrapure water to remove excess dried salt, wicked dry and allowed to fully dry under vacuum before imaging.

2.8 Cell culture: HEK293T and ARPE-19 cells were purchased from ATCC (Manassas, VA) and cultured in high glucose DMEM or DMEM/F12 respectively, supplemented with 10% heat inactivated fetal bovine serum and 1% penicillin/streptomycin. For noted 96-well plate transfection efficacy experiments, cells were plated in CytoOne 96-well tissue culture plates (USA Scientific, Ocala, FL) 24h prior to transfection with 12,000 cells/well in 100 µL complete media. For noted 384-well plate transfection experiments, cells were plated at 2,500 cells/well in 25 µL complete media in 384 well tissue culture plates (Santa Cruz, sc-206081) 24h prior to transfection. Cells were confirmed periodically to be mycoplasma negative via MycoAlert test (Lonza).

2.9 Transfection and cell uptake: For 96 well plate transfections, nanoparticles were formed by dissolving synthesized polymers and eGFP-N1 plasmid DNA in 25 mM sodium acetate (NaAc) pH 5.0 then mixing in a 1:1 volume ratio. Nanoparticles were incubated at room temperature for five minutes, then 20 µL of the nanoparticle solution were added to each well of cells containing 100 µL of complete media and allowed to incubate for two hours, at which point the media was replaced. Transfection efficacy was assessed for percent-transfected cells and geometric mean expression approximately 48h following transfection using flow cytometry with a BD Accuri C6 flow cytometer with HyperCyt autosampler and gated in 2D against untreated cells in FlowJo (Figure 7-S19). Cell viability was assessed using MTS Celltiter 96 Aqueous One (Promega, Madison, WI) cell proliferation assay approximately 24h following transfection. For 384 well plate transfection of low doses of nanoparticles, synthesized polymers in DMSO were dissolved in 25 mM NaAc buffer to a concentration of 7.5 µg/µL then mixed with DNA dissolved in 25 mM NaAc buffer in a 384 polypropylene nanoparticle source plate. Nanoparticles were then dispensed to plates of cells at low volumes using an Echo 550 liquid handler. After two days to allow for reporter expression, plates were scanned and analyzed using Cellomics Arrayscan VTI with live cell imaging module following staining with Hoechst.
Flow cytometry based cell uptake studies were performed in 96 well plates using 20% Cy5 labeled DNA as previously described. To remove associated nanoparticles that were extracellular membrane associated but had not undergone endocytosis, cells were washed once with 50 μg/mL heparin sulfate in 150 mM PBS following trypsinization and transfer to round bottom 96 well plates.

2.10 Confocal microscopy. Cells were plated on Nunc Lab-Tek 8 chambered borosilicate coverglass well plates (155411; Thermo Fisher) at 50,000 cells/well (ARPE-19) or 25,000 cells/well (HEK293T) two days prior to transfection in 250 μL phenol red free DMEM supplemented with 10% FBS and 1% penicillin/streptomycin. Nanoparticles were prepared as described above at 20 or 40 w/w ratios using Cy5 labeled plasmid DNA and eGFP-N1 plasmid DNA at an 0.8/0.2 mass ratio, then added to cells at a total dose of 150 ng DNA/well and incubated for two hours. For imaging, cells were stained for 30 minutes with Hoechst 33342 at a 1:5000 dilution (H3570; Thermo Fisher) for nuclei visualization, as well as Cell Navigator Lysosome Staining dye with pH 4.6 at a 1:2500 dilution (AAT Bio-quest, 22658) in phenol red free DMEM. Cells were then washed twice with phenol red free DMEM and imaged at 37°C in a 5% CO2 atmosphere. Images were acquired using a Zeiss LSM 780 microscope with Zen Blue software and 63x oil immersion lens. Specific laser channels used were 405 nm diode, 488 nm argon, 561 nm solid-state, and 639 nm diode lasers. Laser intensity and detector gain settings were maintained across all image acquisition. All Z-stacks were acquired for entire cell volume over scan area of 140 μm at Nyquist limit resolution.

2.11 Data analysis and figures: FlowJo was used for flow cytometry analysis and Cellomics HCS Studio (Thermo Fisher) was used for image acquisition based transfection analysis. Polymer structures were characterized in ChemDraw (Perkin Elmer, Boston, MA) and Marvin (ChemAxon, Cambridge, MA) to determine logP and logD values. Calculation of normalized 50% serum transfection efficacy was performed by dividing the percent transfection or geometric mean transfection efficacy achieved in 50% serum media by the same nanoparticle (B8% and w/w ratio) formulation percent transfection or geometric mean transfection efficacy achieved in 10% serum. Confocal microscopy colocalization of plasmid DNA with lysosomes was assessed as intensity weighted colocalization in Zen Blue, then normalized by individual image area of plasmid DNA per image for statistical quantification.

2.12 Statistics: Prism 8 (Graphpad, La Jolla, CA) was used for all statistical analyses and curve plotting. Unless otherwise specified, statistical tests were performed with a global alpha value of 0.05. Unless otherwise stated, absence of statistical significance markings where a test was stated to have been performed signified no statistical significance. Statistical significance was denoted as follows: *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001.
3. Results

3.1. Branched poly(Ester Amine) Quadpolymer Synthesis and Characterization

3.1.1. Synthesis of Acrylate Terminated Polymers

A series of Branched poly(Ester Amine) Quadpolymers (BEAQ) with differential degrees of branching was synthesized via step-growth A2 + B2/B3 Michael addition reactions from a small molecule diacylate (BGDA/B7), triacylate (TMPTA/B8) and amino-alcohol (S4) monomers (Figure 7-1 and Table 7-S1). In the synthesis scheme of A2 + B2/B3 + C, A2 corresponds to the primary amine monomer (S4) that can react twice, B2 corresponds to the diacylate monomer (termed B7) that can react twice, B3 corresponds to the triacylate monomer (termed B8) that can react three times and C refers to the end-cap monomer, which reacts once due to its presence in excess. We confirmed that each polymer was primarily acrylate terminated after 24 hours of synthesis via $^1$H NMR (Figure S1) by the presence of acrylate peaks between 5.5-6.5 ppm. Analysis of the acrylate terminated polymer structures with $^1$H NMR also enabled determination of polymer properties including the actual triacrylate mole-fraction of each polymer as well as number of end-cap moieties per polymer molecule (Table 7-1). By precisely varying the triacrylate monomer mole fraction, while maintaining the same 2.2:1 vinyl to amine mole ratio, the degree of branching was able to be carefully modulated in the resulting polymers as assessed by $^1$H NMR. Further, by synthesizing the polymers in each series at the same purity-accounted overall vinyl to amine ratio, the number average ($M_N$) molecular weights within each series of polymers were all very close to 4 kDa as shown by gel permeation chromatography (GPC) (Table 1).
Figure 7-1. Synthesis of Branched poly(Ester Amine) Quadpolymers (BEAQ). A) Diacrylate monomer B7 and triacrylate monomer B8 were mixed with side-chain monomer S4 to synthesize a series of BEAQs with increasing triacrylate mole fraction and degree of branching. B) Linear polymers possess two end-cap structures per molecule (red), while each triacrylate monomer in branched polymers results in an additional end-cap moiety for every branch point. C) One-pot synthesis of acrylate terminated base polymers was performed at 90°C and 200 mg/mL in DMF for 24 hours. Polymers were then end-capped with monomer E6 at room temperature for one hour to yield the final product.

3.1.2 End-cap Modification of Polymers

PBAEs have been “end-capped” with small molecule monomers possessing secondary and tertiary amines that increase the overall polymer amine density, resulting in linear polymers with tertiary amines along the polymer backbone and greater amine density at just the two ends of the linear polymers.12, 21, 34-35 Most of the small molecule end-caps shown previously to increase transfection efficacy with linear PBAE structures21 increase the cationicity of the polymer at both pH 5 and 7 due to the fact that end-capping with primary amine monomers adds at minimum two secondary amines to linear PBAEs. Here, we utilized monomer 2-(3-aminopropylamino)ethanol (termed E6) for end-capping purposes, as it has been shown to be effective as an end-capping group with linear polymers and non-cytotoxic to multiple cell lines.33 38 In contrast to previously reported branched polymer schemes, including branched PBAE schemes, this end-capping molecule exclusively increases the secondary amine content of the polymer. All BEAQs were confirmed to be completely end-capped by 1H NMR and the number average of end-cap moieties per polymer molecule as estimated from NMR spectra ranged from two for the linear polymer to seven for the 90% triacrylate mole fraction polymer (Table 1). Notably, end-cap molecular mass fraction contribution in these polymers reaches near 30% for the high triacrylate mole fraction polymers, whereas linear PBAEs have an end-cap monomer mass fraction of approximately 5%, which reduces further for higher molecular weight linear polymers (Table 1). Polydispersity in moderately branched BEAQs was minimized by synthesizing at a dilute concentration, while high polydispersity of hyperbranched BEAQs with triacrylate mole fraction >60% is consistent with other hyperbranched polymer synthesis schemes.36
Table 7-1. Structural properties of synthesized polymers

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3.1.3 Polymer Series Hydrophobicity

The chemical properties of each polymer in the series with known M<sub>n</sub> and monomer composition were predicted in silico to assess the influence of branching with TMPTA on polymer hydrophobicity. Hydrophobicity was assessed as predicted partition coefficient (logP) and ionization influenced distribution coefficient (logD) at neutral and acidic pH values (Figure 7-2a, 7-S2), demonstrating that branching increases BEAQ hydrophilicity for the monomers utilized here, and that pH sensitive ionization plays an important role in polymer solubility. Branching was hypothesized to reduce both polymer logP and logD values as a greater number of E6 monomer end-cap moieties in branched structures increased the prevalence of hydrophilic hydroxyl groups and charged secondary amines; polymers with a high degree of branching were further subject to reduction in hydrophobicity due to the fact that the mass fraction of the diacrylate monomer B7, which contains a bisphenol group, was likewise reduced. We confirmed this predicted reduction in hydrophobicity experimentally via an absorbance based assay, to show that BEAQS with at least 40% triacrylate mole fraction were over twice as soluble as the linear B8-0% polymer under both low pH and physiological pH conditions (Figure 7-S2).

3.1.4 Polymer Series Buffering Capacity

Titration of the polymers demonstrated buffering capacity in the physiological pH range for hypothesized endosomal escape properties (5 to 7.4), as BEAQs with greater triacrylate mole fraction possessed a larger buffering capacity in this range (Figure 7-2B). Effective pKa in the pH range from 5 to 8 was calculated as the pH at the maximum normalized buffering capacity of the derivative of the titration.

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curves defined as $\Delta(-\text{OH})/\Delta(pH)$ (Figure 7-S2B). Effective pKa was demonstrated to increase moderately with increased branching from approximately 6.0 to 6.75 (Figure 7-2C). These results are due to the combined effects of additional tertiary amine density in the polymer backbone and the presence of additional secondary amines in end-groups as the branching increases.\textsuperscript{18} Tertiary amine density calculated relative to the base polymer structures (Table 7-S4) shows that diacylate B7+S4 polymer repeat units have much lower tertiary amine density than triacylate B8+S4 repeat units and physical spacing of the tertiary amines in high diacylate B7 content polymers is greater than for high triacylate B8 content polymers. But following end-capping with monomer E6, tertiary amine density is similar amongst all synthesized polymers while secondary amine density increased substantially with triacylate mole fraction from 0.851 to 4.194 mMol per gram polymer for B8-0\% and B8-90\% respectively (Table 7-S5).

### 3.1.5 Polymer Series DNA Binding

Assessment of BEAQ/DNA binding strength interactions via Yo-Pro-1 iodide competition binding assays further demonstrated the influence of branching in polymer structure (Figure 7-2D,F). At pH 5, linear and branched polymers were equally effective at binding plasmid DNA, while in isotonic, neutral buffer at pH 7.4, branched polymers statistically outperformed linear polymers for DNA binding (Table S6). To assess if increases in DNA binding strength of the BEAQS were attributable primarily to branched structure or changes in amine content, we calculated Yo-Pro-1 iodide quenching as a function of secondary, tertiary, and total amine content per base-pair DNA from known structural characteristics of each polymer (Figure 7-S3). DNA binding normalized to tertiary amine content effectively condensed the binding assay results at pH 5, while normalization of DNA binding in neutral, isotonic buffer to secondary amine content most effectively condensed the results to fit one curve (Figure 7-2E,G). Gel electrophoresis DNA retention assays were similarly in agreement with these results, demonstrating that branching improved DNA binding particularly in neutral, isotonic buffer (Figure 7-S4). These results indicate that BEAQ backbone tertiary amines play an important role in polymer complexation with DNA at low pH, but secondary amines in BEAQ end-cap structures are primarily responsible for binding plasmid DNA following dilution into neutral solutions.

Further analysis of the difference between binding at low pH and neutral pH do, however, reveal that the increase in end-cap density of branched polymers was not exclusively responsible for increased binding at neutral pH. Scaling the difference in binding efficacy as a function of total amines per base pair DNA revealed that branched polymers were more effective at maintaining DNA binding in a manner that is attributable to structural changes instead of increases in amine content (Figure 7-2H).
Figure 7.2. Polymer properties influenced by triacrylate mole fraction. A) Predicted properties of partition coefficient (logP) and distribution coefficient (logD) for differentially branched polyesters. B) Titration of BEAQs. C) Effective pKa value of maximum buffering point between pH 4.5-8.5 of differentially branched PBAEs. D) Competition binding assay of polymer and Yo-Pro-1 iodide at low pH. (n=3 wells, mean ± SEM) E) DNA binding in low pH buffer normalized to PBAE tertiary amine content. F) Difference in degree of binding between pH 5 and pH 7.4 calculated as a function of total amines per bp DNA. G) Competition DNA binding assay in isotonic, neutral buffer. (n=3 wells, mean ± SEM) H) DNA binding in isotonic, neutral buffer normalized to secondary amine content. I) TEM image of 20% triacrylate mole fraction polymer nanoparticles.

3.2 Nanoparticle Properties

Dynamic light scattering (DLS) measurements of polymer/DNA polyplex nanoparticles to assess hydrodynamic diameter demonstrated effective independence of nanoparticle properties with regards to branching. DLS measurements of polymeric nanoparticles formed in 25 mM NaAc, pH 5.0 at a 40 w/w ratio to DNA showed that all polymers formed nanoparticles with a hydrodynamic diameter of approximately 50-
100 nm that maintained a diameter of approximately 100 nm following a 6-fold dilution into 150 mM PBS (Figure 7-S5A). All nanoparticle formulations showed similar zeta potential values of approximately +15 mV (Figure 7-S5B). Select formulations were analyzed via TEM, which showed dried nanoparticle diameters between 30-60 nm (Figure 7-S5C). Notably, the linear 0% triacrylate mole fraction (B8-0%) particles were the smallest when assessed by TEM at 32±3 nm, compared to a mean of 54±6 nm for B8-50% nanoparticles, which may be attributable to slightly stronger intermolecular polymer interactions driven by increased hydrophobic effect for the less branched polymers with higher B7 fraction / lower triacrylate monomer B8 fraction.

3.3 Cellular Transfection

3.3.1 Nanoparticle Uptake was Not Influenced by Branching Structure

We hypothesized that the increased number of end-cap moieties per polymer molecule would result in increased cellular uptake, as end-capping linear PBAEs has been demonstrated to improve cellular uptake compared to acrylate terminated and side-chain monomer terminated linear PBAEs. Further, end-cap structures have been shown to convey cell type specificity, as well as partially contribute buffering capacity of PBAEs in the physiologically relevant pH range. To assess whether the increased number of end-cap moieties per polymer molecule for BEAQs would yield greater cell uptake relative to linear PBAEs, we assessed cellular uptake by flow cytometry of nanoparticles formed with Cy5 labeled plasmid DNA in HEK293T and ARPE-19 cells at moderate fluorophore labeling density. All polymers were generally effective for mediating cellular uptake of plasmid DNA, with above 95% of cells testing as positive for DNA uptake gated against the untreated cells (Figure 7-S6). These branched polymers showed no significant improvement in cellular uptake at equivalent w/w ratios to the linear polymer. Thus, an increased number of 2-(3-Aminopropylamino) ethanol end-cap moieties per polymer molecule did not mediate higher cellular uptake as hypothesized.

3.3.2 BEAQ Nanoparticles Mediate High Transfection Efficacy

To assess the ability of BEAQs to effectively deliver plasmid DNA to both easier-to-transfect and difficult-to-transfect cell types, HEK293T cells and ARPE-19 retinal pigment epithelial cells were chosen for transfection studies with the reporter gene eGFP-N1. In these two cell lines, the BEAQs nanoparticles achieved up to 99% and 77% transfection efficacy respectively in complete medium as assessed by flow cytometry, which is greater than any reported transfection efficacy using non-viral methods in either cell line to the best of our knowledge (Figure 7-3).

Among commercial reagents we fully tested and optimized, including 25 kDa branched polyethylenimine (BPEI), 4 kDa linear polyethylenimine (LPEI), JetPRIME® and Lipofectamine 2000®
(Figure 7-S7 and 7-S8), JetPRIME gave the highest level of transfection in ARPE-19 cells at approximately 40% transfection with tolerable viability. Linear PEI gave slightly higher transfection, but at the cost of substantial cytotoxicity. The maximum level of transfection achieved in ARPE-19 cells with the reported BEAQ polymers is likewise higher than our previously optimized top linear PBAE 557 formulation, which we found transfected only 40-45% of these cells with keeping cytotoxicity <30%. This formulation was previously shown to lead to transfection in vivo following subretinal injection in mice, making it likely for these BEAQ nanoparticles to function in a similar manner in vivo.37
Figure 7-3. *In vitro* transfection with BEAQs in 10% serum media. HEK239T cells A) percent transfection efficacy, B) normalized geometric mean expression C) viability and D) fluorescence micrograph of cells transfected to express eGFP with the 20 w/w ratio, 50% triacrylate mole-fraction BEAQ. ARPE-19 cells E) percent transfection efficacy, F) normalized geometric mean expression, G) viability and H) fluorescence micrograph of cells transfected to express eGFP with 20 w/w, B8-20% triacrylate mole-fraction BEAQ. (Scale bars 200 µm. n = 4 wells, mean ± SEM).

3.3.3 Moderate Branching in BEAQs Improves Stability in Physiological Serum Conditions

Effective delivery under physiological serum conditions remains a challenge for cationic nanoparticle based gene delivery, due to the shielding and aggregation effects of serum proteins. To assess nanoparticle performance under these conditions, the BEAQs were evaluated for transfection in HEK293T and ARPE-19 cells incubated in 50% serum medium during a two hour nanoparticle incubation (Figure 7-S9). Under these challenging transfection conditions, which more closely model an *in vivo* systemic administration, BEAQs demonstrated remarkably statistically improved transfection efficacy compared to their linear counterparts, which was particularly pronounced at low w/w ratios in both cell lines (Figure 7-4A,B). The optimal BEAQ-50 branched polymer was capable of transfecting 98% and 65% of HEK293T and ARPE-19 cells under 50% serum conditions. After normalizing transfection efficacy results in 50% serum to matched results in 10% serum conditions, BEAQ nanoparticles reported here maintain 80% and 70% geometric mean expression in HEK293T cells and ARPE-19 cells with no reduction in percentage of cells transfected (Figure 7-S10).

3.3.4 Moderate Branching Improves Transfection at Low Plasmid Doses

Transfection at low nanoparticle doses likewise better mimics conditions encountered *in vivo* following administration and dilution into biological fluids. At very low nanoparticle doses, plasmid concentrations between 16-256 pM (0.25-4 pg/cell) in 384 well plates, moderately branched triacrylate mole fraction BEAQs showed statistically higher transfection compared to the optimized corresponding linear PBAE in both cell lines (Figures 7-S11, 7-S12). Overall with statistical assessment at all w/w ratios tested, B8-40% and B8-50% performed the best in both cell lines. Optimal w/w ratio was notably shifted for low DNA dose transfections, such that 60 w/w BEAQ nanoparticles showed better transfection than 20 w/w particles at very low doses (≤5 ng/well). Cell viability was not strongly affected under any of the conditions.
Figure 7.4. The effect of transfection conditions on BEAQs. High serum (50%) transfection of A) HEK293T and B) ARPE-19 cells with 20 w/w nanoparticles (normal DNA dose of 600 ng/well in 96 well plates). Low nanoparticle dose transfection with 40 w/w nanoparticles of C) HEK293T (5 ng) and D) ARPE-19 (10 ng) doses in 384 well plates. (n = 4 wells, mean ± SEM, statistical markings show results of one-way ANOVA with Dunnett corrected multiple comparisons tests to the linear polymer).

3.4 Branching reduces degree of lysosomal accumulation following uptake

Transfection of HEK293T and ARPE-19 cells with Cy5-labeled plasmid DNA followed by assessment of lysosome colocalization with confocal microscopy at 4 and 24 hours following nanoparticle treatment demonstrated that less internalized DNA was colocalized with lysosomes when delivered by B8-50% BEAQs compared to the linear B8-0% polymer (Figure 7-5A). For accurate quantification of lysosomal colocalization throughout the entire cell volume, Z-stacks were acquired at both time-points and nanoparticle area per slice was used to scale the respective contribution to calculated z-stack lysosome correlation coefficient (Figure 7-S13). Representative uncropped maximum intensity projection images of acquired Z-stacks for each condition show a high level of Cy5-DNA uptake with limited lysosome colocalization for all conditions (Figures 7-S14 and 7-S15). All nanoparticle formulations tested demonstrated a statistically significant increase in lysosome colocalization between 4 and 24 hours following nanoparticle treatment (Figure 7-5C), however, the degree of change in lysosome accumulation was lower with the B8-50% BEAQ.
nanoparticles, specifically for the higher 40 w/w ratio tested, which yielded less than 20% of internalized DNA as detectable in lysosomes at 24 hours in either cell type. The degree of lysosome colocalization for the linear B8-0% polymer at 24 hours (0.4) was still far below the colocalization we have previously measured for PLL (0.78) and BPEI (0.7), despite the ability of BPEI to much more effectively buffer protons on a per-unit basis. This result supports the notion that amphiphilic polyesters mediate lysosomal in a different manner than polyethylenimine, as their degree of lysosomal avoidance is not proportional to their buffering capacity. At 24 hours following nanoparticle treatment, cells expressing eGFP from the 20% unlabeled fraction of plasmid DNA were visible for all conditions (Figures 7-S16 and 7-S17). Cy5-labeled plasmid DNA was also detectable in the nucleus of some cells that typically were also strongly expressing eGFP at the 24-hour time point (Figure 7-6). Analysis of single slices from z-stacks did, however, reveal that most plasmid DNA internalized had not localized to the nucleus at 24 hours post-treatment, even when it avoided lysosomal degradation.
**Figure 7-5.** Lysosome colocalization assessment with confocal microscopy. A) Cells transfected with B8-0% and B8-50% at low (20 w/w) and high (40 w/w) nanoparticles and assessed by confocal microscopy show statistically significant differences in the degree of lysosome colocalization. Assessed by one-way ANOVA with Dunnett corrected multiple comparisons to the B8-50%: 40 w/w conditions. B) Representative 2D scattergrams of HEK293T cells at 24 hours post-treatment using 20 w/w nanoparticles. Region 3 represents colocalized pixel intensities. C) All conditions in both cell lines showed statistically significant (Holm-Sidak corrected multiple t-tests) increases in the degree of lysosome colocalization between 4 hours and 24 hours following transfection. (Bars show mean ± SEM of n>100 cells.) D) Representative maximum intensity projection images of cells transfected with 20 w/w nanoparticles 24 hours following transfection, showing lysosome colocalization in white.

**Figure 7-6.** Nuclear localization of plasmid DNA and expression of eGFP assessed by confocal microscopy. HEK293T cells were transfected 24 hours prior with B8-50%: 20 w/w nanoparticles containing 80% non-coding, Cy5-labeled plasmid DNA and 20% coding eGFP-N1 plasmid DNA. A) Maximum intensity projection demonstrating high level of labeled plasmid DNA remaining in the cells with minimal lysosome colocalization. B) Strong eGFP expression from the 20% of unlabeled plasmid DNA. C) A single z-slice shows Cy5-labeled plasmid DNA localized to the nucleus in select cells (white arrows).

**3.5 Trends in Transfection from Differentially Branched BEAQs**

We analyzed transfection efficacy of each polymer over the multiple w/w ratios tested as functions of polymer concentration and known specific buffering capacity as well as secondary, tertiary and total amine content. To account for overall population expression and effects of polymers on viability, we scaled geometric mean expression values by viability and normalized to the maximum geometric mean expression value of each polymer structure to give viability normalized expression. Viability normalized expression was then plotted against each variable of interest (Figure 7-7). All BEAQs demonstrated clear
biphasic trends in normalized geometric mean expression. Upon fitting a single quadratic curve to the data from all polymers, tertiary amine density as a function of tertiary amines per base pair DNA was revealed to be the most important chemical property for predicting optimal w/w ratio for transfection efficacy. Particularly, a single curve quadratic fit for all polymer data across all structures for HEK293T and ARPE-19 cells gave R² values of 0.761 and 0.615 respectively.

**Figure 7.7.** Correlation between BEAQ properties and viability normalized geometric mean expression. Geometric mean expression plots were normalized to the maximum expression for each polymer and scaled by viability at that w/w ratio for A-E) HEK293T cells and F-J) ARPE-19 cells. Dashed-grey curves show a single quadratic fit of all data points for that cell line with calculated R². Plots showing dotted-grey curves in addition to dashed-grey curves were statistically determined to require two fitted quadratic curves to adequately describe the data.

Polyethylenimine did not exhibit the same biphasic trends between amine content and geometric mean expression as BEAQs, but did demonstrate optimal amine content of approximately 30 secondary amines which may be attributable to the greater cytotoxicity encountered with using PEI that limits utilization of high w/w ratios (Figure 7-S18). Interestingly, highly branched 25 kDa BPEI had a much higher optimal total amines per bp DNA similar to the synthesized BEAQs, which may be attributable to the level of interaction...
between amines in linear polymers compared to branched polymers. Spatial accessibility of amines in polymer structures and steric hindrance in branched polymers may necessitate greater overall amine content.

4. Discussion

Branching has been demonstrated to yield enhanced transfection in many cationic polymer systems and studied in PBAEs through the use of monomers with trifunctional amine monomers\textsuperscript{40} or trifunctional triacrylate monomers for generation of branched polymers.\textsuperscript{17} Here, we sought to explore the exact nature by which branching can improve transfection efficacy of these polymers through a fair comparison of fully effective linear PBAEs to equivalent branched species. For this purpose, we synthesized a series of polymers with well-defined degrees of branching that was quantified via NMR and GPC. These BEAQs are notable in part due to the manner in which end-capping with the chosen E6 monomer affected amine density, particularly through adding secondary amines to the polymer structure. We hypothesized that the branching structure and high end-cap moiety mass fraction in BEAQs would show improved DNA binding at neutral pH and would be more effective for delivery at lower w/w ratios as compared to linear PBAEs due to their increased secondary amine cationicity.

BEAQs were shown via computational and experimental methods to be more water soluble due to the increased prevalence of hydrophilic end-cap moieties and more effective at buffering in the physiological pH range. We further calculated the effective pKa value of each polymer to demonstrate that branching influenced the pH point of maximal buffering capacity. Given the long-standing hypothesis that titration capability of polycations in the pH 5-7.4 range is responsible for “proton sponge hypothesis” driven endosomal escape,\textsuperscript{39,41-44} direct variation of the buffering capacity and effective pKa allowed evaluation of the importance of buffering in gene delivery with these polymers. Through quantitative competition DNA binding assays we demonstrated that branching improved DNA binding as a function of both increased secondary amine content via additional end-cap monomers as well as branching structure by normalized binding efficacy to specific amine content of each polymer. Importantly, BEAQs were much more effective at binding nucleic acids compared to the linear polymer following dilution into neutral, isotonic buffer.

Using the two well characterized cell lines human embryonic kidney HEK293T and human retinal pigment epithelium ARPE-19, these polymers demonstrated extremely high transfection efficacy (up to 99\% and 77\% respectively) with no notable cytotoxicity at utilized doses. BEAQs did not demonstrate greater nanoparticle uptake compared to the linear polymer but did improve transfection efficacy and reduce the necessary w/w ratio, effectively improving polymer efficiency of transfection at a given polymer mass. As the highly branched B8-80\% and B8-90\% polymers possessed the greatest buffering capacity and the most
relevant effective pKa values (nearer to pH 7) but the lowest transfection efficacy, our results further indicate that buffering capability and endosomal escape is likely not the rate-limiting step to mediating successful transfection in this polymer system. These results reinforce findings from other groups in alternative polymer systems that polymer buffering capacity between pH 4-7.4 is a necessary, but not on its own a sufficient property for transfection.45

Under more challenging transfection conditions of extremely low nanoparticle doses or under physiological serum conditions, moderately branched BEAQs were statistically shown to outperform the equivalent linear PBAE and possess extremely high transfection efficacy for the reported conditions. At ultra-low plasmid DNA doses, the efficiency of plasmid DNA delivery was rather remarkable compared to previously reported optimal nanoparticles, including PBAE terpolymers that include alkyl side chains for improved colloidal stability that were shown to require roughly 3x the DNA dose used here to transflect HeLa cells with similar efficacy.46 Further, in physiological serum conditions these BEAQ nanoparticles demonstrated an impressive degree of transfection compared to what has been reported in the literature. Fluorinated PAMAM dendrimers were reported to have their transfection efficacy reduced to 30% of what it was in 10% serum when the nanoparticles were added to cells in 50% serum.47 In contrast, BEAQ nanoparticles maintained >70% geometric mean expression under matched conditions to 10% serum transfections. Other non-viral transfection reagents have similarly been reported to facilitate transfection under physiological serum conditions, but often yield only 30-40% the mean expression level of the same particles in 10% serum.48

That being said, even at this relatively high level of efficacy of non-viral transfection, much room is left for improvement in non-viral vector efficiency as compared to viral vectors that have evolved for over a billion years for efficient transduction. At the low doses tested of 5-10 ng plasmid DNA/well, there were approximately 200,000-400,000 plasmids available for every cell in the well (Calculation S1). Based on recent estimates of approximately 10 plasmids per polyplex nanoparticle31,49 there could still be over 20,000 nanoparticles added per cell at this dose, which is a high multiplicity of infection (MOI). With an estimated number of 5,000 plasmids from polyplex nanoparticles being internalized per cell under higher dose transfection conditions and an estimated 1/5 of those plasmids reaching the nuclear envelope, uptake of nanoparticles appears to be a significant hurdle to effective transfection in vitro.32 In comparison to efficient viruses, the low nanoparticle doses tested here are far above the order of magnitude MOI used for adenovirus (1-1,000) and various lentiviruses (1-200) to yield similar levels of expression.50-51 In contrast, naturally occurring AAVs are often used at a much higher MOI of up to 100,000 to achieve similarly detectable reporter gene based levels of transfection in hard-to-transduce cell lines.52-53 Spark Therapeutics recently completed a successful phase III clinical trial using subretinal delivery of AAV for the first FDA approved gene therapy, voretigene neparvovec-rzyl, demonstrating the clinical potential of non-integrating gene
therapy. Given the similar level of MOI for BEAQ and AAV and coupled with challenges in scaling production of AAV for clinical utilization and the limitations of AAV cargo capacity, non-viral delivery of episomal plasmid DNA with this BEAQ system may be a viable strategy for clinical delivery of DNA to RPE cells.

Escape from endosomes and avoidance of lysosomal degradation remains a significant hurdle to nanomaterials aiming to achieve cytosolic delivery. Estimates of endosomal escape of lipid nanoparticles for siRNA has revealed that less than 2% of cargo internalized to endosomes typically reaches the cytosol, which has been improved by some recent lipid nanoparticle formulations yielding up to 15% escape in HeLa cells. Polyplex nanoparticles similarly suffer from low endosomal escape efficacy, with the classic materials such as polyethylenimine and polylysine almost exclusively remaining in acidified vesicles and undergoing lysosomal degradation despite the ability of the former material in particular to buffer hydrogen ions. Transport to acidic lysosomes occurs rapidly following internalization, with nanoparticles typically reaching a lysosomal compartment within one hour following internalization. In contrast to these findings for most other polymeric materials, we demonstrated that BEAQs largely avoid lysosomal degradation with <20% of labeled plasmid DNA being detectable in acidified vesicles at 24 hours post-treatment compared to 40-50% DNA delivered with the linear polymer detected in acidified vesicles. These results are promising in that they demonstrate that branching may improve the ability of these polymers to achieve endosomal escape, which remains a primary hurdle to effective gene delivery.

Finally, we demonstrated how polymer structure, as a function of hydrophobicity and cationicity, related directly to optimal polymer/DNA mass ratio and to transfection efficacy as these variables have been shown repeatedly to be crucial to yielding robust transfection in other polymer systems. To identify structure-function relationships between these polymers and transfection efficacy, we analyzed viability and geometric mean expression as a function of individual polymer properties including buffering capacity, secondary, tertiary and total amine content per bp plasmid DNA. This is the first reported analysis of this type reported to our knowledge and yielded insights into the features of polycations that make them effective for transfection. In particular, we demonstrated that the optimal number of tertiary amines per bp plasmid DNA was near constant across the entire range of branching, while optimal numbers of secondary amines increased with degree of branching. With further knowledge of precise desired polymer structures, solid phase synthesis of alternating copolymers is an option which has been utilized in the synthesis of precisely defined polymers for gene delivery. Degradation rate of polymers could also play a role in the differences in transfection, as differences in constituent monomers can affect the specific degradation rate.

The total possible solution space for BEAQs that may be highly effective for gene delivery is vast, as there are many diacrylate monomers, side-chain amino and end-capping amino monomers available that have been shown to yield linear polymers effective for transfection of diverse cell types. Synthesis of BEAQs via
the guidelines outlined here and in previous publications will enable the rapid prototyping of diverse polymers that may yield further gains to efficient nucleic acid delivery as well as insights into polymeric structure/function relationships. The presented method for generating BEAQs can likewise be easily expanded to include utilization of branching monomers with other triacrylate monomer use as well as quaternary or greater functionality such as pentaerythritol tetraacrylate or dipentaerythritol penta-/hexa-acrylate to further increase structural diversity.

5. Conclusions
Branched poly(ester amine) quadpolymers (BEAQs) were successfully synthesized and characterized and were demonstrated to have multiple enhancements over leading non-viral gene delivery materials including optimized linear PBAEs, BPEI, JetPRIME, and Lipofectamine 2000. BEAQs with a moderate degree of branching were shown to more tightly bind plasmid DNA, maintain DNA binding following dilution in neutral, isotonic buffer, and possess higher solubility in aqueous media compared to linear analogs. Branched polymers formed from diacrylate (B7) and triacrylate (B8) monomers were highly effective for plasmid DNA delivery, and moderately branched BEAQs best maintained efficacy at physiologically relevant high serum concentrations. Analysis of chemical structure highlighted the importance of the ability to buffer pH at approximately 20 nmol H+/µg DNA as well as the key parameter of tertiary amine content at approximately 40 tertiary amines per base pair of DNA. Through differential control of polymer branching, BEAQs were found to be efficient for non-viral gene delivery to difficult-to-transfect human cells. BEAQs are promising as therapeutic gene delivery vehicles and these findings have implications for the design, identification, and optimization of next-generation polymeric materials for nucleic acid delivery.

Associated Content
The Supporting Information is available free of charge on the ACS Publications website. Supporting figures include chemical and binding properties, nanoparticle characteristics and uptake, optimization of transfection conditions and additional confocal microscopy images. Supporting tables include ratios of monomers and calculated statistical analyses.

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**References:**


Supplemental Information

Differentially Branched Poly(Beta-amino ester) Quadpolymers with Amphiphilic and pH Sensitive Properties for Efficient Plasmid DNA Delivery

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A

Echo 550 Nanoparticle Dispense Volume (nL)

<table>
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<th>Polymer w/w</th>
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<th>5.00</th>
<th>10</th>
<th>20</th>
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</thead>
<tbody>
<tr>
<td>20</td>
<td>25</td>
<td>52.5</td>
<td>102.5</td>
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<tr>
<td>40</td>
<td>40</td>
<td>77.5</td>
<td>157.5</td>
<td></td>
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<tr>
<td>60</td>
<td>52.5</td>
<td>105</td>
<td>210</td>
<td></td>
</tr>
</tbody>
</table>

B

C

Transfection Efficacy (%)

Normalized Cell Count

Dunnett's multiple comparisons test Summary Adjusted P Value

0 vs. 10 ns 0.9422
0 vs. 20 ns 0.5227
0 vs. 40 + 0.0276
0 vs. 50 ** 0.0021
0 vs. 60 ns 0.4108
0 vs. 80 ns 0.9996
0 vs. 90 ns 0.9495
**Figure 7-S11.** Low dose BEAQ nanoparticle transfection in ARPE-19 cells. A) Extremely low volume distribution of nanoparticles achieved via Echo 550 acoustic liquid handling with nanoparticle dose titration. B) Transfection efficacy and C) cell counts normalized to untreated for varied w/w ratio and overall nanoparticle dose (as function of total DNA per well). Branched BGDA polymers with 40-50% triacrylate mole-fraction were statistically more effective than the linear BGDA polymer BGDA-0 tested for low dose nanoparticle transfection. No nanoparticle formulations showed high cytotoxicity (>30% reduction in normalized cell count). Values show mean ± SEM of three wells for each condition. Differences in transfection efficacy between polymers were assessed over all tested conditions by One-way ANOVA with multiple comparisons to BGDA-0 using matched values for w/w ratio and DNA dose. One-way ANOVA was performed with Geisser-Greenhouse corrections for sphericity and Dunnet corrections for multiple comparisons. P values shown are multiplicity adjusted. (Error bars show n = 4 wells, mean ± SEM)
Figure 7-S12. Polyethylenimine variants transfection correlation. Linear PEI 4 kDa and branched PEI 25 kDa transfections with varied w/w ratio were assessed for correlation trends between amine content and geometric mean transfection in HEK293T and ARPE-19 cells.
Figure 7-S13. Flow cytometry gating analysis. FlowJo 10 was used for gating cells analyzed using an Accuri C6 flow cytometer. Singlet cell populations were identified and 2D gated for GFP expression or uptake of Cy5 labeled plasmid DNA. For gating, untreated populations were set to be a minimum of <0.5% false positive.
### Table 7-S1. Monomer mole ratios for synthesis of BEAQ series

<table>
<thead>
<tr>
<th>Theoretical Triacrylate Mole Fraction (%)</th>
<th>Diacrylate Ratio</th>
<th>Triacrylate Ratio</th>
<th>Amine Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1.1</td>
<td>0.00</td>
<td>1</td>
</tr>
<tr>
<td>10</td>
<td>0.99</td>
<td>0.07</td>
<td>1</td>
</tr>
<tr>
<td>20</td>
<td>0.88</td>
<td>0.15</td>
<td>1</td>
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<tr>
<td>40</td>
<td>0.66</td>
<td>0.29</td>
<td>1</td>
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<td>50</td>
<td>0.55</td>
<td>0.37</td>
<td>1</td>
</tr>
<tr>
<td>60</td>
<td>0.44</td>
<td>0.44</td>
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<td>80</td>
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<td>1</td>
</tr>
<tr>
<td>90</td>
<td>0.11</td>
<td>0.66</td>
<td>1</td>
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</table>

### Table 7-S2. ¹H NMR integrations for all polymers normalized to acrylate peaks (3H)

<table>
<thead>
<tr>
<th>Theoretical Triacrylate Mole Fraction (%)</th>
<th>BGDA Phenyl (7.11 &amp; 6.8 ppm) (4H each)</th>
<th>B8 methyl (0.83 ppm) (3H)</th>
<th>S4 (2.38 ppm) (2H)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>9.42</td>
<td>0</td>
<td>4.62</td>
</tr>
<tr>
<td>10</td>
<td>7.16</td>
<td>.91</td>
<td>5.05</td>
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<td>20</td>
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<tr>
<td>90</td>
<td>.611</td>
<td>4.16</td>
<td>3.57</td>
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</table>
**Table 7-S3.** Number average GPC calculated mass fraction contributions of monomers

<table>
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<tr>
<th>Theoretic Triacrylate</th>
<th>BGDA</th>
<th>TMPTA</th>
<th>S4</th>
<th>E6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mole Fraction (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>0.815</td>
<td>0.000</td>
<td>0.128</td>
<td>0.057</td>
</tr>
<tr>
<td>10</td>
<td>0.686</td>
<td>0.090</td>
<td>0.133</td>
<td>0.091</td>
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<tr>
<td>20</td>
<td>0.619</td>
<td>0.135</td>
<td>0.132</td>
<td>0.115</td>
</tr>
<tr>
<td>40</td>
<td>0.516</td>
<td>0.204</td>
<td>0.130</td>
<td>0.150</td>
</tr>
<tr>
<td>50</td>
<td>0.418</td>
<td>0.275</td>
<td>0.136</td>
<td>0.171</td>
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<td>60</td>
<td>0.320</td>
<td>0.334</td>
<td>0.127</td>
<td>0.220</td>
</tr>
<tr>
<td>80</td>
<td>0.127</td>
<td>0.468</td>
<td>0.131</td>
<td>0.275</td>
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<td>90</td>
<td>0.063</td>
<td>0.516</td>
<td>0.138</td>
<td>0.282</td>
</tr>
</tbody>
</table>

**Table 7-S4.** Backbone polymer amine density calculations. The molecular weight for polymer repeat units consisting of monomers BGDA+S4, TMPTA+2*S4 and ethylenimine were calculated. Amine density was then determined as the number of amines per polymer backbone molecular weight in Da. The branching monomer TMPTA gives rise to polymers with the highest tertiary amine density while BGDA monomers give rise to polymers with a lower tertiary amine density.

<table>
<thead>
<tr>
<th>Repeat Unit</th>
<th>Molecular Weight (Da)</th>
<th>Amine density (Amines/Da)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BGDA + S4</td>
<td>573</td>
<td>0.00175</td>
</tr>
<tr>
<td>TMPTA + 2*S4</td>
<td>474</td>
<td>0.00422</td>
</tr>
<tr>
<td>Ethylenimine</td>
<td>43</td>
<td>0.02326</td>
</tr>
</tbody>
</table>
**Table 7-S5.** Yo-Pro-1 iodide competition binding assay. RM one-way ANOVA was performed with Geisser-Greenhouse corrections and Dunnett test corrected multiple comparisons to the linear, 0 triacrylate mole fraction polymer. Results are shown for multiple comparisons assessment at the concentration of 75 µg/mL BEAQ concentration tested with an n=3 well replicates for each polymer.

<table>
<thead>
<tr>
<th>Triacrylate mole fraction</th>
<th>BGDA Series pH 5.0, 25 mM</th>
<th>BGDA Series pH 7.4, 150 mM</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td>20</td>
<td>ns</td>
<td>**</td>
</tr>
<tr>
<td>40</td>
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<td>ns</td>
<td>***</td>
</tr>
<tr>
<td>90</td>
<td>ns</td>
<td>*</td>
</tr>
</tbody>
</table>

**Calculation 7-S1.** Plasmids per cell. In 384 well transfection experiments at low nanoparticle doses, moderately branched BEAQs yielded 82% transfection efficacy in HEK293T cells at a dose of 5 ng/well and 42% transfection efficacy in ARPE-19 cells at a dose of 10 ng/well. Cells were seeded at a density of 2500 cells per well and assumed to divide once to yield 5000 cells per well on the day of transfection. The eGFP-N1 plasmid has a size of 4733 bp and molecular weight of approximately 3124 kDa, meaning there were 9.64x10^8 plasmids/well and 192,800 plasmids per cell available at a 5 ng dose.

**Calculation 7-S2.** BPEI buffering capacity. We have demonstrated previously that 25 kDa branched polyethylenimine possesses a buffering capacity of 6.2 mmol H+/g of polymer in the pH range of 7.4 to 5 [2]. This is the equivalent of 6.2 nmol H+ per mg of polyethylenimine, meaning that polyethylenimine would have 6.2 nmol H+ buffering capacity per µg of DNA at a 1 w/w ratio. At the higher than usual optimal w/w ratios of 3 and 4 w/w for HEK293T and ARPE-19 (Figure S6), PEI would have an optimal buffering capacity of either 24.8 or 37.2 nmol H+/µg DNA depending on cell type.

**References**

Chapter 8: Biodegradable STING agonist nanoparticles for enhanced cancer immunotherapy

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Foreword: The following article was a collaborative work with Rupashree Kim, Drew Pardoll and Young Kim that was initially launched by Joel Sunshine and handed off to me at a very early stage. The resulting article was selected by editors at NanoMedicine: NBM as the featured cover article and very well received by the field, accumulating 50 citations in the first two years since publication. The application of endosomal disruptive nanoparticles to facilitate cytosolic delivery of adjuvants for cancer (and other disease) therapy has huge potential and the work presented here may have helped moved the field forward ever so slightly.


Abstract: Therapeutic cancer vaccines require adjuvants leading to robust type I interferon and proinflammatory cytokine responses in the tumor microenvironment to induce an anti-tumor response. Cyclic dinucleotides (CDNs), a potent Stimulator of Interferon Receptor (STING) agonist, are currently in phase I trials. However, their efficacy may be limited to micromolar concentrations due to the cytosolic residence of STING in the ER membrane. Here we utilized biodegradable, poly(beta-amino ester) (PBAE) nanoparticles to deliver CDNs to the cytosol leading to robust immune response at > 100-fold lower extracellular CDN concentrations in vitro. The leading CDN PBAE nanoparticle formulation induced a log-fold improvement in potency in treating established B16 melanoma tumors in vivo when combined with PD-1
blocking antibody in comparison to free CDN without nanoparticles. This nanoparticle-mediated cytosolic
delivery method for STING agonists synergizes with checkpoint inhibitors and has strong potential for
enhanced cancer immunotherapy.

**Key words:** STING agonist, PBAE nanoparticle formulation, cancer immunotherapy

**Abbreviations:** Cyclic dinucleotides (CDN)s; Stimulator of Interferon Receptor (STING); poly(beta-amino
ester)s (PBAEs); interferon (IFN); secreted embryonic alkaline phosphatase (SEAP); cyclic GMP-AMP
synthase (cGAS); pathogen-associated molecular patterns (PAMPs); interferon regulatory factor 3 (IRF3);
peripheral blood mononuclear cells (PBMCs); 1,4-butanediol diacrylate (B4); 4-amino-1-butanol (S4); 1-(3-
aminopropyl)-4-methylpiperazine (E7); transmission electron microscopy (TEM); ISG (interferon-stimulated
genes); Lipopolysaccharide (LPS); sodium acetate (NaAc); secreted embryonic alkaline phosphatase (SEAP);
Nanoparticle Tracking Analysis (NTA);
Introduction

The innate arm of the immune system recognizes pathogen-associated molecular patterns (PAMPs) through pattern recognition receptors (PRRs) to initiate effective immunological responses, and activation of the innate arm of the immune system can be critically important in priming an effective adaptive response. Cyclic dinucleotides (CDNs) have been demonstrated to be highly effective PAMP molecules capable of robust activation of interferon regulatory factor 3 (IRF3). Recently much attention has been focused on STING which localizes to the endoplasmic reticulum, a potent inducer of type I IFNs in response to their binding of CDNs. CDNs are small molecule secondary messengers used by bacteria and produced as endogenous products of cytosolic DNA sensing cyclic GMP-AMP synthase (cGAS) that are recognized by STING. Both the canonical 2,3 linked bacterial cyclic di-GMP and cyclic di-AMP, as well as the non-canonical eukaryotic 2,5 linked cGAMP have been shown to directly bind STING and subsequently initiate TBK1-IRF3 and NFkB dependent type I IFN proinflammatory immune responses. With the recent clinical success of immune checkpoint inhibitors in multiple cancer patients and the overall positive correlations between proinflammatory tumor microenvironment and clinical responses to PD-1 blocking antibodies, intratumoral stimulation of STING may potentially synergize with immune checkpoint inhibitors. While CDNs are currently undergoing clinical trials for safety, there are concerns that simple intratumoral CDN injection is a suboptimal means to stimulate the cytosolic STING signaling pathway.

Since CDN molecules are anionic, we investigated whether cationic polymers that have traditionally been used to deliver DNA and siRNA may enable improved cytosolic delivery of CDNs as well. Nanoparticles have been engineered for the intracellular delivery of DNA particles to facilitate efficient transfection in relevant cells while minimizing toxicity. Poly (beta-amino esters) (PBAEs), a class of synthetic, cationic polymers, have been found to be effective as non-viral gene delivery agents for a wide variety of cell types both in vitro and in vivo. Importantly, PBAEs are relatively easy to synthesize with structural diversity, are effective at binding nucleic acids, and hydrolytically degradable under physiological conditions, which greatly reduces their cytotoxicity despite being cationic in nature. Moreover, they can have specific cellular uptake to target cell types and can also enable effective endosomal escape to the cytoplasm, likely due to their buffering capacity. With these properties we hypothesized that PBAE nanoparticles would improve the cytosolic delivery of anionic CDN molecules, enabling anti-tumor responses at lower CDN doses. We investigated whether PBAEs could enhance CDN delivery to immune cells in vitro, the physical and biological properties of the PBAE/CDN nanoparticles, and their anti-tumor efficacy in vivo.
Methods

Chemicals

All chemicals were of analytical grade and obtained from Sigma Aldrich Chemicals (St Louis, MO, USA). RPMI 1640 medium (Gibco BRL, Grand Island, NY, USA), fluorochrome conjugated antibodies (BD Biosciences, San Jose, CA, USA), MTT Aqueous One cell viability kit (Promega, Madison, WI, USA). Cyclic dinucleotides were provided by Aduro Biotech (Berkeley, CA, USA). QUANTI-blue and THP1-ISG cells were purchased from Invivogen (San Diego, CA, USA). Monocyte isolation kit (Miltenyi Biotec, Auburn, CA, USA) was used to isolate monocytes from peripheral blood mononuclear cells (PBMCs) and Mouse anti-PD-1 antibody (clone G4) was generated in-house using mouse hybridoma cells. Monomers for PBAE synthesis were purchased as specified in the Supplementary Information Table 1.

Polymer Synthesis

Poly(beta-amino ester)s (PBAEs) were synthesized in a two-step Michael addition reaction following protocols previously published. The naming scheme for PBAEs used follows that established by Tzeng et al. with the first digit denoted by base monomer carbon number, the second digit denoted by sidechain monomer carbon number, and the third digit to describe the polymer’s endcapping group. The PBAE chosen for CDN delivery was synthesized at a molar ratio of 1.1:1 from monomers 1,4-butanediol diacrylate (B4) and 4-amino-1-butanol (S4) and then endcapped with a 0.2 M solution of 1-(3-aminopropyl)-4-methylpiperazine (E7) in anhydrous tetrahydrofuran. PBAEs were then precipitated twice in a 10x volume of anhydrous diethyl ether, isolated via centrifugation at 3,000 rcf and stored under vacuum for two days to remove excess diethyl ether. PBAEs were dissolved in anhydrous DMSO at a concentration of 100 µg/µL and stored at -20°C in individual use aliquots. PBAE molecular weight was determined via gel permeation chromatography (GPC; Waters, Milford, MA) using methods previous published. For 1H NMR, approximately 5 mg of diethyl ether precipitated polymer was dissolved in CDCl3 and NMR spectrum was acquired using a Bruker 500 MHz NMR.

Nanoparticle characterization

CDNs and polymer were diluted in sodium acetate buffer (25 mM, pH 5.0) to the same concentrations as used for the in vitro immunostimulatory treatment. Diluted solutions of CDN and polymer were then mixed in a 1:1 volume ratio and allowed to incubate for 10 minutes to formulate nanoparticles. A Nanosight NS500 (Malvern Instruments, UK) was used to determine number-averaged nanoparticle hydrodynamic diameter. A Zetasizer Nano ZS (Malvern Instruments, UK) was used to determine intensity Z-average hydrodynamic diameter and zeta potential of particles diluted six-fold in 150 mM PBS (pH 7.4). For nanoparticle tracking analysis, nanoparticles were diluted 500 fold in 150 mM PBS (pH 7.4) to give 20-80 particles per frame.
Camera and analysis settings were maintained for all comparisons between individually prepared samples. All data points presented are for mean ± standard error of the mean of value of individually prepared samples assessed in triplicate. For transmission electron microscopy (TEM), nanoparticles were formed at a 500 w/w ratio between RR-CDG and PBAE 447 at the same concentration as for in vitro immuno-stimulatory treatments. Ten microliters were then added to a corona plasma treated carbon film 400 square mesh TEM grid and allowed to dry for one hour. TEM images were then acquired using a Philips CM120. Nanoparticles without CDN were prepared in an identical manner, constituting polymer only.

**Mice and Cell Culture**

Female 6-8 weeks old C57BL/6 mice were obtained from The Jackson Laboratory and maintained according to animal care facilities of Johns Hopkins University. THP1-Blue™ ISG (interferon-stimulated genes, Invivogen), THP1 human monocytes were grown at concentrations between 0.2-1.0*10^6 cells/mL in suspension in vertical T25/75 flasks. THP1 cells, B16-F1 melanoma cells, and human monocytes were all cultured in RPM-1640 medium supplemented with 10% FBS and penicillin (100 U/ mL) and streptomycin (100 mg/mL) and maintained in a humidified incubator at 37°C in a 5% CO2 atmosphere. RAW 264.7 murine macrophages were likewise cultured in DMEM medium supplemented with 10% FBS, penicillin (100 U/ mL) and streptomycin (100 mg/mL).

**Immunostimulatory treatment**

Lipopolysaccharide (LPS) was diluted in fresh media from frozen aliquots of 0.1 μg/μL to the concentration required for the specified well dose. CDNs were stored at -20°C in aliquots at 10 μg/μL and were diluted in 25 mM NaAc, pH 5.0, to the required concentration for the specific well dose to be delivered in 20 μL for all treatments. PBAEs were stored at 100 μg/μL in DMSO at -20°C until used. Nanoparticles were formed by diluting PBAEs in 25 mM NaAc buffer, pH 5.0, which was then mixed in a 1:1 v/v ratio with 25 mM NaAc buffer containing CDNs at the concentration required for the doses specified. The nanoparticle solution was incubated for 10 minutes to allow for particle formation after which for freshly prepared particles, 20 μL were added to each well. For lyophilized particles, after particle formation endotoxin free sucrose from a stock of 600 μg/μL was added to each sample to a concentration of 30 μg/μL. Samples were then frozen at -80°C and lyophilized. Lyophilized samples were stored at -20°C with desiccant and resuspended in ultrapure water before being added to wells for treatment. After 3-5 hours of incubation, treatment media was removed from all wells, cells were washed twice and resuspended in 200 μL fresh media.

**Quanti-Blue and Cell Titer Assays**
THP1-Blue™ ISG cells were specifically designed to monitor the interferon (IFN) signaling pathway in a physiologically relevant cell line. They derive from the human THP-1 monocyte cell line by stable integration of an IFN regulatory factor (IRF)- inducible SEAP reporter construct. THP1-Blue™ ISG cells express a secreted embryonic alkaline phosphatase (SEAP) reporter gene under the control of an ISG54 minimal promoter in conjunction with five IFN stimulated response elements. As a result, THP1-Blue™ ISG cells allow the monitoring of IRF activation by determining the activity of SEAP. The levels of IRF-induced SEAP in the cell culture supernatant are readily assessed with QUANTI-Blue™, a SEAP detection reagent (Invivogen). The Quanti-Blue assay was used to measure THP1-Blue cell SEAP activity as a means of assessing IRF3 activation. For the assay, Quanti-Blue detection medium was prepared according to manufacturer’s instruction. Following 18h treatment, 20 µL of supernatants were added to 180 µL of Quanti-Blue detection medium in a new 96 well round bottom plate. The Quanti-Blue assay plate was incubated at 37°C for 1-4h and then the absorbance was read at 630 nm. Mean absorbance of a blank well of Quanti-Blue detection media with fresh RPMI media was subtracted to determine raw Quanti-Blue assay values.

Cell viability assay

Cell viability was assessed using an MTT assay one day following treatment. For the assay, MTT reagent was diluted 10-fold into complete medium and incubated with cells in 96 well plates for 2 h. Absorbance was then analyzed at 490 nm using a multi-plate reader (Synergy 2, Biotek), blank-subtracted and normalized to the untreated cell values to give relative cell viability.

Cellular uptake experiments

Cells were plated at a density of 150,000 cells/mL in complete medium with 100 µL of media per well in 96 well plates. PBAE particles labeled with Cy5 were formed with RR-CDG at a 500 w/w ratio, then were added to cells at a dose of 20 µg polymer/well and incubated for 1h at 37°C after which cells were washed with PBS twice and analyzed via flow cytometry. A BD Accuri C6 (BD Biosciences) flow cytometer with two lasers (488 and 633 nm) with four channels corresponding to green, yellow, red, and far-red fluorescence (FL1 at 530 ± 15 nm, FL2 at 565 ± 10nm, FL3 at 610 ± 10 nm and FL4 at 675 ± 12.5 nm, respectively) was used for all flow cytometry experiments in combination with a HyperCyt autosampler (IntelliCyt). Cell counts per well and nanoparticle concentrations in media were equal between cell types. Statistical significance was assessed between the uptake levels of the treated cells by one-way ANOVA with multiple comparisons corrected for by Tukey’s method.

For confocal microscopy experiments, PBMCs transfected in suspension in 96 well round bottom plates as described above were stained with Hoechst 33342 (H3570; Thermo Fisher) to label nuclei. Cells were resuspended in live cell imaging solution and transferred to 8 well Nunc Lab-Tek chambered
borosilicate coverglass well plates (155411; Thermo Fisher) at 37,500 cells/well for imaging. Images were acquired using a Zeiss LSM 780 microscope with Zen software and 63 oil immersion lens. Specific laser channels used were 405 nm diode, 488 nm argon, 561 nm solid-state, and 639 nm diode lasers.

Tumor growth and Measurement

For in vivo therapeutic experiments, C57BL/6 mice were injected with B16-F1 melanoma cells at a concentration of $2 \times 10^5$ cells/100 µL in the flank. The mice were grouped (n=7) according to the experimental plan. Post days 3, 6, 9 and 12 post-tumor inoculation, mice were treated intratumorally with HBSS, CDN in HBSS, resuspended empty PBAE nanoparticles or resuspended PBAE + CDN nanoparticles via 50 µL intratumoral injection. For anti-PD-1 groups, 100 µg was injected twice weekly on days 3, 7, 10, 14 and 17 after mice were inoculated with tumors.

Statistical analysis

Figures present mean ± standard error of the mean calculated from three replicates unless otherwise noted. All the above experiments were repeated at least twice whereas for experiments with 96 well plates used either three or four well replicates. Statistical significance was designated as follows: ****p<.0001; ***p<.001; **p<.01; *p<.05. Statistical analysis was assessed using Graphpad Prism software (La Jolla, CA) at a global alpha value of 0.05.

Results

**CDNs are potent activators of IRF3 at micromolar doses**

Recent publications have shown CDNs to be potent activators of the adaptive immune system via activation of type I IFN and proinflammatory cytokine response leading to potent efficacy in multiple models of established cancer. As a result, we selected the CDNs shown in Figure 8-1A to act as adjuvant molecules for studies in vitro. ML-RR-CDA is a modified version of cyclic-di-AMP formulated for increased in vivo stability and human STING activation, while RR-CDG was a phosphodiesterase resistant version of cyclic-di-GMP (Figure 8-1A). Since the anti-tumor efficacy of CDN is partly mediated through activity in antigen-presenting cells (APCs), we optimized PBAE formulated CDN nanoparticles in the human monocyte cell line, THP1-Blue. The THP1-Blue human monocyte cell line used for in vitro screening expresses the reporter gene secreted embryonic alkaline phosphatase (SEAP) driven by the IRF3 promoter activation that is activated by ligand binding to the cytosolic protein Stimulator of interferon gene (STING). THP1-Blue cells were shown to respond strongly to extracellular concentrations of 10 µM CDN following a four hour incubation time, while showing minimal response at or below the level of 1000 nM concentrations of CDN (Figure 8-1B/C).
Figure 8-1. CDNs are potent immune activators at μM concentrations. (A) STING activating CDNs ML-RR-CDA and RR-CDG modified for increased stability were used in all studies. (B) ML-RR-CDA was a potent activator of immune response down to 10 μM extracellular concentrations, while (C) RR-CDG was effective down to 10 μM concentrations when assessed using the THP1-Blue cell line with Quanti-Blue assay to detect IRF3 promoter activity.

PBAE nanoparticles improve the efficiency of CDN delivery to human monocytes in vitro

An initial array of biodegradable PBAE structures were assessed for their ability to increase STING activation in the human monocyte cell line (THP1-Blue) at low extracellular doses of CDN (Supplementary Figure 8-1). These structures were selected for having previously been shown to be effective for delivery of plasmid DNA or RNA molecules, which are notably larger and more electronegative molecules than CDNs.\textsuperscript{13,27} While both traditional hydrophobic PBAE and the disulfide backbone polymer tested were
capable of improving the efficiency of CDN delivery to human monocytes and STING activation at low overall CDN doses, they had differential activity. For example, polymers PBAE 446 and PBAE R647 generally had less than half the activity of the lead polymer PBAE 447. On the other hand, polymer 537 demonstrated significant cytotoxicity, even at a relatively low dose of 31.25 nM, whereas the other PBAEs evaluated did not. Overall, PBAE 447 (Figure 8-2A), a structure containing 4 carbons between acrylate groups and 4 carbons between its amine group and its hydroxyl group, was selected for its balance of being most effective at IRF3 activation while maintaining high cell viability among the structures evaluated.

**Figure 8-2. PBAE 447 synthesis and characterization.** (A) PBAEs were synthesized from small molecule monomers B4 and S4 in a Michael addition reaction to yield the base polymer B4S4. The acrylate terminated polymer was then end-capped with small molecule E7 to yield PBAE 447. (B) PBAE nanoparticles assessed using NTA had a hydrodynamic diameter distribution with a mean and SEM of 110 ± 41 nm. (C) TEM of PBAE+CDN nanoparticles at a w/w ratio of 500 showed dried diameters mean and SEM of 77 ± 5 nm. Scale bar 100 nm. (D) Nanoparticle diameter assessed by dynamic light scattering (DLS) Z-average diameter, nanoparticle tracking analysis (NTA) and transmission electron microscopy (TEM) showed varying nanoparticle diameters. Error bars show mean ± SEM of three independently prepared samples (DLS and NTA) or 20 nanoparticles (TEM). (E) Zeta potential mean and SEM of 9.1 ± 1.3 mV for nanoparticles formed at a 500 w/w ratio with CDN in 150 mM PBS. PBAE nanoparticles with CDN had a statistically higher positive zeta potential (*P<0.05).
**PBAE 447 polymer and PBAE 447/CDN nanoparticle physical characterization**

PBAEs were synthesized via Michael addition reaction as our lab has described\textsuperscript{15} and the synthesis scheme is shown in Figure 2A. PBAE 447, selected as optimal for CDN delivery, was measured to have $M_N$ 10,700 g/mol, $M_W$ 38,200 g/mol and a polydispersity of 3.58 via gel permeation chromatography against polystyrene standards. Analysis via $^1$H NMR of the synthesized acrylate and endcapped polymers showed that endcapping was effective (as demonstrated by the disappearance of the acrylate peaks); the resulting endcapped polymers had an $M_N$ of 7 kDa (Supplementary Figure 2). Nanoparticles formed from PBAE 447 fabricated at a w/w ratio of 500:1 with CDN were characterized using Dynamic Light Scattering (DLS), Nanoparticle Tracking Analysis (NTA), and transmission electron microscopy (TEM) (Figure 8-2), demonstrating that they had a diameter of approximately 100 nm and slightly positive zeta-potential of approximately +10 mV. The discrepancy between hydrodynamic diameter reported by DLS and NTA has been noted previously for polyplex nanoparticles\textsuperscript{25} where larger polyplex nanoparticles are over-represented in hydrodynamic measurements calculated by an intensity-averaged measurement (DLS) compared to a number-averaged measurement (NTA). In addition, the nanoparticles were observed to have smaller diameters in dry conditions (TEM) than in hydrated conditions, when hydrodynamic diameters were calculated. Notably, unlike some polyplex nanoparticles such as polyethylenimine, this particular PBAE structure forms nanoparticles via the hydrophobic effect even in the absence of any anionic species for complexation. Although the observed means appeared slightly higher between the diameters of PBAE nanoparticles with the small CDNs compared to those without CDNs as assessed by DLS, NTA, and TEM, no statistically significant differences were found between these measurements. The zeta potential of PBAE nanoparticles with the small CDNs was found to be slightly statistically significantly higher than those without them, presumably because the CDNs would electrostatically attract additional cationic PBAE polymer, which could coat the CDNs and increase cationic surface charge.

**PBAE 447/CDN nanoparticle biological optimization**

Following selection of PBAE 447 as the lead structure, we optimized the dose of PBAE added per well (Figure 8-3A and 8-3B), resulting in an optimized dose of 20 µg for PBAE 447. PBAE 447 was highly effective for delivery of ML-RR-CDA, giving equivalent IRF3 activation to free CDN at a 100-fold lower dose of dinucleotide (Figure 3C and 3D). ML-RR-CDA was also shown to be a significantly more potent activator than RR-CDG in the THP1-Blue cells at lower doses (Figure 8-3E and 8-3F) and was selected for additional studies.
Figure 8-3. Optimization of PBAE 447 delivery of CDN \textit{in vitro}. The dose per well of PBAE polymer 447 doses was screened for (A) efficacy of CDN delivery and (B) cell viability. A dose of 20 µg/well was selected for future \textit{in vitro} experiments to balance efficacy and cytotoxicity. (C) PBAE 447 was able to improve IRF-3 activation at over 100-fold lower extracellular doses compared to free ML-RR-CDA, with minimal effect on (D) cell viability. CDN molecules, ML-RR-CDA and RR-CDG were then tested directly for STING (E) activation and (F) cell viability \textit{in vitro}, showing significantly higher activation for ML-RR-
CDA over RR-CDG in THP1-Blue human monocytes at lower concentrations of CDN when tested with Holm-Sidak corrected multiple T-tests between groups. (**P<0.0001, **P<0.01, *P<0.05). Error bars show mean +/- SEM of three wells.

**PBAE nanoparticles are highly effective for immune cell uptake**

Currently, ML-RR-CDA are being developed for clinical purposes as an intratumoral injectable adjuvant. We hypothesized that PBAE-CDN nanoparticles would enhance the potency of CDNs by selective, efficient cellular uptake into immune cells in the tumor microenvironment. As a result we first examined the efficiency of nanoparticle uptake into monocytes and macrophages as compared to tumor cells *in vitro*. PBAE 447/CDN nanoparticles fluorescently labeled with Cy5 were shown to be taken up very effectively by THP1 human monocytes and RAW 264.7 murine macrophages, showing highly significant uptake over B16-F1 tumor cells cultured and treated under the same conditions (Figure 8-A). Nanoparticle uptake into primary human myeloid cells was also noted, with over 90% of human donor monocytes showing nanoparticle uptake with no significant differences between the degree of uptake between separate donor samples (Figure 8-B). Additionally, fluorescently labeled 447/CDN nanoparticles were clearly internalized following one hour incubation with human donor monocytes as evaluated by confocal microscopy (Figure 8-C).

**Figure 8-4. PBAE/CDN nanoparticles show selective and effective uptake by monocyte and macrophage populations.** (A) PBAE 447+RR-CDG nanoparticles were internalized by THP-1 human monocytes and RAW 264.7 murine macrophages more effectively than B16-F1 murine melanoma cells. Statistics performed as One-way ANOVA with Dunnett corrected multiple comparisons to B16-F1 uptake (****P<0.0001). (B) PBMCs from different donors were shown to take up CDN nanoparticles effectively, with no significant differences between donors. Error bars show mean +/- SEM of the geometric mean uptake of four wells. (C) Confocal microscopy shows RR-
CDG+447-Cy5 labeled nanoparticles (green) effectively internalized by PBMCs. Nuclei stained with Hoechst (blue).

**PB–AE+CDN nanoparticles maintain efficacy post-lyophilization and can be stably stored for at least 9 months**

To facilitate long-term storage of the hydrolysable nanoparticles, lyophilization studies were performed following a protocol that we developed for plasmid DNA nanoparticles to determine if similar stability could be achieved for CDN nanoparticles PBAE+CDN nanoparticles were mixed with sucrose to give a final concentration of 30 mg/mL sucrose, then frozen at -80°C and lyophilized. The particles were then rehydrated with water and tested to compare their relative efficacy to fresh nanoparticles. Lyophilized and stored nanoparticles that showed no detectable changes in efficacy or cell viability compared to the freshly prepared nanoparticles (Figure 8-5A and 8-5B). Impressively, the lyophilized nanoparticles remained highly effective for IRF3 activation in THP1-Blue cells following 9 months of storage at -20°C in containers with desiccant (Figure 8-5C).

![Graphs showing efficacy and cell viability](image)

**Figure 8-5. PBAE/CDN nanoparticles are stable following lyophilization and storage.** Lyophilization had no effect on the (A) efficacy or (B) cell viability of PBAE+CDN nanoparticles when pre-mixed with sucrose as a cryoprotectant. Results analyzed by Holm-Sidak corrected multiple t-tests. (C) Lyophilized PBAE+CDN nanoparticles formulated at a dose of 20 μg polymer and 62.5 nM CDN remained highly effective at IRF3 activation for at least 9 months following lyophilization when stored at -20°C.

*Intratumoral injections of CDN are highly effective at reducing tumor growth in vivo*

Checkpoint inhibition with anti-PD-1 antibodies has become the standard of care for many cancers, but there remains a need to improve upon checkpoint inhibition alone. One attractive approach is combination immunotherapy. To investigate whether PBAE nanoparticle CDN formulations might offer value in the setting of combination immunotherapy, we examined whether co-administration of PBAE+CDN nanoparticles with PD-1 antibody would offer a survival benefit vs combination immunotherapy with free CDN alone in a melanoma treatment model in mice. B16-F1 murine melanoma
tumors were established in C57BL/6 mice with subcutaneous flank injections of 200,000 cells. Starting on
day three, intratumoral injections were performed every three days following the protocol outlined in Figure
6A, with the final injections on day 12. Administration of 2 µg PBAE+CDN nanoparticles with anti-PD-1
antibody resulted in significantly reduced tumor growth compared to combination immunotherapy with
unencapsulated CDN 2 µg dose or empty nanoparticles (Figure 8-6B, P<0.0001 for the comparison).
Additionally, when combined with PD-1 blockade, there was no statistically significant difference between
treatment with 2 µg PBAE+CDN nanoparticles with anti-PD-1 antibody vs CDN 20 µg high dose therapy
(Figure 8-6B) although the high bolus dose of 20 µg CDN was more apparently effective at completely
eliminating tumors.

![Figure 8-6. PBAE+CDN NP therapy reduces tumor growth in vivo in the presence of anti-PD-1.](image)

(A) Tumor growth rate study of B16-F1 melanoma tumors injected subcutaneously in C57BL/6 mice.
Intratumoral injections were started on day three when tumors were palpable in all animals. (B) Twice weekly intraperitoneal administration of anti-PD-1 with the nanoparticle formulation treatment (NP + CDN 2 µg) resulted in statistically reduced tumor growth compared to CDN 2 µg without nanoparticles when compared with Holm-Sidak corrected multiple t-tests. All error bars show mean ± SD of five animals.

Discussion

CDNs are highly potent adjuvants that can impact clinical cancer immunotherapy, leading to robust
activation of the IRF3 transcription factor through STING, which is capable of triggering tumor regression
when activated in the right context.1,26,31 With promising pre-clinical results reported to date, CDNs are
currently under investigation in the FDA Phase I clinical trials for advanced metastatic solid tumors and
lymphomas (NCT02675439) by Aduro Biotech working with Novartis to determine the tolerable dosing
window.31 A clinical trial of intratumoral injected CDN as a combination therapy with anti-PD-1 is likewise
underway in 2017 (NCT03172936). With this rapid advance the clinic, however, there are concerns regarding
their narrow therapeutic window. This can be partly attributed to poor uptake and intracellular delivery of the CDNs into the cytosol of the immune cells in the tumor microenvironment and the need to use high doses to achieve efficacy.

Taking this into consideration, we hypothesized that CDN therapy could be improved at lower overall doses using a nanomedicine formulation that was biodegradable, enabled enhanced cytosolic delivery, and targeted professional APCs. The ER bound STING receptor in APCs does not have access to exogenous CDNs until they either reach the cytosol or are internalized to the endosomal space of activated cells. Because CDN molecules are both low molecular weight and water soluble due to their anionic charge, they are prone to rapid diffusion from the site of the tumor and lack efficient targeting to antigen presenting cells at the tumor site; this could potentially lead to off-target effects, resulting in overstimulation or autoimmunity side-effects that have been problematic for some patients in the case of other immunotherapies.

Here we showed that delivery of CDNs via nanomedicine formulation with cationic and biodegradable PBAEs improves their ability to activate IRF3 in vitro at >100 fold lower extracellular concentrations. The polymeric PBAE/CDN nanoparticles were shown to have a slightly positive surface charge and a particle diameter of approximately 100 nm. Self-assembled PBAE nanoparticles have previously been used for delivery of plasmid DNA, minicircle DNA, and siRNA but have not been previously evaluated for delivery of smaller molecules, including cyclic dinucleotides to immune cells in the tumor microenvironment.

While the small size of CDN molecules impedes the ability to measure their direct cellular uptake as they cannot be fluorescently labeled without affecting their chemical properties, their net negative charge (2-) should enable cationic PBAEs to effectively encapsulate them. We showed that fluorescently labeled PBAE nanoparticles were efficiently internalized to the THP1 human monocyte cell line as well as three human donor monocyte samples, with greater uptake than tumor cells under the same conditions. This degree of selective uptake may be attributable to the endcap utilized for the polymer tested, which has been shown to be able to convey both cell type specificity and endosomal uptake mechanism specificity.

STINGVAX formulations incorporating CDN previously were shown to greatly reduce tumor growth and resulted in complete remission of some tumors for CDN doses of 20 µg, but had limited efficacy at lower CDN doses. Here we showed that a nanoparticle formulation allows for an order of magnitude reduction in the necessary dose to eliminate established poorly immunogenic B16-F1 when administered as a combination therapy with the checkpoint inhibitor anti-PD-1 antibody. This strategy may have implications for pursuing clinical approval of these drug molecules in the future if identification of effective dosing regimens proves to be challenging in current clinical trials. Moving towards this goal, we demonstrated the application of lyophilization and long-term storage over 9 months without loss of efficacy for this nanomedicine formulation. In addition to facilitating the translational potential of this therapy, this
nanoparticle formulation consisted of only two components, unlike some previous STING based therapies incorporating CDN, attenuated tumor cells, and cytokines that could face significant manufacturing and regulatory hurdles and expense. The manufacturing of these nanoparticles, via mixing of two charged components, is amenable to continuous manufacture by a device like the microfluidic device as we have recently described for assembly of PBAE/DNA nanoparticles. Thus, biodegradable STING agonist nanoparticles composed of PBAE 447/CDN are promising for enhanced cancer immunotherapy.

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19. Sunshine JC, DY Peng and JJ Green, Uptake and transfection with polymeric nanoparticles are dependent on polymer end-group structure, but largely independent of nanoparticle physical and chemical properties. Mol Pharm. 2012;9:3375-83


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## Supplementary Information

Supplementary Table 8-S1: Monomer sources

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Supplementary Figure 8-S1. (A) Monomers used for PBAE synthesis of structures tested for CDN delivery. PBAE structures were screened for their ability to (B) induce IRF3 response in THP1-Blue cells with delivery of RR-CDG with (C) minimal toxicity assessed by MTT cell metabolic activity assay. CDN dose was titrated, while PBAE dose remained constant between conditions at 15 µg/well. PBAE 447 was selected as most effective at the doses tested. Bars show mean ± SEM of three wells.
Supplementary Figure 8-S2. PBAE polymer 447 and PBAE B4S4-Acrylate 1H NMR in CDCl₃ was used to verify polymer structure and estimate $M_N$ to be 7 kDa. The mean repeat unit (MW = 287.36 g/mol) was determined to be 23 using the ratio of area between peaks for the endcap secondary amine hydrogen at 0.81 ppm and the hydrogens of the α-carbons of the B repeat units at 4.08 ppm. The peak and satellites at 2.60 ppm were due to DMSO contamination following isolation of the polymer from a DMSO stock.
Supplementary Figure 8-S3. B16 viability following nanoparticle treatment. The nanoparticle formulation of RR-CDG+PBAE 447 at a 500 w/w ratio was shown not to have any effect on B16 cells viability at doses almost 2.5x greater than those used with THP1-Blue cells for immunostimulatory studies.

Chapter 9: Future perspectives in the field of non-viral gene delivery

Foreword: The following is a brief highlight of the most exciting advances in the field of non-viral gene delivery I have seen during my PhD (roughly 2014-2020) and where I believe the field is headed next in a few key areas.

9.a: Recent advances in gene delivery

In the twenty-five years since the publication of polyethylenimine as a delivery vector for plasmid DNA in 1995, non-viral gene delivery has in some ways barely moved forward, while in others has moved forward tremendously. The explosion in number of different polymer and lipid systems for delivery of genetic cargoes has been enormous and this is not the place for an entire review article (of which there have been far too many published in general). What follows is a brief selection of the recent advances in material chemistry, approach or application for non-viral technologies.

On the side of materials engineering, I have been particularly impressed by the inventions of CARTs, use of poly(glutamic acid) conjugated antibodies for specific cell targeting of nanoparticles and the innovation of having lipid molecules function both as a carrier molecule and STING agonist. Charge altering releasable transporters, or CARTs, from Waymouth lab are a very unique recent advancement in that they contain self-immolative polymer chemistry that is cytosolically triggered degradation of the entire polymer molecule; this is contrast to most polymers that rely on degradation at each individual bond level and has great potential for reducing risks of cytotoxicity for highly cationic polymers. The paper from Smith et al. in 2017 for in situ transfection of T-cells in living animals to induce CAR-T cell transfection with a sleeping beauty transposon system using PBAE nanoparticles with surface poly(glutamic-acid)-antibody-Fab targeting was quite incredible as well. Finally, the recent paper from Dan Anderson’s group where heterocyclic lipids were engineered for mRNA delivery that function additionally as STING agonists is incredibly impressive; this paper demonstrates the ability of the delivery vector to be an active drug molecule in and of itself. I also appreciate the work that went into Dan Siegwart’s labs innovation of adding sulfonate moieties to lipidoid materials for improving RNA packing in lipidoid nanoparticle formulations. Finally, I must mention my wife’s recent paper using carboxylic acid ligands for delivery of proteins and ribonucleoprotein complexes for CRISPR/Cas9 editing as what I believe will be a rather transformative advance in the field of cytosolic protein delivery.

Recent advances in assays for development of non-viral gene delivery vectors are also worth mentioning, as these innovations are often as important as new materials themselves in driving the field forward. I particularly admire the work of my friend Kameron Kilchrist to develop a genetically encoded sensor of endosomal disruption using a fusion protein between Galectin-8 and a fluorescent protein.
Galectins are proteins that naturally bind to carbohydrates found on glycosylated proteins that are ordinarily only found on the outside surfaces of cellular membranes; when an endosome has a disruption event occur it exposes those glycosylated proteins to cytosolically expressed Galectin-8-fluorescent-protein construct leading to clustering of the fluorescence into discrete puncta. We are using this assay in Green lab and have sent plasmids out to other groups to facilitate the adoption of the assay as well. Also worthwhile to mention in assay development has been the work of James Dahlman’s lab in barcoding nanoparticles for high-throughput in vivo testing of hundreds or thousands of nanoparticle formulations within the same animal. This technology has been paramount in demonstrating that the most efficient particles in vitro are rarely the most efficient and effective particles in vivo and may limit the utility of in vitro screening.

9.b: The future of non-viral gene delivery

Non-viral delivery for gene therapy and gene editing looks to have a bright future, but that does not mean the path will be easy. Much research is focused on strategies that are inherently near impossible to translate to the clinic and an enormous amount of academic research is effectively wasted as labs are effectively siloed into their own favorite chemical systems. Still, the vast amount of effort poured into developing more efficient delivery vectors will very likely lead to the clinical utilization of some nanoparticle formulations within the next decade (or two). The most exciting areas of development I see for non-viral delivery applications are as follows.

Immuno-oncology is an enormous area of development with huge financial potential due to the large patient population and as a result is one of the primary areas where non-viral delivery is first being tested. Moderna’s recent work (2019) is proof of this, using lipid nanoparticles to deliver mRNA that codes for costimulatory molecules, effectively converting tumor cells to become their own professional antigen presenting cells. Similarly, many companies are interested in using lipid nanoparticles to code for antibodies either at site specific locations or via hepatocyte expression. I expect the first approvals for non-viral delivery of mRNA cargos will come in the immuno-oncology field as the regulatory framework around cancer allows for more rapid approval. The strategy of delivery antibodies as mRNA molecules likely has the ability to usurp much of the profit that pharmaceutical companies have enjoyed that has resulted from development of biologic antibody drugs, so it will be very interesting to see how the landscape changes as mRNA based protein therapeutics reach the clinic.

Similarly, nucleic acids are particularly amenable to developing vaccine formulations. I particularly admire the work spearheaded by the vaccine development group at Novartis (now owned by GSK) in developing the self-amplifying mRNA platform that allows for rapid generation of new protein antigen vaccines, in theory allowing humanity to go from identification of a novel pathogen to creation of a functional intramuscularly administered vaccine in as short of a time span as two weeks. The approach of
delivering self-amplifying mRNA faces many delivery challenges but is immensely promising in the robust immune responses these molecules can administer.

In development of new chemical carriers for gene delivery applications it is difficult to predict the future, but I believe in general that the field of translational nanoparticle development will tend to migrate more to developing defined sequence carriers that have fewer problems with assembly and polydispersity challenges. Some of the materials I utilized during my PhD had molecular weight polydispersity index at or above 3 (MW/MN) which can be fine at a pre-clinical level but is simply not suitable for clinical development. Utilization of solid-phase synthesis and alternative synthesis pathways to generate better defined molecular constructs are needed for true translational potential of many therapies. I believe there is likely to be a trend to more biomimicry in development of non-viral therapeutics as well. I personally feel that the utilization of extracellular vesicles (or exosomes), cell membrane coated nanoparticles or other poorly defined cell derived materials for direct therapeutic applications has little chance of reaching clinical utility despite the large application of effort from the field as whole. Learning what features of extracellular vesicles makes them effective for delivery and recapitulating those features in a synthetic fashion has huge potential to transform the field and yield safer, more effective delivery vectors. Strategies like that taken by Denis Discher’s lab whereby CD47 and a minimal peptide mimicking CD47 improve circulation time of nanoparticles by reconstituting the “don’t-eat-me” signal in a synthetic nanoparticle formulation are the way of the future.

References:


Vita

David Robert Wilson was born in Columbia, MD on October 1, 1991 to Karen K. Wilson and Timothy G. Wilson. He grew up in Hagerstown and Smithsburg, MD, attending Paramount Elementary School, Smithsburg Middle School and Smithsburg High School. Following graduation from high school, he enrolled at Syracuse University where he was recruited to compete on their cross-country and track teams while studying bioengineering. Under the guidance of Dr. Becky Bader, David was introduced to biomedical research in the Syracuse Biomaterials Institute and was encouraged to apply to doctoral programs in biomedical engineering.

David joined the biomedical engineering lab of Dr. Jordan J. Green in August 2014 at Johns Hopkins School of Medicine. In the Green laboratory, David developed polymeric materials to facilitate the cytosolic delivery of nucleic acids and proteins. His doctoral work has resulted in the publication of many research articles and has hopefully helped move the field toward clinical utilization of non-viral delivery ever so slightly. While at Hopkins, David met and married his awesome scientist wife, Yuan Rui, and learned he was in fact a “cat person” when he adopted his first two cats Mavid and Dorkin.
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L.C. Smith College of Engineering and Computer Science
B.S. in Biomedical Engineering
GPA: 3.97

Awards

- **Siebel Scholar**
- **ASGCT Meritorious Abstract Award**
- **Institute for NanoBiotechnology (INBT) Fall Student Research Forum, 2nd place**
- **SAPA-DC Scientific Symposium & 3rd NIH-CSSA Annual Research Symposium: 1st Place Presentation**
- **ASGCT Meritorious Abstract Award**
- **Johns Hopkins BME Retreat Poster Competition, Best poster**
- **NSF Graduate Research Fellow**
- **Syracuse University Scholar**
- **Karen M. Hiiemae Outstanding Achievement Award in Bioengineering (top undergraduate)**
- **Coulter College Design Competition, 2nd Place**
- **SUNY-ESF Biotechnology Symposium, Poster Competition, 3rd Place**
- **Syracuse University Summer Research Symposium, Poster Competition, 4th Place**
- **Syracuse University Founder’s Scholarship**

Leadership / Professional Membership / Service

- **BME Edge (Extramural Development in Graduate Education). Co-Chair (2018-2019); Director of Careers (2017-2018):** Brought biomedical industry affiliated speakers to speak on career transitions and working in industry.
- **Society for Biomaterials (SFB)**

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• American Society of Gene & Cell Therapy (ASGCT) 2015 - Present
• Biomedical Engineering Society (BMES) 2013 - Present
• BME PhD Council webpage manager 2014 - 2015
• Thread Volunteer, Baltimore City high school student mentor 2014 - 2016
• Research presentation for the Syracuse University Board of Trustees on behalf of the Syracuse Biomaterials Institute 2013
• Biomedical and Chemical Engineering Department Faculty Search Committee, Undergraduate Representative 2012

Publications
Research Articles

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Review Articles and Book Chapters


Abstracts

Talks


2. **Wilson DR**, Rui Y, Siddiq K, Routkevitch D, Tzeng SY, Shen J, Campochiaro PA, Green JJ. Differentially Branched Ester Amine Quadpolymers with Amphiphilic and pH Sensitive Properties


Posters


Teaching and Mentoring Experience

- Graduate Research Mentor. Have directly mentored and published research articles with nine undergraduate students in the Green lab. Two have won the JHU PURA award under my mentorship. 2015-Present
- JHU Intersession Course Instructor: Practical Genome Editing and Gene Therapy. 3-week, two credit intersession course taught to 14 undergraduate students. 2019
- Head Teaching Assistant for Molecules and Cells: Semester long, three credit undergraduate core course (136 students) in biomedical engineering under professor Dr. Eileen Haase 2018
- JHU Intersession Course Instructor: Advances in Immunoengineering. 3-week, two credit intersession course taught 25 Johns Hopkins University undergraduate students. 2017
- Johns Hopkins Preparing Future Faculty Teaching Academy. 3 phase teaching program over two years including Teaching Institute 3 day course for doctoral students and postdocs 2017
- Design project mentor at St. Timothy’s high school through the Medical & Educational Perspectives group 2015
- Thread mentor to a student at Dunbar High School 2014–2016
- NSF Research Experience and Mentoring (REM) Program. Research project mentor to an underrepresented student 2013–2014

Mentees


Rahul Upadhya (2016). Co-author on a paper. B.S. Biomedical Engineering, Rutgers University, 2018. Currently pursuing PhD in biomedical engineering at Rutgers University.

Mahita Varanasi (2017-Present). Co-author on 2 papers in review and 2 papers in preparation. B.S. Biomedical Engineering, Johns Hopkins University, 2020 expected.

Shanelle Mendes (2017-Present). Co-author on 2 papers in review and 2 papers in preparation. B.S. Biomedical Engineering, Johns Hopkins University, 2020 expected.


Deepti Sudhakar (2019-Present). Biomedical Engineering, Johns Hopkins University, 2022 expected.

**Intellectual Property: Patents**


**Material Sharing**

- >25 plasmids produced during PhD and deposited to plasmid sharing repository Addgene
- 25 requests of Addgene deposited plasmids since 2018