The Development of Polycationic Materials for Gene Delivery Applications

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ABSTRACT

Peter Benjamin Uthe: The Development of Polycationic Materials for Gene Delivery Applications

(Under the direction of Dr. Valerie Sheares Ashby)

Polycationic materials were designed to serve as vehicles for the delivery of therapeutic DNA into cells. Three areas were explored including ionization and buffering control, degradable scaffolds, and new functionality for enhanced cellular uptake. It was found that the delivery ability was greatly influenced by the ionization state of the materials with a range (24 - 50% ionization at a pH of 7.4) defined as a target in the development of future materials. Use of a degradable scaffold was found to be successful in reducing unwanted toxicity that is present in most delivery vehicles developed to date. Lastly, synthetic strategies were developed for integrating a new functionality, with proven cellular uptake enhancement, into monomers and polymers. Through this work, fundamental studies were conducted that established material design guidelines as well as supported strategies for controlling toxicity and enhancing bioactivity.
To my family and friends for always being there
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<tr>
<td>AIBN</td>
<td>2,2′-Azobis(2-methylpropionitrile)</td>
</tr>
<tr>
<td>BCA</td>
<td>Bicinchoninic acid assay</td>
</tr>
<tr>
<td>Boc</td>
<td>tert-Butoxylcarbonyl</td>
</tr>
<tr>
<td>Boc₂G</td>
<td>1,3-(di-tert-butoxycarbonyl)guanidine</td>
</tr>
<tr>
<td>BPO</td>
<td>Benzoyl peroxide</td>
</tr>
<tr>
<td>CD</td>
<td>β-Cyclodextrin</td>
</tr>
<tr>
<td>mCPBA</td>
<td>meta-Chloroperbenzoic acid</td>
</tr>
<tr>
<td>D-A</td>
<td>Diels-Alder</td>
</tr>
<tr>
<td>DBU</td>
<td>1,8-Diazabicyclo[5.4.0]undec-7-ene</td>
</tr>
<tr>
<td>DMF</td>
<td>N,N-Dimethyl formamide</td>
</tr>
<tr>
<td>DPBS</td>
<td>Dulbecco’s phosphate buffered saline</td>
</tr>
<tr>
<td>DSC</td>
<td>Differential scanning calorimetry</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>GPC</td>
<td>Gel permeation chromatography</td>
</tr>
<tr>
<td>LCST</td>
<td>Lower critical solution temperature</td>
</tr>
<tr>
<td>LDA</td>
<td>Lithium diisopropylamide</td>
</tr>
<tr>
<td>MEM</td>
<td>Minimum essential medium</td>
</tr>
<tr>
<td>MTS</td>
<td>3-(4,5-Dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4- sulfophenyl)-2H-tetrazolium</td>
</tr>
<tr>
<td>NIPAM</td>
<td>N-isopropylacrylamide</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
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<tr>
<td>NLP</td>
<td>Nuclear localizing protein</td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear magnetic resonance</td>
</tr>
<tr>
<td>OD</td>
<td>1,8-Octanediol</td>
</tr>
<tr>
<td>PCL</td>
<td>Poly(ε-caprolactone)</td>
</tr>
<tr>
<td>pCMV-Luc</td>
<td>Plasmid cytomegalovirus; luciferase</td>
</tr>
<tr>
<td>PDI</td>
<td>Polydispersity index</td>
</tr>
<tr>
<td>PAMAM</td>
<td>Poly(amidoamine)</td>
</tr>
<tr>
<td>PBAE</td>
<td>Poly(β-amino ester)</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered solution</td>
</tr>
<tr>
<td>PDMAEMA</td>
<td>Poly[2-(dimethylamino)ethyl methacrylate]</td>
</tr>
<tr>
<td>PEG</td>
<td>Poly(ethylene glycol)</td>
</tr>
<tr>
<td>PEI</td>
<td>Poly(ethyleneimine)</td>
</tr>
<tr>
<td>b-PEI</td>
<td>Branched poly(ethyleneimine)</td>
</tr>
<tr>
<td>l-PEI</td>
<td>linear poly(ethyleneimine)</td>
</tr>
<tr>
<td>PEO</td>
<td>Poly(ethylene oxide)</td>
</tr>
<tr>
<td>PHEMA</td>
<td>Poly(2-hydroxyethyl methacrylate)</td>
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<td>PHPMA-DMAE</td>
<td>poly(carbonic acid 2-dimethylamino-ethyl ester 1-methyl-2-(2-methacryloylamino)- ethyl ester)</td>
</tr>
<tr>
<td>PLA</td>
<td>Poly(lactic acid)</td>
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<tr>
<td>PLGA</td>
<td>Poly(lactic acid-co-glycolic acid)</td>
</tr>
<tr>
<td>PLLA</td>
<td>Poly(L-lactic acid)</td>
</tr>
<tr>
<td>PMMA</td>
<td>Poly(methyl methacrylate)</td>
</tr>
<tr>
<td>PNIPAM</td>
<td>Poly(N-isopropylacrylamide)</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>PS</td>
<td>Polystyrene</td>
</tr>
<tr>
<td>RI</td>
<td>Refractive index</td>
</tr>
<tr>
<td>RLU</td>
<td>Relative light units</td>
</tr>
<tr>
<td>Sn(Oct)$_2$</td>
<td>Stannous 2-ethylhexanoate</td>
</tr>
<tr>
<td>TEA</td>
<td>Triethylamine</td>
</tr>
<tr>
<td>TEG</td>
<td>Tetraethyleneglycol</td>
</tr>
<tr>
<td>TGA</td>
<td>Thermogravimetric analysis</td>
</tr>
<tr>
<td>THF</td>
<td>Tetrahydrofuran</td>
</tr>
<tr>
<td>Tosyl</td>
<td>4-Toluenesulfonyl chloride</td>
</tr>
<tr>
<td>TMP</td>
<td>Tetramethylpiperdine</td>
</tr>
<tr>
<td>TMS</td>
<td>Trimethylsiloxyl</td>
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<tr>
<td>$&lt;M_n&gt;$</td>
<td>Number-average molecular weight</td>
</tr>
<tr>
<td>$&lt;M_w&gt;$</td>
<td>Weight-average molecular weight</td>
</tr>
<tr>
<td>$T_g$</td>
<td>Glass transition temperature</td>
</tr>
<tr>
<td>$T_m$</td>
<td>Crystalline melting temperature</td>
</tr>
<tr>
<td>$F$</td>
<td>Monomer incorporation ratio</td>
</tr>
<tr>
<td>$f$</td>
<td>Monomer feed ratio</td>
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Chapter 1. An Introduction to Gene Therapy
1. Introduction to Gene Therapy

The ability to activate, silence, or introduce gene expression is a powerful tool for the treatment of diseases.\(^1\) One method to accomplish this is to introduce foreign therapeutic DNA to a desired location so that it may affect a target gene. There is a broad range of techniques for delivery including direct introduction of naked DNA, use of modified viruses, or developing synthetic strategies.\(^2\) The first uses an external driving force, such as particle bombardment, magnetic fields, or electric fields, to promote membrane translocation. The latter two approaches incorporate vehicles, or vectors, that contain and protect the DNA, promote cellular uptake, and ultimately release the cargo at the nucleus.

1.1 Delivery.

To frame gene delivery, the biological pathway and barriers need to be introduced. Administration can occur peripherally (e.g. oral, airway, intravenous) or locally (e.g. implant, local injection). If there is global administration of the DNA, it must circulate through the body, avoid premature clearance (e.g. reticuloendothelial system (RES)), resist nuclease degradation, and accumulate near the target location.\(^3,4\) Site accumulation can either go through nonspecific interactions, passive targeting such as the enhanced permeability and retention effect (EPR effect), or active targeting of specific cell types.\(^4,5\) Delivery can also be accomplished by local administration, but complications arise from invasive procedures for implants or injections into the target location. Once at the site, the DNA must associate to the cell membrane via cell receptor/targeting ligands, electrostatic interactions, or hydrophilic interactions, and
translocate into an intracellular compartment (Figure 1.1). If it is taken up through endocytosis, it must escape from the endosome prior to degradation. The next hurdle is passing through the cytosol followed by nuclear uptake and ultimately transcription and translation to produce the therapeutic protein.

Figure 1.1. Specific and non specific cell membrane interactions that lead to cellular uptake; I-II) receptor mediated; III) electrostatic; IV) hydrophobic

1.1.1 Naked DNA Delivery.

The exact pathway for delivery is dependent on the vector chosen, which can be categorized into three main types. The first is direct delivery of naked DNA, the second is viral based delivery, and the third is non-viral delivery. In the case of naked DNA delivery, the DNA enters the cell through an external force that either penetrates the cell membrane or disrupts it to permit the passage of larger molecules. Particle bombardment techniques such as the gene gun or jet injection use high velocity particles or media that
are capable of penetrating a target tissue.\textsuperscript{2} Cellular uptake of naked DNA has also been enhanced by using electroporation, sonoporation, or use of a magnetic field.\textsuperscript{2} The mechanism for electroporation consists of two parts. The first is an electric field that induces accumulation near the cell membrane via electrophoretic mobility. The current also disrupts the cell membrane on a microscopic scale which allows for passage across the membrane. Sonoporation causes cavitation around the target site that enhances membrane permeability.\textsuperscript{2} Lastly, delivery has been enhanced by tethering the cargo to magnetic particles and applying a magnetic field. In naked DNA delivery there is no protection of the DNA from nuclease degradation and these pathways suffer from low efficiency. Furthermore the application of the delivery is often limited to superficial tissues, small target sites, and/or is accompanied with invasive procedures.

\textbf{1.1.2 Viral Delivery.}

Viral vectors are typically derived from retroviruses and adenoviruses, and are formed from partial or complete replacement of the viral wild-type DNA. The viral machinery has evolved to impart high target specificity, cellular uptake, and nuclear localization resulting in high transfection efficiency.\textsuperscript{7} In this system translocation occurs by either a signaling pathway or direct membrane fusion (Figure 1.1). Once taken up into the cell, the vector is contained in an endosome and escape occurs via endosomal disruption proteins or membrane fusion. The vectors then travel through the cytosol to the nucleus, and the genetic material is taken up into the nucleus. Viruses, once again, have evolved to utilize the natural machinery of the cell to be actively taken to the
nucleus through nuclear localizing proteins (NLP). They then utilize nuclear pores to transfer the cargo into the nucleus.

The most common viral vectors that have been developed are the aden-associated virus, herpes virus, pox virus, and lentivirus. Retroviral vectors randomly insert DNA into the host chromosome and results in persistent gene expression. An issue arises however if the insertion occurs at an integral site, interrupting protein expression, and potentially leading to oncogenic effects. Adenoviral vectors do not insert DNA into the host chromosomes, but as a result only yield short term gene expression. In both systems the body can gain natural immunity and elicit inflammatory or immunological responses. Despite the high transfection efficiency, viral type vectors have resulted in cancer and death, and have not been able to move beyond phase III clinical trials.

1.1.3 Synthetic Vector Delivery.

Synthetic vectors are being designed in an attempt to utilize the positive characteristics of the other classes of delivery and avoid the negative effects such as ineffective administration and severe health risks. These materials are designed to form a complex with DNA (polyplex) usually through electrostatic interactions between cationic functional groups on the vector with the anionic phosphate groups along the DNA backbone. Synthetic vectors typically possess primary, secondary, and/or tertiary amino groups that maintain protonated and unprotonated amines. In the polyplex, DNA is highly compacted, allowing for membrane translocation and protection from degradation by nuclease enzymes. Non-viral vectors have the advantage of carrying large DNA structures with lower safety risks. These vectors can also be synthesized in large
quantities, which aids in commercialization. Various topologies have been utilized including linear, branched, hyperbranched, and dendritic materials as well as liposomes and micelles. The greatest disadvantages in synthetic delivery are non-specific toxicity associated with polycationic materials as well as typically low transfection efficiency.

As depicted in Figure 1.2, synthetic vectors go through a similar pathway to viral vectors. Attachment of signaling ligands can induce cellular uptake or they can go through nonspecific membrane association via electrostatic or hydrophobic interactions (Figure 1.1). They are then taken up into the cell through endocytosis. Vectors then escape from the endosome, and to achieve this, endosomal disruption proteins or more prominently the ‘proton sponge’ effect have been utilized (Figure 1.3). Briefly, cationic
materials have a distribution of charged and uncharged functional groups, typically amines. Upon endosomal acidification, the neutral amines become protonated, effectively buffering the endosome and increasing the internal charge potential. This induces chloride diffusion into the vesicle followed by osmotic swelling that leads to membrane rupture and cargo release. Once in the cytosol the cargo must be delivered to the nucleus. Synthetic vectors have been modified with nuclear localizing peptides to take advantage of the natural cellular machinery that actively moves the polyplex to the nucleus. Passive diffusion of large particles in the cytosol is very difficult, and it is generally thought to not be the mode of transport if no NLP is present. To date the exact pathway has not been elucidated to date. It is also important to note that nuclear uptake of synthetic materials typically occurs during cell replication when the nuclear membrane is dissolved, adding another limitation of synthetic vectors by making them cell cycle dependent.

**Figure 1.3.** ‘Proton sponge’ theory of endosomal release; I) early endosome; II) acidification and chloride accumulation; III) osmotic swelling and membrane rupture.
A variety of synthetic methods has been employed to complex and protect DNA including liposomes, micelles, and functionalized polymers. The first two systems utilize amphiphilic materials that encapsulate the DNA. They have the advantage of low toxicity and facile surface functionalization. The greatest disadvantage in these systems is their lower structural stability of the complex and their susceptibility to disruption and premature DNA release. A great focus of gene therapy has been in the research of micelles and liposomes and many reviews have been written on these subjects. The remainder of the research reviewed herein will focus on polymeric systems.

1.2 Non-Degradable Materials

1.2.1 Poly(ethyleneimine)

Polymeric synthetic vectors can be divided into nondegradable synthetic materials, degradable synthetic materials, and biologically derived materials. The most prominent nondegradable material has been poly(ethyleneimine) (PEI). Branched (b-PEI) and linear (l-PEI) topologies have been synthesized, the branched from acid catalyzed polymerization of aziridine, and the linear from ring opening polymerization of 2-ethyl-2-oxazoline followed by hydrolysis (Figure 1.4).
The material is one of the most physically and biologically characterized delivery vehicles, and due to the high efficiency, it has become a standard in the field. The branched form contains $1^\circ$, $2^\circ$, and $3^\circ$ amines in a ratio of 1:1:1 respectively.\textsuperscript{13} The linear form has mainly $2^\circ$ amines. The acid/base profiles for each system show a sloped pH transition between a pH of 5-8 showing multiple pKa values for the amines present in the polymers.\textsuperscript{14} With a range of pK$_a$ values the branched form of PEI has 20% amine protonation at biological pH (pH 7.4) leaving a significant proportion of the amines unprotonated and allowing for a large buffering capacity. This ‘proton sponge’ effect is attributed to efficient endosomal escape and ultimately the high transfection of PEI.

There have been studies of the influence of topology, molecular weight, percentage of amines, and modified chemical composition on the overall transfection efficiency and toxicity. The biological properties and activity of PEI have been shown to be dependent on polymer topology. The branched form, with its array of amine types, formed more stable complexes than the linear form.\textsuperscript{15} Furthermore, b-PEI contains
tertiary amines that impart a greater buffering potential than l-PEI. Despite these properties, l-PEI has shown high in vivo transfection efficiency, and derivatives of this form are more prominent in commercially available gene delivery vehicles, e.g. ExGen500 and jetPEI. The high efficiency has been attributed to the ability of l-PEI to mediate cell-cycle independent nuclear delivery as well as possessing lower complex stability that permits DNA dissociation once the target site has been reached.

The molecular weight of PEI also plays an important role in its transfection efficiency. Godbey et al. demonstrated that in a series of PEIs ranging from 600 to 70,000 Da the transfection efficiency increased as the molecular weight increased. They also reported that the toxicity increased as the molecular weight increased. Kunath et al. demonstrated the use of low molecular weight PEI (5400 Da; LMW-PEI) that had a 2.1-110 fold increase in transfection efficiency (cell line dependent) compared to a commercially available high molecular weight PEI (25,000 Da; HMW-PEI). The toxicity for LMW-PEI was also relatively low. To attain the high transfection efficiencies the concentration of LMW-PEI was optimized at an N/P ratio of 67, where as the optimal N/P ratio of HMW-PEI is 6.7. The lower toxicity of LMW-PEI enabled the use of higher polymer concentration. Werth et al. demonstrated the use of lower molecular weight PEI obtained from fractionation of commercial PEI (25,000 Da) and showed that the low molecular weight fraction was able to efficiently deliver siRNA into a range of carcinoma cell lines, to which the commercially available PEI could not. Once again the concentration for optimized delivery was at an N/P ratio of 66. In general the lower molecular weight materials were less toxic but required much higher effective concentrations.
To capitalize on the high bioactivity and reduce the toxicity, modifications such as $N$-alkylation and $N$-acylation, pegylation, or degradation have been used. Klibanov has shown that $N$-alkylation of the $1^o$ and $2^o$ amines (Figure 1.5) decreased the transfection efficiency of 25 kDa branched PEI, but alkylation of the $3^o$ amines enhanced activity.\textsuperscript{22} This observation was attributed to the position of alkylation with the $1^o$ and $2^o$ amines located at the periphery of the material and the $3^o$ amines more internalized. In solution the hydrophobic alkyl groups placed at the surface collapse the structure, shielding the interior amines. Since the $3^o$ amines are already internalized, alkylation of the $3^o$ amines preserved the overall structure and increased transfection efficiency. Akinc \textit{et al.} demonstrated that quaternization of all of the amines enhances complex formation and cell internalization, but due to the loss of buffering ability, overall transfection was greatly diminished.\textsuperscript{23} The transfection of this material increased when the endosomal buffering compound, chloroquin, was present and was one example that supports the ‘protein sponge’ theory for endosomal release.

In an attempt to decrease the toxicity stemming from the $1^o$ amines, and to a lesser degree $2^o$ amines, $N$-acylation has also been employed (Figure 1.5). Pack has shown that
partial acetylation of up to 57% of the primary amines of b-PEI with acetic anhydride, has a 58-fold increase in transfection efficiency in HEK293 cells.\textsuperscript{24,25} Despite a decrease in buffering capacity, the bioactivity was enhanced. This was attributed to a decrease in binding strength, facilitating DNA dissociation upon delivery. On the other hand, Thomas et al. demonstrated that in commercially available 25 kDa l-PEI, the material is only 89% deacylated as received.\textsuperscript{26} When the remaining 11% of the amides were hydrolyzed, the efficiency was enhanced by 21-fold \textit{in vitro}, and 10,000-fold \textit{in vivo}. The buffering capacity of l-PEI at biological pH was known to be less than b-PEI, and it was concluded that the enhanced transfection efficiency stemmed from its increase. Alkylation and acylation demonstrated the balance of charge, toxicity, binding, and buffering that is required in developing vector. Branched PEI was enhanced by decreasing the number of the more toxic and stronger binding 1° and 2° amines, while linear PEI, with primarily 2° amines, benefited from increasing the buffering capacity.

\begin{figure}
\centering
\includegraphics[width=0.5\textwidth]{figure1.png}
\caption{Acid labile PEG grafts conjugated to branched PEI}
\end{figure}

Modifications such as incorporating PEG blocks or degradability have been used to decrease the cytoxicity of PEI. PEG has been widely used in biological applications due to its biocompatibility, hydrophilicity, and stealth like properties. When a block copolymer with PEG and PEI (PEI-\textit{b}-PEG) is used, the cationic block condenses the
DNA resulting in charge neutralization. PEG then orients itself to the periphery forming a micelle and internally shields the polycation/DNA complex from premature clearance via macrophage or the reticuloendothelial system. When no other modifications to the block copolymers are used such as degradable groups or targeting ligands, PEG decreases surfaces interactions, which results in higher circulation times and lower toxicity, but also prevents membrane association and internalization.\textsuperscript{27} To avoid the limited transfection ability of PEGylated complexes, ether chains have been conjugated to PEI via labile groups that facilitate deshielding of the polyplex once internalized into the cell (Figure 1.6).\textsuperscript{28,29} This strategy coupled with attaching targeting ligands to promote cellular uptake has been a promising for gene delivery. Shuai \textit{et al.} have synthesized a series of PEG-polycaprolactone grafted b-PEIs (Figure 1.7).\textsuperscript{30} The transfection efficiency was directly related to the PEI molecular weight as well as to the grafting density with higher molecular weight PEI (25 kDa vs 800 Da) and lower grafting density showing optimal bioactivity. The toxicity of the degradable materials was less than the nondegradable parent materials, but also higher concentrations were required to reach comparable or better transfection efficiency.

\textbf{Figure 1.7}. a) Linear PEI containing redox active disulfide linkages in the main chain; b) Branched PEI with PEG-b-PCL degradable grafts

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Another strategy for reducing the toxicity of PEI has been crosslinking or chain extension of low molecular weight PEI (< 10 KDa) with a degradable moiety. Lee et al. synthesized linear PEI (800 Da) that contained a reducible disulfide group in the backbone (Figure 1.7). They found that the reducible PEI was nontoxic, but the transfection efficiency was less than the 25 kDa PEI control used in the experiments. There are numerous examples of modifications similar to those reported above, and some of those can be found in recent reviews.

1.2.2 Poly(methacrylate) and Poly(methacrylamide)

A wide range of polymers have been synthesized from chain-growth polymerization of amine bearing acryloyl and acrylamido monomers. The advantages of these systems are the facile polymerization via radical or anionic polymerization, most were commercially available, and access to a large range of functionality. One of the most promising of the acryloyl based systems is poly[(dimethylamino)ethyl methacrylate] (PDMAEMA). Polymerized via aqueous radical polymerization with an
ammonium peroxydisulfate initiator, a $<M_n>$ of $45 \times 10^3$ g/mol and $<M_w>$ of $36 \times 10^4$ g/mol were attained (Figure 1.8). It was found that the transfection efficiency was optimized at a w/w ratio of 6-13/1 and the efficiency was serum independent. At the optimum concentration the transfection was comparable to commercially available Lipofectin®. Despite having promising activity, focus has been on developing copolymers to decrease the toxicity.

Similar to the strategies employed in reducing the toxicity of PEI, acryloyl copolymers have been synthesized that incorporate biocompatible functional groups, and/or degradability. Stolnik developed a series of DMAEMA/PEG-methacrylate (PEGMA) copolymers (Figure 1.8) and studied the effects of architecture and PEGMA chain length on toxicity, cell internalization and transfection. It was found that incorporating the ethylene glycol segments stabilized the complexes with DNA and decreased toxicity compared to PDMEMA. Interestingly, the larger PEGMA (45 repeat units) had higher cell association and internalization compared to the short chain PEGMA (7-8 repeat units) containing material. The overall transfection compared to the parent compound was lower, and this was attributed to a decrease in cell interactions and internalization. Lam et al. developed a biocompatible stabilizer, phosphorylcholin methacrylate (PCMA) (Figure 1.8), which was also designed to shield the cationic charge and stabilize complex formation. The zwitterionic group decreased non-specific cell association and transfection efficiency, and it was proposed that cell targeting ligands functionalized at the periphery would be promising for a low toxicity, cell specific gene delivery vehicle.
Another strategy to partially shield the positive charge and decrease toxicity is incorporation of hydroxyl groups. Jiang et al. synthesized a PDMAEMA grafted poly(hydroxymethyl acrylate) (PHEMA) using a carbonate linkage (Figure 1.9). The PDMAEMA grafts were capable of condensing DNA while PHEMA effectively decreased toxicity. Furthermore, the carbonate linkages imparted degradability and combined with using short chain PDMAEMA grafts the toxicity was decreased. The carbonate linkages hydrolyze rapidly at a pH of 5.0 and are much more stable at a pH of 7.4. Prior to hydrolysis, the material behaves similarly to high molecular weight PDMAEMA, but once degraded in the endosome, low toxicity PDMAEMA oligos are formed. Transfection efficiency was highest with increased PDMAEMA graft density while simultaneously decreasing toxicity.
PDMAEMA has also been used in copolymers with poly(acylamide)s. Kurisawa et al. synthesized a thermoresponsive copolymer poly(N-isoproylacrylamide) (PNIPAM)-co-PDMAEMA-co-poly(butyl methacrylate) (PBMA) that utilized the DNA condensing ability of PDMAEMA with the thermorepsonsive character of PNIPAM (Figure 1.10).\textsuperscript{39}

As a homopolymer, PNIPAM has a lower critical solution temperature (LCST) of 32 °C. At a temperature below the LCST, the polymer remains hydrophilic and water soluble. Above 32 °C water molecules hydrogen bound to the amides disassociate and new hydrogen bonds form between the polymer chains. This causes the polymers to aggregate and fall out of solution. By incorporating a thermoresponsive segment with a cationic polymer, the association/dissociation parameters with DNA were controlled. Water soluble polyplexes were formed below the LCST. The temperature was then raised to promote tight hydrophobic polyplex aggregates. At this step the DNA disassociation remained very low. It was then shown that cells could be transfected above the LCST, and after a sufficient amount of time, the temperature was reduced, causing a thermal switch that promoted DNA dissociation. The efficiency of the
copolymer was enhanced by using the thermal program, and efficiencies greater than poly(L-lysine), but lower than PDMAEMA were observed.

![Thermoresponsive pentablock hydrogel used for persistent DNA delivery](image)

Figure 1.11. Thermoresponsive pentablock hydrogel used for persistent DNA delivery

Mallapragada demonstrated another delivery system utilizing a thermal switch to promote controlled release of a polymer/DNA complex (Figure 1.11). A pentablock was synthesized, poly(diethylaminoethyl methacrylate) (PDEAEMA)-b-poly(ethyleneoxide) (PEO)-b-poly(propyleneoxide) (PPO)-b-PEO-b-PDEAEMA, that took advantage of the condensing ability of PDEAMA and the thermoresponsive property of the PEO-b-PPO-b-PEO central core. The polymer with DNA remained water soluble below 23 °C, and upon heating to 37 °C reversible gelation occurred. The release profile of the polyplexes, after gelation, did not go through an initial burst and remained linear between three and five days, depending on the initial formulation.
Another acrylamide based polymer, poly(carbonic acid 2-dimethylamino-ethyl ester 1-methyl-2-(2-methacryloylamino)-ethyl ester) (PHPMA-DMAE), was developed by Funhoff et al. that incorporated hydrolysable grafts functionalized with tertiary amines (Figure 1.12).\(^{41}\) PHPMA-DMAE gave promising toxicity and transfection results in the absence of serum but had limited transfection efficiency while serum was present. Luten et al. elaborated on this structure by synthesizing acrylamide monomer analogues of HPMA-DMAE to examine how subtle structural changes affected the rate of hydrolysis as well as serum dependence on transfection. (Figure 1.12).\(^{42}\) HPMA-DEAE, HPMA-MPPM, and HPMA-BDMP showed less toxicity than poly(methacrylate) materials that did not contain a rapidly hydrolysable group, and transfection was serum independent.

### 1.3 Degradable Delivery

One tactic for improving synthetic delivery vehicles is increasing efficiency by incorporating different functionalities that take advantage of the natural machinery involved in the extracellular and intracellular delivery pathways. The other tactic is to
decrease toxicity by either masking the cationic sites or by integrating biodegradable groups that leads to formation of less toxic oligomers. Degradable sites can be installed in pendant functionality as seen in some of the examples already discussed but with only limited result in decreasing toxicity. Alternatively, degradable groups can be placed on the main chain of the polymer. Typically this has been done using poly(ester) or poly(amide) based materials. The advantage of chain degradation is that the polymer can be fully digested as opposed to only pendant groups degrading, leaving the main chain intact. The degradation products can be designed to be further metabolized by the body, or the starting monomers can be chosen from a range of biocompatible starting materials. Polymerization typically occurs via ring opening or step-growth polymerization. The former generally yields high molecular weight polymers, and the latter lends itself to facile functionalization and access to large material libraries.

![Figure 1.13](image)

**Figure 1.13.** Conjugative addition polymerization to yield poly(β-amino ester)s utilizing bis-secondary amines or primary amines

### 1.3.1 Poly(β-amino ester)s
The most studied poly(ester)s are poly(β-aminoester) materials. The largest library of approximately 2300 materials was produced by Langer, and these were evaluated for toxicity and transfection ability with high throughput automated screening techniques (Figure 1.13).\textsuperscript{43} Polymerization occurred from conjugate addition of a primary amine or bis-secondary amine with a diacrylate.\textsuperscript{44} The majority of the vectors produced were inefficient at delivery due to issues of solubility, toxicity, and/or poor DNA complex formation. Of the materials, 46 have shown promising delivery ability greater than PEI. The best PBAEs were linear with molecular weights of ~10 kDa. The top 9 materials contained a pendant hydroxyl group and the top three materials were synthesized with a hydroxyl functionalized alkyl amine and a hydrophobic dimethacrylate. As seen in other systems, a material that has hydrophobic groups can enhance transfection due to an increase in membrane interactions, but at the same time, water solubility needs to be maintained. It was also found that the end groups of the PBAEs were important.\textsuperscript{45} When the material was terminated with diemethacrylates, the DNA binding ability as well as transfection decreased. When terminated with primary amines, DNA compaction as well as transfection was enhanced. The best material was shown to have a 5-fold increase in efficiency compared to PEI with the hard to transfect HUVEC cell line and reached levels comparable to lentivirus and adenovirus delivery. This research demonstrated that small structural variations drastically alter bioactivity, and high throughput screening may serve as an essential tool in discovering materials that compete with viral delivery vectors. Despite the decrease in toxicity, the concentrations needed for delivery were much higher than the non-degradable systems, leaving an
opportunity to enhance cellular uptake and activity through new functionality and better controlled degradation.

Figure 1.14. Peptide based delivery vehicles; left: poly(L-Lysine); right: Oligo arginine R7

1.3.2 Poly(amide)s

Poly(amide), peptide, and peptido mimetic materials have also been synthesized to take advantage of enzymatic degradation, while providing greater resistance to premature hydrolytic degradation. The first poly(amide) used was poly(L-lysine) (PLL) which initially was a promising transfection agent. PLL possessed some toxicity, and its efficiency has been far surpassed by other poly(amide) materials. Chen et al. synthesized a series of copolymers of PLL with imidazoleacetic acid. Due to imidazole’s low pKₐ, the pendant group was designed to increase the buffering capability of PLL and ultimately enhance delivery. It was found that the maximum activity was seen in lower molecular weight materials with higher imidazole incorporation. Substitution of the primary amine functionality for imidazole decreased the toxicity of the material as well as increased the buffering capacity. The transfection efficiency increased until an incorporation of 50% imidazole was reached. Above this amount, the DNA complexation was not high enough for effective delivery.
Other functionalities have been used to enhance delivery. One of the greatest adaptations that viral vectors possess is membrane-permeable peptides that enhance membrane association and uptake.\textsuperscript{47,48} A common characteristic of these peptides is a high concentration of cationic amino acids, most notably the guanidine containing arginine. It has been proposed that the cationic domain (protein transduction domain, PTD) on these peptides interacts with heparin sulfate proteoglycans that promote cellular uptake.\textsuperscript{47} To mimic the PTD oligo arginine peptides have been used as delivery vehicles or conjugated onto other delivery vehicles to enhance cellular uptake (Figure 1.14).\textsuperscript{49,50} Utilizing the guanidine rich groups was shown to enhance delivery and oligos of around 7 to 9 arginine repeat units were shown to be optimal.

\textbf{Figure 1.15.} Polyamidoamine (PAMAM) synthesis; a): linear; b): dendritic

One of the most studied classes of amido materials are poly(amido amine)s (PAMAM). These materials have been synthesized in either linear or dendritic topologies (Figure 1.15). Polymerization occurred by hydrogen transfer conjugate
addition to diacrylamides in an analogues synthesis to that of the PBAE materials.\textsuperscript{11} Utilizing this modular synthetic approach, installation of a large range of functionalities, including more amino groups, carboxylic acids, alcohols, as well as peptides, was possible. PAMAMs are less toxic than PLL, and including acids and alcohols further decreases their toxicity.

![Reaction](image)

**Figure 1.16.** Synthesis of a carboxylic acid and guanidine functionalized PAMAM designed as an RGD mimic

Ferruti has developed linear PAMAM materials by reacting an acid functionalized diacrylate with agmatine (Figure 1.16).\textsuperscript{51-53} Agmatine is a decarboxylated analogue of arginine and contains a guanidinium group. The repeat unit containing the acid and guanidine moiety mimics the cell active RGD sequence which promotes cellular uptake. In an acid base titration, three acid dissociation constants were observed, 2.25, 7.45, and $\geq 12.1$ from the carboxylic acid, tertiary amine, and guanidine, respectively. The in vitro toxicity was lower than most amphiphilic PAMAM materials despite the excess cationic groups. This was attributed to the RGD-like structure that behaved more like biologically active RGD than the typical cationic cell surface interactions. The material *in vivo* was also found to be stealth-like without significant preferential accumulation in the liver, and
transfection efficiency was higher than other common PAMAMs and comparable to commercially available JetPEI.

1.4 Biologically Derived Materials

![Structure of the biologically derived chitosan](image)

**Figure 1.17.** Structure of the biologically derived chitosan

### 1.4.1 Chitosan

Biologically derived materials have also been studied to increase biocompatibility and provide a system that could be metabolized by the body. Three main systems have been studied with the most prominent being chitosan. Other materials have been synthesized incorporating β-cyclodextrin rings as well as non-degradable materials with conjugated biologically derived groups. Chitosan delivery vehicles were produced by partial deacylation of chitin to afford glucosamine linked by glycosidic bonds (Figure 1.17).\(^{54}\) Huang *et al.* have shown that delivery efficiency is highly dependent on molecular weight with 213 kDa yielding the highest activity in a range of materials (231 – 17 kDa).\(^{55}\) This was attributed to more stable complex formation. The optimum N/P ratio was 5,\(^{56}\) which was much more comparable to an optimum concentration for nondegradable polymers as opposed to typical degradable materials. Modifications such as N-quaternization and incorporating imidazole bearing groups have been used to
increase efficiency. Quaternization increased transfection efficiency while simultaneously increasing toxicity. The imidazole moiety also increased transfection by increasing the buffering capacity, lending itself to endosomal release via the ‘proton sponge’ effect.\textsuperscript{57}

**Figure 1.18.** Polymerization of $\beta$-cyclodextrin utilizing a cationic diamidine linkage

1.4.2 $\beta$-cyclodextrin
Another carbohydrate derivative, \(\beta\)-cyclodextrin (CD), have also been shown to increase biocompatibility and ultimately efficiency. Synthesis of these polymers was achieved by stepgrowth polymerization between a diaminocyclodextrin and diimidate compounds (Figure 1.18).\(^{58}\) Transfection was optimized at an N/P ratio >10 with low toxicity. The transfection was greatest when the linkage between CD segments was 6-8 methylenes. Greater than 8 methylenes diluted the charge density of the material resulting in solubility issues and low transfection efficiency.\(^{59}\) It was also found that these materials did not escape from the endosome via amine protonation, demonstrating that the transfection pathway is greatly dependent on the material being studied.

![Figure 1.19. Linear PEI with sugar alcohol end groups](image)

**1.4.3 Sugar alcohol functionalized**

To combine the high efficiency of non-degradable materials with the biocompatibility of naturally derived polymers, Rieneke studied the effect of incorporating reduced sugars onto effective delivery vehicles such as PEI (Figure 1.19).\(^{60-63}\) This strategy greatly decreased toxicity compared to PEI. Interestingly the
transfection efficiency was highly dependent on the stereochemistry of the carbohydrates. This was due to changes in the complex stability. The toxicity was also shown to be dependent on the distance from the cationic site and the carbohydrate, with further separation resulting in increased toxicity.

1.5 Bioactive Modifications

Further delivery enhancement has been achieved in the presence of targeting ligands, endosomolytic compounds, and nuclear localizing proteins. A range of targeting ligands has been used such as RGD and folic acid where cellular uptake was initiated by receptor binding. The degree of specificity greatly depends on the ligand chosen. For example the RGD receptor is present on many types of cells and little specificity is gained. Other receptors, such as the folate receptor, are over expressed in some cell lines, and these systems have much higher specificity. The effectiveness of targeting is greatly dependent on the chemistry involved in conjugation to the polymer, with the spacer between the targeting head group and the polymer being important, as well as the density of the ligation. It is critical for the site of conjugation between the polymer and ligand not to interfere with recognition or binding on the cell surface receptors. In polycationic materials there is also nonspecific binding via electrostatic interactions and specificity can only be attained when competition from nonspecific electrostatic interactions is minimized (charge neutrality).

Compounds that promote endosomal escape have also been shown to enhance transfection by a few orders of magnitude. In in vitro studies chloroquin has become commonly added to take advantage of the buffering ability in the endosome. This
Another strategy has been the conjugation of inactivated adenovirus particles. Lastly, natural and synthetic fusogenic peptides have been attached to the delivery vehicle where upon acidification the proteins go through a structural transition. These structural changes lead to membrane interaction and disruption, ultimately enhancing delivery efficiency from one to three orders of magnitude.

Once endosomal escape has been achieved there are many barriers present in the cytosol. Diffusion of large molecules in the cytosol is extremely slow and inefficient. Techniques such as nuclear localizing proteins (NLP) are used to take advantage of the natural cell machinery and promote accumulation at the nucleus. Most NLPs are short cationic protein sequences, usually arginine or lysine rich. The cationic nature of synthetic delivery vehicles are proposed to partially mimic NLPs, but with only limited efficiency. Few complexes reach the nucleus, and little has been elucidated regarding the mechanism post endosomal release, to nuclear localization, and ultimately nuclear uptake and complex dissociation.

6. Conclusions

Gene delivery is a dynamic process that manages many barriers in the extracellular and intracellular environment. Materials are designed to address each of the steps in the delivery process, but in most cases optimizing one step in the delivery process interferes with another. As seen in the characterization of PEI, the molecular weight, charge density, toxicity, buffering capacity, and DNA binding strength must be balanced to achieve optimal transfection. These factors are greatly influenced by the
number, density, type, and pKₐ of the amines. The transfection of b-PEI was enhanced by minimizing binding strength of the material by reducing charge density and number of primary and secondary amines. This showed that the binding strength and buffering capacity of the material was greater than needed for transfection prior to acetylation, and the number of amines could be reduced to limit DNA binding strength without being detrimental to delivery. On the other hand, l-PEI had lower buffering capacity and binding strength than b-PEI, and it was found that deacetylation of its amines enhanced delivery. The molecular weight of vectors has also been shown to influence delivery. Typically, higher molecular weight materials were more efficient until a weight was reached where toxicity minimized the delivery enhancement. To reduce toxicity, degradable and naturally derived materials have been used. These vectors decrease toxicity, but the concentration needed for delivery is greatly increased with N/P ratios typically 20-70, as opposed to an N/P ratio of 5-10 for nondegradable materials.

The balance between positive charge for complex formation and cell association with toxicity is one of the greatest obstacles in synthetic vectors. Furthermore, maintaining a high buffering capacity is important for endosomal release. If there is too much initial charge, complex formation and cell association will be strong but cell toxicity potentially increases. Furthermore, if too many of the amines are protonated before cell internalization, there will be a limited ‘proton sponge’ effect, and the complexes will enter the cell but remain in the endosome and lysosome, eventually leading to degradation in the harsh environment. If the binding strength is too strong the DNA will not dissociate and the genetic replication enzymes will be prevented from transcribing the genes. If there are not enough cationic sites, however, there will be poor
complex formation and cell association, but also potentially lower toxicity. With these parameters in competition there have been seemingly many contradictory examples in the literature and optimized properties for delivery to date has been material specific. Fundamental studies that isolate ionic charge, buffering, and binding strength would be advantageous to the field.
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Chapter 2. Using Ionization Control to Enhance Gene Delivery
Abstract. The degree of ionization of amine containing polymers plays a critical role in gene delivery. In order to develop new materials with optimized properties for transfection, a clear understanding of the influence of ionization needs to be established. We determined the influence of altering pK$_a$ values for structurally similar dialkylaminoisoprene polymers to balance the competing properties of complex formation and buffering capacity. Free radical polymerization was used to generate materials with distinct pK$_a$ transitions. The degree of ionization at physiological pH was determined and found to directly affect DNA binding, transfection efficiency, and toxicity. It was shown that there is a balance between buffering capacity and complex formation with an ideal ionization in the range above 24% to below 50% amine protonation (pH = 7.4). It was also found that a material with 33% protonation yielded a transfection efficiency (N/P ratio of 2) 100 fold higher than the field standard poly(ethyleneimine).

1. Introduction

A major focus of gene therapy research is the development of synthetic materials$^{1-13}$ that aim to avoid the immunogenic or oncogenic$^{14-18}$ effects encountered in viral delivery systems. Despite a decrease in these health risks, synthetic delivery vehicles are generally less efficient than natural vehicles and usually have some degree of cell toxicity due to their cationic nature.$^{19}$ However, synthetic polymers afford a high degree of control over their chemical composition, carrier size, and production. To compete with the natural systems, synthetic polymers must efficiently 1) form a complex with DNA
(polyplex), 2) associate to the cell membrane and become internalized through endocytosis, 3) escape from the endosome, 4) transport the cargo to the nucleus, and 5) dissociate from the DNA.\textsuperscript{20} Most materials to date utilize 1\textdegree, 2\textdegree, and or 3\textdegree amines, which at physiological conditions maintain protonated and unprotonated amines. These properties are fundamental for complex formation and endosomal release, respectively.\textsuperscript{21}

During cell internalization, it is critical for the polyplex to escape from the endosome prior to fusion with a lysosome in order to avoid its harsh degradative environment. The most prominent mechanism for escape is the ‘proton sponge’ effect proposed by Behr in 1995, which asserts upon endosomal acidification the neutral amines become protonated, followed by an influx of chloride ions to balance the charge. Ultimately, there is an increase in osmotic pressure and eventual membrane rupture.\textsuperscript{22-24} From the early endocytic vesicle to the late endosome, the pH ranges from 7.4 to \textasciitilde5.\textsuperscript{25} Poly(ethyleneimine) (PEI) has become a standard in the field due to the material’s high delivery efficiency which is attributed to the range of amines that allow it to buffer over the entire lifetime of the endosome. The result is efficient endosomal escape.\textsuperscript{24} Many of the reported materials attempt to mimic PEI’s buffering ability by integrating functionality, such as an imidazole moiety\textsuperscript{26-31}, to impart varied pK\textsubscript{a} values and gain enhanced buffering characteristics.

Previously, we reported a series of dialkylamino functionalized dienes that were shown to have higher transfection efficiency than PEI at low concentrations.\textsuperscript{10} Unlike the varied types of amines and broad buffering capability of PEI, these diene based materials contain only one type of tertiary amine, imparting a distinct buffering range. With this in mind, it was not obvious why the aminodiene’s relative efficiency was high. This
research focuses on the characterization of the buffering capability, DNA/polymer complex formation, and resulting transfection efficiency to further understand structure/biological activity relationships. To this end, homopolymers and copolymers were synthesized with discrete pKₐ ranges to compare the effects of ionization on delivery efficiency. Through this work insights will be gained into the effects of charge on transfection and property targets will be established for the design and screening of new gene delivery materials.

2. Experimental Details

2.1 Materials. Plasmid DNA (pCMV-Luc) was purchased from Elim Biopharmaceuticals, Inc. (Hayward, CA). Minimum essential medium (MEM), Hanks’ balanced salt solution (HBSS), Dulbecco’s phosphate buffered saline (PBS), fetal bovine serum (FBS), and a 1 kb DNA ladder were purchased from Invitrogen. CellTiter 96 AQueous one solution cell proliferation assay and luciferase assay system were purchased from Promega Corp. (Madison, WI). BCA protein assay kit was purchased from Pierce (Rockford, IL). Branched poly(ethyleneimine) with a molecular weight of 25,000 g/mol was used as a control in the characterization experiments. All other chemicals were purchased from Sigma-Aldrich and were used without further purification.

2.2 Instrumentation. ¹H and ¹³C NMR spectra were acquired in deuterated chloroform on a Bruker 400 AVANCE spectrometer. Molecular weights were measured by a Waters GPC system using polystyrene standards. The measurements were taken using 5% triethylamine/THF as the solvent on four columns (Waters Styragel HR0.5,
HR2, HR4, and HR5). Glass transition temperatures were measured with a Seiko 220C differential scanning calorimeter, using a heating and cooling rate of 10 °C/min. The glass transition temperatures were reported based on the second heating. Thermogravimetric analysis was carried out using a Perkin Elmer TGA 7 with a heating rate of 10 °C/min in a N\textsubscript{2} atmosphere. Zeta potential measurements were completed on a Malvern Zeta Sizer Nano Series Nano-ZS instrument using Dispersion Technology Software version 4.20 at a wavelength of 633 nm using a 4.0 mW, solid state He-Ne laser at a scattering angle of 173 °C. Cell culture plates were analyzed on a Molecular Devices SpectraMax M5. Ethidium bromide stained agarose gels were visualized on a Bio Rad VersaDoc imaging system.

### 2.3 Diene Synthesis.  

*General synthesis of 2-bromo-N,N-dialkylprop-2-en-1-amine.*  

To a solution of \(N,N\)-dialkylamine in diethyl ether was added 2,3-dibromopropene dropwise at 0 °C while stirring. The reaction was allowed to warm to room temperature and to react for 18 h. NaOH (1 M) was added until the formed salt dissolved, and the solution was extracted with diethyl ether (3x, 50 mL). The organic layers were combined, washed with brine, dried with MgSO\textsubscript{4}, and concentrated. The product was purified and isolated by distillation as a colorless liquid.

*2-bromo-N,N-dimethylprop-2-en-1-amine.*  \(^{1}\text{H} \text{NMR (400 MHz, CDCl}	extsubscript{3})\): \(\delta 5.77\) (s, 1H), \(5.53\) (s, 1H), \(3.07\) (s, 2H), \(2.23\) (s, 6H). \(^{13}\text{C} \text{NMR (400 MHz, CDCl}	extsubscript{3})\): \(\delta 131.60\) (CH\textsubscript{2}=CBrCH\textsubscript{2}), \(119.12\) (CH\textsubscript{2}=CBrCH\textsubscript{2}), \(68.20\) (CH\textsubscript{2}N), \(45.14\) (N(CH\textsubscript{3})\textsubscript{2})

*2-bromo-N,N-diethylprop-2-en-1-amine.*  \(^{1}\text{H} \text{NMR (400 MHz, CDCl}	extsubscript{3})\): \(\delta 5.90\) (s, 1H), \(5.55\) (s, 1H), \(3.24\) (s, 2H), \(2.58\) (q, 4H, \(J = 7.2\) Hz), \(1.03\) (t, 6H, \(J = 7.2\) Hz). \(^{13}\text{C} \text{NMR (400 MHz, CDCl}	extsubscript{3})\):
NMR (400 MHz, CDCl$_3$): $\delta$ 132.64 (CH$_2$=CBrCH$_2$), 116.80 (CH$_2$=CBrCH$_2$), 61.46 (CH$_2$N), 46.67 [N(CH$_2$CH$_3$)$_2$], 11.73 [N(CH$_2$CH$_3$)$_2$]

2-bromo-N,N-dipropylprop-2-en-1-amine. $^1$H NMR (400 MHz, CDCl$_3$): $\delta$ 5.90 (s, 1H), 5.53 (s, 1H), 3.23 (s, 2H), 2.43 (t, 4H, $J = 7.6$), 1.45 (m, 4H, $J = 7.6$), 0.88 (t, 6H, $J = 7.2$). $^{13}$C NMR (400 MHz, CDCl$_3$): $\delta$ 133.60 (CH$_2$=CBrCH$_2$), 117.80 (CH$_2$=CBrCH$_2$), 63.00 (CH$_2$N), 55.92 [N(CH$_2$CH$_2$CH$_3$)$_2$], 20.78 [N(CH$_2$CH$_2$CH$_3$)$_2$], 12.16 [N(CH$_2$CH$_2$CH$_3$)$_2$]

General synthesis of 2-(N,N-diakylaminomethyl)-1,3-butadiene. To a flame dried 1 L three neck flask was added [1,3-bis(diphenylphosphino)propane]dichloronickel (0.5 mol%), dialkylaminobromopropene, and dry THF under a N$_2$ atmosphere. Vinyl magnesium bromide (1 M in THF) was added dropwise at 0 °C while stirring. The reaction was allowed to warm to room temperature and to react for 24 h. The reaction was quenched with NH$_4$Cl (satd.) and extracted with diethyl ether (3x, 50 mL). The organic layers were combined, washed with brine, dried with MgSO$_4$, and concentrated. The product was purified and isolated by distillation as a colorless liquid.

$N,N$-dimethyl-2-methylenebut-3-en-1-amine (Dimethylaminoisoprene; DMAI). $^1$H NMR (400 MHz, CDCl$_3$): $\delta$ 6.36 (dd, 1H, $J_1 = 17.6$ Hz, $J_2 = 11.2$ Hz), 5.41 (d, 1H, $J = 17.6$), 5.14 (s, 1H), 5.11 (m, 1.5H), 5.09 (s, 0.5H), 3.01 (s, 1H), 2.21 (s, 1H). $^{13}$C NMR (400 MHz, CDCl$_3$): $\delta$ 143.31 (CH$_2$=CCH$_2$N), 137.56 (CH$_2$=CHCCH$_2$N), 117.73 (CH$_2$=CHCCH$_2$N), 114.44 (CH$_2$=CCH$_2$N), 61.86 (CH$_2$N), 45.54 [N(CH$_3$)$_2$]

$N,N$-diethyl-2-methylenebut-3-en-1-amine (Diethylaminoisoprene; DEAI). $^1$H NMR (400 MHz, CDCl$_3$): $\delta$ 6.39 (dd, 1H, $J_1 = 17.6$ Hz, $J_2 = 10.8$ Hz), 5.46 (d, 1H, $J =
17.6), 5.204 (s, 1H), 5.142 (s, 1H), 5.08 (d, 1H), 3.16 (s, 1H), 2.51 (q, 4H, J = 7.2), 1.02 (t, 6H, J = 7.2). $^{13}$C NMR (400 MHz, CDCl$_3$): $\delta$ 144.07 (CH$_2$=CCH$_2$N), 137.92 (CH$_2$=CHCCH$_2$N), 116.98 (CH$_2$=CHCCH$_2$N), 113.96 (CH$_2$=CCH$_2$N), 55.25 (CH$_2$N), 47.03 [N(CH$_2$CH$_3$)$_2$]), 11.67 [N(CH$_2$CH$_3$)$_2$]

$N,N$-dipropyl-2-methylenebut-3-en-1-amine (Dipropylaminoisoprene; DPAI). $^1$H NMR (400 MHz, CDCl$_3$): $\delta$ 6.38 (dd, 1H, $J_1$ = 17.6 Hz, $J_2$ = 10.8 Hz), 5.45 (d, 1H, $J$ = 17.6), 5.22 (s, 1H), 5.12 (s, 1H), 5.06 (d, 1H, $J$ = 10.8), 3.15 (s, 1H), 2.35 (t, 4H, J = 7.6 Hz), 1.46 (m, 4H, J = 7.6 Hz), 0.87 (t, 6H, J = 7.6). $^{13}$C NMR (400 MHz, CDCl$_3$): $\delta$ 144.22 (CH$_2$=CCH$_2$N), 137.94 (CH$_2$=CHCCH$_2$N), 116.99 (CH$_2$=CHCCH$_2$N), 113.92 (CH$_2$=CCH$_2$N), 56.66 (CH$_2$N), 56.22 [N(CH$_2$CH$_2$CH$_3$)$_2$], 20.18 [N(CH$_2$CH$_2$CH$_3$)$_2$], 11.95 [N(CH$_2$CH$_2$CH$_3$)$_2$]

2.4 Polymer Synthesis. Free Radical Polymerization of 2-(N,N-dialkylaminomethyl)-1,3-butadiene. Monomer and AIBN were added to an ampoule with a magnetic stir bar. After three freeze-pump-thaw cycles, the ampoule was sealed under nitrogen and placed in an oil bath preheated to 70 °C. After 24 h, the polymer was precipitated into acetone at -78 °C and dried under vacuum at room temperature for 5 d.

poly(2-(N,N-dimethylaminomethyl)-1,3-butadiene) (PMAI). $^1$H NMR (400 MHz, CDCl$_3$): $\delta$ 5.26 (s, 1H), 2.83 and 2.74 (s, 2H from trans and cis CH$_2$N, respectively), 2.13 [broad, 10H, 6 from 2(CH$_3$) and 4 from the polymer backbone]. $^{13}$C NMR (400 MHz, CDCl$_3$): $\delta$ 136.99 (CH$_2$CH=CCH$_2$), 128.36 (CH$_2$CH=CCH$_2$), 66.52 [CH$_2$N(CH$_3$)$_2$, cis], 58.37 [CH$_2$N(CH$_3$)$_2$, trans], 45.40 [N(CH$_3$)$_2$], 35.80 (CH$_2$CH=CCH$_2$), trans), 28.75 (CH$_2$CH=CCH$_2$, cis), 26.51 (CH$_2$CH=CCH$_2$)
poly(2-(N,N-diethylaminomethyl)-1,3-butadiene) (PEAI). \(^1\)H NMR (400 MHz, CDCl\(_3\)): \(\delta\) 5.27 (s, 1H), 2.92 (s, 2H, trans), 2.85 (s, 2H, cis), 2.41 (m, 4H), 2.10 (m, 4H), 0.96 (m, 6H). \(^1\)C NMR (400 MHz, CDCl\(_3\)): \(\delta\) 137.57 (CH\(_2\)CH=CH\(_2\)), 127.72 (CH\(_2\)CH=CH\(_2\)), 59.94 [CH\(_2\)N(CH\(_2\)CH\(_3\))\(_2\), cis], 51.84 [CH\(_2\)N(CH\(_2\)CH\(_3\))\(_2\), trans], 46.43 [N(CH\(_2\)CH\(_3\))\(_2\), 36.13 (CH\(_2\)CH=CH\(_2\), trans), 27.97 (CH\(_2\)CH=CH\(_2\), cis), 26.61 (CH\(_2\)CH=CH\(_2\)), 11.67 [N(CH\(_2\)CH\(_3\))\(_2\)]

poly(2-(N,N-dipropylaminomethyl)-1,3-butadiene (PPAI)). \(^1\)H NMR (400 MHz, CDCl\(_3\)): \(\delta\) 5.28 (s, 1H), 2.93 (s, 2H, trans), 2.85 (s, 2H, cis), 2.27 (m, 4H), 2.12 (m, 4H), 1.42 (m, 4H), 0.85 (m, 6H). \(^1\)C NMR (400 MHz, CDCl\(_3\)): \(\delta\) 138.00 (CH\(_2\)CH=CH\(_2\)), 127.80 (CH\(_2\)CH=CH\(_2\)), 61.29 [CH\(_2\)N(CH\(_2\)CH\(_2\)CH\(_3\))\(_2\), cis], 55.65 [N(CH\(_2\)CH\(_2\)CH\(_3\))\(_2\)], 53.09 [CH\(_2\)N(CH\(_2\)CH\(_2\)CH\(_3\))\(_2\), trans], 36.02 (CH\(_2\)CH=CH\(_2\), trans), 28.04 (CH\(_2\)CH=CH\(_2\), cis), 26.42 (CH\(_2\)CH=CH\(_2\)), 20.21 [N(CH\(_2\)CH\(_2\)CH\(_3\))\(_2\)], 12.04 [N(CH\(_2\)CH\(_2\)CH\(_3\))\(_2\)]

**Free Radical Copolymerization.** The density for each monomer was determined, and varied amounts of monomer were added via micropipette to an ampoule with a magnetic stir bar. After three freeze-pump-thaw cycles, the ampoule was sealed under nitrogen and placed in an oil bath preheated to 70 °C. After 24 h, the polymer was precipitated into acetone at -78 °C and dried under vacuum at room temperature for 5 d.

poly(2-(N,N-dimethylaminomethyl)-3-methyl-1,3-butadiene-co-2-(N,N-dipropylaminomethyl)-3-methyl-1,3-butadiene) (MePr). \(^1\)H NMR (400 MHz, CDCl\(_3\)): \(\delta\) 5.28 (s, 1H), 2.94-2.74 (m, 2H), 2.26 (broad, 4H), 2.14 [m, 10H, 6 from 2(CH\(_3\)) and 4 from the polymer backbone], 1.42 (broad, 4H), 0.84 (broad, 6H). \(^1\)C NMR (400 MHz,
CDCl$_3$): $\delta$ 137.10 (CH$_2$=CCH$_2$), 128.25 (CH$_2$=CCH$_2$), 66.53 [CH$_2$N(CH$_3$)$_2$, cis], 61.21 [CH$_2$N(CH$_2$CH$_2$CH$_3$)$_2$, cis], 58.23 [CH$_2$N(CH$_3$)$_2$, trans], 55.73 [N(CH$_2$CH$_2$CH$_3$)$_2$], 53.04 [CH$_2$N(CH$_2$CH$_2$CH$_3$)$_2$, trans], 45.77 [N(CH$_3$)$_2$], 35.91 (CH$_2$CH=CCH$_2$, dimethyl, trans), 34.56 (CH$_2$CH=CCH$_2$, dipropyl, trans), 29.09 (CH$_2$CH=CCH$_2$, dimethyl, cis), 28.81 (CH$_2$CH=CCH$_2$, dipropyl, cis), 27.98 (CH$_2$CH=CCH$_2$, dimethyl), 26.39 (CH$_2$CH=CCH$_2$, dipropyl), 20.23 [N(CH$_2$C$_2$H$_3$)$_2$], 12.04 [N(CH$_2$CH$_2$CH$_3$)$_2$]

poly(2-(N,N-diethylaminomethyl)-1,3-butadiene-co-2-(N,N-dipropylaminomethyl)-1,3-butadiene) (EtPr). $^1$H NMR (400 MHz, CDCl$_3$): $\delta$ 5.28 (s, 1H), 2.94-2.88 (m, 2H), 2.42 (broad, 4H), 2.26 (broad, 4H), 2.11 (broad, 4), 1.43 (broad, 4H), 0.978 (broad, 6H), 0.85 (broad, 6H). $^{13}$C NMR (400 MHz, CDCl$_3$): $\delta$ 137.90 (CH$_2$CH=CCH$_2$), 127.61 (CH$_2$CH=CCH$_2$), 61.23 [CH$_2$N(CH$_2$CH$_2$CH$_3$)$_2$, cis], 60.16 [CH$_2$N(CH$_2$CH$_3$)$_2$, cis], 55.73 [N(CH$_2$CH$_2$CH$_3$)$_2$], 53.04 [CH$_2$N(CH$_2$CH$_2$CH$_3$)$_2$, trans], 51.92 [CH$_2$N(CH$_2$CH$_3$)$_2$, cis], 46.95 [N(CH$_2$CH$_3$)$_2$], 34.60 (CH$_2$CH=CCH$_2$, diethyl, trans), 34.62 (CH$_2$CH=CCH$_2$, dipropyl, trans), 29.21 (CH$_2$CH=CCH$_2$, diethyl, cis), 28.85 (CH$_2$CH=CCH$_2$, dipropyl, cis), 28.04 (CH$_2$CH=CCH$_2$, diethyl), 26.50 (CH$_2$CH=CCH$_2$, dipropyl), 20.24 [N(CH$_2$CH$_2$CH$_3$)$_2$], 12.00 [N(CH$_2$CH$_3$)$_2$], 11.72 [N(CH$_2$CH$_2$CH$_3$)$_2$]

Reactivity Ratios. Reactivity ratios were determined by $^1$H NMR with low conversion (< 10%) copolymerizations of DMAI and DPAI. The polymerization setup was identical to the copolymer procedure. Reactions were run for 2 h and samples were added directly to NMR tubes containing CDCl$_3$.

2.5. Polymer/Polypelex Characterization. pH Titration. The pH of the solution was monitored using a Pasco pH meter calibrated with 4.0 and 10.0 buffers and verified with a 7.4 phosphate buffer. Polymer solutions were made by dissolving approximately
20 mg of material into 10 mL of 0.1 M HCl. The solution was titrated with 0.1 M NaOH, and the pH was recorded after equilibration (30 s). A derivative of the curve was used to determine the acid base equilibrium points. A line of best fit was taken through the buffering plateau, and the average of the two equilibrium points was used to determine the inflection point of the titration curve.

*Standard Polyplex Formation.* To 1 mg of each polymer sample was added 15 µL of glacial acetic acid followed by 485 µL of either opti-MEM or MEM media to make polymer solutions at a concentration of 2 mg/mL. Once dissolved the polymer samples were diluted with the appropriate media to obtain the polymer solutions at concentrations of 0.1 mg/mL. To solutions of 2 µg/mL pCMV-Luc plasmid in media was added the polymer solution and media to form complexes at desired N/P ratios with total volumes of 200 µL.

*Agarose Gel Electrophoresis.* The standard polyplex formation was used to generate complexes at desired N/P ratios. Electrophoresis was conducted on a 0.75% agarose gel in TAE buffer containing 0.5% ethidium bromide for 45 min. at 120 V. The bands were visualized using ethidium bromide staining.

*Cytotoxicity Assay.* HeLa cells were grown in 96-well plates at a density of 5×10³ cells/well in 150 µL of growth medium (89% MEM, 10% FBS, 1% L-glutamine). Cells were grown for 24 h. The media was removed, and the cells were washed with 100 µL of DPBS and incubated with 15 µL of polyplex solution and 135 µL of MEM for 4 h. The solutions were removed, and the cells were washed with DPBS. Following the addition of 150 µL of MEM, the cells were allowed to recover for 20 h. The solutions
were removed, and the cells were washed with DPBS. To the cells were added 100 µL of MEM and 20 µL of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazoleum, inner salt (MTS, from CellTiter 96 AQueous One Solution Cell Proliferation Assay kit). The cells were incubated for 4 h and the absorbance at 490 nm was monitored on a Molecular Devices SpectraMax M5 plate reader.

Transfection Efficiency Assay. HeLa cells were grown in 48-well plates at a density of 1.5×10^4 cells/well in 300 µL of growth medium (89% MEM, 10% FBS, 1% L-glutamine). Cells were grown for 24 h. The media was removed, and the cells were washed with 300 µL of HBSS and incubated with 450 µL of opti-MEM and 50 µL of polyplex solution for 4 h. The solutions were removed, and the cells were washed with HBSS and incubated with 500 µL of opti-MEM (89% MEM, 10% FBS, 1% L-glutamine) for 32 h. The growth medium was removed and the cells were washed with HBSS and treated with 150 µL of cell lysis buffer (1X). The resulting mixtures were centrifuged and 20 µL of supernatant were added directly to white opaque 96-well plates. Luciferase activity was quantified by adding 100 µL of luciferase assay reagent, followed by a 2 second delay. Luminescence was quantified over a 1 second read time. The result was normalized to the total protein content using a BCA protein assay whose absorption was monitored at 562 nm on a Molecular Devices SpectraMax M5 plate reader.

Zeta Potential Measurements. Plasmid solutions were made by diluting 80 µL of pCMV-Luc (1 mg/mL) with 9.92 mL of 100 mM HEPES buffer to give a final DNA concentration of 8 µg/mL. Polymer solutions were made by dissolving 1 mg of material with 15 µL of glacial acetic acid and 9.985 mL of 100 mM HEPES buffer. The final pH
of the polymer was verified to be 7.4. Using an autotitrator, the polymer concentration was increased by an N/P ratio of 0.25 up to a total ratio of 10, and the ζ-potentials were monitored.

3. Results and Discussion

Scheme 2.1. Synthesis of dialkylaminoisoprenes and resulting polymers; \textsuperscript{a}MePr-X (X = percent DMAI); \textsuperscript{b}EtPr-Y (Y = percent DEAI)
Table 2.1. Radical Homo- and Copolymerizations of DMAI, DEAI, and DPAI

<table>
<thead>
<tr>
<th>Sample</th>
<th>Feed (%)</th>
<th>Actual Incorp. (%)</th>
<th>$\langle M_n \rangle$ (× 10^{-3} g/mol)</th>
<th>PDI</th>
<th>$T_g$ (°C)</th>
<th>Decomposition (°C)</th>
<th>DP</th>
</tr>
</thead>
<tbody>
<tr>
<td>PMAI</td>
<td>-</td>
<td>-</td>
<td>16</td>
<td>1.50</td>
<td>-29</td>
<td>353 365 109</td>
<td></td>
</tr>
<tr>
<td>PEAI</td>
<td>-</td>
<td>-</td>
<td>15</td>
<td>2.29</td>
<td>-46</td>
<td>337 359 120</td>
<td></td>
</tr>
<tr>
<td>PPAI</td>
<td>-</td>
<td>-</td>
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<td>1.67</td>
<td>-55</td>
<td>313 352 122</td>
<td></td>
</tr>
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<td>1.59</td>
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<td></td>
</tr>
<tr>
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<td>17</td>
<td>1.66</td>
<td>-47</td>
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<td></td>
</tr>
<tr>
<td>MePr-54</td>
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<td>1.74</td>
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</tr>
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<td>345 356 136</td>
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<td>20</td>
<td>1.92</td>
<td>-47</td>
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<td></td>
</tr>
<tr>
<td>EtPr-60</td>
<td>60</td>
<td>58</td>
<td>18</td>
<td>2.15</td>
<td>-46</td>
<td>346 360 114</td>
<td></td>
</tr>
<tr>
<td>EtPr-80</td>
<td>80</td>
<td>78</td>
<td>17</td>
<td>2.50</td>
<td>-46</td>
<td>345 360 109</td>
<td></td>
</tr>
</tbody>
</table>

$^a$ amine monomer relative to the DPAI; $^b$ determined by $^1$H NMR; $^c$ determined by GPC; $^d$ determined by DSC; $^e$ determined by TGA; $^f$ based on the average MW of the monomers incorporated into the polymer; $^g$ designed as a pK$_a$ mimic of PEAI

3.1 Synthesis

The monomers, dimethylaminoisoprene (DMAI), diethylaminoisoprene (DEAI), and dipropylaminoisoprene (DPAI) were synthesized (Scheme 2.1) in high purity as previously described.$^{32}$ Poly(dimethylaminoisoprene) (PMAI), poly(diethylaminoisoprene) (PEAI), and poly(dipropylaminoisoprene) (PPAI) homopolymers with molecular weights of 16.0, 15.2, 20.4 kg/mol and polydispersities (PDIs) of 1.50, 2.29, 1.67, respectively, were synthesized using AIBN at 70 °C for 24 h. The molecular weights of the copolymers ranged from 16 to 20 kg/mol with high fidelity between the feed and actual monomer incorporation. All of the polymers were comparable molecular weight and PDI to the materials previously synthesized in our group for gene delivery studies.$^{10}$ For each of the materials, there was one glass transition indicating formation of random copolymers (Table 2.1).
To further verify random copolymer formation, the reactivities of DMAI and DPAI monomers were determined by $^1$H NMR (Table 2.2). Monomer concentrations were varied by 0.1 equivalent of DMAI relative to DPAI. The ratio of monomers was determined by normalizing the region of 2.8 to 3.2 ppm for the methylenes extending from the diene or polymer backbone and integrating the peaks at 3.13 and 3.01 ppm, which correspond to DMAI and DPAI, respectively. The normalized monomer concentration ($A_t^M$) at time $t$ and the starting monomer concentration ($A_0^M$) were used to determine the mole fraction of DMAI ($F_t^M$) and DPAI ($F_t^P$) incorporated into the copolymers (equations 1 and 2; Table 2.2).

$$F_t^M = \frac{A_0^M - A_t^M}{[A_0^M - A_t^M] + [A_0^P - A_t^P]}$$
$$F_t^P = \frac{A_0^P - A_t^P}{[A_0^P - A_t^P] + [A_0^M - A_t^M]}$$

Table 2.2. Calculated values for DMAI and DPAI reactivity ratio determination

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<th>MePr-10</th>
<th>0.15</th>
<th>1.52</th>
<th>0.15</th>
<th>0.85</th>
<th>8.5</th>
<th>0.18</th>
<th>0.11</th>
<th>-0.51</th>
<th>0.07</th>
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<td>1.37</td>
<td>0.28</td>
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<td>7.5</td>
<td>0.39</td>
<td>0.25</td>
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<tr>
<td>MePr-40</td>
<td>0.67</td>
<td>1.05</td>
<td>0.46</td>
<td>0.54</td>
<td>7.5</td>
<td>0.87</td>
<td>0.67</td>
<td>-0.10</td>
<td>0.51</td>
</tr>
<tr>
<td>MePr-50</td>
<td>0.86</td>
<td>0.86</td>
<td>0.50</td>
<td>0.50</td>
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<td>1.00</td>
<td>1.00</td>
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<td>1.00</td>
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<tr>
<td>MePr-60</td>
<td>1.06</td>
<td>0.70</td>
<td>0.58</td>
<td>0.42</td>
<td>6.0</td>
<td>1.40</td>
<td>1.50</td>
<td>0.43</td>
<td>1.61</td>
</tr>
<tr>
<td>MePr-70</td>
<td>1.25</td>
<td>0.52</td>
<td>0.65</td>
<td>0.35</td>
<td>7.5</td>
<td>1.87</td>
<td>2.33</td>
<td>1.09</td>
<td>2.90</td>
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<td>MePr-80</td>
<td>1.46</td>
<td>0.35</td>
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<td>0.26</td>
<td>5.5</td>
<td>2.80</td>
<td>4.00</td>
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<tr>
<td>MePr-90</td>
<td>1.67</td>
<td>0.18</td>
<td>0.87</td>
<td>0.13</td>
<td>5.5</td>
<td>6.50</td>
<td>9.00</td>
<td>7.62</td>
<td>12.46</td>
</tr>
</tbody>
</table>

* Determined by $^1$H NMR
Copolymer incorporation ratios \( F \) and monomer feed ratios \( f \) were then determined using the following equations:

\[
F = \frac{F_t^M}{F_t^P} \quad (3) \\
f = \frac{A_0^M}{A_0^P} \quad (4)
\]

The value of \( F \) and \( f \) were used to determine \( G \) and \( H \) (Finemann-Ross method; equations 5 and 6; Table 2.2).

\[
G = \frac{f}{F}(F - 1) \quad (5) \\
H = \frac{f^2}{F} \quad (6) \\
G = Hr_m - r_p \quad (7)
\]

A plot of equation 7 gave the ratios for DMAI \( (r_m = 0.64) \) and for DPAI \( (r_p = 0.60) \). This, in addition to thermal data, indicated the generation of random copolymers. The structural similarities of DEAI and DPAI prevented accurate reactivity ratios from being determined, but due to the high incorporation fidelity and single glass transition, it is believed that these materials are also random copolymers.
It is thought that the range of amines or pK$_a$ values contained in PEI results in efficient buffering at various pH values, and this property leads to high transfection efficiency. Previously, we have seen high efficiency in a diene based material that contains one unique tertiary amine. This finding deviates from the ideology that varied pK$_a$ values lead to higher transfection efficiency. Given that a broad buffering capacity

**Figure 2.1** Acid/base titrations for the (a) MePr and (b) EtPr series

3.2 Ionization Studies

It is thought that the range of amines or pK$_a$ values contained in PEI results in efficient buffering at various pH values, and this property leads to high transfection efficiency. Previously, we have seen high efficiency in a diene based material that contains one unique tertiary amine. This finding deviates from the ideology that varied pK$_a$ values lead to higher transfection efficiency. Given that a broad buffering capacity
range is not required, the question remains, what are the optimum buffering properties that lead to high transfection efficiency?

![Graph showing pH as a function of monomer feed for EtPr and MePr series]

**Figure 2.2.** pK<sub>a</sub> as a function of monomer feed; (♦) EtPr series and (■) MePr

To examine buffering capacity and ionization, acid-base titrations were carried out to determine the average pK<sub>a</sub> of the series of aminoisoprene homopolymers in solution and to compare their buffering profiles with the profile for PEI (Figure 2.1). The titration experiments began with dissolution of ~20 mg of material in 10 mL of 0.1 N HCl and slowly titrating the solution with 0.1 N NaOH. The acid/base equilibrium points were then used to determine the inflection point of the curve, giving pK<sub>a</sub> values for PMAI, PEAI, and PPAI of 7.9, 6.9, and 5.8, respectively. One of the first observations that came from the initial experiments was the small difference of pH between the onset and endpoint of amine protonation. A more drastic change in pH was expected due to the electrostatic depression of amine protonation as the number of charged sites increased. The result of the titration gave an almost distinct buffering transition for each material,
and the mechanism for which is under further investigation. These titration results (Table 2.3) help to better understand the results of our previous work where it was determined that PMAI and PPAI had low transfection efficiency, but PEAI showed higher efficiency than PEI at an N/P ratio of 2 (N/P ratio = protonizable amines of the polymer to phosphates of the DNA). Polyplex formation for PMAI was the most efficient in the series with moderate to low transfection efficiency. With an average pK$_a$ of 7.9, PMAI had 76% of its amines protonated at physiological pH, explaining the low concentration needed for complex formation. As such, this left only a small percentage of free amines available for endosomal buffering and release. On the other hand, PPAI had 2.4% of its amines protonated, and it was shown to be an ineffective DNA binder at concentrations below an N/P of 20. PEAI, with a pK$_a$ of 6.9, had 24% of its amines protonated and offered the best balance of charge for polyplex formation and buffering for endosomal release. Similar observations have been made by Ahmad et al. where they observed a

<table>
<thead>
<tr>
<th>Polymer</th>
<th>pK$_a$</th>
<th>Ionization pH = 7.4 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PMAI</td>
<td>7.9</td>
<td>76</td>
</tr>
<tr>
<td>PEAI</td>
<td>6.9</td>
<td>24</td>
</tr>
<tr>
<td>PPAI</td>
<td>5.8</td>
<td>2.4</td>
</tr>
<tr>
<td>MePr-20</td>
<td>6.3</td>
<td>7.4</td>
</tr>
<tr>
<td>MePr-40</td>
<td>6.7</td>
<td>16</td>
</tr>
<tr>
<td>MePr-54</td>
<td>6.9</td>
<td>24</td>
</tr>
<tr>
<td>MePr-60</td>
<td>7.1</td>
<td>33</td>
</tr>
<tr>
<td>MePr-80</td>
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<td>EtPr-60</td>
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<td>14</td>
</tr>
<tr>
<td>EtPr-80</td>
<td>6.8</td>
<td>20</td>
</tr>
</tbody>
</table>
bell-shaped curve that defined the relationship of charge density and transfection efficiency in a series of liposomes. Our data agree with their observation that there is a region that balances buffering capacity and complex stability to optimize transfection.

With this understanding, we wanted to further define an optimal window of ionization that balances the competing properties of charge and buffering potential. To answer this question, copolymers (PMAI-co-PPAI and PEAI-co-PPAI) were designed with varying ratios of monomer incorporation, shifting the pK\textsubscript{a}, and thereby percent ionization, to access pK\textsubscript{a} values above and below 6.9 (PEAI; 24% ionization). The feed ratios were varied by 20% up to homopolymers. Similar to the homopolymers, titration experiments revealed a single distinct transition for each copolymer (Figure 2.1), and the resulting values had a linear relationship with regard to the monomer feed (Figure 2.2). A line of best fit in a plot of pK\textsubscript{a} as a function of feed afforded an equation that can be used to synthesize a material with a programmed pK\textsubscript{a} between 5.8 and 7.9. To take advantage of this tool the material MePr-54 was designed with a target pK\textsubscript{a} of 6.9 to directly compare with the ionization of PEAI. In doing so, the structural differences between these two materials are separated from the charge, giving a more accurate assessment of ionization and structure on overall transfection efficiency. To further investigate the properties of these materials in reference to charge, DNA binding agarose gels and transfection experiments were conducted.

### 3.3 DNA Binding Agarose Gels and Transfection

DNA binding ability was investigated using agarose gel electrophoresis to determine the polymer concentrations that each material could effectively bind DNA.
Transfection efficiency (Figure 2.4) was also evaluated and then related to ionization/buffering and complex formation to establish a target for material design. In the DNA binding experiments complexes were formed at various N/P ratios (0.1 – 100; depending on the charge neutralization). For the transfection experiments, a 6000 base pair pCMV-luc plasmid containing the luciferase reporter gene was used, and each material was tested at N/P ratios of 2, 4, and 10.

At an ionization of 76%, PMAI was shown to have efficient complex formation with quenching at an N/P ratio of 1. Meanwhile, it had low buffering capacity and only nominal transfection, which may be from the inability of the material to serve as an efficient ‘proton sponge’. PPAI, on the other hand, with an ionization of 2.4% had poor complex formation with quenching at an N/P of 20, but simultaneously maintained high buffering capacity. With the inability to form a neutral complex until high polymer concentrations, this material had poor transfection efficiency. When looking at the MePr copolymer series, the percent ionization was shifted to examine whether a balance between complex formation and buffering capacity could be reached. In this series, as the DMAI incorporation decreased the complexation efficiency decreased. Much like PPAI, MePr-40 (16.6% ionization) and MePr-20 (7.6% ionization) possessed high buffering capacity, but had the lowest binding ability, with complexation occurring above an N/P of 4. These materials showed low transfection, with the exception of MePr-40 which had some efficiency at an N/P of 10. MePr-80 and MePr-60 had ionizations of 50 and 33%, respectively, and showed DNA binding near an N/P ratio of 2. These materials had drastically improved transfection at this low polymer concentration, with the efficiencies 100 fold higher than any of the diene homopolymers or PEI. MePr-60 was
the highest performing polymer in this study, and with 33% ionization, balances complex formation with strong buffering ability.

PEAI was less efficient at DNA binding than PMAI with quenching at an N/P ratio of 2, and similarly, the complexation efficiency in the EtPr series decreased as DEAI incorporation decreased. PEAI, with ionization of 24%, showed transfection twenty fold higher than plasmid DNA at an N/P ratio of 4, but at an N/P of 2 and 10 there was low efficiency. On the other hand, EtPr-80, with an ionization of 20%, had low efficiency until an N/P of 10. This followed the same trend as the MePr series where the materials below 16% ionization did not effectively complex the DNA until an N/P of 10 or greater and also showed no transfection ability at the lower concentrations tested. Based on these results it appears that a window above 24% and below 50% charge optimizes the balance between complex formation and buffering capacity and is a reasonable target for designing materials that transfect at low polymer concentrations.

MePr-54 was designed to mimic the pK\textsubscript{a} of PEAI which was 6.9. PEAI had more efficient binding than its analogue, MePr-54, despite having the same degree of ionization. The transfection efficiency however, for MePr-54 and PEAI were similar with only small variations in efficiency at low concentrations. At an N/P of 10, MePr-54 maintained higher transfection efficiency than PEAI which is possibly the result of requiring slightly higher concentrations to form a neutral complex with DNA. Despite having an identical charge and buffering capacity, the structural property of the material played an important role, and the design of future materials will probe the influence of binding affinity on transfection; taking into account contributions from electrostatic, steric, hydrophobic, hydrogen bonding, and van der Waals interactions.
Figure 2.3. Agarose gel electrophoresis assay; Lanes correspond to various N/P ratios; Lane L: DNA molecular ladder; lane P: Naked DNA; All other lanes correspond to various N/P ratios; a) MePr series and b) EtPr series
3.4 Complex Studies

The polyplexes were also characterized by surface charge measurements (ζ-potential; Appendix A). For nonspecific transfection, it is important for the complex to maintain a positive surface charge to interact with the cell surface. The zeta potential revealed that the surface charge of the complexes closely followed the pKₐ trends of the materials and transfection only occurred at concentrations for each material that resulted in positive surface charge; however, the isoelectric point occurred at lower concentrations than the percent ionization would have predicted. This shows that the effective charge is not only governed by the pKₐ of the materials but also that there is a cooperative effect of binding which results in further protonation of the amines. It was previously reported that binding can induce proton transfer from the surrounding media. This occurs from a

**Figure 2.4.** Transfection experiments of selected materials with HeLa cells at N/P ratios of 2, 4, and 10; Values represent mean + SD (n = 3)
decrease of electrostatic repulsion upon cation-phosphate charge neutralization, effectively increasing the pK_a of the polymer.\textsuperscript{37,38}

![Figure 2.5](image.png)

**Figure 2.5.** Cytotoxicity of polyplexes *in vitro*; Values represent mean ± SD (n = 3)

### 3.5 Cytotoxicity

The *in vitro* cytotoxicity was then evaluated using an MTS assay (Figure 2.5). The polyplexes were tested at N/P ratios ranging from 1 to 100 with a DNA concentration of 0.5 µg/mL. PPAI was shown to have the lowest toxicity followed by the copolymers with higher incorporation of DPAI. The DEAI series also showed less toxicity when compared to the DMAI series. As expected, the materials with the highest charge density had the highest toxicity potentially stemming from membrane perturbation of the cell.\textsuperscript{19,39,40} However, the toxicity of PEAI was slightly higher than that of MePr-54. PEAI was also slightly more efficient at complexation than MePr-54, which may result
from the incorporation of the dipropylamine that may decrease surface interactions compared to the diethylamine functionality.

4. Conclusions

A series of tertiary amine functionalized homopolymers and copolymers have been synthesized with high control over their ionization potential. A combination of the buffering capacity as well as binding affinity governs the transfection efficiency of these diene based delivery vehicles. We have shown that delivery is possible with materials that have distinct buffering ranges as opposed to the broad range that occurs with PEI. MePr-60 maintains 33% amine protonation at a pH of 7.4, whereas PEI has only 20% protonation.22 This enabled MePr-60 to form a complex at low concentrations while maintaining a high buffering capacity. Ultimately, this led to the ability of MePr-60 to transfect at a concentration (N/P of 2) that PEI was not able. In this study a window from 24% to 50% ionization was found to be optimal for transfection with the best material having 33% amine protonation. This range may serve as a target for designing future delivery systems. While the facile acid/base titration proved useful as an initial test, based on the differences between PEAI and MePr-54 continued work is needed to examine the ionization properties of these materials in an environment that better mimics that of the endosome. To this end, more sophisticated titration experiments have been developed41 and will be utilized for further biophysical characterization. Future studies will also focus on quantifying DNA binding affinity in order to identify a desired binding strength, determine the contribution of various forces involved in complex formation, and ultimately further establish a guideline in the design of new materials.
References

(22) Behr, J. P. *Chimia* **1997**, *51*, 34-36.
Chapter 3. Synthesis of Degradable Gene Therapy Materials with Decreased Toxicity
Abstract. A series of amine functionalized polyesters were synthesized to examine the influence of a degradable scaffold on cytotoxicity. To achieve high amine density, a new monomer, 4-((diethylamino)methyl)-5-methylcyclohex-4-ene-1,2-dicarboxylic acid, was designed to avoid unwanted crosslinking found in materials previously developed by our group. Polymerization of this monomer was successful, and amine homopolymers as well as copolymers were produced. The resulting materials were then evaluated for their toxicity, DNA binding, and transfection efficiency. The toxicity was found to be much less than an industry standard, poly(ethyleneimine), but transfection efficiency was nominal even at elevated concentrations.

1. Introduction

Despite the advantages of synthetic gene delivery materials, one of the most pervasive and limiting properties is cytotoxicity.\textsuperscript{1-6} This in part stems from the cationic nature of materials that ultimately induces membrane perturbation upon cell association and leads to death. To minimize these effects, two main strategies have been employed. The first is to shield the charge by incorporating biocompatible hydrophilic groups such as ether blocks, grafts, or main chain segments.\textsuperscript{7-9} As in other areas of biomedical research, creating poly(ethylene glycol) (PEG) blocks and grafts (often referred to as pegylation) leads to micelle formation that reduces nonspecific cell interactions.\textsuperscript{10,11} Upon DNA complexation, electrostatic neutralization results in hydrophobic aggregation of the polycation/DNA complex, and these aggregates are stabilized by the hydrophilic PEG chains extending into the aqueous environment. This shielding enables the
complexes to avoid premature clearance from the immune or RES systems, yielding long blood circulation times. The decrease in toxicity is from a reduction of cell interactions, but unfortunately this also prevents uptake at the target site, and low delivery efficiency is common.

Another shielding strategy has been incorporating electronegative or anionic groups onto grafts or the main chain of the polymer.\textsuperscript{12-14} Alcohol or carboxylic acid functionalized materials have shown great promise in reducing toxicity without being detrimental to the bioactivity. However, the concentration (N/P ratio; number of amines of the polymer divided by the number of phosphates on the DNA) needed to reach competitive transfection efficiency with the nonshielded systems was much higher, typically well above an N/P ratio of 20. The functional groups also impose limitations on the synthesis of the materials mandating mild reaction conditions and/or the use of protecting groups. These materials were also sensitive to the proximity of the shielding group from the amine, with a shorter distance being more effective at decreasing toxicity. This can lead to limitations in the design and synthesis of materials.

The second strategy for decreasing toxicity has been to integrate the cationic functionality onto a degradable scaffold.\textsuperscript{15} Degradation can be a result of bioreduction (disulfides)\textsuperscript{16,17}, enzymatic degradation (amides, carbohydrates)\textsuperscript{14,18-24}, or hydrolytic degradation (acetals, carbonates, esters)\textsuperscript{3,12,13,25}. Generally degradable systems, not unlike those with charge shielding, have decreased toxicity, but also require higher N/P ratios to compete with the nondegradable systems. This is more pronounced in materials with more labile functionalities such as the poly(esters), but simultaneously these systems generally have the least amount of toxicity. Likewise, the more stable functionalities
used in the poly(amide) and carbohydrate vectors deliver at concentrations lower than their less stable counterparts, but also typically have higher toxicity. Ultimately, the decrease in toxicity enables high dose concentrations which are required to compete or exceed the transfection efficiency of their nondegradable counterparts. For example, the poly(β-aminoester) shown in Figure 3.1 had efficiency (Polymer:DNA ratio = 60:1) greater than the field standard poly(ethylenemine) (Polymer:DNA ratio = 1:1) and rivaled that of adenovirus efficiency, but also required 60 times more material.²⁶

Figure 3.1. Polymer structures; a) poly(β-aminoester), b) poly(ethyleneimine) (PEI), c) poly(diethylaminoisoprene) (PEAI)
**Scheme 3.1.** top: Diels Alder synthesis of the diacid monomer (D1) and new target monomer (D2); bottom: amine containing degradable copolymer synthesis

Our group has developed a novel delivery vector (Figure 3.1), poly(diethylaminoisoprene) (PEAI), that showed competitive results to PEI with an N/P ratio of 4, and greater efficiency at a ratio of 2.\(^\text{27,28}\) Despite these promising results, the materials showed some degree of toxicity, and in an attempt to minimize this property, it was desired to install the allylic dialkylamino functional group of PEAI into a degradable system. Our group has developed a strategy to incorporate diene monomers into a biodegradable scaffold via a Diels Alder cycloaddition with fumaric acid followed by a polycondensation polymerization (Scheme 3.1).\(^\text{29}\) As a proof of concept the monomer diethylaminoisoprene, which was used in the delivery material PEAI, was reacted with fumaric acid to achieve an amine containing diacid (D1). This was then copolymerized with an unfunctionalized dimethylcyclohexene diacid and octanediol, and a series of materials with varied diacid feed ratios was synthesized. It was found that the amino diacid could only be incorporated up to 50% relative to the unfunctionalized monomer. Above 50% incorporation, the polymer was insoluble and only swelled in solvent; likely
due to a crosslinking reaction through the unsaturated portion of the ring. At 50% amine incorporation, the functionality was too dilute for a delivery vehicle. As a response, in the current research a methylated analogue of D1 was designed to create a tetrasubstituted double bond in order reduce its reactivity and prevent crosslinking (Scheme 3.1). By avoiding network formation, homopolymers of an amine containing diacid could be synthesized and then evaluated for its toxicity, DNA binding, and transfection ability. The goal of this work was the creation of a material that incorporated functionality with proven high transfection ability that also minimized unwanted cytotoxicity.

2. Experimental Details

2.1 Materials. Plasmid DNA (pCMV-Luc) was purchased from Elim Biopharmaceuticals, Inc. (Hayward, CA). Minimum essential medium (MEM), Hanks’ balanced salt solution (HBSS), Dulbecco’s phosphate buffered saline (PBS), fetal bovine serum (FBS), and a 1 kb DNA ladder were purchased from Invitrogen. CellTiter 96 AQueous one solution cell proliferation assay and luciferase assay system were purchased from Promega Corp. (Madison, WI). BCA protein assay kit was purchased from Pierce (Rockford, IL). Branched poly(ethyleneimine) with a molecular weight of 25,000 g/mol was used as a control in the characterization experiments. All other chemicals were purchased from Sigma-Aldrich and were used without further purification.

2.2 Instrumentation. $^1$H and $^{13}$C NMR spectra were acquired in deuterated chloroform or deuterated water on a Bruker 400 AVANCE spectrometer. Molecular
weights were measured by a Waters GPC system using polystyrene standards. The measurements were taken using THF as the solvent on four columns (Waters Styragel HR0.5, HR2, HR4, and HR5). Glass transition temperatures were measured with a Seiko 220C differential scanning calorimeter, using a heating and cooling rate of 10 °C/min. The glass transition temperatures were reported based on the second heating. Thermogravimetric analysis was carried out using a Perkin Elmer TGA 7 with a heating rate of 10 °C/min in a N₂ atmosphere. The size and polydispersity of polypplexes was analyzed via dynamic light scattering (DLS) using a 90Plus Particle Size Analyzer (Brookhaven Instruments Corporation). Cell culture plates were analyzed on a Molecular Devices SpectraMax M5. Ethidium bromide stained agarose gels were visualized on a Bio Rad VersaDoc imaging system.

2.3 Monomer Synthesis. 2-bromo-N,N-diethylprop-2-en-1-amine. To a solution of N,N-diethylamine in diethyl ether was added 2,3-dibromopropene dropwise at 0 °C while stirring. The reaction was allowed to warm to room temperature and to react for 18 h. NaOH (1 M) was added until the formed salt dissolved, and the solution was extracted with diethyl ether (50 mL, 3x). The organic layers were combined, washed with brine, dried with MgSO₄, and concentrated. The product was purified and isolated by distillation as a colorless liquid. ¹H NMR (400 MHz, CDCl₃): δ 5.90 (s, 1H), 5.55 (s, 1H), 3.24 (s, 2H), 2.58 (q, 4H, J = 7.2 Hz), 1.03 (t, 6H, J = 7.2 Hz). ¹³C NMR (400 MHz, CDCl₃): δ 132.64 (CH₂=CBR₂), 116.80 (CH₂=CBR₂), 61.46 (CH₂N), 46.67 [N(CH₂CH₃)₂], 11.73 [N(CH₂CH₃)₂]

2-(N,N-diethylaminomethyl)-3-methyl-1,3-butadiene. Prop-1-ene 2-magnesium bromide (1 M in THF, 0.24 mol) was added dropwise to 2-bromo-3-
(N,N-diethylamino)propene (0.20 mol) in the presence of [1,3-bis(diphenylphosphino)propane]dichloronickel (7.40 x 10⁻⁴ mol) at 0 °C. The reaction mixture was allowed to slowly warm to room temperature and stirred for 16 h. The reaction mixture was then quenched with 300 mL of saturated ammonium chloride solution, extracted with diethyl ether (50 mL, 30x), washed once with brine, and dried over anhydrous magnesium sulfate. After evaporation of the solvent, the residue was distilled to give 2-(N,N-diethylaminomethyl)-1,3-butadiene in 67-70% yield. 

**1H NMR** (400 MHz, CDCl₃): δ 5.21 (s, 1H), 5.16 (s, 2H), 4.95 (s, 1H), 3.15 (s, 3H), 2.47 (q, 4H, J = 8.0 Hz), 1.89 (s, 3H), 0.98 (t, 6H, J = 8.0 Hz)

**Synthesis of 4-((diethylamino)methyl)-5-methylcyclohex-4-ene-1,2-dicarboxylic acid.** 2-(N,N-diethylaminomethyl)-3-methyl-1,3-butadiene (2.0 g, 13.04 mmol) was added to a flask containing a solution of fumaric acid in ethanol (1.82 g, 15.65 mmol in 20 mL ethanol). The mixture was brought to reflux and stirred for 5 days. The solid product precipitated out of solution and was filtered and rinsed with ethanol to yield the pure product in 51.2% yield. 

**1H NMR** (300 MHz, D₂O) δ ppm 6.04 (s, 1H), 3.78 (dd, 2H), 3.18 (tt, J = 13.34, 13.34, 6.55, 6.55 Hz, 4H), 2.76-2.72 (m, 2H), 2.40-2.19 (m, 4H), 1.75 (s, 3H), 1.27 (t, J = 7.30, 7.30 Hz, 6H). 

**13C NMR** (100.61 MHz, D₂O) δ (ppm) 181.31 and 181.28 (-CO₂H), 138.47 (=C-CH₂N-), 118.15 (=C-CH₃), 109.99 (CH₂=CHCCH₂N), 54.57 (-CH₂-N-), 47.33 and 46.99 (-N(CH₂CH₃)₂, 34.22(-CH-CO₂H), 30.96 (-CH₂-CH=), 19.01 (-CH₃), 8.51 and 8.49 (-N(CH₂CH₃)₂

4,5-dimethylcyclohex-4-ene-1,2-dicarboxylic acid. 2,3-dimethylbuta-1,3-diene (10.0 g, 0.121 mol) was added to a flask containing a solution of fumaric acid (14.13 g, 0.121 mol) in 50 mL ethanol. The mixture was brought to reflux and stirred for 5 days.
The solvent was removed and the product was partitioned between water and diethyl ether (50 mL, 30x). The organic layers were combined, washed once with brine, and dried over anhydrous magnesium sulfate. Pure product was isolated after recrystallization from water and ethanol. $^1$H NMR (400 MHz, D$_2$O) $\delta$ (ppm) 2.69 (m, 2H), 2.07-2.26 (m, 4H), 1.55 (s, 6H). $^{13}$C NMR (100.61 MHz, D$_2$O) $\delta$ (ppm) 177.56 (-CO$_2$H), 125.62 (=C(CH$_3$)CH$_2$-), 43.45 (-CH- CO$_2$H), 35.75 (-CH$_2$-C(CH$_3$)=), 19.72 (-CH$_3$)

2.4 Polyester Synthesis. A 10 mL round bottom flask was charged with the dicarboxylic acids (1.0 eq total) and 1,8-octanediol (OD, 1 eq) or tetraethyleneglycol (TEG, 1 eq). For each material approximately 1 g of diol was used. The flask was sealed, evacuated, and refilled with nitrogen gas. A homogenous melt was formed by heating the flask to 165 °C while stirring. Tin octanoate (0.01 equiv) was added to the melt. The reaction mixture was allowed to stir for 1 h. The temperature was increased to 170 °C and the pressure was reduced to 20 torr for 17 h, followed by 2 torr for 6 h (OD containing materials) or 30 h (TEG containing materials). At this time, the reaction mixture was allowed to cool and dissolved in chloroform. This solution was precipitated into stirring methanol (OD containing) or hexanes (TEG containing) at -78 °C. Solvent was decanted from the polymer, and the solid was dried in a vacuum oven at 50 °C for 24 h.

Poly(octanediol 4,5-dimethylcyclohex-4-ene-trans-1,2-dicarboxylate-co-octanediol 4-(N,N-diethylaminomethyl)-5-methyl-cyclohex-4-ene-trans-1,2-dicarboxylate). $^1$H NMR data in the series of copolymers varies only by the integration area for peaks corresponding to protons in the diacid portion of the molecule. $^1$H NMR
Poly(tetraethyleneglycol 4,5-dimethylcyclohex-4-ene-trans-1,2-dicarboxylate-co-tetraethyleneglycol 4-(N,N-diethylaminomethyl)-5-methyl-cyclohex-4-ene-trans-1,2-dicarboxylate). \( ^1\)H NMR data in the series of copolymers varies only by the integration area for peaks corresponding to protons in the diacid portion of the molecule. \( ^1\)H NMR (400 MHz, CDCl\(_3\)) \( \delta \) ppm 4.09 (br, 8H), 3.55 (s, 3H), 3.52 (br, 12H), 2.91 (br, 2H), 2.72 (br, 4H), 2.37 (br, 4H), 2.24-2.03 (m, 2H), 1.57 (s, 3H), 1.49 (s, 6H), 1.10 (m, 8H), 0.89 (br, 6H). \( ^{13}\)C NMR (100.61 MHz, CDCl\(_3\)) \( \delta \) ppm 178.88 (-CO\(_2\)-) 123.71 (=C-), 70.42 (-OCH\(_2\)CH\(_2\)OCH\(_2\)CH\(_2\)-), 68.92 (-OCH\(_2\)CH\(_2\)OCH\(_2\)CH\(_2\)O), 61.39 (-OCH\(_2\)CH\(_2\)OCH\(_2\)CH\(_2\)O), 54.06 (-OCH\(_2\)CH\(_2\)OCH\(_2\)CH\(_2\)O), 47.76 (N-CH\(_2\)CH\(_3\)), 41.76 (-CHCO\(_2\)-), 40.18 (-CHCO\(_2\)-), 33.89 (-CH\(_2\)-CHCO\(_2\)-), 30.88 (-CH\(_2\)-CHCO\(_2\)-), 18.55 (-CH\(_3\)), 11.11 (NCH\(_2\)CH\(_3\))

2.5 Polymer/Polyplex Characterization. Standard Polyplex Formation. To 1 mg of each polymer sample was added 15 \( \mu \)L of glacial acetic acid followed by 485 \( \mu \)L of either opti-MEM or MEM media to make polymer solutions at a concentration of 2
mg/mL. Once dissolved the polymer samples were diluted with the appropriate media to obtain the polymer solutions at concentrations of 0.1 mg/mL. To solutions of 2 µg/mL pCMV-Luc plasmid in media was added the polymer solution and media to form complexes at desired N/P ratios with total volumes of 200 µL.

**Agarose Gel Electrophoresis.** DNA quenching was monitored over a range of DNA to polymer ratios (w/w). The polymer solutions were made as described above. Then desired amounts were titrated into a solution containing 10 µg of plasmid DNA. Electrophoresis was conducted on a 0.75% agarose gel in TAE buffer containing 0.5% ethidium bromide for 45 min. at 120 V. The bands were visualized using ethidium bromide staining.

**Cytotoxicity Assay.** HeLa cells were grown in 96-well plates at a density of 5×10³ cells/well in 150 µL of growth medium (89% MEM, 10% FBS, 1% L-glutamine). Cells were grown for 24 h. The media was removed, and the cells were washed with 100 µL of DPBS and incubated with solutions containing MEM and polymer for 4 h. The solutions were removed, and the cells were washed with DPBS. Following the addition of 150 µL of MEM, the cells were allowed to recover for 4 h. The solutions were removed, and the cells were washed with DPBS. To the cells were added 100 µL of MEM and 20 µL of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazoleum, inner salt (MTS, from CellTiter 96 AQueous One Solution Cell Proliferation Assay kit). The cells were incubated for 4 h and the absorbance at 490 nm was monitored on a Molecular Devices SpectraMax M5 plate reader.
**Transfection Efficiency Assay.** HeLa cells were grown in 48-well plates at a density of $1.5 \times 10^4$ cells/well in 300 µL of growth medium (89% MEM, 10% FBS, 1% L-glutamine). Cells were grown for 24 h. The media was removed, and the cells were washed with 300 µL of HBSS and incubated with 450 µL of opti-MEM and 50 µL of polyplex solution for 4 h. The solutions were removed, and the cells were washed with HBSS and incubated with 500 µL of opti-MEM (89% MEM, 10% FBS, 1% L-glutamine) for 32 h. The growth medium was removed and the cells were washed with 300 µL of HBSS and treated with 150 µL of cell lysis buffer (1X). The resulting mixtures were centrifuged and 20 µL of supernatant were added directly to white opaque 96-well plates. Luciferase activity was quantified by adding 100 µL of luciferase assay reagent, followed by a 2 second delay. Luminescence was quantified over a 10 second read time. The result was normalized to the total protein content using a BCA protein assay whose absorption was monitored at 562 nm on a Molecular Devices SpectraMax M5 plate reader.

**DLS and Zeta Potential Measurements.** Plasmid solutions were made by diluting 130 µL of pCMV-Luc (1 mg/mL) with 1.37 mL of 0.9% saline to give a final DNA concentration of 100 µg/mL. Polymer solutions were made by dissolving 1 mg of material with 15 µL of glacial acetic acid and then diluted with 0.9% saline to reach a final concentration of 0.1 µg/mL. The final pH of the polymer was verified to be 7.4. Polyplexes were formed at N/P ratios of 1, 2, 4, 10, and 20, allowed for a 20 minute equilibration time, and the complex diameters and ζ-potentials were monitored.
3. Results and Discussion

Scheme 3.2. a) Synthesis of 2-(N,N-diethylaminomethyl)-3-methyl-1,3-butadiene and of 4-((diethylamino)methyl)-5-methylcyclohex-4-ene-1,2-dicarboxylic acid (D2); b) amine containing degradable copolymer synthesis containing octanediol; c) amine containing degradable copolymer synthesis containing tetraethyleneglycol; (X = percent D2 feed)

3.1 Synthesis. The aminodiene 2-(N,N-diethylaminomethyl)-3-methyl-1,3-butadiene was synthesized as seen in Scheme 3.2. This diene as well as 2,3-dimethylbutadiene were then reacted with fumaric acid via a Diels Alder cycloaddition to
afford the target monomers 4-((diethylamino)methyl)-5-methylcyclohex-4-ene-1,2-dicarboxylic acid (D2) and 4-((dimethylamino)methyl)cyclohex-4-ene-1,2-dicarboxylic acid (D3) in high purity. The diacid monomers were then copolymerized in bulk with octanediol (OD) or tetraethyleneglycol (TEG), using a tin octanoate catalyst at elevated temperatures and reduced pressure (Scheme 3.2). The monomer ratios were varied from 20% up to formation of the amine functionalized homopolymer with octanediol (Table 3.1). To increase solubility a shorter series of materials were made, replacing the octanediol with the more hydrophilic TEG and materials with 0, 60, and 100% amino diacid feed were synthesized.

In both series of materials there was good fidelity between the feed and incorporation, with the amine monomer always slightly lower than expected (Table 3.1). As the incorporation of the D2 increased, the molecular weights decreased. The PDI of the OD containing systems were between 1.3-1.8, showing some molecular weight fractionation during work up. The PDI of the TEG containing materials however increased with amine incorporation with no apparent fractionation. Purification of the latter materials was carried out in hexanes at -78 °C due to the increased hydrophilicity as opposed to methanol used in the OD systems. To reach comparable molecular weights of the OD materials, the reaction time for the TEG materials was increased from 24 to 48 hours with the time at full vacuum being increased from 6 to 30 hours. The difference in polymerization may stem from the ether diol interacting with the coordination sites of the tin catalyst decreasing its effectiveness in catalyzing ester formation.
There was one glass transition between -5 and -13 °C for the OD materials and -13 to -18 °C for the TEG materials. The combination of high fidelity between the feed, as well as the single \( T_g \) transition, supports the formation of random copolymers. Due to the poor solubility of the OD containing material, all further characterization (toxicity, complex size and charge, DNA binding, and transfection) was done only on the TEG materials.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Feed(^a) (%)</th>
<th>Incorp.(^b) (%)</th>
<th>Yield (%)</th>
<th>( M_n )^(c) 10(^{-3}) (g/mol)</th>
<th>PDI(^c)</th>
<th>( T_g )(^d)</th>
<th>5(^e) %</th>
<th>10(^e) %</th>
</tr>
</thead>
<tbody>
<tr>
<td>PI-0</td>
<td>0</td>
<td>0</td>
<td>81</td>
<td>16.3</td>
<td>1.7</td>
<td>-13</td>
<td>359</td>
<td>372</td>
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<td>PI-20</td>
<td>20</td>
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<td>72</td>
<td>14.1</td>
<td>1.8</td>
<td>-10</td>
<td>337</td>
<td>357</td>
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<tr>
<td>PI-40</td>
<td>40</td>
<td>33</td>
<td>76</td>
<td>9.4</td>
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<td>39</td>
<td>6.3</td>
<td>1.7</td>
<td>-7</td>
<td>281</td>
<td>326</td>
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<tr>
<td>PI-80</td>
<td>80</td>
<td>65</td>
<td>39</td>
<td>5.7</td>
<td>1.3</td>
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<td>278</td>
<td>320</td>
</tr>
<tr>
<td>PI-100</td>
<td>100</td>
<td>100</td>
<td>33</td>
<td>6.1</td>
<td>1.5</td>
<td>-8</td>
<td>288</td>
<td>326</td>
</tr>
<tr>
<td>PHI-0</td>
<td>0</td>
<td>0</td>
<td>75</td>
<td>12.0</td>
<td>1.9</td>
<td>-13</td>
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<tr>
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<td>58</td>
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<td>4.0</td>
<td>3.0</td>
<td>-18</td>
<td>235</td>
<td>293</td>
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</tbody>
</table>

\(^a\) amine monomer relative to the DPAI; \(^b\) determined by \(^1\)H NMR; \(^c\) determined by GPC; \(^d\) determined by DSC; \(^e\) percent decomposition determined by TGA
3.2 Polyplex/Biological Characterization. The in vitro cytotoxicity was evaluated to determine if utilizing a degradable scaffold yielded materials with a low toxicity profile. To do this, an MTS assay (Figure 3.2) was performed with PII-60, PII-100, and PEI at concentrations ranging from 1 to 1000 µg/mL. The polyesters were less toxic compared to the nondegradable material, with PII-100 only showing significant toxicity at concentrations greater than 50 µg/mL. Interestingly, PII-100 was less toxic than PII-60, which has less cationic sites. This was not expected since toxicity of gene delivery materials is usually associated with the charge density, and future studies will focus on evaluating the toxicity of the nonfunctionalized monomer (D3).
Table 3.2. Complex size and surface charge measurements

<table>
<thead>
<tr>
<th>N/P</th>
<th>PEI</th>
<th>PII-60</th>
<th>PII-100</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Size (^a) (nm)</td>
<td>(\zeta)-potential (^a)</td>
<td>Size (^a) (nm)</td>
</tr>
<tr>
<td>1</td>
<td>146</td>
<td>-25.6</td>
<td>309</td>
</tr>
<tr>
<td>2</td>
<td>314</td>
<td>-19.6</td>
<td>220</td>
</tr>
<tr>
<td>4</td>
<td>347</td>
<td>20.9</td>
<td>272</td>
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<tr>
<td>10</td>
<td>346</td>
<td>22.6</td>
<td>222</td>
</tr>
<tr>
<td>20</td>
<td>358</td>
<td>27.2</td>
<td>322</td>
</tr>
</tbody>
</table>

\(^a\) determined by a 90Plus Particle Size Analyzer

The properties of the polyplexes were then evaluated for surface charge (\(\zeta\)-potential) and complex size (Table 3.2). For nontargeted transfection, it is important for the complex to maintain a positive surface charge to interact with the cell surface. Complexes were formed between the TEG materials (PII) or PEI and a 6000 base pair plasmid, pCMV-Luc. The \(\zeta\)-potentials revealed that both of the polyesters reached a near neutral charge around an N/P ratio of 2, and PEI was between 2 and 4. Requiring a lower concentration to create a positive surface for the PII materials was promising since they could potentially transfect at lower concentrations than PEI. Complex sizes were then measured by dynamic light scattering. The average diameter of the unbound plasmid was ~750 nm, and it was shown that each material was able to complex DNA and form a more compact structure.
Figure 3.3. Transfection experiments of PII-60, PII-100, and PEI with HeLa cells at an N/P ratio of 20 for the polyesters and N/P of 4 for PEI

Based on the results of low toxicity, ability to compact DNA, and positive surface charge at low concentrations, the materials were evaluated for their transfection ability. For the transfection experiments, the same pCMV-Luc plasmid containing the luciferase reporter gene was used, and PEI served as the positive control. As seen in Figure 3.3, at an N/P of 20 the polyesters showed poor transfection ability, with PII-100 performing 1000 fold lower than PEI (whose optimum performance was taken at an N/P of 4). Despite concentrations well above charge neutrality and complexation, there was no bioactivity. The DLS experiments, however, only gave limited insight into complex formation without distinguishing the nature of the cation/anion interaction. To further characterize the complex formation, DNA binding gels were used.
The DNA binding ability was investigated using agarose gel electrophoresis to determine the polymer concentrations that PII-100 could effectively bind DNA (Figure 3.4). In this test when a current was applied, naked DNA migrated down the gel. As aliquots of cationic material were added, the electrostatic interactions resulted in charge neutralization and migration of the DNA bind was quenched. Polymer/DNA complexes were formed at ratios from 0 µg to 50 µg of polymer with 10 µg of DNA. As seen in Figure 3.4, even at high polymer concentrations, there was no effective DNA binding, or complex formation. With a molecular weight of the repeat unit near 500 g/mol, a material with only a total molecular weight of 4000 g/mol would only have eight amines per polymer chain. Transfection efficiency is greatly dependent on the amine density in
order to complex DNA and to serve as a ‘proton sponge’ for endosomal release, and the poor results of these materials may stem from their low amine density.

4. Conclusions

A facile Diels Alder reaction between a functionalized diene and fumaric acid was exploited to synthesis an amine functionalized monomer. Previously, materials utilizing this chemistry were limited to an upper limit of 50% incorporation of the amine monomer with higher feed ratios resulting in crosslinking. A new monomer was developed that utilized a tetrasubstituted double bond to prevent crosslinking. After successful synthesis of this monomer, a series of degradable tertiary amine containing copolymers and a homopolymer were synthesized. Installment of the methyl group on the double bond was successful in limiting crosslinking and permitted development of the amine homopolymer. Two types of polyesters were synthesized, the first containing octanediol, and the second containing tetraethyleneglycol. The former was found to have poor solubility, and the latter materials were used in the biological characterization experiments. It was shown that the degradable system was much less toxic than the control (PEI), but only formed weak complexes with DNA and ultimately had low transfection efficiency. Only low molecular weight material was synthesized, resulting in low amine density. This most likely led to the materials poor delivery performance.
References

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(27) Yang, Y.; Lee, J.; Cho, M.; Sheares, V. V. Macromolecules 2006, 39, 8625-8631.
(28) Yang, Y.; Sheares, V. V. Polymer 2007, 48, 105-109.
Chapter 4. Developing Guanidine Functionalized Materials for Enhanced Cellular Uptake
Abstract. To date, the most common functionalities used in synthetic gene therapy materials are 1°, 2°, and 3° amines. These groups, however, are generic, serving as cationic sites for DNA complexation and cell membrane association, as well as providing a buffering potential for endosomal release. In this research, a specialized functionality, guanidine, was identified to enhance cell association and uptake. Herein, multiple strategies have been developed for synthesizing a guanidine functionalized diene. The most promising of which utilizes a tosylated hydroxyisoprene intermediate that can be rapidly synthesized and is stable at 4 °C. This intermediate has also been used to produce tertiary amine functionalized dienes and could potentially serve a general method for the preparation of 2-functionalized isoprene.

1. Introduction

Viral based gene delivery vehicles offer the most efficient method for cell transfection.\(^1,2\) Viral machinery has evolved to impart target specificity, cellular uptake, endosomal release, and nuclear localization that results in enhanced bioactivity.\(^3\) Unfortunately, there are inherent problems with the use of viruses. After repeated administration, the body may gain natural immunity and diminish the effectiveness of delivery. Furthermore, natural vectors have been shown to elicit immune or inflammatory responses. Depending on the viral type, some vectors have the ability to insert their DNA directly into the host’s chromosome which may disrupt expression of integral proteins. These issues have limited viral delivery, resulting in cancer and death in phase III clinical trials.\(^4,5\)
As a response, synthetic materials are being designed that are not recognized by the immune system and have shown limited inflammatory response.\textsuperscript{6-18} To compete with the natural systems, synthetic polymers must efficiently 1) form a complex with DNA (polyplex), 2) associate to the cell membrane and become internalized through endocytosis, 3) escape from the endosome, 4) transport the cargo to the nucleus, and 5) dissociate from the DNA.\textsuperscript{19} To date few materials have been developed with efficiency that competes with viral delivery. One strategy to increase efficiency is incorporating cationic functionalities beyond the typical $1^\circ$, $2^\circ$, and $3^\circ$ amines with the potential to decrease toxicity, increase buffering capacity (endosomal release), and enhance cellular uptake. Two functionalities that have been commonly utilized are pyridine and imidazole groups.\textsuperscript{13,20-22} It has been shown that cationic groups with the charge in resonance typically had decreased toxicity.\textsuperscript{23} Due to the lower pK\textsubscript{a} values, pyridine and imidazole also increased the buffering capacity of the polymer by remaining unprotonated until acidification inside the endosome. While these groups focus on the issues of toxicity and buffering, they do not address increasing cellular uptake efficiency; one of the key aspects of viral vectors.

One way viruses achieve their efficiency is by utilizing membrane permeable proteins that associate to the cell membrane and activate cellular uptake. In a series of these proteins, the amino acids that that have been identified as critical for uptake have a high occurrence of cationic amino acids, most notably arginine (Table 4.1).\textsuperscript{18} The functional group of arginine is a guanidine moiety which has been shown to form electrostatic interactions and two hydrogen bonds between anionic groups such as phosphates and sulfates on the cell surface. Binding of these groups can signal for
uptake. This binding also results in a neutral charge, promoting condensation on the cell surface, also increasing the rate of cellular uptake.

**Table 4.1.** Membrane permeable peptides, the active sequences, and relative translocation efficiency (amino acid position)

<table>
<thead>
<tr>
<th>Peptides Sequences</th>
<th>Translocation Efficiency</th>
</tr>
</thead>
<tbody>
<tr>
<td>HIV-1 Tat(48–60)</td>
<td>GRKKRRQRRPPQ</td>
</tr>
<tr>
<td>R9-Tat</td>
<td>GRRRRRRRRPPQ</td>
</tr>
<tr>
<td>HIV-1 Rev(34–50)</td>
<td>TRQRARRRWRERQRRRRR</td>
</tr>
<tr>
<td>FHV Coat(35–49)</td>
<td>RRRNRTRRRRVR</td>
</tr>
<tr>
<td>CCMV Gag(7–25)</td>
<td>KLTRAQRAAARKNKRNTTR</td>
</tr>
<tr>
<td>P22 N(14–30)</td>
<td>NAKTRRHERRKLAIER</td>
</tr>
<tr>
<td>1 N(1–22)</td>
<td>MDAQTTRRRRAEKQAQWKAAN</td>
</tr>
<tr>
<td>j 21 N(12–29)</td>
<td>TAKTRYKARRLIAERR</td>
</tr>
<tr>
<td>Yeast PRP6 (129–144)</td>
<td>TRRNKRNRRIQEQNLNRK</td>
</tr>
<tr>
<td>Human U2AF(142–153)</td>
<td>SQMTTRQARRLYV</td>
</tr>
</tbody>
</table>

Materials have been previously developed incorporating guanidine groups with success in increasing cellular uptake and transfection. The most basic of these were oligo-arginine peptides, with which efficient transfection was successful with optimal chain lengths between 7 and 9 amino acids. Another material was developed by Ferrutti, where a poly(amidoamine) was synthesized with a carboxylic acid and guanidine moiety in every repeat unit. This material was shown to have low toxicity and
efficiencies comparable to jetPEI, a commercially available linear poly(ethyleneimine) delivery system. The functional group has also been placed at the periphery of poly(L-lysine) dendrimers. When compared to the nonfunctionalized dendrimer, the membrane transport was greatly enhanced. Guanidine has repeatedly proven to be an integral functional group in natural and synthetic delivery vehicles.

Previously, we reported a series of dialkylamino functionalized dienes with one, poly(diethylaminoisoprene) (PEAI), that was shown to have higher transfection efficiency than PEI at low concentrations. This material showed efficiency better than PEI at an N/P ratio of 2 (N/P ratio = the number of amines of the polymer divided by the number of phosphates of the DNA). Incorporating a guanidine moiety may increase cellular uptake and enhance its bioactivity. A 2-substituted diene would be an analogous monomer to that used in our initial studies with PEAI, but difficulty arises in functionalizing a diene in this position. Current methods are tedious and no general procedure exists. Incorporation of the functionality can be achieved through substitution by a guanidine moiety (guanidinylation) or by conversion of a 1° or 2° amine to a guanidine moiety (guanylation). Current research focuses on developing strategies to create a guanidine functionalized isoprene. Once obtained, a series of copolymers with diethylaminoisoprene (DEAI) will be synthesized and an optimized monomer feed for transfection will be established. Through this work, an enhanced delivery vehicle may be produced and the value of functionalizing existing and future materials with the guanidine group may be further supported. Furthermore, this work will also develop a facile path to synthesizing dienes functionalized in the 2-position.
2. Experimental

2.1 Materials and Instrumentation. Chloroprene was purchased from Pfaltz and Bauer Inc, and used after distillation. All of the other reagents were purchased from Aldrich. Isoprene was freshly distilled before each reaction. All other chemicals were used without further purification. $^1\text{H}$ and $^{13}\text{C}$ NMR spectra were acquired in deuterated chloroform or dimethylsulfoxide on a Bruker 400 AVANCE spectrometer or Bruker 300 AMX spectrometer. Molecular weights were measured by a Waters GPC system using polystyrene standards. The measurements were taken using THF as the solvent on four columns (Waters Styragel HR0.5, HR2, HR4, and HR5).

2.2 Bromoisoprene. 3-Methyl-2,5-dihydrothiophene-1,1-dioxide. Liquid sulfur dioxide (~ 50 mL) was collected using an acetone/dry ice cold finger. A 300 mL Parr reactor, cooled to -10 °C, was then charged with isoprene (68 g.), methanol (40 mL), SO$_2$(l), and hydroquinone (2.5 g). The vessel was sealed and then heated to 85 °C while stirring for 4h. The system was allowed to return to room temperature, and the product was poured into 300 mL of dIH$_2$O and cooled to 4 °C overnight. The mixture was filtered to yield 64.6 g (64% yield) of off-white crystals. $^1\text{H}$ NMR (300 MHz, CDCl$_3$): δ 5.68 (s, 1H), 3.79 (s, 2H), 3.68 (s, 2H), 1.89 (s, 4H)

3-Bromomethyl-2,5-dihydrothiophene-1,1-dioxide. To a 1 L round bottom flask equipped with a condenser was added 3-methyl-2,5-dihydrothiophene-1,1-dioxide, benzoyl peroxide, N-bromosuccinimide (57 g, 0.32 mol, NBS) and chloroform (500 mL). The mixture was stirred at reflux for 24 h. The solvent was then concentrated by rotary evaporation, cooled to 4 °C for 12 h, followed by vacuum filtration to remove the succinimide byproduct. The remaining solvent was removed via rotary evaporation, and
the resulting solid was purified by recrystallization from 95% ethanol yielding 8.21 g (12.2% yield) of product as an off-white powder. $^1$H NMR (300 MHz, CDCl$_3$): $\delta$ 6.10 (s, 1H), 4.03 (s, 2H), 3.88 (s, 4H)

2-Bromomethyl-1,3-butadiene (1). To a 100 mL round bottom flask equipped with a distillation apparatus was added 3-bromomethyl-2,5-dihydrothiophene-1,1-dioxide (8.21 g, 0.039 mol) and hydroquinone (~ 10 mg). The flask was heated to 170 °C with continuous distillation at 200 torr. The product (1.4 g, 24%) was obtained as a greenish brown liquid. $^1$H NMR (400 MHz, CDCl$_3$): $\delta$ 6.30–6.34 (dd, 1H), 5.46 and 5.40 (d, 1H), 5.40 (s, 1H), 5.28 and 5.25 (d, 1H), 5.25 (s, 1H), 4.11 (s, 2H)

1,3-(di-tert-butoxycarbonyl)guanidinoisoprene (2). To a 50 mL round bottom flask equipped with a drying tube was added sodium hydride (0.51, 0.21 mol) followed by dry DMF (6 mL) over ice. A solution of 1,3-(di-tert-butoxycarbonyl)guanidine (2.88 g, 0.011 mol) dissolved/suspended in dry THF (10 mL) was added dropwise and allowed to stir for 30 min. 1 was then slowly added and the reaction was stirred at room temperature for 18 h. To quench the remaining NaH, dIH$_2$O was added and the product was extracted with diethyl ether (50 mL, 3x). The organic layer was then washed with dIH$_2$O (50 mL, 3x) to remove the remaining DMF. The organic layers were then combined, washed with brine, dried with MgSO$_4$, and concentrated. The product was then purified by column chromatography with 5% EtOAc/Hexane as the eluent to yield 2 (2.62 g, 72 % yield). $^1$H NMR (400 MHz, CDCl$_3$): $\delta$ 6.37–6.45 (dd, 1H), 5.28 and 5.22 (d, 1H), 5.11 and 5.08 (d, 1H), 5.06 (s, 1H), 4.83 (s, 1H), 4.78 (s, 2H), 1.49 (s, 9H), 1.44 (s, 9H). $^{13}$C NMR (100.61 MHz, CDCl$_3$): $\delta$ 163.66 (C=O), 160.65 (C=O), 154.82 (C=N), 142.41 (CH$_2$=CCH$_2$N),
137.00 (CH$_2$=CHCCH$_2$N), 113.54 (CH$_2$=CHCCH$_2$N), 112.66 (CH$_2$=CCH$_2$N), 83.59 (CH$_2$N), 78.53 ([C(CH$_3$)$_3$]$_2$), 28.17 and 27.65 ([([CH$_3$]$_3$)$_2$]

poly(1,3-(di-tert-butoxycarbonyl)guanidinoisoprene). AIBN (5.05 mg, 0.03 mmol, monomer 2 (1g, 0.003 mol), and dioxane (0.5 mL) were added to an ampoule with a magnetic stir bar. After three freeze-pump-thaw cycles, the ampoule was sealed under nitrogen and placed in an oil bath preheated to 50 °C. After 5 d, methylene chloride (2 mL) was added to the polymer solution and the polymer was precipitated into methanol at -78 °C and dried under vacuum at room temperature for 5 d. $^1$H NMR (400 MHz, CDCl$_3$): δ 5.04 (s, 1H), 4.71 and 4.53 (s, 2H from trans and cis CH$_2$N, respectively), 2.05 and 1.90 (s, 4H from trans and cis respectively), 1.46 (s, 18H)

2.3 Hydroxyisoprene. 1-((2-bromoallyl)oxy)-4-methylbenzene (4). To a 100 mL round bottom equipped with a drying tube was added sodium hydride (0.51, 0.21 mol) followed by dry DMF (20 mL) over ice. To the solution was added 4-methoxyphenol (13.7 g, 0.11 mol) dropwise and allowed to stir for 30 min. 2,3-dibromopropene (25 g, 0.1 mol) was then slowly added and the reaction was stirred at room temperature for 18 h. To quench the remaining NaH, dIH$_2$O was added and the product was extracted with diethyl ether (50 mL, 3x). The organic layer was then washed with dIH$_2$O (100 mL, 3x) to remove the remaining DMF. The organic layers were combined, washed with brine, dried with MgSO$_4$, and concentrated. The purified product 1-((2-bromoallyl)oxy)-4-methylbenzene (4) was purified by distillation at 130 °C and 2 torr to yield 18.3 g of 4 (63 % yield). $^1$H NMR (400 MHz, CDCl$_3$): δ 6.83-6.90 (m, 4H), 6.0 (s, 1H), 5.67 (s, 1H), 4.60 (s, 2H), 3.78 (s, 3H)
(2-bromoallyloxy)trimethylsilane (6). To a 25 mL round bottom flask containing 2-bromoalcohol (5 g, 0.036 mol) in DMF (7 mL) was added imidazole (6.2 g, 0.091 mol). Chlorotrimethylsilane (5.8 mL, 0.0457) was then added dropwise at 0 °C and allowed to stir for 18 h. The reaction mixture was added to diethyl ether (25 mL) and extracted with dIH2O. The water layer was extracted with diethyl ether (50 mL, 3x). The organic layers were combined and the back extracted with dIH2O (25 mL, 2x), washed with brine, dried with MgSO4, and concentrated via rotary evaporation. The product was then purified by distillation at 85 °C and 30 torr to yield 6 (4.8g, 63 % yield). 1H NMR (400 MHz, CDCl3): δ 5.95 (s, 1H), 5.56 (s, 1H), 4.21 (s, 2H), 0.18 (s, 3H)

2,5-dihydrothiophene-3-carboxylic acid 1,1-dioxide (7). A 300 mL Parr reactor was charged with 3-sulfolene (12.06 g, 0.102 mol), 1,8-diazabicyclo[5,4,0]undec-7-ene (30.6 mL, 0.205 mol) and dimethylsulfoxide (DMSO, 15 mL). The reactor was sealed and pressurized with CO2 (50 psi) and left stirring at room temperature for 3 d. The mixture was diluted with acetone (50 mL) and filtered. The solid was then dissolved in methylene chloride (500 mL) and dry HCl was bubbled through the solution until precipitate stopped forming. The HCl gas was formed in situ by slow addition of H2SO4 into mixture of NaCl and concentrated HCl. The gas was dried through H2SO4 prior to reaching the reaction flask. The solid was isolated by filtration, dissolved in acetone (75 mL), and passed through a short silica column. The organic fractions were combined and the solvent was removed by rotary evaporation to afford 7 as an off-white solid (9.7g, 0.06 mol, 59.8 % yield). 1H NMR (400 MHz, DMSO-d6): δ 6.96 (s, 1H), 4.13 (s, 2H), 3.99 (s, 2H). 13C NMR (100.61 MHz, DMSO-d6): δ 164.12 (C=O), 134.78 (CH=C), 130.58 (CH=C), 58.02 (CHC), 55.01 (CHC=C)
hydroxyisoprene (5) (sulfone method). To a 500 mL round bottom containing 7 (10 g, 0.062 mol) in THF (100 mL) was added 1M diisobutylaluminum hydride in THF (215.8 mL, 0.216 mol) dropwise at 0 °C. The reaction was allowed to return to room temperature and stirred for 18 h. Excess methanol (200 mL) was added to the flask followed by dIH$_2$O (0.2158 mol) and the formed solid was filtered off. The filtrate was then concentrated by reduced pressure. Crude: $^1$H NMR (400 MHz, DMSO-d$_6$): δ 6.00 (s, 1H), 4.26 (s, 2H), 3.85 (s, 2H), 3.80 (s, 2H). Deprotection was then carried out at 150 °C with continuous distillation at 400 torr and briefly reduced to 100 torr in an attempt to collect any substantial amount of product. Only a small amount of 5 was isolated and immediately analyzed by proton NMR. $^1$H NMR (400 MHz, CDCl$_3$): δ 6.30–6.40 (dd, 1H), 5.30 (s, 1H), 5.26 (d, 1H), 5.15 (s, 1H), 5.12 (d, 1H), 4.34 (s, 2H)

hydroxyisoprene (5) (chloroprene method). To a 25 mL round bottom was added dry THF (10 mL) followed by magnesium turnings (0.21 g, 0.0085 mol). The turnings were activated by scratching. To the solution was added dibromoethane (1 mL) and the system was cooled to 0 °C. ZnCl$_2$ (0.023 g, 0.169 mmol) was added and a small amount of freshly distilled chloroprene for activation. The distilled chloroprene (0.5 g, 0.006 mol) was then added dropwise to maintain slow reflux of the solvent. The formaldehyde was generated by addition of 2,2,6,6-tetramethylpiperdine (1.92 mL, 0.0113 mol) to a solution of dry THF (10 mL) and 2 M $n$-butyllithium in THF (5.65 mL, 0.0113 mol).$^{42}$ The solution was cooled to 0 °C and benzotriazolemethanol (1.68 g, 0.0113 mol) was added. The temperature was then reduced to -78 °C and the chloroprene Grignard was added dropwise. The reaction was kept at this temperature for 3 h and then allowed to warm to room temperature for 18 h. The reaction was quenched with diH$_2$O and
extracted with diethyl ether (50 mL, 3x). The organic layers were combined, washed with 4 N NaOH (15 mL), brine (15 mL), and dried over MgSO₄. The product mixture was concentrated and hydroxyisoprene 5 along with the allene isomer 9 were isolated by distillation. Further separation could not be achieved.

*hydroxyisoprene (5) (ring opening method).* Lithium diisopropylamide (LDA, 18.2 g, 0.17 mol) was added to an oven dried 1 L round bottom flask. To the flask was then added freshly distilled diethyl ether (300 mL) that was dried over sodium. The epoxide, 2-methyl-2-vinylloxirane (11 g, 0.131 mol) was added dropwise maintaining a gentle reflux upon addition. The solution was then stirred for 3 h and added to 2 N HCl (200 mL). The organic layer was isolated, washed with 5% sodium bicarbonate, brine, and then dried over MgSO₄.

The organic layers were concentrated via rotary evaporation yielding the crude product 5 (5.24 g, 48% yield). The product was further purified by distillation at 50 °C and 2 torr, but the typical percent yields were below 10%. For this reason the crude product was used without further purification. ¹H NMR (400 MHz, CDCl₃): δ 6.35–6.42 (dd, 1H), 5.29 (s, 1H), 5.27 (d, 1H), 5.15 (s, 1H), 5.11 (d, 1H), 4.33 (s, 2H)

tosylisoprene (3). To a 100 mL round bottom was added methylene chloride (50 mL) p-toluenesulfonyl chloride (8.3 g, 0.434 mol), and triethylamine (6.05 mL, 0.0434). 5 was added dropwise at 0 °C for 4 h. The reaction was poured into hexanes and the solid removed by filtration. The solution was concentrated under reduced pressure to yield the crude product 3 (5.09 g, 68 % yield). ¹H NMR (400 MHz, CDCl₃): δ 7.80 (s, 2H), 7.34 (s, 2H), 6.24–6.31 (dd, 1H), 5.25 (s, 1H), 5.21 (s, 1H), 5.15 (d, 1H), 5.10 (s, 1H), 4.68 (s, 2H), 2.46 (s, 3H). Subsequent small scale substitution reactions were carried out with
1,3-(di-tert-butoxycarbonyl)guanidine or diethylamine and initial proton NMR spectrum can be found in the supplemental section.

3. Results and Discussion

![Scheme 4.1. Synthesis of Boc protected guanidinoisoprene via a bromoisoprene intermediate](image)

**3.1 Bromoisoprene**

A few methods have been used for developing 2-substituted dienes. The first is by synthesis of 2-bromoisoprene (1) followed by nucleophilic substitution. Synthesis of 1 entails either dehydrohalogenation of brominated isoprene, or bromination of sulfur dioxide protected isoprene with N-bromosuccinimide (NBS). The dehydrohalogenation pathway traditionally has low yields, and to avoid the use of excess Br₂ (which is both toxic and not atom economical) the second method was used to produce the key intermediate 1 (Scheme 4.1). Synthesis began with the protection of isoprene with sulfur dioxide, producing an off-white crystalline solid in moderate yields. The protected
isoprene was then brominated in the allylic position with NBS, and after purification by repeated recrystallizations, the product was isolated as an off white powder in low yields. The target intermediate was then obtained by thermal deprotection at 170 °C while under continuous distillation at 200 torr. Due to the intermediate’s instability and high volatility, the yields for this step were also low (24 %). Once 1 was obtained, it was immediately used without further purification. Guanidinylation was carried out by deprotonation of 1,3-(di-tert-butoxycarbonyl)guanidine (Boc₂G) with NaH followed by slow addition of 1. Pure product (2) was isolated after column chromatography as an off white crystalline powder in moderate yields. Boc₂G-isoprene was polymerized using a radical initiator, AIBN, in dioxane at low temperature. Higher temperature polymerizations resulted in insoluble solid. The Boc protecting group can be removed by thermal cleavage, and it is thought that the t-butyl carbocation intermediediate formed during removal may initiate a cationic polymerization through the double bond. The isolated polymer was found to have a molecular weight of 35,000 g/mol and a PDI of 1.7. Subsequent deprotections of the polymer resulted in crosslinked material. Due to an overall yield of 1%, few attempts were made to vary the deprotection conditions. This pathway also had numerous steps, was time demanding, and utilized an unstable precursor 1. As a response, a synthon to 1 was targeted which replaced the bromide with a tosylate leaving group (3, Scheme 4.1).
3.2 Hydroxyisoprene

The target tosylisoprene intermediate could be formed from hydroxyisoprene (5). Our lab has previously synthesized dienes functionalized at the two position. The synthesis employed substitution of 2,3-dibromopropene with the appropriate nucleophile. The diene was then formed by coupling with vinyl magnesium bromide using Kumada-Corriu conditions. Using this strategy, the synthesis of 3 began with installing a hydroxyl protecting group, p-methoxyphenylether (Scheme 4.2). This began with deprotonation of p-methoxyphenol with NaH followed by slow addition of 2,3-dibromopropene. The product was purified by distillation and isolated in 63% yield. The subsequent Kumada coupling only yielded the initial protecting group with no product seen by proton NMR. Allylic aryl ethers have been shown to cleave in the presence of Ni(II)/Ni(0) systems which are thought to be present in the coupling reaction.

Scheme 4.3. Preparation of hydroxyisoprene utilizing a silyl ether protecting group
An alternate protecting group (trimethylsilylether; TMS) was then used to avoid deprotection during the coupling step. The protecting group was installed by reacting trimethylsilyl chloride with 2-bromoallyl alcohol, and after distillation afforded the pure protected bromopropene (6) in moderate yields (Scheme 4.3). Once again, upon coupling, there was no product detected, and the focus was then placed on functionalization of a preexisting diene.

Three pathways were used toward hydroxylation and subsequent tosylation of a preformed diene. The first targeted reduction of isoprene carboxylic acid (7). As shown in Scheme 4.4, synthesis began with carboxylation of sulfolene with 50 psi of CO\(_2\)\(_{(g)}\) in the presence of 8-diazabicyclo[5,4,0]undec-7-ene (DBU). This step occurred selectively to afford the target 7. The acid was then reduced with diisobutylaluminum hydride (Dibal) followed by deprotection at 150 °C while under continuous distillation. The yield of the purified product was low, possibly from heat promoted polymerization of the monomer prior to distillation.
Scheme 4.5. Synthesis of hydroxyisoprene through a grignard reaction between chloroprene and formaldehyde

The second pathway was a one step reaction of a Grignard, 2-(1,3-butadienyl)magnesium chloride (8) with formaldehyde to afford 5 in one step without protection and deprotection steps (Scheme 4.5). Synthesis began with Grignard formation of chloroprene. Simultaneously formaldehyde was generated in situ by addition of benzotriazolylmethanol to a solution of LiTMP at 0 °C. The temperature was reduced to -78 °C, the Grignard added slowly, and the system stirred at this temperature for 3 hours. An inseparable mixture of the target monomer and the allene isomer (9) was isolated by distillation. It was previously reported that a reaction between 2-(1,3-butadienyl)magnesium chloride with formaldehyde yields a mixture of diene and allene isomers.45

Scheme 4.6. Synthesis of tosylisoprene from ring opening of 2-methyl-2-vinylisoxirane
The third strategy targeted selective ring opening of 2-methyl-2-vinyloxirane, 10, with a sterically hindered base (Scheme 4.6). To this end, 10 was slowly added to a diethyl ether solution containing lithium diisopropylamide at room temperature and allowed to react for 3 hours. The product was washed with dilute HCl and concentrated to afford the crude product 5 in moderate yields. Purification was possible by distillation, but yields were low despite a low temperature distillation. For this reason, the crude product was typically used without purification. Tosylation of 5 with p-toluenesulfonyl chloride afforded the target monomer 3. Initial substitution of the tosylate was successful with guanidine in a similar procedure to the guanidinylation steps previously discussed. Furthermore, substitution with diethylamine was also successful. To date, only small scale reactions have been attempted to gain an understanding of the breadth of nucleophiles that can be used in the substitution, and current research is focusing on optimization and increasing the reaction scale.

4. Conclusions

Guanidine functionalized materials may potentially enhance the transfection efficiency of delivery vehicles used currently. Synthetic strategies have been developed to produce a guanidine functionalized isoprene to be a comonomer with DEAI to enhance an efficient gene delivery material, PEAI, that was developed in our lab. In an effort towards a faster method of producing isoprene substituted off of the 2-postion, two monomer intermediates were identified, the first being bromoisoprene and the second tosylisoprene. Through both pathways guanidine functionalized monomers were successfully created. Previous diene syntheses were between 5 to 7 days, and through
this work, a method was developed to obtain a monomer intermediate in 2 days. Furthermore, this intermediate could react with a wide range of nucleophiles and could lead to rapid development of a library of dienes functionalized at the 2-position. Future work will focus on optimization of this pathway and further elaboration of the diene library.
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Chapter 5. General Conclusions
The work described was focused on the development of polycationic materials for gene delivery applications. The variables or properties of these polymers that participate in delivery are quite complex and are often in competition. To date, there have been attempts to create guidelines into designing new materials, but there remain no clear rules or targets for polymer development. Three aspects of delivery were targeted to fundamentally study the process including pK$_a$ and ionization control for endosomal release, degradability for lowered toxicity, and a new functionality for enhanced cellular uptake. Control over ionization by programming materials with unique pK$_a$ transitions afforded insights into optimizing the complexation/buffering potential for designing new materials. In this study the acid/base inflection points for a range of materials were varied from an average pK$_a$ of 5.8 up to 7.9 through the synthesis of a series of copolymers. It was determined that the materials near an inflection of 7.1 had the best balance of charge for DNA complexation (at low polymer concentrations) with buffering capacity for endosomal release. A window from 24% to 50% amine protonation in solution was identified, with the highest bioactivity at 33% ionization. The best material was a copolymer of dimethylaminoisoprene (DMAI) and dipropylaminoisoprene (DPAI) (60:40 feed ratio) where the homopolymers of each of the monomers showed no transfection ability. It would be interesting in future experiments to quaternize the homopolymers, particularly poly(diethylaminoisoprene) PEAI, in the range of 24% to 50% amine quaternization to explore if the enhancement that the copolymers benefited from could be transferred to the homopolymers. In doing so an effective material may be
synthesized, while simultaneously verifying the defined ionization window (24-50%) that could be applied towards future material design (Scheme 5.1). With the high level of control over the ionization of these materials, they may also be used in applications that range from smart materials for drug delivery or integrated into environmentally sensitive microfluidic devices.

![Scheme 5.1. Quaternization of PEAI from 24% to 50% of the amines](image_url)

Another aspect of gene delivery that was explored was decreasing the toxicity of a material that possessed functionality already proven to impart high bioactivity in previous delivery materials developed by our lab. This research utilized a Diels Alder cycloaddition of diethylaminoisoprene (DEAI) with fumaric acid to afford a monomer that could be polymerized in step growth fashion with a diol or diamine. Previous attempts to incorporate an amino diacid into a material via this route were only successful as a 50% copolymer with an unfunctionalized diacid, where above this feed the material crosslinked. By designing a diacid with a tetrasubstituted doublebond, the crosslinking reaction was prevented, and an amine containing homopolymer was synthesized. The solubility of the resulting polymer could also be controlled by incorporating a hydrophobic or hydrophilic diol. When compared to a nondegradable commercially
available delivery vector, use of the polyester scaffold was successful in lowering the materials toxicity. Unfortunately, the transfection ability was nominal, despite elevated polymer concentrations. This result was attributed to the low amine content in a low molecular weight homopolymer.

The last focus was placed on enhancing the cellular uptake of the polyplex by using a peptide mimic of transmembrane proteins found in viral based delivery systems. These natural peptides have higher concentrations of arginine at their active site. The functionality of arginine is a guanidine moiety and has been shown in some materials to increase membrane translocation. In an attempt to further enhance an efficient delivery material, poly(diethylaminoisoprene) (PEAI), previously developed in our lab, an analogue to diethylaminoisoprene (DEAI) that replaced the diethylamine with guanidine was designed. Numerous strategies were employed, and two intermediate targets proved successful to ultimately synthesizing the guanidine monomer. The first was bromoisoprene, where nucleophilic substitution with a diboc protected guanidine afforded the target monomer. This strategy, however, was plagued with low yields and instability of the bromoisoprene. The second pathway targeted a tosylated hydroxyisoprene that would be reactive towards an identical nucleophilic substitution with diboc protected guanidine. The hydroxyisoprene and subsequent tosylation were successful in 2 days, and the monomers were stable at 4 °C for over a month. Utilizing either strategy yielded the target monomer. The polymerization of this monomer was successful, but deprotection steps have yielded crosslinked materials. Future studies will focus on varying the deprotection conditions, specifically utilizing carbocation scavengers to prevent cationic polymerization when the t-butyl carbocation is produced.
The hydroxyisoprene tosylate has proven to be a powerful intermediate for developing 2-functionalized isoprenes (Figure 5.1), which previously had no general method for synthesis. Preliminary studies have shown that substitution was possible with guanidine as well as a secondary amine nucleophile. It is thought that a wide range of nucleophiles may be selected, and a library of 2-functional dienes could be rapidly synthesized. Not only can the tosylate electrophile be used, but the hydroxyisoprene itself could be used as a nucleophile to generate ethers, esters, etc. Another potential study with the hydroxyisoprene monomer is the polymerization to form a polyol and from which grafting from the backbone would generate cylindrical brushes. Two possible targets for initial grafting studies could be poly(caprolactone) (degradable) and poly(N-isopropylamide) (thermally responsive) (Scheme 5.2). Preliminary radical
polymerization attempts of hydroxyisoprene resulted in crosslinking reactions. Further attempts will focus on protecting the hydroxyl group as an acetate or as a silyl ether followed by polymerization. The cylindrical brushes would be valuable due to their unique solution properties for fundamental physical studies as well as potentially useful drug delivery systems.

Scheme 5.2. Cylindrical brush formation by a) ring opening of caprolactone or b) ATRP polymerization of N-isopropylamide
$^1$H of 2-bromo-$N,N$-dimethylprop-2-en-1-amine
$^1$H of $N,N$-dimethyl-2-methylenebut-3-en-1-amine (Dimethylaminoisoprene; DMAI)
$^{13}$C of $N,N$-dimethyl-2-methylenebut-3-en-1-amine (Dimethylaminoisoprene; DMAI)
$^1$H of poly(2-($N,N$-dimethylaminomethyl)-1,3-butadiene) (PMAI)
$^{13}$C of poly(2-(N,N-dimethylaminomethyl)-1,3-butadiene) (PMAI)
$^1$H of 2-bromo-$N,N$-diethylprop-2-en-1-amine
$^{13}$C of 2-bromo-$N,N$-diethylprop-2-en-1-amine
$^1$H of $N,N$-diethyl-2-ethylenebut-3-en-1-amine (Diethylaminoisoprene; DEAI)
$^{13}$C of $N,N$-diethyl-2-ethylenebut-3-en-1-amine (Diethylaminoisoprene; DEAI)
$^1$H of poly(2-(N,N-diethylaminomethyl)-1,3-butadiene) (PEAI)
$^{13}$C of poly(2-$(N,N$-diethylaminomethyl)-1,3-butadiene) (PEAI)
$^1$H of 2-bromo-$N,N$-propylprop-2-en-1-amine
$^{13}$C of 2-bromo-$N,N$-propylprop-2-en-1-amine
$^1$H of $N,N$-dipropyl-2-ethylenbut-3-en-1-amine (Dipropylaminoisoprene; DPAI)
$^{13}$C of $N,N$-dipropyl-2-ethylenbut-3-en-l-amine (Dipropylaminoisoprene; DPAI)
$^1$H of poly(2-(N,N-dipropylaminomethyl)-1,3-butadiene) (PPAI)
$^{13}$C of poly(2-(N,N-dipropylaminomethyl)-1,3-butadiene) (PPAI)
$^1$H of poly(2-(N,N-dimethylaminomethyl)-3-methyl-1,3-butadiene-co-2-(N,N-dipropylaminomethyl)-3-methyl-1,3-butadiene) (20:80) (MePr-20)
$^1$H of poly(2-(N,N-dimethylaminomethyl)-3-methyl-1,3-butadiene-co-2-(N,N-dipropylaminomethyl)-3-methyl-1,3-butadiene) (40:60) (MePr-40)
$^1$H of poly(2-(N,N-dimethyaminomethyl)-3-methyl-1,3-butadiene-co-2-(N,N-dipropylaminomethyl)-3-methyl-1,3-butadiene) (54:46) (MePr-54)
$^1$H of poly$(2$-(N,N-dimethylaminomethyl)-3-methyl-1,3-butadiene-co-2-(N,N-dipropylaminomethyl)-3-methyl-1,3-butadiene) (60:40) (MePr-60)
$^{13}$C of poly(2-(N,N-dimethylaminomethyl)-3-methyl-1,3-butadiene-co-2-(N,N-dipropylaminomethyl)-3-methyl-1,3-butadiene) (60:40) (MePr-60)
$^1$H of poly(2-($N,N$-dimethylaminomethyl)-3-methyl-1,3-butadiene-co-2-($N,N$-dipropylaminomethyl)-3-methyl-1,3-butadiene) (80:20) (MePr-80)
$^1$H of poly(2-(N,N-diethylaminomethyl)-1,3-butadiene-co-2-(N,N-dipropylaminomethyl)-1,3-butadiene) (20:80) (EtPr-20)
$^1$H of poly(2-(N,N-diethylaminomethyl)-1,3-butadiene-$\text{co}$-2-(N,N-dipropylaminomethyl)-1,3-butadiene) (40:60) (EtPr-40)
$^1$H of poly(2-(N,N-diethylaminomethyl)-1,3-butadiene-co-2-(N,N-dipropylaminomethyl)-1,3-butadiene) (60:40) (EtPr-60)
$^{13}$C of poly(2-(N,N-diethylaminomethyl)-1,3-butadiene-co-2-(N,N-dipropylaminomethyl)-1,3-butadiene) (60:40) (EtPr-60)
$^1$H of poly(2-(N,N-diethylaminomethyl)-1,3-butadiene-\textit{co}-2-(N,N-dipropylaminomethyl)-1,3-butadiene) (80:20) (EtPr-80)
DSC of poly(2-(N,N-dimethylaminomethyl)-1,3-butadiene) (PMAI)
DSC of poly(2-(N,N-diethylaminomethyl)-1,3-butadiene) (PEAI)
DSC of poly(2-((N,N-dipropylaminomethyl)-1,3-butadiene) (PPAI)
DSC of poly(2-(N,N-diethylaminomethyl)-1,3-butadiene-co-2-(N,N-dipropylaminomethyl)-1,3-butadiene) (20:80) (EtPr-20)
DSC of poly(2-(N,N-diethylaminomethyl)-1,3-butadiene-co-2-(N,N-dipropylaminomethyl)-1,3-butadiene) (40:60) (EtPr-40)
DSC of poly(2-(N,N-diethylaminomethyl)-1,3-butadiene-co-2-(N,N-dipropylaminomethyl)-1,3-butadiene) (60:40) (EtPr-60)
DSC of poly(2-(N,N-diethylaminomethyl)-1,3-butadiene-co-2-(N,N-dipropylaminomethyl)-1,3-butadiene) (80:20) (EtPr-80)
DSC of poly(2-(N,N-dimethylaminomethyl)-3-methyl-1,3-butadiene-co-2-(N,N-dipropylaminomethyl)-3-methyl-1,3-butadiene) (20:80) (MePr-20)
DSC of poly(2-(N,N-dimethylaminomethyl)-3-methyl-1,3-butadiene-co-2-(N,N-dipropylaminomethyl)-3-methyl-1,3-butadiene) (40:60) (MePr-40)
DSC of poly(2-(N,N-dimethylaminomethyl)-3-methyl-1,3-butadiene-co-2-(N,N-dipropylaminomethyl)-3-methyl-1,3-butadiene) (54:46) (MePr-54)
DSC of poly(2-(N,N-dimethylaminomethyl)-3-methyl-1,3-butadiene-co-2-(N,N-dipropylaminomethyl)-3-methyl-1,3-butadiene) (60:40) (MePr-60)
DSC of poly(2-(N,N-dimethylaminomethyl)-3-methyl-1,3-butadiene-co-2-(N,N-dipropylaminomethyl)-3-methyl-1,3-butadiene) (80:20) (MePr-80)
TGA of poly(2-(N,N-dimethylaminomethyl)-1,3-butadiene) (PMAI)
TGA of poly(2-(N,N-diethylaminomethyl)-1,3-butadiene) (PEAI)
TGA of poly(2-(N,N-dipropylaminomethyl)-1,3-butadiene) (PPAI)
TGA of poly(2-(N,N-diethylaminomethyl)-1,3-butadiene-co-2-(N,N-dipropylaminomethyl)-1,3-butadiene) (20:80) (EtPr-20)
TGA of poly(2-(N,N-diethylaminomethyl)-1,3-butadiene-co-2-(N,N-dipropylaminomethyl)-1,3-butadiene) (40:60) (EtPr-40)
TGA of poly(2-(N,N-diethylaminomethyl)-1,3-butadiene-co-2-(N,N-dipropylaminomethyl)-1,3-butadiene) (60:40) (EtPr-60)
TGA of poly(2-(N,N-diethylaminomethyl)-1,3-butadiene-co-2-(N,N-dipropylaminomethyl)-1,3-butadiene) (80:20) (EtPr-80)
TGA of poly(2-(N,N-dimethylaminomethyl)-3-methyl-1,3-butadiene-co-2-(N,N-dipropylaminomethyl)-3-methyl-1,3-butadiene) (20:80) (MePr-20)
TGA of poly(2-\(N,N\)-dimethylaminomethyl)-3-methyl-1,3-butadiene-\(co\)-2-\(N,N\)-dipropylaminomethyl)-3-methyl-1,3-butadiene) (40:60) (MePr-40)
TGA of poly(2-(N,N-dimethylaminomethyl)-3-methyl-1,3-butadiene-co-2-(N,N-dipropylaminomethyl)-3-methyl-1,3-butadiene) (54:46) (MePr-54)
TGA of poly(2-(N,N-dimethylaminomethyl)-3-methyl-1,3-butadiene-co-2-(N,N-dipropylaminomethyl)-3-methyl-1,3-butadiene) (60:40) (MePr-60)
TGA of poly(2-(N,N-dimethylaminomethyl)-3-methyl-1,3-butadiene-co-2-(N,N-dipropylaminomethyl)-3-methyl-1,3-butadiene) (80:20) (MePr-80)
GPC of poly(2-(N,N-dimethylaminomethyl)-1,3-butadiene) (PMAI)
GPC of poly(2-(N,N-diethylaminomethyl)-1,3-butadiene) (PEAI)
GPC of poly(2-(N,N-dipropylaminomethyl)-1,3-butadiene) (PPAI)
GPC of poly(2-(N,N-diethylaminomethyl)-1,3-butadiene-co-2-(N,N-dipropylaminomethyl)-1,3-butadiene) (20:80) (EtPr-20)
GPC of poly(2-(N,N-diethylaminomethyl)-1,3-butadiene-co-2-(N,N- dipropylaminomethyl)-1,3-butadiene) (40:60) (EtPr-40)
GPC of poly(2-(N,N-diethylaminomethyl)-1,3-butadiene-co-2-(N,N-dipropylaminomethyl)-1,3-butadiene) (60:40) (EtPr-60)
**GPC of poly(2-(N,N-diethylaminomethyl)-1,3-butadiene-co-2-(N,N-dipropylaminomethyl)-1,3-butadiene) (80:20) (EtPr-80)**

<table>
<thead>
<tr>
<th>Dist. Name</th>
<th>Mn</th>
<th>Mw</th>
<th>ZP</th>
<th>MW</th>
<th>MW1</th>
<th>Polydispersity</th>
<th>K alpha</th>
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<tr>
<td>1</td>
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<td>21656</td>
<td>21554</td>
<td>20552</td>
<td>39503</td>
<td>2.4832</td>
<td>2.4854</td>
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</table>
GPC of poly(2-(N,N-dimethylaminomethyl)-3-methyl-1,3-butadiene-co-2-(N,N-dipropylaminomethyl)-3-methyl-1,3-butadiene) (20:80) (MePr-20)
GPC of poly(2-((N,N-dimethylaminomethyl)-3-methyl-1,3-butadiene-co-2-(N,N-dipropylaminomethyl)-3-methyl-1,3-butadiene) (40:60) (MePr-40)
GPC of poly(2-(N,N-dimethylaminomethyl)-3-methyl-1,3-butadiene-co-2-(N,N-dipropylaminomethyl)-3-methyl-1,3-butadiene) (60:40) (MePr-60)
GPC of poly(2-(N,N-dimethylaminomethyl)-3-methyl-1,3-butadiene-co-2-(N,N-dipropylaminomethyl)-3-methyl-1,3-butadiene) (80:20) (MePr-80)
Appendix B
DLS and $\zeta$-potential measurements of the homo- and copolymers complexed with pCMV-Luc

<table>
<thead>
<tr>
<th>Polymer</th>
<th>Complex Diameter$^a$ (nm)</th>
<th>$\zeta$-Potential$^b$ (mV)</th>
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<tbody>
<tr>
<td></td>
<td>2$^c$</td>
<td>4$^c$</td>
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<tr>
<td>PMAI</td>
<td>244</td>
<td>219</td>
</tr>
<tr>
<td>PEAI</td>
<td>529</td>
<td>403</td>
</tr>
<tr>
<td>PPAI$^d$</td>
<td>-</td>
<td>-</td>
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<tr>
<td>MePr-20</td>
<td>144</td>
<td>184</td>
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<tr>
<td>MePr-40</td>
<td>381</td>
<td>265</td>
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<tr>
<td>MePr-54</td>
<td>473</td>
<td>410</td>
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<tr>
<td>MePr-60</td>
<td>200</td>
<td>100</td>
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<tr>
<td>MePr-80</td>
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<td>305</td>
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<tr>
<td>EtPr-20$^d$</td>
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<td>-</td>
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<tr>
<td>EtPr-40</td>
<td>368</td>
<td>367</td>
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<tr>
<td>EtPr-60</td>
<td>460</td>
<td>245</td>
</tr>
<tr>
<td>EtPr-80</td>
<td>373</td>
<td>310</td>
</tr>
</tbody>
</table>

$^a$: Determined by dynamic light scattering at 173$^o$. $^b$: Determined on a zetasizer with a low volume flow cell. $^c$: N/P ratio. $^d$: Polymer did not remain soluble during the DLS and $\zeta$-potential experiments.

DLS was done to examine if the complex size would allow for an endocytotic pathway. The experiments showed that multiple populations formed with one population being 150-250 nm and the other > 1000 nm. The smaller population is most likely the group that translocates into the cell and labeling studies will be conducted in the future to further explore the uptake pathway. For each material there was also a rapid complex size increase at low concentrations where the polymer seems to induce aggregation.

This clustering effect has been seen in other polymer systems, most notably poly(lysine), and is strongly dependent on the characteristics of the individual polymer.$^a$

$^1$H of 2-($N,N$-diethylaminomethyl)-3-methyl-1,3-butadiene
\(^1\text{H}\) of 4-((diethylamino)methyl)-5-methylcyclohex-4-ene-1,2-dicarboxylic acid
$^{13}$C of 4-((diethylamino)methyl)-5-methylcyclohex-4-ene-1,2-dicarboxylic acid
$^1$H of 4,5-dimethylcyclohex-4-ene-1,2-dicarboxylic acid
$^{13}$C of 4,5-dimethylcyclohex-4-ene-1,2-dicarboxylic acid
$^1$H of Poly(octanediol 4,5-dimethylcyclohex-4-ene-trans-1,2-dicarboxylate-co-octanediol 4-(N,N-diethylaminomethyl)-5-methyl-cyclohex-4-ene-trans-1,2-dicarboxylate) (80:20) (PI-20)
$^1$H of Poly(octanediol \textit{4,5-dimethylcyclohex-4-ene-trans-1,2-dicarboxylate-co-octanediol 4-}(N,N-diethylaminomethyl)-5-methyl-cyclohex-4-ene-trans-1,2-dicarboxylate) (60:40) (PI-40)
$^1$H of Poly(octanediol 4,5-dimethylcyclohex-4-ene-trans-1,2-dicarboxylate-co-octanediol 4-($N,N$-diethylaminomethyl)-5-methyl-cyclohex-4-ene-trans-1,2-dicarboxylate) (40:60) (PI-60)
$^1$H of Poly(octanediol 4,5-dimethylcyclohex-4-ene-trans-1,2-dicarboxylate-co-octanediol 4-(N,N-diethylaminomethyl)-5-methyl-cyclohex-4-ene-trans-1,2-dicarboxylate) (20:80) (PI-80)
$^1$H of Poly(octanediol 4,4-($N,N$-diethylaminomethyl)-5-methyl-cyclohex-4-ene-trans-1,2-dicarboxylate) (PI-100)
$^1$H of Poly(tetraethyleneglycol 4,5-dimethylcyclohex-4-ene-trans-1,2-dicarboxylate) (P11-0)
$^1$H of Poly(tetraethyleneglycol 4,5-dimethylcyclohex-4-ene-trans-1,2-dicarboxylate-co-tetraethyleneglycol 4-$(N,N$-diethylaminomethyl)$)$-5-methyl-cyclohex-4-ene-trans-1,2-dicarboxylate) (40:60) (PII-60)
$^1$H of Poly(tetraethyleneglycol 4-($N,N$-diethylaminomethyl)-5-methyl-cyclohex-4-ene-trans-1,2-dicarboxylate) (PII-100)
DSC of Poly(octanediol 4,5-dimethylcyclohex-4-ene-trans-1,2-dicarboxylate) (PI-0)
DSC of Poly(octanediol 4,5-dimethylcyclohex-4-ene-trans-1,2-dicarboxylate-co-octanediol 4-(N,N-diethylaminomethyl)-5-methyl-cyclohex-4-ene-trans-1,2-dicarboxylate) (80:20) (PI-20)
DSC of Poly(octanediol 4,5-dimethylcyclohex-4-ene-trans-1,2-dicarboxylate-co-octanediol 4-(N,N-diethylaminomethyl)-5-methyl-cyclohex-4-ene-trans-1,2-dicarboxylate) (60:40) (PI-40)
DSC of Poly(octanediol 4,5-dimethylcyclohex-4-ene-trans-1,2-dicarboxylate-co-octanediol 4-(N,N-diethylaminomethyl)-5-methyl-cyclohex-4-ene-trans-1,2-dicarboxylate) (40:60) (PI-60)
DSC of Poly(octanediol 4,5-dimethylcyclohex-4-ene-trans-1,2-dicarboxylate-co-octanediol 4-(N,N-diethylaminomethyl)-5-methyl-cyclohex-4-ene-trans-1,2-dicarboxylate) (20:80) (PI-80)
DSC of Poly(octanediol 4-(N,N-diethylaminomethyl)-5-methyl-cyclohex-4-ene-trans-1,2-dicarboxylate) (PI-100)
DSC Poly(tetraethyleneglycol 4,5-dimethylcyclohex-4-ene-trans-1,2-dicarboxylate) (PII-0)
DSC of Poly(tetraethyleneglycol 4,5-dimethylcyclohex-4-ene-trans-1,2-dicarboxylate-co-tetraethyleneglycol 4-(N,N-diethylaminomethyl)-5-methyl-cyclohex-4-ene-trans-1,2-dicarboxylate) (40:60) (PII-60)
DSC of Poly(tetraethyleneglycol 4-(N,N-diethylaminomethyl)-5-methyl-cyclohex-4-ene-trans-1,2-dicarboxylate) (PII-100)
TGA of Poly(octanediol 4,5-dimethylcyclohex-4-ene-trans-1,2-dicarboxylate) (PI-0)
TGA of Poly(octanediol 4,5-dimethylcyclohex-4-ene-trans-1,2-dicarboxylate-co-octanediol 4-(N,N-diethylaminomethyl)-5-methyl-cyclohex-4-ene-trans-1,2-dicarboxylate) (80:20) (PI-20)
TGA of Poly(octanediol 4,5-dimethylcyclohex-4-ene-trans-1,2-dicarboxylate-co-octanediol 4-(N,N-diethylaminomethyl)-5-methyl-cyclohex-4-ene-trans-1,2-dicarboxylate) (60:40) (PI-40)
TGA of Poly(octanediol 4,5-dimethylcyclohex-4-ene-trans-1,2-dicarboxylate-co-octanediol 4-(N,N-diethylaminomethyl)-5-methyl-cyclohex-4-ene-trans-1,2-dicarboxylate) (40:60) (PI-60)
TGA of Poly(octanediol 4,5-dimethylcyclohex-4-ene-trans-1,2-dicarboxylate-co-octanediol 4-\(N,N\)-diethylaminomethyl)-5-methyl-cyclohex-4-ene-trans-1,2-dicarboxylate) (20:80) (PI-80)
TGA of Poly(octanediol 4-(N,N-diethylaminomethyl)-5-methyl-cyclohex-4-ene-trans-1,2-dicarboxylate) (PI-100)
TGA of Poly(tetraethyleneglycol 4,5-dimethylcyclohex-4-ene-trans-1,2-dicarboxylate-co-tetraethyleneglycol 4-(N,N-diethylaminomethyl)-5-methyl-cyclohex-4-ene-trans-1,2-dicarboxylate) (40:60) (PII-60)
TGA of Poly(tetraethyleneglycol 4-(N,N-diethylaminomethyl)-5-methyl-cyclohex-4-ene-trans-1,2-dicarboxylate) (PII-100)
TGA of Poly(tetraethyleneglycol 4,5-dimethylcyclohex-4-ene-trans-1,2-dicarboxylate-)
(PII-0)
GPC of Poly(tetraethyleneglycol 4,5-dimethylcyclohex-4-ene-trans-1,2-dicarboxylate-<i>co</i>-tetraethyleneglycol 4-(N,N-diethylaminomethyl)-5-methyl-cyclohex-4-ene-trans-1,2-dicarboxylate) (40:60) (PII-60)
GPC of Poly(tetraethyleneglycol 4-(N,N-diethylaminomethyl)-5-methyl-cyclohex-4-ene-trans-1,2-dicarboxylate) (PII-100)
Appendix C
$^1$H of 3-Methyl-2,5-dihydrothiophene-1,1-dioxide
$^1$H of 3-Bromomethyl-2,5-dihydrothiophene-1,1-dioxide
$^1$H of 2-Bromomethyl-1,3-butadiene
$^1$H of 1,3-(ditertbutoxycarbonyl)guanidinoisoprene
$^{13}$C of 1,3-(ditertbutoxycarbonyl)guanidinoisoprene
$^1$H of poly(1,3-(ditertbutoxycarbonyl)guanidinoisoprene)
$^1$H of 1-((2-bromoallyl)oxy)-4-methylbenzene
$^1$H of ((2-bromoallyl)oxy)trimethylsilane
$^1$H of 2,5-dihydrothiophene-3-carboxylic acid 1,1-dioxide
$^{13}$C of 2,5-dihydrothiophene-3-carboxylic acid 1,1-dioxide
$^1$H of hydroxyisoprene (sulfone method)
$^1$H of hydroxyisoprene (chloroprene method)
$^1$H of hydroxyisoprene (crude) (ring-opening method)
$^1$H of tosylisoprene (ring-opening method)
$^1$H of crude poly(1,3-(ditertbutoxycarbonyl)guanidinoisoprene) (ring-opening method)