Dual-Stimuli Responsive Poly(ethylenimine)s with a Tunable LCST for Gene Delivery

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DUAL-STIMULI RESPONSIVE POLY(ETHYLENIMINE)S WITH A TUNABLE LCST FOR GENE DELIVERY

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science

By:

MARY C. ABRAHAM
B.A., Cedarville University, 2011

2013
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I HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER MY SUPERVISION BY Mary C. Abraham ENTITLED Dual-Stimuli Responsive Poly(ethylenimine)s with a Tunable LCST for Gene Delivery BE ACCEPTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF Master of Science.

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ABSTRACT

Abraham, Mary C. M.S., Department of Chemistry, Wright State University, 2013.
Dual-Stimuli Responsive Poly(ethylenimine)s with a Tunable LCST for Gene Delivery.

Hyperbranched poly(ethylenimine)s (HPEI) were modified with hydrophobic isobutyryl amide groups (HPEI-IBAm$_{0.60}$) to cause an LCST. These modified polymers were then further substituted with hydrophobic alkyl chains (HPEI-IBAm$_{0.60}$-R$_{0.40}$), a mixture of alkyl chains and hydroxyethyl groups (HPEI-IBAm$_{0.60}$-EtOH$_{0.20}$-R$_{0.20}$), and a mixture of alkyl chains and low molecular weight PEG (HPEI-IBAm$_{0.60}$-PEG$_{0.20}$-R$_{0.20}$) to determine the effect of different functional groups on solubility behavior. At pH 7.4, all but three LCSTs were below body temperature (37 °C), while at pH 5, all but three LCSTs were above body temperature. The effect of the concentration of amines per gram of polymer (APG) on the solubility behavior was also investigated. The expected decrease in cytotoxicity from modifying primary amines to secondary amines, as well as the sensitivity to temperature and pH stimuli, makes these polymer systems strong candidates for non-viral gene delivery vectors.
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DEDICATION

I would like to dedicate this thesis to my family. I am inexpressibly grateful to my parents, Kamel and Kathleen Abraham, for the energy and love they have poured into my life. I am especially thankful for all the effort my mother spent teaching me at home for twelve years. I am also grateful for my siblings, Phoebe and Charles Horton, Heidi Abraham, Matthew Abraham, and Amy Abraham. They have encouraged me, motivated me to do my best, supported me, teased me, and laughed with me every step of the way. I cannot imagine life without them.

(Pepper and Homer, you have been the best pets. I am grateful to God for giving me you.)
1. INTRODUCTION

1.1 Gene Delivery

Effective gene delivery provides scientists with the unique opportunity to actually correct mutated or disease-causing genes, rather than simply treating the effects of those genes. Gene delivery can either correct defective or mutated DNA by inserting an accurate strand of DNA or reactivate apoptosis in cells where the programmed-cell death pathway is not functioning properly, as in the case of cancer\(^1\). Drug delivery, on the other hand, transports drugs directly to cells, thus releasing the drugs only in cells. The detrimental and toxic side effects of drug therapy, caused by potentially harmful drugs dispersed equally among sick and healthy cells, are thereby decreased\(^2\).

1.1.1 Applications

Gene delivery is broadly applicable. Not only can gene delivery be used for disorders caused by genetic mutations, such as sickle cell anemia, it also offers possible cures for brain disease, like Parkinson’s and Alzheimer’s, and autoimmune issues, such as HIV\(^3\). Another prime area of interest is cancer, as gene delivery provides the ability to deliver apoptosis-inducing genes directly to tumors. Gene delivery is also being explored as a way to distribute DNA vaccines.

1.2 The mechanism of gene delivery: Endocytosis

While there are many mechanisms whereby particles traverse a cell wall, such as diffusion, through channels, or via active transport, endocytosis is the easiest pathway for
molecules that cannot enter via channels or diffusion. Endocytosis occurs when a cell extends a portion of its membrane around foreign objects, engulfing them in a vesicle, which then pinches off from the membrane and is released into the cytoplasm.

Phagocytosis generally refers to endocytosis of large molecules and, at the mammalian level, is generally only performed by macrophages, cells related to the immune system. Pinocytosis, sometimes referred to as cell drinking, is an indiscriminate engulfing of extracellular fluid and solutes. This is the most likely method by which gene delivery vectors enter the cell. Entrance into the cell can also be effected through receptor-mediated endocytosis. In this case, the exterior of the cell membrane is lined with receptors for various molecules, such as proteins or carbohydrates. Thus, if the gene delivery vector was functionalized with molecules that had corresponding receptors on targeted cells, the vector could enter specific cells through this mechanism.

![Diagram of Endocytosis]

**Scheme 1. Endocytosis**

Once the vesicle detaches from the cell wall, it joins with an endosome. As the endosome proceeds through the processing of received particles, the pH of the endosome decreases to between 6.0 and 4.8, with a typical value of pH 5, in a late endosome. At
the end of the pathway, an endosome either delivers its contents to a lysosome, which destroys the particles, or the DNA can escape from the endosome into the cytoplasm, whence DNA can enter the nucleus\(^1\) (Scheme 1).

1.3 Gene delivery vectors

While the first instinct may be to use naked DNA directly for gene therapy, gene delivery via naked DNA has many difficulties, caused primarily by the properties of DNA. Since DNA has a negatively charged backbone, due to the repeating phosphate groups throughout its structure (Figure 1), its journey through the hydrophobic interior of a cell membrane is severely hindered. The bulk added by its helical structure only further impedes DNA’s passage\(^7\).

![Figure 1. The structure of DNA, illustrating the negative charge on the backbone](image)

In addition to the problems caused by its structure, the ratio of DNA administered to DNA successfully delivered is fairly low\(^1,8,9\). DNA is often degraded by nucleases before it can reach the appropriate cells or integrate into the cellular DNA\(^1\). Finally, naked DNA allows no opportunity for targeting specific cells\(^9\). Thus, research into gene
and drug delivery has been largely devoted to discovering or designing effective carriers, or vectors, for transporting DNA into cells.

1.3.1 Viral vectors

Viral vectors are of especial interest for gene delivery, because they are already designed to penetrate the cell wall, release the viral DNA/RNA, and then hijack the host cell to copy the viral genes and synthesize the viral proteins\(^7\). In order to be used in the human body, the genes causing repeated replication must first be removed from the virus and replaced with the therapeutic DNA/RNA\(^{10}\). Because the natural ability to enter the cell and replicate the genes is retained, the viral vectors show high transfection efficiencies. In other words, a high percentage of the DNA carried in the virus is successfully integrated into the host DNA and expressed in the cell.

However, using a modified infection agent as a vector carries many risks. First, the body’s immune system views viruses, modified or unmodified, as foreign bodies that must be eliminated. Therefore, viral vectors are highly immunogenic\(^{1,9,11,12}\), which means they tend to induce a strong immune response with potentially serious side effects for the patients. Second, because a virus’s genome is fairly small, the size of DNA/RNA that can be integrated into a virus is rather limited\(^{8,9,11,12}\). Thus, viral vectors are not effective therapeutic agents in cases concerning large, mutated DNA sequences. Third, some types of viral vectors can cause insertional mutagenesis\(^{10,12}\), which occurs when a base – adenine, cytosine, guanine, or thymine – is inserted into the DNA sequence. As a result, the code for the amino acids is completely changed, leading to the production of incorrect proteins and, possibly, cancer. Finally, preparing the viral vectors is complicated, making them poor candidates for mass production\(^{8,9,11,12}\).
Some viruses overcome a few of these limitations, but the other drawbacks still remain prohibitive. For example, adeno-associated viruses (AAVs) and retroviruses have low immunogenicity. But AAVs can only carry very small DNA lengths, while retroviruses are unstable and much more likely to cause insertional mutagenesis\textsuperscript{9,10}. Retroviruses are able to transfect only cells that are not proliferating, while other viruses, such as adenoviruses, AAVs, and lentiviruses have the advantageous capability of transfecting cells in any stage of reproduction, whether dividing or non-dividing\textsuperscript{8-10}. Conversely, adenoviruses have high immunogenicity\textsuperscript{9,10}, and lentiviruses can cause insertional mutagenesis\textsuperscript{10}.

1.3.2 Non-viral vectors

In order to avoid the problems currently associated with viral vectors, many non-viral vectors have been investigated. Principal non-viral carriers include cationic lipids, cationic polymers, and inorganic nanoparticles. Cationic lipids possess a hydrophilic, cationic head and long, hydrophobic carbon chains\textsuperscript{11} with an example shown in Figure 2.

\textbf{Figure 2.} An inorganic nanoparticle modified with polymers and 1,2-dioleyloxy-3-trimethylammonium propane, a typical cationic lipid

Cationic polymers usually contain positive charges directly on or pendent to the backbone. Typical inorganic nanoparticles investigated for gene delivery include gold, silicon oxide, iron oxide, and calcium phosphate particles, sometimes modified with
organic groups to improve transfection ability. These methods offer many advantages for application in gene delivery.

First, unlike viral vectors, non-viral vectors are easy to manufacture, making large scale production a viable possibility. Non-viral carriers are also easily modified to adjust the physical properties for optimum delivery capability. By changing the functional groups on cationic lipids or polymers, the charge density, toxicity, and degradability can be increased or decreased. Bulky or non-bulky moieties or changes to the surface of a nanoparticle can alter the final morphology of a vector. Modifications can also affect the transfection ability. For example, by conjugating gold nanoparticles and poly(ethylenimine) (PEI), the transfection efficiency of PEI was significantly increased. The ability to easily attach new groups means that targeting groups can be readily added to non-viral vectors, making the vectors particularly attractive to certain cells and allowing specific delivery of genes.

Second, non-viral vectors have low immunogenicity, provoking little response from the immune system. Cationic polymers and lipids are often either natural polymers or resemble biological molecules and thus trick the immune system into treating them as non-threatening molecules. Most nanoparticles are highly biocompatible, especially if they are already found in the biological system. Calcium phosphate particles are commonly found in bone tissue and so appear normal to the immune system.

Third, directly related to DNA–vector interactions, non-viral vectors place no limit on the length of DNA that can be delivered, since the vectors can effectively surround the DNA or the DNA strand can coil up to fit inside the vector, rather than
actually integrating into a small viral genome\textsuperscript{8,9}. Thus, a far broader range of diseases and mutations can be treated than is possible with viral delivery. For cationic polymers and lipids, because the DNA is packaged inside of the vector, it has good protection for its journey through the body, ensuring that it arrives intact within the cell\textsuperscript{1}.

Unfortunately, non-viral vectors do have some disadvantages. First, while they do not provoke an immune response, some vectors, especially cationic lipids and polymers, are toxic to cells\textsuperscript{11,13}. It is specifically the cationic nature of these vectors that seems to cause the toxicity, as the positive charges interact with slightly anionic components inside cells to prevent normal function\textsuperscript{11}. Inorganic nanoparticles, however, tend to have fairly low toxicity\textsuperscript{13}.

Second, transfection efficiencies \textit{in vivo} for all non-viral vectors are significantly lower than for viral vectors or even naked DNA\textsuperscript{1,8,9,13}. Since they lack the natural abilities of viruses to cross the cell wall and cause their genes to be processed into proteins, the difficulty lies in entering the cell and integrating the delivered DNA into cellular DNA.

Finally, because the DNA simply adheres to the surface of many inorganic nanoparticles, rather than being encapsulated inside them, the DNA is rather unprotected for its journey to the cell and faces many of the same problems as naked DNA\textsuperscript{13}. Thus, even though nanoparticles have low toxicity, many still fail to effectively deliver genes.

\textbf{1.3.3 Characteristics of good non-viral gene delivery vectors}

While non-viral vectors hold much promise for gene delivery, they still lag behind viral vectors in many areas. In order, then, to work towards improving non-viral vectors, the characteristics of good non-viral vectors must first be established. First, non-viral vectors must possess high transfection efficiencies. This includes being able to enter a
cell via endocytosis and escape the endosomal pathway and degradation within the endosome\textsuperscript{15}. From this point, in order to demonstrate a high gene expression, the DNA must be equipped to enter the nucleus and successfully produce the proteins encoded in it\textsuperscript{1}. Second, both prior to entering the cell and within the cell, the vector must protect the DNA from enzymatic degradation\textsuperscript{1,3}. Third, the non-viral vector should have low immunogenicity and low toxicity, and be biodegradable\textsuperscript{1}. With these qualities the vector can effectively deliver the genes without harming the cell or remaining in the cell long-term. Fourth, since a cell’s surface is anionic, vectors should be able to at least neutralize the negative charge on DNA’s backbone in order to avoid being repelled by the negative charge on the cell\textsuperscript{3}. Finally, a non-viral vector must be able to condense the bulky DNA to a size small enough to enter the cell via endocytosis\textsuperscript{3,11}.

### 1.4 Cationic polymer vectors

Because they already meet some of these requirements, cationic polymers as non-viral vectors are of particular interest. First, because of the positive charge on their backbone, when they condense with DNA through electrostatic interactions, cationic polymers neutralize the anionic DNA and overcome the repulsion of the cell’s surface. Second, while cationic lipids can also neutralize DNA, these lipids are unstable, while cationic polymers are stable\textsuperscript{9}. Third, cationic polymers can collapse the DNA into smaller complexes than cationic lipids can\textsuperscript{11,16,17}.

Finally, due to their size, cationic polymers can take advantage of an occurrence in cancerous tumors referred to as the “enhanced permeability and retention” (EPR) effect\textsuperscript{2,18}. The EPR effect results from changes in the vascular and lymphatic systems within a tumor. While capillaries normally do not allow macromolecules to diffuse across
their walls into surrounding tissue, in both tumors and inflamed areas within the human body, the permeability of the capillaries is increased\textsuperscript{2,18}. Thus, macromolecules are able to cross from within the capillaries into the interstitial spaces. This, in itself, is not necessarily problematic, as the lymphatic generally cleans these areas, carrying away the macromolecules. However, in tumors specifically, the lymphatic system does not function properly allowing the macromolecules to accumulate in the diseased tissue\textsuperscript{2,18}. Therefore, the EPR effect, observed for macromolecules larger than 20 kDa\textsuperscript{2}, provides natural targeting for gene delivery systems aimed particularly at cancer cells.

1.4.1 Natural cationic polymers

Cationic polymers can generally be divided into two categories. “Natural” polymers refer to those derived from molecules already found in nature, while synthetic polymers are discovered in laboratories. Of the many natural polymers studied for gene delivery, there are two that attract frequent attention: poly(L-lysine) and chitosan (Figure 3).

![Figure 3](image)

**Figure 3.** Common natural cationic polymers
Poly (L-lysine), one of the first polymers investigated for gene delivery, is polymerized from the amino acid, lysine, and is biodegradable. However, it possesses fairly low transfection efficiencies, even though it has a high cellular uptake, probably because it cannot effectively protect the genes while inside the endosome\textsuperscript{8,12}. Chitosan, also nontoxic and biodegradable, is a linear aminopolysaccharide derived from chitin\textsuperscript{12}. While chitosan does condense DNA to a very small size, like poly(L-lysine), it still has low transfection efficiency even though it effectively transports DNA into the cell due to the positive charges on the polymer backbone\textsuperscript{8,11,12,19}.

1.4.2 Synthetic cationic polymers

There are many synthetic polymers also studied for gene delivery. In order to be cationic, they generally contain amine groups, either in the backbone or pendent. The amines also provide two additional benefits. First, they cause the polymers to look like legitimate biological molecules, such as amino acids, to the immune system, thus making them effective stealth molecules. Second, primary and secondary amines are reasonably good nucleophiles, thus cationic polymers are easy to modify via nucleophilic substitution. Most cationic polymers are related to one of three main groups; poly(dimethylaminoethyl methacrylate) (PDMAEMA), poly(amidoamine) (PAMAM) dendrimer, and poly(ethylenimine) (PEI) (Figure 4).
PDMAEMA is of particular interest, because it contains tertiary amines. Tertiary amines are stronger bases than primary or secondary amines, and so are better able to protect the DNA from the acidic environment within the endosome. PDMAEMA has also demonstrated higher transfection efficiencies than PEI. However, PDMAEMA is still cytotoxic and the polyplexes remain in the endosome even after 24 hours. PAMAM is often found in dendrimer form, generally with cations capping the branches. Thus it more effectively encapsulates and protects the DNA from nucleases. However, as the amount of branching, and thus the cationic character, increases, so does the toxicity.

1.5 Poly(ethylenimine)

There are two types of PEI typically used for gene delivery – linear and branched (HPEI). Of the two, HPEI generally transfects cells better than linear PEI, and so is more often studied. Due to the high concentration of amines – one amine for every two carbons – HPEI has a high charge-to-mass ratio, giving it three important advantages as a gene delivery vector.

First, HPEI is especially good at condensing DNA due to the strong attraction between the many cations on the polymer and the anions on the DNA backbone. Second,
the excess positive charge increases the attraction of the polyplexes to the anionic cell membrane surface, which facilitates high rates of endocytosis. Finally, HPEI also excels in helping the genes to escape from the acidic environment within endosomes. The large quantity of amines makes HPEI an effective buffer, allowing it to act as a proton sponge. Once an endosome is formed, proton pumps in its wall start pushing in large quantities of protons to create an acidic environment, which is the ideal surrounding for nucleases to function. Since HPEI is a buffer, as protons enter the endosome, the neutral amines act as bases and become protonated, thus moderating the acidity and allowing only a slight drop in pH. Because the pH is not low enough, protein folding within the vacuole might be altered, preventing the normal function of nucleases and thus protecting the DNA/RNA from degradation. In order to reach the desired pH, the proton pumps continue to bring in more protons, which HPEI continues to absorb. As a result, the positive charges build up on the polymer and repel each other, causing HPEI to expand like a sponge (Scheme 2).

Scheme 2. The proton sponge effect of HPEI acting as a buffer.

At the same time, chloride anions are entering the endosome as counter ions. In response to the higher than usual concentration of charges within the endosome, water diffuses across the endosomal membrane to decrease the concentration (Scheme 2). The
combination of swelling HPEI and excess water within the endosome ruptures it, releasing the DNA into the cytoplasm\textsuperscript{22,23}.

The molecular weight of HPEI affects both the transfection efficiency and the toxicity of the polymer. As the molecular weight increases, so does the overall transfection efficiency\textsuperscript{21,24}, probably due to the increasing amount of positive charge. However, an augmentation in toxicity also accompanies the increase in molecular weight and cationic nature\textsuperscript{21,25}. Thus, a balance must be struck between transfection efficiency and toxicity, which explains the attraction of the commercially available molecular weight of 25kDa. While this size still shows high toxicity, modifications to the amines on HPEI, especially primary amines, can help to decrease the toxicity\textsuperscript{12}.

1.5.1 Modifications to PEI

There are two common group types used to modify HPEI. First, hydrophobic groups bring many potential advantages to gene delivery vectors, though the effects tend to vary depending on the polymer and hydrophobic group. Due to the length and flexibility of hydrophobic portions, they help to physically surround the DNA, rather than protecting it only via electrostatic attractions. Hydrophobic moieties also increase the attraction of the polyplex to the lipid cell membrane, thus increasing the transfection efficiency\textsuperscript{26}. Depending on the percent modification, hydrophobicity, and structure of the added functional groups, the cytotoxicity of the polymer vector can be decreased. However, there does not seem to be a linear correlation of cytotoxicity decreasing as hydrophobicity increases\textsuperscript{12,26}. Second, poly(ethyleneglycol) (PEG) modified HPEI has also been shown to increase transfection efficiency and decrease toxicity, as the PEG chains cover the positive surface charge on the polyplexes\textsuperscript{12}.
1.6 Stimuli responsive polymers

Stimuli responsive polymers, or smart polymers, are another subset of non-viral gene delivery vectors that offers the additional advantage of controlled release of therapeutic materials, whether genes or drugs. Smart polymers are capable of changing their chemical and physical, or conformational, properties in response to small external signals\textsuperscript{27,28}. Their abilities to bind and encapsulate or release molecules, and alter their shape are singularly applicable to gene delivery. Light, pH, and temperature responsive polymers are typically studied as non-viral vectors.

1.6.1 Light responsive polymers

There are two common responses to ultraviolet light – either a conformational change or cleavage of a bond. When irradiated with ultraviolet light, azobenzene absorbs a photon and is excited from the lower energy trans form to the higher energy cis form (Scheme 3).

![Scheme 3. Reversible switch from trans to cis of azobenzene](image)

Azobenzene derivatives can be used in conjunction with polymers to change the physical properties of the host polymer. Pillar[6]arene is a cyclic oligomer, which when modified with triethylene oxide groups (Figure 5) becomes water-soluble and develops a
lower critical solution temperature (LCST). An LCST is a reverse switching temperature for solubility – below the LCST a material is soluble, while above the LCST it is insoluble. When \textit{trans} azobenzene molecules, modified with two 1,4-diazabicyclo[2.2.2]octane (DABCO) (\textbf{Figure 5}), were added to the solution, the LCST increased as the azobenzene derivatives threaded through the cyclic oligomers and increased the order of the solution. However, when the solution was irradiated with ultraviolet light, the azobenzene derivatives switched to the \textit{cis} form, and no longer fit through the rings. Thus, aggregation occurred sooner and the LCST decreased\textsuperscript{29}.

\textbf{Figure 5.} A) Azobenzene modified with DABCO and B) Pillar[6]arene
If azobenzene could be functionalized with drugs, in the same fashion that it was functionalized with DABCO, this system could be used for drug delivery. At one temperature, the drug could be both protected inside the pillar[6]arene and later released upon site specific exposure to ultraviolet light.

1.6.2 pH responsive polymers

pH responsive polymers are created by synthesizing poly(acid)s or poly(base)s. Thus, as the pH of the solution is changed, the polymers become protonated or deprotonated creating positive and negative charges along the backbone and affecting both conformation and solubility. First, the charges repel each other, pushing the polymer apart and changing the conformation from a compact globule to an elongated coil (Scheme 4).

Scheme 4. Transition from globule to coil

Second, the solubility is also affected as the increasing charge makes the polymers, in effect poly(electrolyte)s, more soluble in aqueous solutions. The solubility can also be influenced by functional groups on the polymers, as hydrophobic groups will decrease solubility while hydrophilic groups will increase solubility in water.

Poly(β-amino ester), a poly(base) with a pKₐ around 6.5\(^{30}\), is soluble in solutions below pH 6.5, because it is protonated. However, as the pH becomes basic, the polymer
is deprotonated and becomes insoluble in water. Poly(ethyleneglycol) methyl ether (MPEG) was added as block copolymer (Figure 6) in order to provide a protective hydrophilic shell to the globule formed in basic conditions$^{30}$. The shell helps to protect the polyplex of polymer and DNA from degradation within the body$^{30,31}$.

![Figure 6. pH responsive MPEG-poly(β-amino ester) block copolymer](image)

Because the MPEG is hydrophilic, it also increases the switching pH to at least pH 7.2, depending on the ratio of MPEG added$^{30}$. Thus, this polymer can be used for gene delivery since at physiological pH it is clamped down in the globule form, capable of protecting DNA, but at the lower pH within endosomes the polymer can open up to the coil form, releasing the DNA.

### 1.6.3 Temperature responsive polymers

Like pH responsive polymers, many temperature responsive polymers also experience a conformational change from globule to coil depending on temperature. The switch occurs as a result of an LCST, and depends on the ratio of hydrophobic to hydrophilic groups. Poly($N$-isopropylacrylamide) (PNIPAAM), a common example of a thermo-responsive polymer, demonstrates an LCST between 30-34°C$^{32}$, because of the presence of the hydrophobic alkyl group on the amide moiety (Figure 7).
Since PNIPAAM is in the globule form at normal body temperature (37°C), it can effectively encapsulate DNA or drugs for delivery and rely on degradation in extremely acidic environments, such as the stomach, to release its contents.

1.7 Mechanism of LCST behavior

An LCST usually arises in polymers functionalized with hydrophobic groups, introducing two competing forces into solubility. While the hydrophilic portions are attracted to the water molecules, at the same time the water molecules repulse the hydrophobic portions of the polymer. Which of these forces will determine the solubility changes depending on the temperature of the solution and is related to the Gibbs’ free energy of mixing ($\Delta G = \Delta H - T\Delta S$). The formation of hydrogen bonds and polar attractions with water stabilizes the polymer and releases energy, making a negative (favorable) contribution to the enthalpy of mixing. However, while the hydrophilic portion is solvated, the solution is more orderly and makes an unfavorable entropy contribution to the free energy of mixing. As temperature increases break hydrogen bonds, the entropy term carries more weight and eventually offsets the enthalpy term, causing the solution to phase separate and become more disorderly. The phase separation occurs as the hydrophilic portion of the polymer is no longer hydrated and collapses from an extended coil to a globule. These globules then aggregate together, separating from the water.
1.7.1 Factors affecting LCST

There are different factors that can change the temperature at which the LCST occurs. First, the ratio of hydrophilic groups to hydrophobic groups plays a major role. If the ratio is larger, then there are more hydrogen bonds that can be formed and greater polar interactions with water, thus increasing the LCST as the favorable enthalpy contribution is larger. Conversely, if the ratio is smaller, the polymer will have fewer attractions to water and will become insoluble at a lower temperature. Second, pH can affect the LCST if the polymer contains acid or base moieties. For example, a basic polymer would become more protonated as the pH decreases, thus developing positive charges and becoming more ionic in character. As a result, the cations will be more strongly attracted to water than the neutral polymer, thus increasing the LCST.

This phenomenon allows the interactions between the basic polymer and the DNA to be altered at a single temperature by varying the pH (Scheme 5).

![Scheme 5. Effect of temperature and pH on polyplexes](image)

As the temperature increases, the polymer collapses to the globule form and clamps down on the DNA. However, if the pH is decreased, the LCST increases, re-expanding the polymer to the coil conformation and releasing the genes.
1.8 Previous work

Liu et al. worked to design a structural isomer of PNIPAAM by the addition of isobutyryl amide groups to HPEI (Figure 8)\(^{34}\).

![Figure 8. Amidated HPEI](image)

They found that amidated HPEI (HPEI-IBAm), like PNIPAAM, is a temperature responsive polymer that demonstrates an LCST. Unlike PNIPAAM, due to the nitrogens in the backbone that can be protonated, HPEI-IBAm is also responsive to pH changes (Scheme 5). The LCST of this polymer can also be tailored by modifying the percent of amide groups added. Liu et al. studied 70% and 100% amidated HPEI at varying pHs, which led to LCSTs ranging from approximately 12°C – 65°C.

Previous members of our group synthesized a series of polymers with varying percent alkylation and percent amidation. Polymers with increasing hydrophobicity from alkyl chains generally manifested a decrease in their LCST\(^{35}\). It was also shown that as the percent of amide groups increased, the LCST decreased due to the increasing hydrophobic character of the polymer\(^{36}\). Solution properties also affected the LCST. As the weight percent of the polymer in solution decreased, the LCST increased since the lower concentration made it more difficult for the polymer to aggregate\(^{36}\). The concentration of salt in the solution decreased the LCST, as less of the water molecules were free to solvate the polymer, being more strongly attracted to the ionic particles\(^{36}\).
One polymer was also made that was amidated and hydroxyethylated. As would be expected from the increasing hydrophilicity, the LCST did increase slightly compared to the amidated polymer\textsuperscript{36}.

### 1.9 Current work

The goal of this project was to improve transfection efficiency, decrease toxicity, and tune the LCST of HPEI-IBAm through modification with different functional groups. Part of the goal in investigating the LCST was to determine what effect solubility behavior has on transfection efficiency. For example, does an abrupt release caused by a rapid change in solubility transfect cells better than a gradual release cause by a slow change in solubility? A second part of improving transfection is related to the ability of the polymer vector to carry the genes across the cell wall. Since the cell wall contains a hydrophobic interior, modification with hydrophobic functional groups, such as alkyl chains, could improve the endocytosis process. The addition of long functional groups, whether hydrophobic or hydrophilic, can also improve cytotoxicity by shielding the positive charges on HPEI. Third, modification with long chains allows the opportunity to add a targeting group that will be more exposed to the cell receptors than if it were hidden on the polymer backbone. However, the beneficial effects of targeting groups and decreased cytotoxicity must be balanced with the effect of these functional groups on the LCST.

As the first step in this project, then, the effect on solubility behavior of increasing hydrophobicity was to be determined. Next, the hydrophobic groups were to be tempered with a small hydrophilic group in order to provide greater control over solubility.
behavior. Finally, the effect of PEG and PEG mixed with hydrophobic groups was to be studied.
2. EXPERIMENTAL

2.1 Instrumentation

$^1$H and $^{13}$C Nuclear Magnetic Resonance (NMR) spectra were recorded on a Bruker AVANCE 300 MHZ instrument operating at 300 and 75.5 MHz, respectively. Samples were dissolved in D$_2$O or CDCl$_3$ at a concentration of (~ 30 mg / 0.7 mL). Samples were dried using a Savant Speed-Vac (SC110) with attached Refrigerated Vapor Trap (RVT4104). Transmittance data were obtained on a Hewlett Packard 8453 Ultraviolet-visible spectrometer with an external heating source.

2.2 Materials

Hyperbranched polyethylenimine (HPEI), Lupasol® WF (BASF, $M_n = 10,000$g/mol), was dried in vacuo using the speed vacuum prior to use. Triethylamine (TEA, Sigma-Aldrich, 99%) and dimethyl sulfoxide (DMSO, Sigma-Aldrich, 99%) were dried over CaH$_2$ and distilled under nitrogen. Isobutyryl chloride (Sigma-Aldrich, 98%) was distilled under nitrogen. Anhydrous tetrahydrofuran (THF, Sigma-Aldrich, 99.9%), 2-bromoethanol (Sigma-Aldrich, 95%), 1-bromobutane (Sigma-Aldrich, ReagentPlus 99%), 1-bromohexane (Sigma-Aldrich, 98%), 1-bromoctane (Sigma-Aldrich, 99%), 1-bromododecane (Sigma-Aldrich, 97%), and poly(ethylene glycol) methyl ether tosylate (TPEG, Sigma-Aldrich, $M_n = 900$g/mol) were used as received. Reagent-grade anhydrous potassium carbonate powder ($\text{K}_2\text{CO}_3$, Sigma-Aldrich, 99%) was dried at 130 °C in an oven prior to use. Ethanol (EtOH, Decon Laboratories) and methanol (MeOH, Fischer
Scientific) were used as received. Sodium hydroxide (NaOH, VWR) and hydrochloric acid (HCl, Macron) were used as received. All water utilized was ultra-purified via a three-step purification process. Initially, the water went through Wright State’s reverse osmosis system, followed by an ion-exchange-membrane provided by Dayton Water Company. Finally, it was passed through a filter (carbon, deionization, deionization, organic absorption, and ultra violet) Labconco Water Pro Plus system.

2.3 Synthesis of HPEI-IBAm

To an oven-dried 500 mL RBF with septum inlet equipped with water condenser, nitrogen inlet, and stir bar were added 2.00 g (33.9 mmol of modifiable amines) of dried HPEI, 40.0 mL (287 mmol) TEA, and 300 mL anhydrous THF. The flask was cooled to 0°C in an ice bath. Then 2.13 mL (20.3 mmol) of distilled isobutyryl chloride were added dropwise under vigorous stirring. The mixture was allowed to warm to room temperature overnight and then heated to 50°C for 30 minutes. The solvent was evaporated in vacuo, the residue was dissolved in DDI H2O and placed in aqueous dialysis for 48 hours. The water was then evaporated using the speed-vacuum to afford 2.70 g (79%) of a pale yellow, glassy material. 1H-NMR (D2O, δ): 0.98 (s, 6H), 2.33-3.54 (broad, 13H). 13C-NMR (D2O, δ): 18.9, 30.0, 35.0, 37.5, 45.4-51.0.

2.4 Synthesis of HPEI-IBAm-Bu

In an oven-dried 5 mL RBF, equipped with a stir-bar, water condenser, and nitrogen inlet, 0.150 g (0.590 mmol of modifiable amines) of HPEI-IBAm were dissolved in 0.90 mL of THF. The RBF was placed in a 65°C preheated oil bath and 67 µL (0.619 mmol) of 1-bromobutane were added. After 48 hours the reaction was quenched with 0.0252 g (0.619 mmol) of NaOH dissolved in 0.25 mL of MeOH. After 24
hours the solvent was evaporated and the residue dissolved in DDI H$_2$O. Dialysis was done in DDI H$_2$O for 48 hrs. The water was then evaporated using the speed-vacuum to give 0.118 g (64.5%) of a dark yellow glassy solid. $^1$H-NMR (D$_2$O, $\delta$): 0.87 (s, 3H), 1.01 (s, 6H), 1.31 (s, 2H), 1.62 (s, 2H), 2.36-3.59 (broad, 15H).

### 2.5 Synthesis of HPEI-IBAm$_{0.60}$-Bu$_{0.40}$ delayed addition of K$_2$CO$_3$

In an oven-dried 5 mL RBF, equipped with water condenser, nitrogen inlet, and stir bar, 0.156 g (2.11 mmol of modifiable amines) of HPEI-IBAm$_{0.60}$ were dissolved in 0.90 mL of EtOH. Then 0.128 g (0.926 mmol) of K$_2$CO$_3$ were added, and the RBF was placed in a 65°C preheated oil bath. Then 68 µL (0.573 mmol) of 1-bromobutane were added. After 72 hours the reaction mixture was cooled, dissolved in DDI H$_2$O, and placed in aqueous dialysis for 48 hours. The water was then evaporated using the speed-vacuum to afford 0.0759 g (33.5%) of a yellow glass solid. $^1$H-NMR (D$_2$O, $\delta$): 0.92 (s, 3H), 1.03 (s, 6H), 1.37 (s, 2H), 1.68 (s, 2H), 2.39-3.67 (broad, 15H).

### 2.6 Synthesis of HPEI-IBAm$_{0.60}$-Bu$_{0.40}$ delayed addition of K$_2$CO$_3$

In an oven-dried 5 mL RBF, equipped with water condenser, nitrogen inlet, and stir bar, 0.136 g (1.84 mmol of modifiable amines) of HPEI-IBAm$_{0.60}$ were dissolved in 0.78 mL of EtOH. The RBF was placed in a 65°C preheated oil bath and then 60 µL (0.538 mmol) of 1-bromobutane were added. After 48 hours, 0.111 g (0.806 mmol) of K$_2$CO$_3$ were added. After 24 hours, the reaction mixture was cooled, dissolved in DDI H$_2$O, and placed in aqueous dialysis for 48 hours. The water was then evaporated using the speed-vacuum to afford 0.145 g (88%) of a solid yellow gel. $^1$H-NMR (D$_2$O, $\delta$): 0.87 (s, 3H), 1.01 (s, 6H), 1.24 (s, 2H), 1.57 (s, 2H), 2.40-3.59 (broad, 15H). $^{13}$C-NMR (D2O, $\delta$): 13.21, 18.92, 20.15, 27.67, 29.95, 35.05, 36.37-37.79, 43.37-53.94.
2.7 Synthesis of HPEI-IBAm$_{0.60}$-He$_{0.28}$

In an oven-dried 5 mL RBF, equipped with water condenser, nitrogen inlet, and stir bar, 0.145 g (1.96 mmol of modifiable amines) of HPEI-IBAm$_{0.60}$ were dissolved in 0.85 mL of EtOH. Then 0.119 g (0.860 mmol) of K$_2$CO$_3$ were added and the RBF was placed in a 65°C preheated oil bath. Then 83 µL (0.573 mmol) of 1-bromohexane were added. After 72 hours the reaction mixture was cooled, dissolved in DDI H$_2$O, and placed in aqueous dialysis for 48 hours. The water was then evaporated using the speed-vacuum to afford 0.178 g (98%) of a yellow glassy solid. $^1$H-NMR (D$_2$O, δ): 0.80 (s, 3H), 1.01 (s, 6H), 1.26 (s, 6H), 1.68 (s, 2H), 2.38-3.69 (broad, 15H).

2.8 Synthesis of HPEI-IBAm$_{0.60}$-He$_{0.40}$ delayed addition of K$_2$CO$_3$

In an oven-dried 5 mL RBF, equipped with water condenser, nitrogen inlet, and stir bar, 0.128 g (1.74 mmol of modifiable amines) of HPEI-IBAm$_{0.60}$ were dissolved in 0.74 mL of EtOH. The RBF was placed in a 65°C preheated oil bath. Then 75 µL (0.507 mmol) of 1-bromohexane were added. After 48 hours, 0.105 g (0.764 mmol) of K$_2$CO$_3$ were added. After 24 hours, the reaction mixture was cooled, dissolved in DDI H$_2$O, and placed in aqueous dialysis for 48 hours. The water was then evaporated using the speed-vacuum to afford 0.0321g (14%) of a solid yellow gel. $^1$H-NMR (D$_2$O, δ): 0.80 (s, 3H), 1.01 (s, 6H), 1.25 (s, 6H), 1.68 (s, 2H), 2.38-4.05 (broad, 15H). $^{13}$C-NMR (D$_2$O, δ): 13.76, 18.90, 22.04, 23.16, 25.30, 30.00, 35.03, 37.58, 41.84-54.84.

2.9 Synthesis of HPEI-IBAm$_{0.60}$-Oc$_{0.24}$

In an oven-dried 5 mL RBF, equipped with a stir-bar, water condenser, and nitrogen inlet, 0.149 g (0.587 mmol of modifiable amines) of HPEI-IBAm$_{0.60}$ were dissolved in 0.90 mL of THF. The RBF was placed in a 65°C preheated oil bath. 0.107
mL (0.617 mmol) of 1-bromooctane were added. After 48 hours the reaction was quenched with 0.0244 g (0.617 mmol) of NaOH dissolved in 0.24 mL of MeOH. After 24 hours the solvent was evaporated and the residue dissolved in DDI H₂O. Dialysis was done in DDI H₂O for 48 hrs. The water was then evaporated using the speed-vacuum to give 0.194 g (90.7%) of a dark yellow reddish glassy solid. \(^1\)H-NMR (D₂O, δ): 0.81 (s, 3H), 1.01 (s, 6H), 1.22 (s, 10H), 1.59 (s, 2H), 2.42-3.86 (broad, 15H). \(^{13}\)C-NMR (D₂O, δ): 13.91, 19.07, 22.56, 25.68, 29.95, 35.00-35.16, 35.50-37.70, 43.32-53.09.

2.10 Synthesis of HPEI-IBAm\(_{0.60}\)-DoDec\(_{0.24}\)

In an oven-dried 5 mL RBF, equipped with a stir-bar, water condenser, and nitrogen inlet, 0.137 g (0.539 mmol of modifiable amines) of HPEI-IBAm\(_{0.60}\) were dissolved in 0.83 mL of THF. The RBF was placed in a 65°C preheated oil bath. 0.137 mL (0.567 mmol) of 1-bromododecane were added, causing the polymer to form a ball. The following day, 0.3 mL of EtOH were added to fully dissolve the polymer. After 48 hours the reaction was quenched with 0.0227 g (0.567 mmol) of NaOH dissolved in 0.25 mL of MeOH. After 24 hours the solvent was evaporated and the residue dissolved in DDI H₂O. Dialysis was done in DDI H₂O for 48 hrs. The water was removed with the speed-vacuum to give 0.165 g (73%) of a yellow brown viscous gel. \(^1\)H-NMR (D₂O, δ): 0.82 (s, 3H), 1.01 (s, 6H), 1.23 (s, 18H), 1.75 (s, 2H), 2.41-3.69 (broad, 15H). \(^{13}\)C-NMR (CDCl₃, δ): 14.1, 19.7, 22.6, 29.4, 31.9, 35.3, 46.0-52.3, 178.1.

2.11 Synthesis of HPEI-IBAm\(_{0.64}\)-EtOH\(_{0.18}\)-Bu\(_{0.18}\)

In an oven-dried 5 mL RBF, equipped with water condenser, nitrogen inlet, and stir bar, 0.250 g (2.42 mmol of modifiable amines) of HPEI-IBAm\(_{0.64}\) were dissolved in 1.5 mL THF. The RBF was placed in a 65°C preheated oil bath. Then, 50 µL (0.467
(0.467 mmol) of 1-bromobutane were added. After 48 hours the reaction was quenched with 0.0187 g (0.467 mmol) of NaOH in 0.19 mL of MeOH and allowed to stir for 24 hours. After 24 hours the solvent was evaporated and the residue was redissolved in EtOH in the same flask. Then, 0.033 mL (0.467 mmol) of 2-bromoethanol were added. After 48 hours the reaction was quenched with 0.0190 g (0.467 mmol) of NaOH in 0.19 mL of MeOH and allowed to stir for 24 hours, at which point the solvent was evaporated, the residue was dissolved in DDI H$_2$O and placed in aqueous dialysis for 48 hours. The water was then evaporated using the speed-vacuum to afford 0.0990 g (33.4%) of a dark yellow-brownish glassy solid. $^1$H-NMR (D$_2$O, $\delta$): 0.91 (s, 3H), 1.02 (s, 6H), 1.33 (s, 2H), 1.65 (s, 2H), 2.42-4.02 (broad, 20H).

2.12 Synthesis of HPEI-IBAm$_{0.60}$-EtOH$_{0.20}$-He$_{0.20}$

In an oven-dried 5 mL RBF, equipped with water condenser, nitrogen inlet, and stir bar, 0.143 g (1.42 mmol of modifiable amines) of HPEI-IBAm$_{0.60}$ were dissolved in 0.83 mL EtOH. The RBF was placed in a 65°C preheated oil bath and 20 µL (0.284 mmol) of 2-bromoethanol were added. After 48 hours the reaction was quenched with 0.0113 g (0.281 mmol) of NaOH in 0.11 mL of MeOH. After 24 hours 40 µL (0.284 mmol) of 1-bromohexane were added to the same flask. After 48 hours the reaction was quenched with 0.0113 g (0.284 mmol) of NaOH in 0.11 mL of MeOH and allowed to stir for 24 hours, at which point the reaction solution was dissolved in DDI H$_2$O, and placed in aqueous dialysis for 48 hours. The water was then evaporated using a Savant speed-vacuum system to afford (72.5%) of a yellow glassy solid. $^1$H-NMR (D$_2$O, $\delta$): 0.81 (s, 3H), 1.00 (s, 6H), 1.25 (s, 6H), 1.63 (s, 2H), 2.37-4.09 (broad, 20H). $^{13}$C-NMR (D$_2$O, $\delta$): 13.39, 18.95, 21.79, 25.47, 29.95, 30.53, 35.03, 36.09-37.85, 43.32-59.96.
2.13 Synthesis of HPEI-IBAm$_{0.60}$-EtOH$_{0.20}$-Oc$_{0.20}$

In an oven-dried 5 mL RBF, equipped with water condenser, nitrogen inlet, and stir bar, 0.142 g (1.41 mmol of modifiable amines) of HPEI-IBAm$_{0.60}$ were dissolved in 0.48 mL EtOH. The RBF was placed in a 65°C preheated oil bath and 20 µL (0.281 mmol) of 2-bromoethanol were added. After 48 hours the reaction was quenched with 0.0112 g (0.281 mmol) of NaOH in 0.11 mL of MeOH. After 24 hours 49 µL (0.281 mmol) of 1-bromooctane were added to the same flask. After 48 hours the reaction was quenched with 0.0112 g (0.281 mmol) of NaOH in 0.11 mL of MeOH and allowed to stir for 24 hours, at which point the solvent was evaporated, the residue was dissolved in DDI H$_2$O, and placed in aqueous dialysis for 48 hours. The water was then evaporated using a Savant speed-vacuum system to afford 0.153 g (82.7%) of a dark yellow-brownish viscous gel. $^1$H-NMR (D$_2$O, δ): 0.79 (s, 3H), 0.97 (s, 6H), 1.20 (s, 10H), 1.40 (s, 2H), 2.37-4.04 (broad, 20H). $^{13}$C-NMR (D$_2$O, δ): 14.0, 19.0, 22.6, 29.3, 30.5, 31.9, 35.0, 29.3-37.8, 43.7-59.2, 62.6, 72.0, 180.7.

2.14 Synthesis of HPEI-IBAm$_{0.63}$-EtOH$_{0.19}$-DoDec$_{0.18}$

In an oven-dried 5 mL RBF, equipped with water condenser, nitrogen inlet, and stir bar, 0.145 g (1.43 mmol of modifiable amines) of HPEI-IBAm$_{0.63}$ were dissolved in 0.48 mL of ethanol. The RBF was placed in a 65°C preheated oil bath. Then 19 µL (0.267 mmol) of 2-bromoethanol were added. After 48 hours the reaction was quenched with 0.0107 g (0.267 mmol) of NaOH in 0.107 mL of MeOH. After 24 hours 61 µL (0.253 mmol) of 1-bromododecane were added. After 48 hours the reaction was quenched with 0.0101 g (0.253 mmol) of NaOH in 0.101 mL of MeOH. After 24 hours the solvent was evaporated and the residue dissolved in DDI H$_2$O and placed in aqueous
dialysis for 48 hours. The water was then evaporated using a Savant speed-vacuum system to afford 0.153 g (77%) of a yellow glassy solid. \(^1\text{H-NMR}\) (D\(_2\)O, \(\delta\)): 0.81 (s, 3H), 1.00 (s, 6H), 1.21 (s, 20H), 2.41-4.08 (broad, 20H).

2.15 Synthesis of HPEI-IBAm\(_{0.60}\)-PEG\(_{0.25}\)

In an oven-dried 5 mL RBF, equipped with water condenser, nitrogen inlet, and stir bar, 0.158 g (2.14 mmol of modifiable amines) of HPEI-IBAm\(_{0.60}\) were dissolved in 1.3 mL of DMSO. Then 0.130 g (0.939 mmol) of K\(_2\)CO\(_3\) were added and the RBF was placed in a 65°C preheated oil bath. Then 0.599 g (0.664 mmol) of TPEG in 0.2 mL of DMSO were added. After 72 hours the reaction mixture was cooled, dissolved in DDI H\(_2\)O, and placed in aqueous dialysis for 48 hours. The water was then evaporated using the speed-vacuum to afford 0.246 g (57%) of a brownish viscous gel. \(^1\text{H-NMR}\) (D\(_2\)O, \(\delta\)): 1.02 (s, 6H), 2.34 (s, 1H), 2.41-4.16 (broad, 16H), 3.32 (s, 3H), 3.64 (s, 64H). \(^{13}\text{C-NMR}\) (D\(_2\)O, \(\delta\)): 18.93, 29.93, 35.08, 37.21-38.79, 41.98-53.54, 58.09, 63.71-66.52, 69.62, 71.03.

2.16 Synthesis of HPEI-IBAm\(_{0.60}\)-PEG\(_{0.23}\)-Bu\(_{0.17}\)

In an oven-dried 5 mL RBF, equipped with water condenser, nitrogen inlet, and stir bar, 0.127 g (0.513 mmol of modifiable amines) of HPEI-IBAm\(_{0.60}\)-PEG\(_{0.23}\) were dissolved in 0.3 mL of EtOH. Then 0.0213 g (0.154 mmol) of K\(_2\)CO\(_3\) were added. The RBF was placed in a 65°C preheated oil bath. Then 11 \(\mu\)L (0.103 mmol) of 1-bromobutane were added. After 72 hours the reaction mixture was cooled, dissolved in DDI H\(_2\)O, and placed in aqueous dialysis for 48 hours. The water was then evaporated using the speed-vacuum to afford 0.121 g (85%) of a brownish viscous gel. \(^1\text{H-NMR}\) (D\(_2\)O, \(\delta\)): 0.89 (s, 3H), 1.02 (s, 6H), 1.26 (s, 2H), 1.38 (s, 2H), 2.41-4.16 (broad, 15H),
3.32 (s, 3H), 3.64 (s, 64H). $^{13}$C-NMR (D$_2$O, δ): 13.35, 18.96, 25.17, 29.97, 35.01, 36.59-37.77, 42.49-47.51, 49.14-51.67, 52.16-53.88, 58.01, 69.6, 71.03.

2.17 Synthesis of HPEI-IBAm$_{0.60}$-PEG$_{0.25}$-Oc$_{0.15}$

In an oven-dried 5 mL RBF, equipped with water condenser, nitrogen inlet, and stir bar, 0.162 g (0.595 mmol of modifiable amines) of HPEI-IBAm$_{0.60}$-PEG$_{0.25}$-Oc$_{0.15}$ were dissolved in 0.3 mL of EtOH. The RBF was placed in a 65°C preheated oil bath. Then 15 µL (0.0893 mmol) of 1-bromooctane were added. After 48 hours, 0.0195 g (0.0893 mmol) of K$_2$CO$_3$ were added. After 24 hours, the reaction mixture was cooled, dissolved in DDI H$_2$O, and placed in aqueous dialysis for 48 hours. The water was then evaporated using the speed-vacuum to afford 0.143 g (81%) of a dark brown glassy solid. $^1$H-NMR (D$_2$O, δ): 0.83 (s, 3H), 1.00 (s, 6H), 1.23 (s, 12H), 3.30 (s, 3H), 3.62 (s, 64H), 2.36-4.14 (broad, 15H).

2.18 Synthesis of HPEI-IBAm$_{0.60}$-PEG$_{0.23}$-DoDec$_{0.17}$

In an oven-dried 5 mL RBF, equipped with water condenser, nitrogen inlet, and stir bar, 0.106 g (0.387 mmol of modifiable amines) of HPEI-IBAm$_{0.60}$-PEG$_{0.23}$-DoDec$_{0.17}$ were dissolved in 0.3 mL of EtOH. The RBF was placed in a 65°C preheated oil bath. Then 16 µL (0.0658 mmol) of 1-bromobutane were added. After 48 hours, 0.0135 g (0.0658 mmol) of K$_2$CO$_3$ were added. After 24 hours, the reaction mixture was cooled, dissolved in DDI H$_2$O, and placed in aqueous dialysis for 48 hours. The water was then evaporated using the speed-vacuum to afford 0.102 g (90%) of a medium brown glassy solid. $^1$H-NMR (D$_2$O, δ): 0.84 (s, 3H), 1.01 (s, 6H), 1.22 (s, 20H), 3.31 (s, 3H), 3.62 (s, 64H), 2.36-4.17 (broad, 20H).
2.19 Characterization

2.19.1 General procedure for determining LCST

Transmittance data were obtained at 500 nm, at a heating rate of 1 °C min\(^{-1}\) under air. Polymers were dissolved in DDI water at a concentration of 2 wt. %. Dilute HCl and NaOH were used to adjust the pH to 7.4 or 5. The LCST was defined as the temperature corresponding to the 50% transmittance in the resulting transmittance versus temperature curves.
3. RESULTS AND DISCUSSION

Three different polymer series were synthesized in order to study the effect of different functional groups on solubility behavior and to pave the way for further transfection studies and eventual modification with targeting groups. First, an amidated HPEI polymer was synthesized and used as the foundation for all further modifications. Second, a series of polymers modified with increasing lengths of hydrophobic alkyl chains, namely butyl, hexyl, octyl, and dodecyl, was prepared and characterized. Third, another series was synthesized containing an equal split between the same alkyl chains and a hydroxyethyl group. Finally, the polymer form of hydroxyethyl, poly(ethyleneglycol) (PEG), was used to prepare an analogous series of mixed alkyl and PEG modifications.

The HPEI used from BASF contains primary, secondary, and tertiary amines in a ratio of 1:1:20:0.76\textsuperscript{37}. Tertiary amines are not available for modification, because attack at a tertiary amine would lead to an unstable quaternary ammonium species. Since only primary and secondary amines are available for modification via S\textsubscript{N}2 reactions, 73% of the amines in HPEI can be modified. All percent modifications are calculated as percentages of this initial 73%.

3.1 Characterization and transmission of HPEI-IBAm\textsubscript{0.60}

The first step was to create the structural isomer of PNIPAAM by amidating HPEI with isbutyryl chloride. The goal was to introduce enough hydrophobicity to cause a LCST below 37°C at pH 7.4, but leave sufficient primary and secondary amines available
for further modification with other functional groups. The procedure from Liu et al.\textsuperscript{34} was followed with minor modifications, as shown in Scheme 6, to prepare 60% amidated HPEI (HPEI-IBAm\textsubscript{0.60}). HPEI was dissolved in a mixture of TEA and anhydrous THF, cooled to 0°C in an ice bath, and then isobutyryl chloride was added dropwise. After warming to room temperature overnight, the reaction mixture was heated to 50°C for 30 minutes to finalize the reaction. After cooling, the solvent was evaporated and the residue dissolved in deionized water and purified by dialysis.

\begin{center}
\textbf{Scheme 6.} Synthesis of HPEI-IBAm\textsubscript{0.60} (1)
\end{center}

\begin{center}
\textsuperscript{1}H-NMR and \textsuperscript{13}C-DEPT 135 NMR spectroscopy were used to characterize the product. Compared to the \textsuperscript{1}H-NMR spectrum of HPEI (Figure 9), two differences are seen in the peaks from the backbone hydrogens at 2.56-2.63 ppm. First, the peaks broaden, due to the presence of slightly different chemical environments. Second, a new set of backbone peaks develops slightly downfield at 3.05-3.57 ppm, as the hydrogens are more deshielded by the electron withdrawing amide groups. A new peak appears at 0.98 ppm from the methyl hydrogens on the amide groups.
\end{center}
The percent modification of HPEI was calculated by comparing the actual integration ratios from the $^1$H-NMR spectrum to the theoretical integration ratios for 100% amidated HPEI, as demonstrated in Equation 1. For the theoretical ratio, the six methyl hydrogens on the amide group are multiplied by the ratio of amines available for modification, 0.73, and then divided by the four hydrogens from the polymer backbone. The actual integration ratio is found by dividing the six methyl hydrogens by the integration of the backbone peaks, minus one hydrogen from the methine carbon in the amide group.

**Figure 9.** 300 MHz $^1$H-NMR spectral (D$_2$O) overlay of HPEI and HPEI-IBAm$_{0.60}$ (1)
Theoretical Ratio: \[
\frac{(6)(0.73)}{4} = 1.095
\]

Actual Ratio: \[
\frac{6}{10.2 - 1} = 0.652
\]

Percent Amidation: \[
\frac{0.652}{1.095} \times 100\% = 60\%
\]

Equation 1. Calculation of percent amidation

Of particular interest in the $^{13}$C-DEPT 135 spectrum (Figure 10), which shows $–$CH$_2$– carbons down, and $–$CH– and $–$CH$_3$– carbons up, are the three positive peaks. There are only two possibilities for these three peaks – the primary carbons and the methine $\alpha$ carbon in the isobutryl amide groups. The peak at 18.9 ppm represents the methyl carbon. Thus, the two remaining peaks at 30.0 ppm and 35.0 ppm must be the methine carbons in two different environments; namely, bonded to either a tertiary amide or a secondary amide. Because a tertiary amide is bonded to two electron-donating groups, it is more willing to share its electrons by resonance with the carbonyl group. Thus, the inductive effect of the carbonyl group on the $\alpha$ carbon is reduced, leaving the $\alpha$ carbon with more electron density. In contrast, the secondary amide only has one electron-donating group and therefore does resonance with the carbonyl group to a lesser extent. As a result, the carbonyl group has a stronger inductive effect on the $\alpha$ carbon, increasing the partial positive charge on this carbon. Therefore, in the carbon NMR spectrum, the $\alpha$ carbon adjacent to the tertiary amide will be more shielded and farther upfield than the $\alpha$ carbon in the secondary amide group$^{38}$. 
The last step in creating this thermo- and pH- responsive polymer was to determine the LCST at pH 7.4 (pH of the bloodstream) and pH 5 (pH within a lysosome). A UV/VIS spectrometer was used to determine the LCST, since the % transmittance decreased as the polymer became insoluble. The LCST was taken as the midpoint of the drop in transmittance. As can be seen in Figure 11, the LCST at pH 7.4 was at 24.0 °C and well below body temperature. As the acidity of the solution increased to pH 5, more amines became protonated, increasing the water solubility and leading to a significant increase in the LCST to 41.5 °C, a few degrees above body temperature.

**Figure 10.** 75.5 MHz $^{13}$C DEPT 135 NMR spectrum (D$_2$O) of HPEI-IBA$_m$$_{0.60}$ (1)
3.2 Effects of increasing hydrophobicity on transmission

The next step was to study the effect of increasing alkyl chain length, and thus increasing hydrophobicity, on the LCST. The LCST was expected to decrease as alkyl chain length increased, but some previous research saw a reversal in that trend for longer chains. When Simons studied polymers that had been propoxylated and alkylated, he observed that polymers with decyl functional groups had a higher LCST than polymers with butyl, pentyl and hexyl groups. Winnik et al. incorporated octadecyl chains, either as block copolymers or pendant functional groups, into PNIPAAM and found that the LCST of the hydrophobically modified polymers remained fairly close to the LCST of PNIPAAM. The long alkyl chains seem to be capable of folding up into micelles, with
the hydrophilic portion of the polymer on the outside protecting and keeping in solution the hydrophobic portion on the inside of the micelle\textsuperscript{39}.

A series of four polymers with increasing hydrophobicity were synthesized, generally following the procedures used by Simons and Skidmore\textsuperscript{35,36} (Scheme 7). In the initial procedures, HPEI-IBAm\textsubscript{0.60} was dissolved in THF for these reactions. However, due to solubility issues, later reactions were done in EtOH, and EtOH was also added to the initial reactions using octyl and dodecyl groups. The reactions were done at 65 °C, allowing 48 hours for the initial S\textsubscript{N}2 reaction of the bromoalkanes and 24 hours for quenching the reactions with NaOH. Unfortunately, complete 40\% alkylation did not occur, as \textsuperscript{1}H-NMR spectra showed alkylation at roughly 20\%. For the latter, the calculation process will be shown later.

Due to the lower than expected alkylation of the reactions quenched with NaOH, the later alkylations, using 1-bromobutane and 1-bromohexane, were attempted using K\textsubscript{2}CO\textsubscript{3} as a proton trap. The reactions were done two ways – with K\textsubscript{2}CO\textsubscript{3} present at the outset, and with K\textsubscript{2}CO\textsubscript{3} added after 48 hours – with no significant difference. However, using K\textsubscript{2}CO\textsubscript{3} did result in alkylation at the intended percentage.

\textbf{Scheme 7.} Synthesis of alkylated and amidated HPEI
$^1$H-NMR and $^{13}$C-DEPT 135 NMR spectroscopy were used to confirm the structure of the functionalized HPEI. As shown in the $^1$H-NMR spectrum of 2a (Figure 12), while the peak at 1.01 ppm attributed to the methyl carbons on the amide functional group remained the same, new peaks developed in the aliphatic region. The peak at 0.87 ppm corresponds to the methyl group at the end of the alkyl chain, the hydrogens farthest away from electron withdrawing groups and thus the most shielded. The peaks at 1.24 ppm and 1.57 ppm represent two hydrogens, each on the carbons closer to the nitrogen. The last two hydrogens on the butyl chain at 1.57 ppm (peak h) – those adjacent to the nitrogen – are farther downfield and overlap with the peaks from the backbone hydrogens.

![Figure 12](image_url)

**Figure 12.** 300 MHz $^1$H-NMR spectrum (D$_2$O) of HPEI-IBAm$_{0.60}$-Bu$_{0.40}$ (2a)
The integration values from this proton NMR spectrum can also be used to determine percent alkylation, by calculating the theoretical values from the expected percentage and comparing those to the actual integration values. The theoretical values are determined according to **Equation 2**, by multiplying the number of hydrogens in each functional group times the expected modification ratio. The downfield hydrogens were set to the calculated value, and then the upfield hydrogens were integrated, giving an integration value of 4.88, a difference of only 0.21 from the theoretical value.

\[
\begin{align*}
\text{Upfield Hydrogens:} & \quad (6)(0.73)(0.60) + (7)(0.73)(0.40) = 4.67 \\
\text{Downfield Hydrogens:} & \quad 4 + (1)(0.73)(0.60) + (2)(0.73)(0.40) = 5.02
\end{align*}
\]

**Equation 2.** Example calculation of percent alkylation

The same peak trends are seen in the \(^1\text{H}-\text{NMR}\) spectra for 2b, 2c, and 2d, as shown in **Figure 13**. The peak at roughly 1.24 ppm becomes more intense, compared to the other alkyl peaks, as the length of the carbon chain increases.
Figure 13. 300 MHz $^1$H-NMR spectral (D$_2$O) overlay of HPEI-IBAm$_{0.60}$-He$_{0.40}$ (2b), HPEI-IBAm$_{0.60}$-Oc$_{0.24}$ (2c), and HPEI-IBAm$_{0.60}$-DoDec$_{0.24}$ (2d)

Looking at the representative $^{13}$C-DEPT 135 NMR spectrum of 2a (Figure 14), two sets of peaks are significant. First, within the peaks in the aliphatic region corresponding to the alkyl carbons, there is considerable information. As would be expected, there is a positive peak at 13.2 ppm from the terminal methyl carbons, followed by two sets of negative peaks, at 20.2 ppm and 27.7 ppm, that represent the two middle carbons in the butyl chain. Second, the two peaks from the two different types of $\alpha$ carbons adjacent to the amide group are still present at 30.0 ppm and 35.0 ppm. It is difficult to determine from this spectrum if all the primary amines have been functionalized, since secondary carbons adjacent to primary amines typically give rise to peaks at ~ 38 ppm and ~ 40 ppm. While there is no negative peak at 40 ppm, there is still
a peak at ~ 38 ppm, which could be due either to a carbon adjacent to a primary amine or to carbon g, the alkyl carbon attached to the amine.

Figure 14. 75.5 MHz $^{13}$C-DEPT 135 NMR spectral overlay (D$_2$O) of HPEI, HPEI-IBAm$_{0.60}$ (1), and HPEI-IBAm$_{0.60}$-Bu$_{0.40}$ (2a)

An $^{13}$C inverse gated NMR spectrum, such as the one of 2a in Figure 15, allows these two peaks to be integrated, so that the percentage of modified primary and secondary amines can be determined. The ratio of 1° to 2° was found to be 1:0.747 by integration. So of the total primary and secondary amines available for modification, 57% of the amidations occurred at primary amines and 43% occurred at secondary amines. Even though there were a greater number of secondary amines available, the primary
amines are better nucleophiles since they are less sterically hindered. Thus, it is reasonable that a greater percentage of primary amines were modified.

**Figure 15.** 75.5 MHz inverse gated $^{13}$C NMR spectrum (D$_2$O) of HPEI-IBAm$_{0.60}$-Bu$_{0.40}$ (2a)

The solubility behavior of the alkylated polymers was analyzed, and only the butylated polymer was soluble above 4 °C at either pH 7.4 or pH 5. Compared to HPEI-IBAm$_{0.60}$, the LCST of HPEI-IBAm$_{0.60}$-Bu$_{0.40}$ decreased slightly from 24.0 °C to 22.8 °C at pH 7.4, and from 41.5 °C to 39.6 °C at pH 5 (Figure 16). The butyl chains are long enough to increase the hydrophobicity and cause aggregates to form faster, yet not sufficiently lengthy to coil up, form micelles, and either increase or not affect the LCST. However, the LCST values are still adequate for gene delivery, since the LCST at pH 7.4 is below body temperature, meaning the polymers will form globules encapsulating the
genes. Yet, at pH 5, the LCST is above body temperature, allowing the polymer to open up and release the genes.

![Figure 16: Transmission plots at pH 7.4 and 5 of HPEI-IBAm\(_{0.60}\) (1) and HPEI-IBAm\(_{0.60-}\)Bu\(_{0.40}\) (2a)](image)

Since the amines constitute the hydrophilic part of the polymer and play an important role in solubility through hydrogen bonding with water, calculating the concentration of amines in the polymer allows further insight into the solubility behavior of the modified HPEI. The first step in this process was to calculate the repeat unit formula weight (RUFW) of the polymer, which was done by multiplying the molecular weight of each type of repeat unit by the ratio of that unit in the polymer. To find the RUFW of 1, there were two types of repeat units, amidated and unmodified HPEI, with molecular weights of 113.16 g/mol and 43.07 g/mol, respectively (Equation 3).
**Formula**

\[ \text{Formula} = (%1^° + 2^° \text{amines})(% \text{modification})(MW \ of \ amidated \ RPU) + (1 - \% \text{modified})(MW \ of \ unmodified \ RPU) \]

\[ RUFW \ of \ 1 = (0.73)(0.60)(113.12 \ g/mol) + (0.56)(43.1 \ g/mol) = 73.8 \ g/mol \]

**Equation 3.** Example calculation of formula weight for HPEI-IBAm\(_{0.60}\) (1)

The concentration of amines was then calculated by finding the ratio of millimoles of amine per gram of polymer (APG), which was found simply by taking the inverse of the RUFW (Equation 4).

\[ \text{Formula} = \frac{1}{MW} \]

\[ APG \ of \ 1 = \frac{1 \text{mol}}{73.8 \ g} \left( \frac{1000 \text{mmol}}{1 \text{mol}} \right) = 13.6 \text{mmol/g} \]

**Equation 4.** Example calculation of APG for HPEI-IBAm\(_{0.60}\) (1)

It was postulated that an increasing APG would increase the LCST, and a plot of LCST vs. APG was made to visually represent the trend. The chart in Figure 17 demonstrates that at both pH 7.4 and pH 5, as APG increases, the LCST increases as well.
Figure 17. Plot of LCST vs APG for 1 and 2a at pH = 7.4 and 5.

To include in the comparison the alkylated polymers which did not demonstrate an LCST above 4 °C, Table 1 was made to summarize the solubility and APG data. All of the polymers appear to follow the expected trend except for 2b, which has an APG of 11.3 mmol/g. This is 0.2 mmol/g higher than 2a, yet 2a shows a significantly higher LCST. Part of the discrepancy is explained by the difference in % alkylation, since 2c is only 24% alkylated compared to the 40% alkylation of 2a, it will yield a higher than expected APG, since the molecular weight is lower. It is also likely that the longer octyl and dodecyl chains, while not long enough to form micelles, are shielding the amines from the water, preventing all the free amines from interacting with water.
Table 1. Table of the APGs and LCSTs of the alkylated polymers at pH 7.4 and pH 5

<table>
<thead>
<tr>
<th>Polymer</th>
<th>APG (mmol/g)</th>
<th>LCST (°C) pH 7.4</th>
<th>LCST (°C) pH 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>HPEI-IBAm$_{0.60}$ (1)</td>
<td>13.6</td>
<td>24.0</td>
<td>41.5</td>
</tr>
<tr>
<td>HPEI-IBAm$<em>{0.60}$-Bu$</em>{0.40}$ (2a)</td>
<td>11.1</td>
<td>22.8</td>
<td>39.6</td>
</tr>
<tr>
<td>HPEI-IBAm$<em>{0.60}$-He$</em>{0.40}$ (2b)</td>
<td>10.4</td>
<td>&lt; 4</td>
<td>&lt; 4</td>
</tr>
<tr>
<td>HPEI-IBAm$<em>{0.60}$-Oc$</em>{0.24}$ (2c)</td>
<td>11.3</td>
<td>&lt; 4</td>
<td>&lt; 4</td>
</tr>
<tr>
<td>HPEI-IBAm$<em>{0.60}$-DoDec$</em>{0.24}$ (2d)</td>
<td>10.2</td>
<td>&lt; 4</td>
<td>&lt; 4</td>
</tr>
</tbody>
</table>

3.3 Effects of rebalancing with a hydrophilic group

Since modification with only hydrophobic groups reduced the LCST far below body temperature at both pH 7.4 and pH 5, the next step was to rebalance the excessive hydrophobicity with a hydrophilic group. While previous work done by Skidmore found that the LCST increased upon addition of a hydroxyethyl group, this group was not studied in conjunction with hydrophobic groups. Thus, the goal was to discover what and how much of an effect mixing hydroxyethyl with alkyl groups would cause.

The addition of the hydrophilic and hydrophobic groups was accomplished step-wise in a one-pot synthesis (Scheme 8). The synthesis of the butylated and hydroxyethylated polymer was done by first adding the butyl group in THF and quenching, then evaporating the solvent, redissolving in EtOH, and then adding the hydroxyethyl group and quenching. The product was purified by dialysis in DDI water. However, due to solubility issues, all later reactions were done completely in EtOH. Because previous members of this group found that alkylations occurred unevenly in
EtOH\textsuperscript{25}, the hydroxyethylation was done first in these later syntheses. Thus, when the bromoalkanes were added second, they alkylated all remaining primary and secondary amines, obviating the problem of uneven substitution. All reactions were done at 65 °C, allowing 48 hours for the substitution by bromoalkanes or bromohydroxyethyl and 24 hours for the quenching with NaOH.

**Scheme 8.** Synthesis of amidated HPEI modified with alkyl and hydroxyethyl groups

The structure of the polymers was confirmed with \textsuperscript{1}H-NMR and \textsuperscript{13}C-DEPT 135 NMR spectroscopy. Using the proton spectrum of HPEI-IBAm\textsubscript{0.60}-EtOH\textsubscript{0.20}-Oc\textsubscript{0.20} (Figure 18) as a specific example, two features point to the expected structure. First, the peaks previously seen in the aliphatic region for the octylated polymer are present again at 0.79 ppm, 1.20 ppm, and 1.40 ppm. Second, the magnitude and multiplicity of the broad peaks from 2.37-4.04 ppm increases, indicating a greater number of deshielded
hydrogens are present. This would be consistent with where the protons from the hydroxyethyl group would appear.

Figure 18. 300 MHz $^1$H-NMR spectrum (D$_2$O) of HPEI-IBAm$_{0.60}$-EtOH$_{0.20}$-Oc$_{0.20}$ (3e)

The integration values from the proton spectra were again utilized to verify expected percent modification, following the same formula employed for calculations for the alkyl series. The hydrogen on the alcohol group was assumed to appear in the downfield peaks. The theoretical values were found to be 4.82 for the upfield peaks, and 5.46 for the downfield peaks. The difference between the theoretical and actual value for the downfield hydrogens is only 0.10, confirming that the expected percent modification was achieved.
An overlay of $^1$H-NMR spectra of 3a, 3b, and 3d shows the same general trends, as seen in Figure 19. The appearance of the alkyl hydrogens in the aliphatic region is consistent with their previous appearance in the alkylated polymers’ proton spectra.

Figure 19. 300 MHz $^1$H-NMR spectral (D2O) overlay of HPEI-IBAm$_{0.64}$-EtOH$_{0.18}$-Bu$_{0.18}$ (3a) and HPEI-IBAm$_{0.60}$-EtOH$_{0.20}$-Oc$_{0.20}$ (3c)

$^{13}$C-DEPT 135 NMR spectroscopy was used to further elucidate the structure of the polymers by differentiating between primary, secondary, and tertiary carbons. The spectrum of 3c (Figure 20) shows three important peaks sets. First, the different carbons in the octyl chain are more separated in the carbon spectrum than in the proton spectrum and give rise to three peaks. The sharp peak at 22.6 ppm is likely due to just one carbon, while the broader peaks at 29.3 ppm and 31.9 ppm represent the intermediary carbons in the alkyl chain. Second, the two different peaks attributed to the tertiary carbons bound to
secondary and tertiary amides are still present at 30.5 ppm and 35.0 ppm. Finally, the carbon adjacent to the alcohol in the hydroxyethyl group is sufficiently deshielded to be farther downfield than the backbone carbons, with a sharp peak at 62.6 ppm. Again, the peak at ~ 38 ppm is still present, while the peak at ~ 40 ppm has disappeared. It is unclear whether there are still primary amines present or whether the peak at ~ 38 ppm is due to a different type of carbon.

**Figure 20.** 75.5 MHz $^{13}$C-DEPT 135 NMR spectral overlay (D$_2$O) of HPEI, HPEI-IBAm$_{0.60}$ (1), and HPEI-IBAm$_{0.60}$-EtOH$_{0.20}$-He$_{0.20}$ (3b)

Again, the $^{13}$C inverse gated NMR spectrum (**Figure 21**) allows the two methine $\alpha$ carbons to be integrated. From the integral values, the modification ratio of primary to
secondary amines can be calculated. The ratio is 1:0.975, which means 51% of the substitution reactions occurred at primary amines and 49% at secondary amines.

Figure 21. 75.5 MHz inverse gated $^{13}$C NMR spectrum (D$_2$O) of HPEI-IBAm$_{0.60}$-EtOH$_{0.20}$-Oc$_{0.20}$ (3c)

Solutions of these polymers were then analyzed to determine the effect of the hydroxyethyl group on the LCST. Figure 22 depicts the surprising change in the behavior of the polymers containing octyl and dodecyl groups. Both polymers become significantly more soluble, manifesting an increase in the LCST from <4 °C to 18.5 °C and 19.5 °C respectively. There are two possible explanations for this behavior. First, it could simply be that the increased amount of hydrophilic groups capable of hydrogen bonding is sufficient to overcome the extreme hydrophobicity of the lengthy alkyl chains. Second, hydroxyethyl groups, in combination with the amines, repel the hydrophobic chains to a greater extent than simply the amines. Thus, in order to “hide” from the
hydrophilic groups, the alkyl groups could form micelles, allowing the polymer to stay in solution at higher temperatures.

**Figure 22.** Transmission plot at pH 7.4 of HPEI-IBAm_{0.60} (1), HPEI-IBAm_{0.64}-EtOH_{0.18}-Bu_{0.18} (3a), HPEI-IBAm_{0.60}-EtOH_{0.20}-He_{0.20} (3b), HPEI-IBAm_{0.60}-EtOH_{0.20}-Oc_{0.20} (3c), and HPEI-IBAm_{0.63}-EtOH_{0.19}-DoDec_{0.18} (3d)

The solubility behavior in solutions at pH 5 shows a complete reversal of the expected trend (**Figure 23**). Rather than the LCST increasing as the alky chain length decreases, the exact opposite occurs, with the dodecylated and hydroxethylated polymer demonstrating the highest LCST at 45.0 °C, compared to 41.8 °C for 3c and 40.1 °C for 3a. In addition, compared with the curves at pH 7.4, the LCST changes more gradually at pH 5, with the lines manifesting slightly gentler slopes.
To determine if this reversal was caused by a difference in APG, a chart was made plotting LCST vs APG at both pH 7.4 and pH 5 for the hydroxyethylated series as well (Figure 24). While at pH 7.4, the LCST increases as the APG increases, with the exception of 3c and 3d. These two have almost identical LCSTs, with 3c becoming insoluble at 18.5 °C and 3d becoming insoluble at 19.5 °C. However, at pH 5 the LCST decreases as APG increases for 3a, 3c, and 3d, the opposite of the trend seen in the alkyl series.

Figure 23. Transmission plot at pH 5 of HPEI-IBAm\textsubscript{0.60} (1), HPEI-IBAm\textsubscript{0.64}-EtOH\textsubscript{0.18}-Bu\textsubscript{0.18} (3a), HPEI-IBAm\textsubscript{0.60}-EtOH\textsubscript{0.20}-He\textsubscript{0.20} (3b), HPEI-IBAm\textsubscript{0.60}-EtOH\textsubscript{0.20}-Oc\textsubscript{0.20} (3c), and HPEI-IBAm\textsubscript{0.63}-EtOH\textsubscript{0.19}-DoDec\textsubscript{0.18} (3d)
As tabulated in Table 2, there is a significant difference between the APGs of the hydroxyethyl series. 3d is 0.73 mmol/g less than 3c, and 1.53 mmol/g less than 3b, so some factor other than APG must be affecting the solubility behavior. The polymers are more cationic at pH 5, since both the amines and the alcohol groups are protonated. Thus, the hydrophilic groups, to an even greater degree than in a solution of pH 7, repel the nonpolar hydrophobic chains. So the repulsive forces either force the polymer to remain in the extended coil conformation for longer, or they again encourage the formation of micelles, concealing the alkyl chains and allowing the enthalpic forces to retain control at higher temperatures.
<table>
<thead>
<tr>
<th>Polymer</th>
<th>APG (mmol/g)</th>
<th>LCST (°C) pH 7.4</th>
<th>LCST (°C) pH 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>HPEI-IBAm$_{0.60}$ (1)</td>
<td>13.6</td>
<td>24.0</td>
<td>41.5</td>
</tr>
<tr>
<td>HPEI-IBAm$<em>{0.64}$-EtOH$</em>{0.18}$-Bu$_{0.18}$ (3a)</td>
<td>11.2</td>
<td>22.7</td>
<td>40.1</td>
</tr>
<tr>
<td>HPEI-IBAm$<em>{0.60}$-EtOH$</em>{0.20}$-He$_{0.20}$ (3b)</td>
<td>10.8</td>
<td>22.1</td>
<td>45.4</td>
</tr>
<tr>
<td>HPEI-IBAm$<em>{0.60}$-EtOH$</em>{0.20}$-Oc$_{0.20}$ (3c)</td>
<td>10.4</td>
<td>18.5</td>
<td>41.8</td>
</tr>
<tr>
<td>HPEI-IBAm$<em>{0.63}$-EtOH$</em>{0.19}$-DoDec$_{0.18}$ (3d)</td>
<td>9.67</td>
<td>19.5</td>
<td>45.0</td>
</tr>
</tbody>
</table>

**Table 2.** Table of the APGs and LCSTs of the hydroxyethylated and alkylated polymers at pH 7.4 and pH 5

### 3.4 Effects of PEG on transmission

As mentioned previously, PEI modified with PEG tends to have reduced cytotoxicity. So the final polymer series was functionalized with low molecular weight PEG, containing 16 repeat units, and alkyl chains. The goal was to lower cytotoxicity and to determine what effect the long hydrophilic PEG chains would have on solubility behavior. Burgy *et al.* performed a substitution reaction with PEG and sodium azide in the microwave using an EtOH/H$_2$O mix as the solvent$^{40}$. In the current research, the reaction to substitute PEG was first done in EtOH and quenched with NaOH, following the same procedure for hydroxyethylation and using poly(ethylene glycol) methyl ether tosylate (TPEG) as the substrate. However, this resulted in only ~10% PEGylation, rather than the intended 40%. Since TPEG is highly susceptible to hydrolysis, the reaction was probably wet and TPEG was hydrolyzed before the amine could react with it.

Rivera *et al* first converted the tosyl group on TPEG to an iodide group and then performed the substitution reaction in DMF with aniline as the nucleophile and Na$_2$CO$_3$.
as the proton trap\textsuperscript{41}. However, an analogous reaction with HPEI-IBAm\textsubscript{0.60} as the nucleophile is not possible as the polymer is not soluble in DMF, nor most other common anhydrous solvents, except for DMSO. Since DMSO is aprotic and polar, it facilitates S\textsubscript{N}2 reactions. In the current research, the reaction was next performed in distilled DMSO, using K\textsubscript{2}CO\textsubscript{3} as a proton trap, at 65 °C for 72 hours (\textbf{Scheme 9}). The product was purified in aqueous dialysis. While PEGylation at the intended 40% was never achieved, the percent modification did improve significantly to 25%.

\textbf{Scheme 9.} Synthesis of PEG-modified HPEI-IBAm\textsubscript{0.60}

\textsuperscript{1}H-NMR spectroscopy was used to confirm the structure and to calculate percent modification with PEG. Since all the hydrogens in PEG are adjacent to oxygen, they are deshielded by the electron withdrawing nature of oxygen and appear downfield from the aliphatic region. In the spectrum of HPEI-IBAm\textsubscript{0.60}-PEG\textsubscript{0.25} (\textbf{Figure 25}), a very intense, sharp peak appears at 3.64 ppm, representing the hydrogens in PEG. Slightly upfield at 3.32 ppm is another sharp peak, but with a much weaker signal, which is attributed to the methyl group attached to an oxygen atom at the end of PEG.
Percent modification was calculated by finding the ratio of the actual integration for the main PEG peak to the theoretical integration value for this peak (Equation 5).

The methyl peak at 1.02 ppm was a known value, which was calculated by multiplying the six hydrogens it represents by their known percent modification (Equation 5).

\[
\text{Integration value of methyl hydrogens} : (0.73)(0.60)(6 \text{ Hs}) = 2.63
\]

\[
\text{Theoretical integration value of PEG peak} : (0.73)(0.40)(64 \text{ Hs}) = 18.7
\]

\[
\% \text{ modification with PEG} : \left( \frac{11.9}{18.7} \right) (40\% \ \text{theoretical modification})(100\%) = 25\%
\]

Equation 5. Calculation of percent modification with PEG
A $^{13}$C-DEPT 135 NMR spectrum was also obtained to confirm the structure (Figure 26). The intense peak at 69.6 ppm represents the secondary carbons in the PEG chain, confirming successful modification with PEG. The methoxy signal is also present at 58.1 ppm, as are the two peaks attributed to the two different tertiary carbon atoms of isobutyryl amide groups at 29.9 ppm and 35.1 ppm.

![NMR Spectrum](image)

**Figure 26.** 75.5 MHz $^{13}$C-DEPT 135 NMR spectrum (D$_2$O) of HPEI-IBAm$_{0.60}$-PEG$_{0.25}$ (4)

Next, the PEG-functionalized amidated HPEI was modified with alkyl groups to finish covering the primary amines and to determine if the series of PEG-alkyl polymers would follow the same trends as the hydroxyethyl-alkyl polymers. The reactions followed the same general procedure as previous alkylations (Scheme 10) except that EtOH was
used as the solvent, and K$_2$CO$_3$ was used as the base, rather than NaOH. For the addition of butyl, the proton trap was added from the beginning. However, for the additions of octyl and dodecyl, K$_2$CO$_3$ was added after 48 hours to protect against deprotonation and substitution at the amide nitrogen. The reaction continued for 24 hours after the addition of the proton trap, and then was purified in aqueous dialysis.

Scheme 10. Synthesis of HPEI-IBAm$_{0.60}$ modified with PEG and alkyl groups

The structures were confirmed with $^1$H-NMR spectroscopy, which showed a combination of the peaks seen previously from alkyl groups and PEG groups (Figure 27). The expected two new aliphatic peaks are present at 0.84 ppm and 1.23 ppm, while the methoxy peak from PEG appears at 3.31 ppm and the PEG chain hydrogens give rise to a peak at 3.62 ppm. The proton spectrum also confirmed the expected percent alkylation. The integration value of the PEG peak at 3.62 ppm was adjusted to the same
number it was integrated at in the spectrum of 4. Then the aliphatic peaks were integrated in reference to the PEG peak, and that value was compared to the calculated value, found following the formula in **Equation 2**. The theoretical value was 5.48, only 0.06 less than the actual value. So the actual percent modification with dodecyl was very close to the theoretical percentage.

![Diagram of molecular structure](image)

**Figure 27.** 300 MHz $^1$H-NMR spectrum (D$_2$O) of HPEI-IBAm$_{0.60}$-PEG$_{0.23}$-DoDec$_{0.17}$ (4d)

The proton spectra of 4a and 4c (Figure 28) follow the same pattern, showing both the uniquely sharp and intense PEG peaks downfield and the broader alkyl peaks upfield. As the ratio of PEG hydrogens to alkyl hydrogens increases, the magnitude of the aliphatic peaks gradually decreases, making the individual peaks more difficult to distinguish.
Figure 28. 300 MHz \(^1\text{H-NMR} \) spectral (D\(_2\)O) overlay of HPEI-IBAm\(_{0.60}\)-PEG\(_{0.23}\)-Bu\(_{0.17}\) (4a) and HPEI-IBAm\(_{0.60}\)-PEG\(_{0.25}\)-Oc\(_{0.15}\) (4d).

The \(^{13}\text{C-DEPT} \) 135 NMR spectrum (Figure 29) of 4a manifests the characteristic peaks of both PEG and butyl. The butyl methyl group is at 13.4 ppm and the two middle carbons in a broad peak at 25.2 ppm. The two carbons associated with the secondary and tertiary amides are found at 30.0 ppm and 35.0 ppm. Finally, the secondary carbons in the PEG chain give rise to a sharp peak at 70.0 ppm, while the methoxy group causes a smaller peak at 58.0 ppm.
Figure 29. 75.5 MHz $^{13}$C-DEPT 135 NMR spectrum (D$_2$O) of HPEI-IBAm$_{0.60}$-PEG$_{0.23}$-Bu$_{0.17}$ (4a)

Again, the $^{13}$C inverse gated NMR spectrum (Figure 30) allows the two methine \( \alpha \) carbons to be integrated. From the integral values, the modification ratio of primary to secondary amines can be calculated. The ratio is 1:1.18, which means 46% of the substitution reactions occurred at primary amines and 54% at secondary amines.
When the solubility behavior of the PEGylated polymers was studied at pH 7.4, all of the polymers in this series had an LCST higher than that of HPEI-IBAm$_{0.60}$, as would be expected given the great increase in hydrophilicity caused by PEG (Figure 31). However, somewhat surprisingly, with LCSTs at 29.2 °C and 26.4 °C respectively, the butylated and dodecylated polymers remained in solution longer than either the octylated polymer or the polymer functionalized with PEG only, with LCSTs at 25.2 °C and 25.5 °C. It would seem that the butyl chain is small enough that the PEG chains effectively hide the nonpolar group from the surrounding solution. The dodecyl group, repelled by the increasing hydrophilicity caused by the PEG chain, is long enough to coil inwards and form micelles protected by the hydrophilic segments. The octyl group is caught in
between, neither short enough to be hidden while extended, nor long enough to coil as efficiently as the dodecyl chain. There does still seem to be some shielding of the octyl chain, since its LCST is barely lower than the LCST of 4. Compared to the hydroxyethylated series, the micellar effect seems to be enhanced, probably due to the greater hydrophilicity of PEG compared to the short hydroxyethyl group.

Figure 31. Transmission plot at pH 7.4 of HPEI-IBAm$_{0.60}$ (1), HPEI-IBAm$_{0.60}$-PEG$_{0.23}$ (4), HPEI-IBAm$_{0.60}$-PEG$_{0.23}$-Bu$_{0.17}$ (4a), HPEI-IBAm$_{0.60}$-PEG$_{0.25}$-Oc$_{0.15}$ (4c), and HPEI-IBAm$_{0.60}$-PEG$_{0.23}$-DoDec$_{0.17}$ (4d)

The solubility was also studied at pH 5, and the results are seen in Figure 32. As the alkyl character of the polymers increased, the slopes of the lines became much smaller as the change in solubility occurred more gradually. Here again, as was seen in the hydroxyethylated series, the trend reverses and 4d has the highest LCST at 74.0 °C, then 4c at 62.8 °C, then 4a at 55.5 °C, followed by 4 with the lowest LCST at 46.5 °C. The increasing cationic, and thus hydrophilic, nature of the polymer at pH 5 repulses the
longer hydrophilic chains to a greater degree than at pH 7.4. Thus, while butyl was hidden by virtue of being short at the lower pH, at pH 5 it is too short to coil and thus cannot be concealed as easily as the octyl and dodecyl chains. At pH 5, the increasing difference between polar and nonpolar groups gives the octyl and dodecyl groups added impetus to form micelles, thus increasing the formation of micelles and increasing the LCST as the hydrophobic groups are more hidden.

**Figure 32.** Transmission plot at pH 5 of HPEI-IBAm\(_{0.60}\) (1), HPEI-IBAm\(_{0.60}\)-PEG\(_{0.23}\) (4), HPEI-IBAm\(_{0.60}\)-PEG\(_{0.23}\)-Bu\(_{0.17}\) (4a), HPEI-IBAm\(_{0.60}\)-PEG\(_{0.25}\)-Oc\(_{0.15}\) (4c), and HPEI-IBAm\(_{0.60}\)-PEG\(_{0.23}\)-DoDec\(_{0.17}\) (4d)

To determine if the unexpected trends might be due to the concentration of amines, APG values were calculated and plotted against the LCSTs (Figure 33). The concentration of amines in the PEGylated series is much lower than in previous series due to the great increase in molecular weight caused by the addition of PEG.
Figure 33. Chart of LCST vs APG for HPEI-IBAm\textsubscript{0.60} (1), HPEI-IBAm\textsubscript{0.60}-PEG\textsubscript{0.23} (4), HPEI-IBAm\textsubscript{0.60}-PEG\textsubscript{0.23}-Bu\textsubscript{0.17} (4a), HPEI-IBAm\textsubscript{0.60}-PEG\textsubscript{0.25}-Oc\textsubscript{0.15} (4c), and HPEI-IBAm\textsubscript{0.60}-PEG\textsubscript{0.23}-DoDec\textsubscript{0.17} (4d).

Looking at the actual values for the APG in Table 3, the APG generally decreases as the alkyl chain length increases. 4c has a slightly higher APG than 4d, because it contains a slightly greater percentage of PEG. At pH 7.4, there is not a clear trend in the relation between APG and LCST, due to the varying behavior of the hydrophobic groups. At pH 5, the APG and LCST values are inversely related, contrary to expectation. However, in solution at both pH 7.4 and pH 5, as micelles form, the actual concentration of amines would appear greater than the theoretical values. Thus, the solubility behavior is still dependent on the ability and freedom of the amines to form hydrogen bonds with the water molecules.
<table>
<thead>
<tr>
<th>Polymer</th>
<th>APG (mmol/g)</th>
<th>LCST (°C)</th>
<th>LCST (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HPEI-IBAm(_{0.60}) (1)</td>
<td>13.6</td>
<td>24.0</td>
<td>41.5</td>
</tr>
<tr>
<td>HPEI-IBAm(<em>{0.64})-PEG(</em>{0.23}) (4)</td>
<td>5.55</td>
<td>25.5</td>
<td>46.5</td>
</tr>
<tr>
<td>HPEI-IBAm(<em>{0.60})-PEG(</em>{0.23})-Bu(_{0.17}) (4a)</td>
<td>4.93</td>
<td>29.2</td>
<td>55.5</td>
</tr>
<tr>
<td>HPEI-IBAm(<em>{0.60})-PEG(</em>{0.25})-Oc(_{0.15}) (4c)</td>
<td>4.57</td>
<td>25.2</td>
<td>62.8</td>
</tr>
<tr>
<td>HPEI-IBAm(<em>{0.60})-PEG(</em>{0.23})-DoDec(_{0.17}) (4d)</td>
<td>4.61</td>
<td>26.4</td>
<td>74.0</td>
</tr>
</tbody>
</table>

**Table 3.** Table of the APGs and LCSTs of the PEGylated and alkylated polymers at pH 7.4 and pH 5
4. CONCLUSION

Three series of modified HPEI polymers were prepared. First, a series of polymers with increasing hydrophobicity, 2a-2d, were synthesized. The influence of the alkyl chain length on solubility behavior was analyzed. Of the four polymers, only the butylated polymer demonstrated an observable LCST at either pH 7.4 or pH 5. Since the LCST at pH 7.4 was below body temperature and at pH 5 was above body temperature, this polymer is suitable for gene delivery as it displays the desired change in conformation. However, for the remaining three polymers, while the majority of primary amines were successfully hidden, the polymers are not good candidates for gene delivery, since they do not exhibit a change in conformation in response to temperature or pH.

Second, a series of polymers tempering the hydrophobicity with a hydroxyethyl group, 3a-3d, were prepared. Finally, a series of polymers modified with a mixture of PEG and alkyl chains, 4-4d, were synthesized. Again, the effect of temperature on the LCST was studied. All of the polymers in both series, at both pH 7.4 and pH 5, exhibited LCSTs that were below body temperature in the neutral environment and above body temperature in the acidic environment. So all of the polymers from these two series have potentially low cytotoxicity and are high efficiency gene delivery vectors, since the majority of the primary amines are modified to secondary amines and they do switch conformation from extended coil to globule in response to temperature and pH stimuli.
5. FUTURE WORK

In order to verify the ability of these modified HPEI polymers to serve as effective gene delivery vectors, transfection studies need to be performed, with different cell lines and varying polymer/DNA ratios. A better method for introducing PEG onto HPEI-IBAm\textsubscript{0.60}, in order to accomplish complete 40% PEGylations, should be developed. Also, an amidated polymer modified with 20% PEG and 20% hexyl should be synthesized for further elucidation of the relationship between APG and LCST. Finally, since overexpression of folate and glucose receptors is common in cancer cells, methods for introducing targeting groups, such as glucose and folic acid, should be explored.
6. REFERENCES


