Temperature and pH Responsive Polyethylenimine Systems as Potential Nonviral Gene Vectors

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TEMPERATURE AND pH RESPONSIVE POLYETHYLENIMINE SYSTEMS AS
POTENTIAL NONVIRAL GENE VECTORS

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

By

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B.A., Case Western Reserve University, 2008

2010
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Abstract

Skidmore, S. Chad. M.S., Department of Chemistry, Wright State University, 2010.
Temperature and pH responsive Polyethylenimine Systems as Potential Nonviral Gene Vectors.

Modified HPEI was used to determine if the LCST could be controlled by varying multiple variables, including: percent modification, pH, salt solution concentration, and weight percent. Two modifications were done with butylene oxide (Butox) and isobutyryl amide (IBAm). Specifically, 100% (HPEI-Butox_{1.00}) butoxylated and 10% (HPEI-IBAm_{0.10}), 54% (HPEI-IBAm_{0.54}), and 70% (HPEI-IBAm_{0.70}) amidated samples were synthesized. Additionally, HPEI-IBAm_{0.54} was further modified with hydroxyl ethyl groups (HPEI-IBAm_{0.54-EOx_{0.46}}) and ethyl groups (HPEI-IBAm_{0.54-Et_{0.44}}). The LCSTs of all amidated systems were below body temperature, 37 °C, at pH = 7.4. This indicates an ability of these systems to be used as a vector for gene delivery. A PBS salt solution lowered the LCST, while weight percent had a non linear effect on the LCST. Transfection data on the 54% amidated HPEI system showed transfection efficiency equivalent to control transfectant, LT1. These data suggest a strong potential for using modified HPEI systems as nonviral gene vectors.
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Acknowledgements

I would like to thank my committee members Dr. Kenneth Turnbull, Dr. Ioana Pavel, and Dr. Eric Fossum. Additionally, I would like to thank Dr. J. Ashot Kozak for conducting transfection studies as well as the other members of Dr. Fossum’s research group. Lastly, I would like to thank Dr. Fossum for guidance and dedication in my pursuit of a Masters degree.
1. Background

1.1 Gene Delivery

Gene therapy is the process of restoring mutated DNA back to their “normal” state by the integration of calibrated DNA. The four hallmarks of successful gene therapy are: 1) targeting the right cells, 2) activation of the gene, 3) integration of the gene into the cells, and 4) limited side effects. The process of delivering the gene into the cells can determine the outcome of all four of those areas. Ideally, a “carrier”, or vector, for gene delivery should use the naturally occurring cellular mechanisms to increase the integration and activation of the gene as well as limit harmful side effects.

Mammalian cells have developed multiple intricate mechanisms for uptake of extracellular material, collectively called endocytosis (Figure 1). These mechanisms include phagocytosis (“cell eating”) and pinocytosis (“cell drinking”), as well as receptor dependent and independent endocytosis.

![Figure 1. The Mechanism of Endocytosis.](image)

As part of these processes, the phospholipid bilayer surrounding the cell engulfs extra-cellular material, forming an endosome. However, allowing access of any molecule into the cell can be detrimental to the cell and as part of a method of defense, inside the
endosome, the pH drops to $\approx 2-4$ in an attempt to degrade potentially harmful substances. Therefore, a vector for gene delivery, which can protect the DNA from degradation, would be highly valued.

In the world changing discovery by Wolff and colleagues, unassisted, or naked, genes were expressed following direct injection into skeletal muscle. (2) This proved to be a useful protector against live infectious agents and eventually lead to “DNA vaccines”. However, the uncontrolled expression of such genes limited the use to immunizations only. If gene expression could be controlled, applications could be extended to gene delivery in tumors. Two possible vectors for controlled expression are viral and nonviral.

### 1.1.1 Viral Vectors

Viral vectors have a natural advantage into transfection efficiency over nonviral vectors. The natural role of a virus is delivering typically harmful DNA into cells. Therefore, by removing the harmful DNA and replacing it with genetically specific DNA, viruses could have extraordinary potential as gene vectors. However, viral vectors have the potential to revert back to their harmful natural state. Additionally, most viral vectors are recombinant viruses such as retrovirus, lentivirus and pox virus, making their natural, harmful state potentially deadly and thus too dangerous for a gene delivery vector. (3) Finally, the cost of manufacturing large quantities of viral vectors is exceptionally high. Therefore, while viral vectors have shown promising transfection efficiencies, the dangers and cost of using viruses in humans have made nonviral vectors promising alternatives. (4)

### 1.1.2 Nonviral Vectors

Cationic polymers have shown the ability to form a polyplex, complex using a polye, with DNA and thus are strong candidates for nonviral gene vectors. For a polycation to be used in gene delivery, it must be able to meet the needs and withstand the challenges
presented by the cellular mechanism shown in Figure 1. As the polyplex is brought into the cell through endocytosis, the polycation must be able to withstand the degrading enzymes and low pH without damage to the DNA. Additionally, the process of endocytosis requires the polyplex to be small and tightly formed. Once inside the cell, the polymer must be able to release the DNA and be removed without damaging the cell. The integration of DNA requires the tight polyplex to relax and release the DNA into the cell. The responsive nature of the polymer to form a tight complex with the DNA and then release on command is a difficult but crucial aspect for polycationic vectors. One of the initial polycationic vectors was poly(lysine), which showed low levels of transfection. Transfection using poly(lysine) could be improved, but only in conjunction with another substance, such as chloroquine. Therefore, a strong push has been made to develop a more efficient, nonviral vector system. One such polycationic system is polyethylenimine (PEI), which, due to its unique characteristics, has become the focus of gene delivery.

1.2 Polyethylenimine (PEI)

PEI can either be branched or linear (Figure 2). However, significantly greater success in transfection studies have made branched PEI the standard form of PEI used for gene delivery. Branched PEI consists of 3 subunits, linear, dendritic, and terminal, in approximately a 1:2:1 ratio, respectively. Each subunit consists of either a primary, secondary or tertiary amine connected by an ethylene group. Variations in molecular weight can drastically change the ratio of subunits. Since the first reported use of PEI as a nonviral gene vector, there has been much research focused on different aspects of PEI systems, including buffering capacity, molecular weights, branching, and modification reactions. A variety of PEI systems with a range of molecular weights and structures are commercially available, which has made PEI an inexpensive material for nonviral gene vector studies.
1.2.1 Branched vs. Linear

There is strong uncertainty in the effect of linear vs. branched on transfection. Not only are branched polymers actually smaller in solution than their linear counterparts, which could decrease the ability to enter and exit the cell, but also there has been some concern that primary amines are the source of toxicity in cell studies. Therefore, branched PEI, with more primary amines per polymer would conceivably be more toxic than the linear analogue. However, such a direct correlation has not been found. These structural differences have a potentially, albeit unclear, effect on transfection efficiency. Both branched and linear PEIs have been shown to have better transfection efficiency than the other. In particular, Wiseman et al have shown linear PEI to have a higher transfection efficiency, both in vitro and in vivo, compared to branched PEI of a similar molecular weight.\textsuperscript{(15)}

1.2.2 Buffering of Polyethylenimine (PEI)

PEI has an extreme cationic-charge to density ratio.\textsuperscript{(11)} The presence of nitrogen at every third backbone atom, allows for a wide range of pK\textsubscript{a} values and a remarkable buffering capacity. In 1995, Behr et al reported the first use of PEI as a nonviral gene vector. The results indicated good transfection efficiencies both in vitro and in vivo. The proposed
mechanism of transfection (Figure 1) included the influx of protons, along with chloride counter-ions, into the endosome giving rise to the “proton sponge effect”. The increased volume eventually ruptures the endosome thereby releasing the gene. (12) Therefore the buffering capacity of PEI is essential in gene delivery given the “proton sponge effect”. (4)

1.2.3 Effects of Molecular Weight

The molecular weight of PEI is potentially the most important factor for gene delivery. As stated before, the ratio of subunits can vary with molecular weight. By changing the ratio of subunits, the overall buffering capacity could change. Additionally, the molecular weight can affect toxicity and complex formation with DNA. Larger polymers would have a larger charge per polymer ratio. This could allow for stronger polyplex formation. However, this could be problematic inside the cell, when DNA needs to be released. Lastly, the sheer size could decrease the ability of the polyplex to enter the cell. These factors will certainly affect the transfection efficiency, however, current studies differ on the overall effect. (5) (10) (13)

The effects of molecular weights were first studied by Godbey et al who looked at branched PEI systems ranging from 600 to 70,000 Daltons. (10) Low molecular weights (less than 1,800 Daltons) showed little to no cellular transfection. PEI systems with molecular weights between 10,000 and 70,000 Daltons had a marked increase in transfection efficiency. Conversely, Kunath et al showed 5,000 Daltons was the optimum molecular weight for cellular transfection. (14) However, the differences in molecular weight not only affect the size, but moreover the toxicity and buffering capacity, which could allow for variations in transfection efficiency in different cell lines and solvents.
1.3 Smart Polymers – Thermo and pH Responsive Systems

There are a wide variety of polymeric systems that are capable of responding, typically by a conformational change, to an external stimulus, such as temperature, pH, or salt concentration (Scheme 1). The resulting conformational change is often reversible and may result in an alternative controlled event. The conformational change may also result in a lower critical solution temperature (LCST). Contradictory to traditional solubility behavior, the LCST is the temperature below which the polymer is soluble and above which it is insoluble. As a result, these types of systems are considered “smart” polymers.

Scheme 1. Conformational Change of a “Smart” Polymer Responding to an External Stimulus.

One widely studied “smart” polymer system is poly(N-isopropylacrylamide) or PNIPAM (Figure 3). As part of its responsive nature, PNIPAM exhibits a LCST of 32 °C. The LCST is due to a balance of hydrophobic and hydrophilic groups, backbone and isopropyl amide, respectively for PNIPAM.

Figure 3. Structure of Poly(N-Isopropylacrylamide), PNIPAM.
Subsequent heating above the LCST results in a coil to globular transition and concurrent precipitation of the polymer. Additionally, the LCST can be dependent on the pH. As the pH decreases the H\(^+\) concentration increases, thus increasing PNIPAM’s ionic characteristic making it more soluble in water, and raising the LCST. Therefore, the LCST of PNIPAM is dependent on the pH and ionic strength of the aqueous solution, but only to a limited degree, a typically 1 or 2 °C range.\(^{(17)}\)

1.3.1 LCST Effects on Transfection Efficiencies

Gene delivery applications using polymers that possess an LCST were first reported by Yokoyama\(^{(18)}\) and more recently by Alexander,\(^{(19),(20)}\) Gorecki,\(^{(21)}\) and Oupicky\(^{(22)}\). Yokoyama’s gene vector system was based on the thermoresponsive behavior of PNIPAM in conjunction with cationic (amine) and hydrophobic comonomers. By preparing copolymers with varying percentages of the cationic and hydrophilic monomers they were able to tune the LCST of the resulting copolymer to approximately 21 °C, well below the physiological temperature of 37 °C. After formation of copolymer: DNA complexes at 37°C a series of incubation studies were carried out with COS1 cells at varying temperatures. When a 3-hour incubation time, below the LCST of the copolymer (21 °C), was utilized, followed by return to 37 °C there was a threefold increase in β-galactosidase, or gene, expression, compared with an incubation procedure in which the temperature was maintained at 37 °C. This showed that the concept of thermoresponsive polymers being used as efficient gene vectors was confirmed.

1.3.2 Effect of Salts on LCST

The LCST of a “smart” polymer is based on multiple factors participating in the interaction of the polymer and the surrounding water molecules. Although transmission
studies are typically carried out in water, transfection studies are carried out in a phosphate buffer saline solution (PBS). Therefore, the effect of the salt solution (PBS) on the LCST is crucial to producing a viable nonviral gene vector.

The introduction of salts into this system could have many different effects on these interactions, including effects on the backbone, polar groups, and hydrogen bonding. (23) There are two types of salts: “salting-in” and “salting-out” ions. These salts are categorized by the reported Hofmeister series. Salting-in ions, or salts later in the series, decrease the hydrophobic effect, thus increasing solubility. Conversely, salting-out ions, which are early in the Hofmeister series, are considered structure makers for water, and as a result increase the hydrophobic interactions. (24) The interactions with salts and polymer systems are dependent on the anionic species, but independent of the cationic species. (24)

The PBS solution used as a solvent for transfection studies contains NaCl, KCl, Na₂HPO₄, and KH₂PO₄. Salting-out ions are CO₃²⁻ > SO₄²⁻ > S₂O₃²⁻ > H₂PO₄⁻ > F⁻ > Cl⁻ > Br⁻ and the salting-in ions are NO₃⁻ > I⁻ > ClO₄⁻ > SCN⁻. (23) Therefore, while different salts have different effects of the LCST, transmission studies conducted in PBS should lower the LCST. This will be looked at in greater detail later.

1.4 Working Hypothesis

Although PEI does not exhibit a LCST itself, modified hyperbranched PEIs (HPEIs) can. Depending on the hydrophilic and hydrophobic nature of the modification, it could be possible to control the resulting LCST. Very few modified HPEI systems have been designed, particularly ones with a LCST at biological pH and temperature. One such system was proposed by Liu et al. He proposed amidating 100% and 70% of the primary and secondary amines in HPEI. This produced two LCSTs, both of which were below 37 °C at a pH of 7.4. (25) Additionally, some modified HPEI systems have been synthesized by previous members of the Fossum group (Table 1). (23), (26) Although, most possess a relatively high
LCST, which makes them not ideal for gene delivery, the understanding of different effects resulting from different hydrophobic groups gave useful insight as to future types of modification, which might lead to a lower LCST.

<table>
<thead>
<tr>
<th>Sample</th>
<th>% Alkyl</th>
<th>% 2-hydroxypropyl</th>
<th>LCST (°C)</th>
<th>pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>7 Butyl</td>
<td>65</td>
<td>62</td>
<td>9.50</td>
</tr>
<tr>
<td>2</td>
<td>6.5 Pentyl</td>
<td>72</td>
<td>59</td>
<td>10.12</td>
</tr>
<tr>
<td>3</td>
<td>5 Hexyl</td>
<td>75</td>
<td>59</td>
<td>9.63</td>
</tr>
<tr>
<td>4</td>
<td>10 Hexyl</td>
<td>70</td>
<td>50</td>
<td>9.17</td>
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<tr>
<td>5</td>
<td>15 hexyl</td>
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<td>7</td>
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<tr>
<td>8</td>
<td>4.9 Dodecyl</td>
<td>79</td>
<td>66</td>
<td>9.81</td>
</tr>
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</table>

Table 1. LCST Values and pHs of a Series of Modified PEI Systems.

As depicted in Scheme 2, this project is based on the working hypothesis that cellular uptake will be enhanced by a “tight” polyplex formed during an initial incubation below the LCST, followed by a rise in temperature above the LCST. The dual-responsive nature of the HPEI “smart” polymer will allow the formation of a “loose” polyplex with the decrease of pH inside the endosome. This “loose” polyplex should facilitate release and subsequent transcription of the gene.
Scheme 2. Representation of a Dual-Stimuli Responsive PEI as a Nonviral Gene Vector.

1.5 Project Outline

To use a cationic polymer as a vector for gene delivery one must consider many issues, including ease of production, transfection efficiency, and toxicity. By modifying HPEI (mHPEI) with hydrophobic/hydrophilic groups, it may be possible to get the mHPEI to respond to temperature and/or pH changes with the desired conformational changes. This could give rise to a lower critical solution temperature (LCST), which may help increase the transfection efficiency. Therefore, the main objective was to determine what modifications to HPEI would produce a LCST appropriate for enhancing gene delivery.

The research presented in this thesis can be divided into three objectives: 1) gene vector development and characterization, 2) LCST studies, and 3) *in vitro* transfection and cytotoxicity studies of modified HPEI. A feedback loop will be established, after the initial formation of a polymer with a workable LCST, to provide design criteria for subsequent polymer syntheses, based on cytotoxicity, polyplex formation, and transfection results. Additionally, the effects of varying pH and weight percent will be studied to determine their effects on the LCST of synthesized polymer.
2. Experimental

2.1 Materials, Characterization, and Synthesis for Modified HPEI

2.1.1 Materials

Hyperbranched polyethylenimine HPEI10K (Sigma Aldrich, $M_n = 10,000$ g/mol) and Lupasol® WF (BASF, $M_n = 10,000$ g/mol) were dried under vacuum prior to use. Triethylamine (TEA, Sigma Aldrich, 99%) was dried over CaH₂ and distilled under nitrogen. Isobutyril chloride (Sigma Aldrich, 98%), 2-bromoethanol (Sigma Aldrich, 95%), and iodoethane (Sigma Aldrich, 99%) were used as received. Phosphate buffer saline solution (PBS) was made in our laboratory, pH = 7.4. Tetrahydrofuran (THF) was dried over sodium and distilled under nitrogen. 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 5 filter (carbon, deionization, deionization, organic absorption, and ultra violet) Labconco Water Pro Plus system.

2.1.2 Characterization

$^1$H and $^{13}$C NMR spectra were recorded on a Bruker AVANCE 300 MHZ instrument operating at 300 and 75.5 MHz, respectively. Samples were dissolved in D₂O or CDCl₃ at a concentration of 100 mg/mL. Samples were dried using a Savant Speed-Vac SC110 with attached Refrigerated Vapor Trap RVT4104. Transmittance data were obtained (500 nm) on a Hewlett Packard 8453 Ultraviolet-visible spectrometer with an external heating source and a heating rate of 1 °C/min under N₂. Transmittance data were acquired on polymers dissolved in either DDI water or PBS (2 wt. % unless otherwise stated) at varying pHs. The LCST was defined as the temperature corresponding to the 50% transmittance in the resulting transmittance versus temperature curves.
2.1.3 *Synthesis of 75% Butoxylated HPEI, HPEI-Butox0.75*

19.5 g (453 mmol) of HPEI was placed into a Parr-reactor, purged with nitrogen, and dissolved in 200 mL of nitrogen sparged ethanol. Next, 31.5 mL (362 mmol) of butylene oxide was slowly added via syringe and the Parr-reactor was heated to 60°C for 3 hours. The reaction mixture was allowed to cool in the Parr-reactor to room temperature over night. The ethanol was removed by evaporation. In increments of 2.0 g, the resulting product was dissolved in 20 mL of DDI water and purified by dialysis against DDI water to remove any salts and other low molecular weight impurities. The water was removed under vacuum using the speed-vac to afford 39.8 g (90%) of a light green residue.

$^1$H NMR (D$_2$O, δ): 0.89 (s); 1.47 (s); 2.9 - 3.8 (broad, backbone H’s); $^{13}$C NMR (D$_2$O, δ): 9.05 (s); 27.32 (s); 44.80 – 68.16 (broad, backbone C’s).

2.1.4 *Synthesis of 94% Amidated HPEI, HPEI-IBAm0.94*

The synthesis of 94% amidated HPEI was carried out via a procedure similar to that reported by Liu et al. (25) In an oven-dried and nitrogen purged, 100 mL three necked RB flask (RBF), equipped with a water condenser and nitrogen inlet, were placed 0.11 g (1.9 mmol of terminal groups) of HPEI, 2.6 mL (19 mmol) of TEA, and 20 mL of THF. The resulting mixture was cooled to 0 °C using an ice bath, and 0.14 mL (1.3mmol) of isobutyryl chloride was added dropwise under vigorous stirring. The mixture was allowed to warm to room temperature and stirred for 12 hours. Finally, the reaction temperature was raised to 50 °C for half an hour. The reaction mixture was cooled to room temperature, the salt was removed via filtration, and the filtrate was concentrated under vacuum. The residue was dissolved in 2 mL of methanol and purified by dialysis against methanol for 2 days to remove any salts and other low molecular weight impurities. The methanol was removed under vacuum using the speed-vac to afford 0.16 g (80%) of a yellowish residue.
\[ ^1\text{H NMR} \quad (\text{CDCl}_3, \delta): \begin{align*} &1.045 \,(s); \, 2.16-3.00 \,(\text{broad, 6H's}); \, 3.11-3.71 \,(\text{broad, 3H's}); \quad ^{13}\text{C NMR} \quad (\text{CDCl}_3, \delta): \begin{align*} &18.83 \,(s); \, 29.06 \,(s); \, 34.27 \,(s); \, 36.1-55.0 \,(\text{broad, backbone C's}); \, 176.61 \,(s); \, 177.0 \,(s). \end{align*} \]

\[ ^{13}\text{C NMR} \text{ (CDCl}_3, \delta): \begin{align*} &18.83 \,(s); \, 29.06 \,(s); \, 34.27 \,(s); \, 36.1-55.0 \,(\text{broad, backbone C's}); \, 176.61 \,(s); \, 177.0 \,(s). \end{align*} \]

2.1.5 **Synthesis of 54% Amidated HPEI, HPEI-IBAm\(_{0.54}\)**

The synthesis of 54% amidated HPEI was carried out via a slightly modified literature procedure.\(^{(25)}\) In an oven-dried and nitrogen purged 1L three necked RB flask (RBF) equipped with a water condenser and nitrogen inlet, were placed 2.0 g (34.0 mmol of terminal groups) of HPEI, 47.3 mL (340 mmol) of TEA, and 300 mL of THF. The resulting mixture was cooled to 0 °C, using an ice bath and 0.89 mL (8.5 mmol) of isobutyryl chloride was added dropwise under vigorous stirring. The mixture was allowed to warm to room temperature and stirred for 12 hours. Finally, the reaction mixture temperature was raised to 50 °C for half an hour. The reaction was cooled to room temperature and the salt removed via filtration. The resulting filtrate was evaporated to dryness under vacuum and the residue was dissolved in 36 mL of DDI water and purified by dialysis against DDI for 48 hours in a refrigerator to remove any salts and other low molecular weight impurities. The DDI water was removed under vacuum using the speed-vac to afford 2.20 g (67%) of a yellowish residue.

\[ ^1\text{H NMR} \text{ (D}_2\text{O, }\delta): \begin{align*} &1.00 \,(s); \, 1.96-2.82 \,(\text{broad, 6H's}); \, 2.82-3.80 \,(\text{broad, 3H's}); \quad ^{13}\text{C NMR} \text{ (D}_2\text{O, }\delta): \begin{align*} &19.0 \,(s); \, 29.95 \,(s); \, 35.00 \,(s); \, 36.87-52.7 \,(\text{broad, backbone C's}); \, 180.47 \,(s). \end{align*} \]

2.1.6 **Synthesis of 10% Amidated HPEI, HPEI-IBAm\(_{0.10}\)**

The synthesis of 10% amidated HPEI was a modified procedure from the one reported by Liu et al.\(^{(25)}\) In an oven-dried and nitrogen purged 1L three necked RB flask (RBF) equipped with a water condenser and nitrogen inlet, was placed 2.0 g (34.0 mmol of terminal groups) of HPEI, 47.3 mL (340 mmol) of TEA, and 300 mL of THF. The resulting mixture was
cooled to 0 °C, using an ice bath, and 0.69 mL (6.57 mmol) of isobutyryl chloride was added dropwise under vigorous stirring. The resulting mixture was allowed to warm to room temperature and stirred for 12 hours. Finally, the reaction temperature was raised to 50 °C for 30 minutes. The reaction mixture was cooled to room temperature, the salts removed via filtration, and the filtrate was concentrated under vacuum. The residue was dissolved in 36 mL of DDI water and purified by dialysis against DDI for 48 hours in a refrigerator to remove any salts and other low molecular weight impurities. The DDI water was removed under vacuum using the speed-vac to afford 2.00 g (61.1%) of a yellowish residue.

\[ ^1H \text{ NMR} (\text{D}_2\text{O}, \delta): 1.00 (s); 1.96-2.82 \text{ (broad, 6H's)}; 2.82-3.80 \text{ (broad, 3H's)}; \]

\[ ^{13}C \text{ NMR} (\text{D}_2\text{O}, \delta): 19.0 (s); 29.95 (s); 35.00 (s); 36.87-52.7 \text{ (broad, backbone C's)}; 180.47 (s). \]

2.1.7 Synthesis of 54% Amidated and 46% Hydroxyethylated HPEI, HPEI-IBAm\textsubscript{0.54} – EtOH\textsubscript{0.46}

The hydroxyethylilation reaction followed the synthesis of HPEI-IBAm\textsubscript{0.54}, as described above. In an oven-dried and nitrogen purged 50 mL three necked RB flask (RBF), equipped with a water condenser and nitrogen inlet, 0.99 g (14.05 mmol of terminal groups) of HPEI-IBAm\textsubscript{0.54} was dissolved in 3.5 mL ethanol. The RBF was placed in a 65 °C preheated oil bath. Next, 0.35 mL (4.76 mmol) of 1-bromoethanol was added in 3 equal increments. Each increment was stirred for 48 hours followed by the addition of 0.04 g (1.07 mmol) of NaOH in 0.43 mL of methanol, which was stirred for 24 hours with a constant temperature of 65 °C. The solvent was removed under vacuum. The residue was dissolved in methanol and purified by dialysis for 48 hours to remove any salts and other low molecular weight impurities. The methanol was removed under vacuum using the speed-vac to afford 1.03 g (85.8%) of a brown residue.
\textbf{2.1.8 Synthesis of 54\% Amidated and 46\% Ethylated HPEI, HPEI-IBAm\textsubscript{0.54-Et\textsubscript{0.46}}}

Following the synthesis of HPEI-IBAm\textsubscript{0.54}, ethyl groups were added to the polymer. In an oven-dried and nitrogen purged 50 mL three necked RB flask (RBF), equipped with a water condenser and nitrogen inlet, 0.658 g (9.35 mmol of terminal groups) of HPEI-IBAm\textsubscript{0.54} was dissolved in 4 mL of THF. The RBF was placed in a 65 °C preheated oil bath. Next, 0.25 mL (3.14 mmol) of Iodoethane was added and stirred for 48 hours. This was followed by 0.13 g (3.14 mmol) of NaOH in 1.2 mL of methanol, which stirred for 24 hours with a constant temperature of 65 °C. The solvent was removed under vacuum. The residue was dissolved in methanol and purified by dialysis for 48 hours to remove any other salts and low molecular weight impurities. The methanol was removed under vacuum using the speed-vac to afford 0.556 g (74\%) of a yellowish residue.

\textbf{1H NMR (D\textsubscript{2}O, \(\delta\))}: 1.01 (s); 1.26 (s); 2.23-3.93 (broad, 10H's); \textbf{\textsuperscript{13}C NMR (D\textsubscript{2}O, \(\delta\))}: 19.0 (s); 29.95 (s); 35.00 (s); 36.87-52.7 (broad, backbone C’s); 180.47 (s).

\textbf{2.2 Materials and Characterization for Transfection Studies}

\textbf{2.2.1 Materials}

A Human Embryonic Kidney 293 (HEK293) cell line was used for transfection studies. HEK293 cells were grown at 37°C in standard Dulbecco’s Modified Essential Medium (DMEM) growth medium supplemented with 10\% fetal bovine serum (FBS). The control transfectant, LT1, was purchased from Mirus. The reporter construct is a pTarget plasmid with an enhanced green fluorescent protein (pTarget-eGFP plasmid).
2.2.2 Characterization

Transfection studies were characterized with an Olympus epifluorescence microscope using GFP filter sets and a halogen arc lamp for illumination. Magnification was consistent at 20x.

2.2.3 Transfection Studies

Transfection studies were conducted by J. Ashot Kozak, Ph.D. in the Department of Neuroscience, Cell Biology, and Physiology at Wright State University. DNA-polymer complexes were formed at 14 °C for 2 hours, followed by a warm up step to room temp over 4 minutes. DNA-polymer formation was done in DMEM. Transfections were done in 6-well polystyrene plates, with HEK293. DNA-polymer complexes were added to HEK293 and incubated for 2 hours. DNA was consistent at 1 µg per well, while polymer varied from 4 to 8 µg, producing an N/P ratio ranging from 10-15.
3. Results and Discussion

3.1 Characterization of Commercially Available HPEI

Before the synthesis of any modified HPEI can be done, the average repeat unit molecular weight (AMW) of commercially purchased unmodified HPEI must be determined to allow for correct stoichiometric calculations. Determination of the AMW requires the ratio of primary, secondary, and tertiary amines within the unmodified HPEI. Additionally, since the modification reactions are carried out via a SN2 mechanism, tertiary amines do not participate. First, any tertiary amine is hindered by the surrounding polymer. Moreover, modification at a previous tertiary amine would result in formation of an unstable, cationic nitrogen. Consequently, tertiary amines do not lead to permanent C-N bonds and are thus not considered possible sites of modification.

Figure 4. Inverse-Gate $^{13}$C NMR Spectrum of Sigma Aldrich HPEI.
Therefore, determining the amount of tertiary amines in unmodified HPEI is crucial for proper stoichiometric modification calculations as well as characterization of the resulting modified HPEI. It is for this reason, that the first step is to characterize unmodified HPEI to determine the ratio of primary, secondary and tertiary amines. This characterization was carried out via an inverse-gated $^{13}$C NMR spectroscopic technique (Figure 4).

While a typical $^{13}$C NMR spectrum cannot give peak integration values, the inverse-gated pulse sequence allows the peaks to be integrated for quantitative determination of the ratio of primary, secondary and tertiary amines using the following equation (Equation 1), respectively: (27)

$$
i(g+h):\frac{i(d+e+f)}{2}:\frac{i(a+b+c)}{3}
$$

Equation 1. Equation For Determining The Ratio of Primary, Secondary, and Tertiary Amines.

From the $^{13}$C NMR spectrum shown in Figure 4, it was determined that the commercially purchased HPEI consisted of 33% primary, 40% secondary, and 27% tertiary amines. Based on this ratio it was then possible to determine the average repeat unit molecular weight of 43.1 g/mol. Additionally, with 33% and 40% primary and secondary amines, respectively, only 73% of the total amines are available for a nucleophilic-type reaction such as butoxylation or amidation. With the average repeat unit molecular weight and percentage of possible reaction sites known, the synthesis and characterization of amidated HPEI was now possible.


3.2 Characterization and LCST Determination of HPEI-Butox$_{0.75}$

The synthesis of butoxylated HPEI is shown in Scheme 3. The percentage of butoxylation was controlled by the stoichiometric amount of butylene oxide added in the reaction. The AMW used for this calculation was determined from the characterization of unmodified HPEI, as described earlier. Since the tertiary amines are not considered a site of modification, only 73% of the amines can be modified. However, the primary amines can be modified twice, thus making the total number of reaction sites equal to the total number of N-H bonds. Characterization was done with $^1$H NMR and confirmed a 75% modification.

Following the synthesis and characterization of HPEI-Butox$_{0.75}$, transmission studies were conducted. Before determining the effect of pH, weight percent, salts, or other modification on the LCST, a baseline was first established. Therefore, HPEI-Butox$_{0.75}$ was dissolved in DDI water to 2 weight %, with no addition of acid or base. This resulted in a pH = 9.47 and the temperature dependent transmission plot is shown in Figure 5.
By convention the LCST is defined as the temperature correlating to 50% transmittance, thus HPEI-Butox$_{0.75}$ possessed an LCST of 19.5°C.

To fully utilize the dual-responsive nature of “smart” polymers, the LCST should be below the temperature of the body, 37°C. This allows for complex formation with DNA below the LCST, and when placed inside the body, the polymer will “close” around the DNA, forming a tight complex and increasing the ease with which the polyplex can enter the cell. However, for a modified HPEI system to be ideal for gene delivery, the LCST should be below 37°C at biological pH, 7.4.

As the pH decreases, more amines will be protonated and the overall cationic nature of the polymer will increase. This increase in ionic nature will increase the polymer’s solubility in water, thus raising the LCST. So, although HPEI-Butox$_{0.75}$ has an ideal LCST at a pH = 9.47, it may not at pH = 7.4. Therefore, the next step was to determine the effect of
lowering the pH on the LCST of HPEI-Butox0.75, to determine if it is still a potential vector for gene delivery.

HPEI-Butox0.75 was again dissolved in DDI water, however this time 0.1M HCl was added to lower the pH to 8.00 and then 7.00. The temperature dependence of the transmission results is shown in Figure 6.

Figure 6. Transmission Plot for HPEI-Butox0.75 at Varying pH in H2O.

From Figure 6, it can be seen there is a strong relationship between the pH and the LCST. Recall, an ideal vector for gene delivery would possess an LCST below 37°C at pH = 7.4. HPEI-Butox1.00 at pH = 8.00 has an LCST of 38°C and it still does not reach a transmittance of zero. The same polymer at pH = 7.00 never reaches 50% transmittance. From these results it can be concluded that HPEI-Butox1.00 is not an ideal vector for gene delivery.
The addition of butylene oxide added a hydrophobic alkyl chain along with a hydrophilic alcohol group. When the pH was lowered, every amine in the polymer had the ability to be protonated, regardless of the location of butoxylation. With such a vast number of amines to be protonated, small changes in pH resulted in large changes in LCST. However, if a modification to a particular amine could reduce its ability to protonate in acidic solutions, then small changes in pH might result in small changes in LCST. Therefore, amidation was the next modification to be tested.

### 3.3 Characterization of Varying Percent Amidation of HPEI

The synthesis of amidated HPEI is shown in Scheme 4. The percentage of amidation was controlled by the stoichiometric amount of isobutryl chloride added in the reaction. Additionally, the AMW used in the stoichiometric calculations for the varying percent amidation was determined from the characterization of unmodified HPEI. The percent of primary and secondary amines, or available reaction sites for isobutryl chloride, was important.

Scheme 4. Synthesis of Amidated HPEI.
Since this reaction follows a typical nucleophilic acyl substitution reaction, a tertiary amine is an unlikely sight for nucleophilic substitution. In addition, upon amidation, the primary amines are not likely to undergo a second amidation. It is for these reasons that tertiary amines are not considered in the percent amidation, as will be seen later.

Characterization of the amidated HPEI was done with $^1$H, inverse gated and dept $^{13}$C NMR spectroscopy. The $^1$H NMR spectrum for the 54% amidated HPEI, HPEI-IBAm$_{0.54}$ system is shown in Figure 7. Additionally, the two $^{13}$C NMR spectra for the same modified HPEI system are shown in Figure 8.

![Figure 7. $^1$H NMR Spectrum of 54% Amidated HPEI, HPEI-IBAm$_{0.54}$](image-url)
As can be seen in Figure 7, with the exception of the six –CH$_3$ protons, the protons in HPEI-IBAm$_{0.54}$ cannot be readily distinguished by $^1$H NMR spectroscopy. However, using Equation 2 below, the percent modification was determined.$^{(25)}$

$$\% \text{ Modification} = \frac{4i\left(\frac{a}{6}\right)}{i\left(b + c + d + e + f + g\right) - i\left(\frac{a}{6}\right)}$$

Equation 2. Equation For Determining The Percent Amidation of HPEI.

The 73% is the percent of primary and secondary amines in the unmodified HPEI as determined from Figure 4 and Equation 1. Therefore, Equation 2, gives the percent amidation of only the possible reaction sites, i.e. excluding the tertiary amines. In addition to Equation 2, the percent of amidation was determined from the integration of the inverse-
gated $^{13}$C NMR spectrum. The dept $^{13}$C NMR spectrum helped to distinguish the backbone methylenes (-CH$_2$-) from the other peaks. Then the carbonyl peak was compared to that of the backbone. Moreover, the inverse-gated $^{13}$C NMR spectrum allowed for determination of the percent amidation at primary as well as secondary amines as labeled in Figure 8B.

3.4 Transmission of Amidated HPEI

HPEI was modified with isobutyryl chloride, producing 54% amidated HPEI, HPEI-IBAm$_{0.54}$. As previously stated, the addition of hydrophobic or hydrophilic groups to HPEI gives rise to an LCST, above which temperature the polymer becomes insoluble. Before determining if the percent modification, pH, presences of salts, weight percent, or other modifications have any effects on the LCST, a baseline must first be established.

![Transmission Plot for HPEI-IBAm$_{0.54}$ in H$_2$O.](image)

Figure 9. Transmission Plot for HPEI-IBAm$_{0.54}$ in H$_2$O.
HPEI-IBAM$_{0.54}$ was dissolved in DDI water with no addition of acid or base to determine the LCST. The transmission plot for this sample (pH = 7.66) is shown in Figure 9, while the pictures of the actual sample, both above and below the LCST are shown in Figure 10. The LCST was defined as the temperature correlating to 50% transmittance. As such HPEI-IBAm$_{0.54}$ was determined to have an LCST of 23.5°C.

Recall that an LCST below body temperature (37°C) theoretically has properties ideal for gene delivery. Complex formation with DNA can occur below the LCST, but when placed in the body it will “close” around the DNA. Therefore HPEI-IBAm$_{0.54}$ was considered the baseline for further amidated HPEI studies.

While the LCST was below body temperature, it was not at biological pH, 7.4. As the proton concentration increases, the ionic characteristic of the polymer increases, thus increasing solubility in water. This could have a drastic effect on the LCST. Therefore, the next step was to determine the effect of pH on the LCST as well as determine if at pH 7.4, the LCST is still below 37°C.
3.5 Effect of pH on Transmission

With decreasing pH, the H⁺ concentration increases. Higher H⁺ concentrations should lead to greater levels of protonated amines. With more amines protonated, the cationic nature of the polymer increases, thus increasing the polymer’s solubility in water. If the polymer is more soluble in a given solvent, the LCST, temperature above which the polymer becomes insoluble, will increase; keeping the polymer in solution, longer. Therefore, as the pH decreases, LCST increases, Figure 11.

The HPEI-IBAm0.54 sample was dissolved in DDI water and the pH was lowered with HCl to pHs of 7.4, 6.0, and 5.0. These are compared to the original sample at pH = 7.66. Figure 11. Transmission Plots of HPEI-IBAm0.54 at Varying pH’s in H₂O.

The LCST data from Figure 11 are summarized below in Table 2. From Table 2, it is clear that as the pH decreases the LCST increases.
<table>
<thead>
<tr>
<th>Polymer System</th>
<th>pH</th>
<th>LCST (˚C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HPEI-Butox₁₀₀</td>
<td>8.00</td>
<td>53.5</td>
</tr>
<tr>
<td>HPEI-Butox₁₀₀</td>
<td>7.00</td>
<td>Never Reached</td>
</tr>
<tr>
<td>HPEI-IBAm₀₅₄</td>
<td>7.66</td>
<td>23.5</td>
</tr>
<tr>
<td>HPEI-IBAm₀₅₄</td>
<td>7.40</td>
<td>24</td>
</tr>
<tr>
<td>HPEI-IBAm₀₅₄</td>
<td>6.00</td>
<td>33.7</td>
</tr>
<tr>
<td>HPEI-IBAm₀₅₄</td>
<td>5.00</td>
<td>50.1</td>
</tr>
</tbody>
</table>

Table 2. LCST Values at Varying pHs for HPEI-Butox₁₀₀ and HPEI-IBAm₀₅₄.

It also confirms that at a biological pH, the LCST is still below 37˚C. Therefore, HPEI-IBAm₀₅₄ is still a possible vector for gene delivery. When HPEI-IBAm₀₅₄ is compared to the HPEI-Butox₁₀₀, also in Table 2, at similar pH, a clear distinction can be seen.

3.6 Effect of Varying Percent Modification on Transmission

Increasing or decreasing the percent amidation increases or decreases the number of amines which can be readily protonated. Any protonated amine is cationic, increasing the polymer’s ionic character, and making it more soluble. However, as isobutyryl chloride is added, any newly formed amide group will no longer likely be protonated. Therefore, increasing the percent modification should decrease the modified HPEI’s solubility in water, lowering the LCST, while the inverse is true for decreasing the percent amidation, as seen in Figure 12.
Figure 12. Transmission Plots for Varying Percent Amidations of HPEI.

The results for varying percent amidation are not as expected. With 44% and 40% variation, greater changes in LCST were expected. One possible explanation for this is that although tertiary amines are the most hindered, they are also the most electron rich. Since steric hindrance does not play as much of a role with H^+, when dissolved in a solution with an acidic pH, the tertiary amines will be the first to be protonated, followed by the secondary and primary amines, respectively. This could explain the unusually small changes in LCST with varying percent amidation. Recall that the amidation is a typical nucleophilic acyl substitution reaction and therefore does not take place at tertiary amines. So although the amide groups are reducing the ability of primary and secondary amines to be protonated, it is the tertiary amines that are being protonated in solution. Thus, the overall effect of increasing
amidation on the LCST is small. However, with such a large variation in percent amidation, a great range of LCST would still be expected.

3.7 Effect of Salts on Transmission

The next step was to determine the effect of salts on the cloud point, or LCST. HPEI-IBAm$_{0.54}$ was dissolved in phosphate buffer saline solution (PBS) followed by a pH adjustment to 7.4. This sample should give the most accurate LCST as compared to what is expected during transfection studies. As previously stated, it was expected that the salt ions in the PBS solution would lower the LCST. Figure 13 shows a comparison between two HPEI-IBAm$_{0.54}$ samples, both at pH = 7.4, with one in PBS and the other in DDI water.

![Figure 13. Transmission Plots of HPEI-IBAm$_{0.54}$ in H$_2$O and PBS.](image_url)
The LCST for HPEI-IBAm\textsubscript{0.54} dropped 3.6 °C, from 24 °C in water to 20.4 °C in PBS. This confirms that the salts in PBS are salting-out ions. More importantly, the LCST is still below 37 °C and thus it remains a possible vector for gene delivery.

### 3.8 Effects of Weight Percent on Transmission

Determining the effects of weight percent on the LCST is essential to fully characterize these systems. Again, looking ahead, as a vector for gene delivery, certain concentrations may be more ideal than others. However if the LCST is affected in a negative manner, then this polymer system could not be used. Therefore, the next step was to determine the effect of weight percent on the LCST, not only at varying pHs, but also in PBS. Figure 14 shows the transmission of HPEI-IBAm\textsubscript{0.54} (pH = 7.4) at weight percents ranging from 1% to 5%.

![Figure 14. Transmission Plots of HPEI-IBAm\textsubscript{0.54} (pH = 7.4) at Varying Weight Percents in H\textsubscript{2}O.](image)
The weight percent clearly has an effect on the LCST. However, this could change when more than one variable is involved. Therefore, the weight percent was also varied with samples at a pH of 6.00 as well as samples in PBS, as shown in Figures 15 and 16, respectively.

Figure 15. Transmission Plots of HPEI-IBAm$_{0.54}$ (pH = 6.00) at Varying Weight Percents in H$_2$O.
Figure 16. Transmission Plots of HPEI-IBAm\textsubscript{0.54} (pH = 7.40) at Varying Weight Percents in PBS.

The relationship between weight percent and LCST is not linear. Figure 17 shows the correlation of weight percent and LCST for the HPEI-IBAm\textsubscript{0.54} samples at pH 7.40 and 6.00, as well as in PBS (pH = 7.40).
Figure 17. Overview of the Effect of Weight Percent on the LCST for Multiple HPEI-IBAm0.54 Samples.

The trend for increasing weight percent (wt%) is not linear, however it is consistent. Additionally, at low wt%, both the PBS and water (pH = 7.4) samples were still below 37 °C. This is encouraging for its applications in gene delivery.

3.9 Effects of Hydroxyethylation on Transmission

Primary amines are very good sites for side reactions, therefore, covering up any remaining primary amines could help prevent side reactions with the DNA or any other species. However, any further modification could also alter the LCST. So the next step was to cover the amines with hydroxyethyl groups according to the procedure outlined in Scheme 5.
Scheme 5. Synthesis of Hydroxyethylated of HPEI-IBAm.

Hydroxyethylation was determined as a possible “protector” of the primary amines, due to its balance of hydrophobic and hydrophilic species. If possible, these would balance each other out and not affect the LCST, whilst covering any remaining primary amines. The transmission plot for HPEI-IBAm$_{0.54}$-EtOH$_{0.46}$, along with HPEI-IBAm$_{0.54}$ for comparison, is given in Figure 18. Both samples were at a pH of 7.40.

![Graph](image-url)

Figure 18. Transmission Plots of HPEI-IBAm$_{0.54}$-EtOH$_{0.46}$ and HPEI-IBAm$_{0.54}$ at pH 7.40 in H$_2$O.
Although the hydroxyethyl group did increase the LCST (26.6 °C), it remained below 37 °C. Additionally, it slowed the conformational change from extended to coiled. There is also some possibility this slower conformational change could enhance transfection of DNA into cells. However, in this regard, the presence of an alcohol will also have to be determined.

3.10 Effects on Ethylation on Transmission

As a last modification, ethyl groups were also used to cover up primary amines, as opposed to hydroxyl ethyl groups. This helped determine if the presences of an alcohol had any effect on the LCST or the cellular transfection. Ethylation of HPEI-IBAm\textsubscript{0.54} was carried out according to Scheme 6.

![Scheme 6. Synthesis of Ethylated HPEI-IBAm](image)

Similar to hydroxyethylation, ethylation was done to protect the primary amines. However, without the alcohol group, there is much less hydrophilic nature, which may decrease the LCST. Figure 19 shows the comparison of HPEI-IBAm\textsubscript{0.54-Et\textsubscript{0.46}} and HPEI-IBAm\textsubscript{0.54} at a pH of 7.4 in H\textsubscript{2}O.
The alkyl group had very little effect on the LCST. It may have slightly increased the LCST, which does not correlate to the increase in hydrophobic nature. However, it still possesses an LCST below 37 °C at pH 7.4. Therefore, it can still be used in transfection studies.

3.11 Transfection Results

Transfection studies were carried out on the 54% amidated sample as well as the hydroxyethylated sample. The transfection studies consisted of a DNA complex formation at 14 °C, followed by the placement of the DNA-polymer complex on cells at 37 °C. Complex formation with DNA time was varied to determine the ideal length of polyplex formation. The HPEI-IBAm\textsubscript{0.54} system was tested with 20-minute, 1 hour and 2 hour DNA-polymer complex formation times, all at 14 °C. The samples were warmed to room temperature, and then placed on cells at 37 °C. The 20 minute complex formation time had some transfection,
but not to the level of the control. However, the 1 hour and 2 hour complex formation time samples reached the same level, or possibly even exceeded, the control levels of transfection. Additionally, the intensity of fluorescence in the 1 and 2 hour samples surpassed that of the control. The HPEI-IBAm$_{0.54}$-EtOH$_{0.46}$ sample was also run with a 14 °C complex formation of 20 minutes. Unfortunately, there was very little transfection compared to the control.
4. Conclusions

By modifying commercially available HPEI with hydrophobic and hydrophilic groups, the LCST was controlled. Not only was the LCST changed between butoxylation and amidation, but also with multiple different variables, including pH, percent modification, salt concentration, and percent weight. The pH had a very clear and consistent effect on the LCST; as the pH decreased, the LCST increased. This was expected, with the decrease in pH, more amines are protonated increasing the cationic characteristic of the polymer, making it more soluble and increasing the LCST. On the other hand, the percent modification was not a clear cut. Increasing the percent amidation should decrease the ionizable amines, and thus decrease the solubility and LCST in H₂O. While this overall trend was observed, it was not to the extent expected. Additionally, in concentrated salt solution, PBS, the LCST was lowered, indicating the presences of salting-out ions. Lastly, the weight percent showed a non linear correlation with LCST. This correlation was consistent between samples at different pH as well as in different solutions. The HPEI-IBAm₀.₅₄, HPEI-IBAm₀.₅₄-EtOH₀.₄₆, and HPEI-IBAm₀.₅₄-Et₀.₄₆ samples were all considered possible vectors for gene delivery.

Although HPEI-IBAm₀.₅₄-EtOH₀.₄₆ did not show promising transfection efficiency, HPEI-IBAm₀.₅₄ showed strong promise. Complex formation and gene expression was at least at control levels. Additionally, the increase in fluorescence intensity could indicate a more efficient delivery of the gene into the cell.
References


