Abstract

Ischemic stroke can be associated with varying degrees of neuronal damage and death. To help combat the negative impact of cerebral ischemic events, research has turned its attention to the delivery of plasmids coding for key growth factors to improve neuronal survival. Delivery through the blood brain barrier, however, complicates drug delivery. The use of antibodies presents a unique opportunity to selectively target key receptors on the brain and gain access to this highly selective organ. In this study, a stable formulation for an antibody-conjugated polyplex system was established using polyethylene glycol-polyethylenimine copolymers complexed with plasmid DNA. These polyplexes offer the ability to deliver plasmid DNA through a self-assembling delivery system that has decreased clearance and selective localization to the brain. In establishing a foundation for such polyplexes, this study hopes to demonstrate the utility of this system in drug delivery to the brain.

Introduction

Ischemic stroke is a serious and debilitating condition that is a leading cause of death in the United States. Although the extent of damage can vary, studies have shown that post-cerebral ischemic event, there is an increase in activity in the ischemic area starting minutes after the incident. Part of this increased activity is related to growth factors, which play a role in angiogenesis, neurogenesis and neuroplasticity.[1] Brain derived neurotrophic factor (BDNF) is a growth factor that plays a role in both neuronal and synaptic development and survival. It is known that BDNF is a part of the natural pathophysiology that occurs post-ischemic event, however, the naturally expressed levels of BDNF are not enough to fully combat the damage of ischemia.[2] Thus, a theory has originated that the supplementation of BDNF to the brain can aid in the recovery process after a cerebral ischemic event.[1,2] A study by Schäbitz et al. (2007) showed that animals treated within five days of induced ischemic stroke with intravenous bolus doses of BDNF versus vehicle alone had improved functional recovery.[3] Despite the potential benefits of BDNF supplementation, a major concern for its use in cerebral ischemia is successful delivery to the brain.

Delivery of drugs through the blood brain barrier (BBB) is a significant limitation to pharmacological innovations that seek to implement treatments such as BDNF. While it is possible to deliver drug directly into the brain, this method is highly invasive and associated with increased risk for unwanted complications. The intravenous route is preferred, but delivery across the BBB is complicated by tight extracellular junctions, low pinocytic activity, extensive efflux mechanisms and enzymatic activity.[4] Although it is possible that the BBB may become leaky in areas of cerebral damage, depending on this mechanism of delivery alone is not reliable. A potential method for delivery across the BBB is the utilization of receptor-mediated transport or transcytosis. Delivery through this system can be further improved by using a polymer drug carrier. Limiting carrier size to 100 nm or smaller as well as polymer PEGylation can improve stability and help minimize drug carrier clearance through the reticuloendothelial system.[4] Zhang et al. (2003) showed that a specific receptor could be successfully targeted using a PEGylated liposomal system carrying plasmid DNA using a monoclonal antibody for guided localization to the target receptor.[5] While antibodies offer the advantage of selective binding, Yu et al. (2011) looked at the relationship between antibody binding affinity and the extent of uptake into

the brain. After comparing high- to low-affinity anti-transferrin receptor antibodies at trace and therapeutic doses, they found an inverse relationship between antibody affinity and uptake. While high-affinity antibodies were shown to bind to the target receptor well, they had a low rate of release into the brain after receptor-mediated transcytosis. The low-affinity antibodies, however, if pushed by a concentration gradient, both bound to the target receptor and dissociated after transcytosis.[6] Given this information, the goal of this study was to establish a stable formulation for an antibody-conjugated polyplex system composed of polyethylene glycol-polyethylenimine (PEG-PEI) copolymers complexed with plasmid DNA.

Materials and Methods

Materials

Branched polyethylenimine (PEI, average MW 25 kDa), Anti-goat IgG (Fc specific) antibody produced in rabbit (2.12 mg/mL) and Anti-human IgG (γ-chain specific)-gold antibody produced in goat (5 nm colloidal gold, 171 μg/mL) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Maleimide-polyethylene glycol-succinimidyl carboxyl methyl ester (MAL-PEG-SCM, average MW 5 kDa) was purchased from Creative PEGWorks (Chapel Hill, NC, USA). 2,4,6-trinitrobenzenesulfonate solution (TNBS, 5 % w/v), N-succinimidyl S-acetylthioacetate (SATA, MW 231), NAP-5 Columns (1.5 mL sample volume), Ellman's reagent (MW 396), Cysteine HCl (MW 175), and Micro BCA™ Protein Assay Kit were obtained from ThermoFisher Scientific (Hanover Park, IL, USA). 4–20 % Criterion™ TGX™ Gel (18 well, 30 μl each), Precision Plus Protein™ All Blue Prestained Protein Standards and Bio-Safe™ Coomassie Stain were purchased from Bio-Rad (Hercules, CA, USA). Folded Capillary Zeta Cells were obtained from Malvern Instruments (Malvern WR14 1XZ, UK). Absorbance assays were done using Softmax Pro software. All other materials were existing property of the Kabanov Lab (Chapel Hill, NC, USA).

All buffers and solvents were filtered through a 200 nm pore size filter prior to use.

Synthesis of PEG-PEI Conjugate

The preparation technique for the PEG-PEI conjugate was adapted from Zhang et al. (2010). All solutions were prepared in 100 mM HEPES, pH 7.4. A 3.6 mg/mL solution of branched PEI was prepared; half of the PEI solution was mixed in equal portions with a 15 mg/mL solution of MAL-PEG-SCM. The remaining PEI solution was used as a control. The PEG-PEI solution was dialyzed through a 25 kDa dialysis membrane against two 3 hour rounds of 100 mM HEPES, pH 7.4, followed by two 3 hour rounds of deionized water (DI water) after it had incubated at room temperature (r.t.) for 2.5 hours.[7]

TNBS Assay

TNBS assay was used to determine free amino groups content. A 15 mg/mL solution of TNBS was prepared using 5 % w/v TNBS solution and DI water. Two samples were analyzed: the PEI control solution (1.8 mg/mL) and the resultant PEG-PEI conjugate. Using a 96 well plate, 4 μL of TNBS solution was added to 1 μL, 2 μL, and 5 μL of each sample, with 3 replicas each. Borate buffer, pH 9.3, was the reaction buffer; enough borate buffer was added to each well to bring all wells to the same final volume. Borate buffer, pH 9.3, alone and this buffer with 4 μL of TNBS solution, each with 3 replicas, served as the assay controls. The covered 96 well plate was allowed to incubate at r.t. for 40 minutes. The absorbance was measured at 420 nm.

NMR

The structure of PEI and the PEG-PEI conjugate was analyzed by NMR. A sample of the PEI solution (1.8 mg/mL) and the PEG-PEI solution was lyophilized. The lyophilized powders were then each dissolved in enough deuterium oxide (D_2O) to create 700 μL of solution for each sample.

Ethidium Bromide (Et-Br) Exclusion Assay

An Et-Br exclusion assay was used to determine the concentration of PEI and PEG-PEI polymer needed to cause complete condensation, or compaction, of cow thalamus (ct)-DNA. A 0.5 mg/mL solution of PEI and of PEG-PEI was prepared; the concentration was based on amine content. A 20 μg/mL solution of ct-DNA was prepared using 10 mM HEPES buffer, pH 7.4. The ct-DNA solution was allowed to stand at r.t. for 10 minutes.

Enough Et-Br was added to the DNA solution to achieve a final Et-Br concentration of 1 μg/mL. Half of the DNA solution was transferred to a plastic UV cuvette and using a fluorimeter with appropriate filters (540 nm for excitation and 590 nm for emission), the PEI solution was slowly added to the DNA solution in the cuvette. After each addition, the relative fluorescence units, or RFUs, were recorded. This process was repeated for PEG-PEI using the other half of the DNA solution in a new UV cuvette. The RFU data was used to determine the molar ratio of polymer amines to DNA phosphates (N/P ratio) needed to produce stable polyplexes. The collected data was validated by a second experiment completed in the same fashion with luciferase DNA.

Agarose Gel Electrophoresis

Agarose gel electrophoresis was used to determine the stability of polyplexes prepared using N/P ratios above and below the determined N/P ratio for successful condensation of plasmid DNA. Enough tris base, borate, and ethylenediaminetetraacetic acid (EDTA) were combined in DI water to create a 10X TBE buffer. This buffer solution was used to create all other TBE buffers used. A 0.8 % agarose gel solution was prepared using agarose powder and 1X TBE buffer. Prior to pouring the solution into a gel cast, enough Et-Br was added to the solution to produce a final Et-Br concentration of 0.5 μg/mL. The gel was allowed to solidify for 1 hour. Eleven samples were prepared for electrophoresis; each had 20 μg/mL of ct-DNA and the same total volume (20 μL). For both PEI and PEG-PEI, polyplexes were created with a N/P ratio of 0.5, 1, 1.7, 3 and 5. Additionally, a DNA control and a ladder were prepared; the ladder contained 6X DNA loading dye with DNA ladder. All samples were prepared in 10 mM HEPES, pH 7.4. Once the samples were loaded, the gel cast was filled with 0.5X TBE buffer. The agarose gel was allowed to run for 1 hour at 120 V and 60 mA. After electrophoresis was complete, the gel was imaged under UV light to identify ct-DNA migration.

Particle Size and Zeta Potential Characterization

Using a Malvern Zetasizer Nano machine, dynamic light scattering (DLS) was used to determine polyplex size and zeta potential at different N/P ratios. All samples were prepared in 10 mM HEPES, pH 7.4. The 7 samples prepared included ct-DNA alone, and for both PEI and PEG-PEI, polyplex samples with a N/P ratio of 0.5, 1.7 and 5. Samples

were filtered through a 200 nm pore size filter prior to their transfer to a folded capillary zeta cell for analysis.

Sulfhydryl Modification of IgG Antibody and Coupling to PEG-PEI Polyplexes

SATA was used to introduce sulfhydryl groups onto IgG antibody for subsequent antibody reaction with the PEG-PEI maleimide. The protocol for IgG modification with SATA was based on the manufacturer's product instructions. This process was first performed and verified using polyplexes of ct-DNA; luciferase DNA was chosen as a model plasmid for further application. Phosphate-buffered saline (PBS), pH 7.2-7.5, was prepared as the reaction buffer. Enough SATA was dissolved in dimethyl sulfoxide (DMSO) to produce a 25:1 molar ratio of SATA: IgG; 2 mg of IgG were combined with SATA. The solution was allowed to incubate at r.t. for 1 hour. After incubation, the solution was purified using a NAP-5 column. The column was washed with PBS and the produced acylated antibody was collected in PBS. Ellman's assay was used to ensure successful acetylation of sulfhydryl groups. The protocol for assay of sulfhydryl groups with Ellman's reagent was based on the manufacturer's instructions using cysteine standards and a reaction buffer of 0.1 M sodium phosphate with 1 mM EDTA. The acylated antibody was then deacetylated to generate free sulfhydryl groups per the manufacturer's instructions using 0.5 M hydroxylamine with 25 mM EDTA in PBS, pH 7.2-7.5, as the deacetylation solution and a NAP-5 column. Deacetylated IgG was then mixed with 1 mL of PEG-PEI polyplexes, prepared in 10 mM HEPES, pH 7, using luciferase DNA at a N/P ratio of 10. The pH of the mixture was adjusted to a pH of 7.0 and the mixture was allowed to incubate at r.t. for 12 hours. Agarose gel electrophoresis was performed using the method outlined earlier to compare the stability of unmodified luciferase DNA containing PEG-PEI polyplexes to the generated IgG modified luciferase DNA containing PEG-PEI polyplexes.

Transmission Electron Microscopy (TEM)

TEM imaging was performed to characterize differences in binding affinity, size and shape between IgG modified PEG-PEI polyplexes and unmodified PEG-PEI polyplexes. Binding affinity was determined by the extent of gold particle aggregation visualized when each polyplex sample was exposed to anti-human IgG containing 5 nm gold particles. Each polyplex sample was combined with IgG containing gold in a 1:1.71 ratio, respectively.

Imaging was accomplished through equipment provided by the Chapel Hill Analytical and Nanofabrication Laboratory, Chapel Hill, NC.

MicroBCA Protein Assay with IgG Standards

MicroBCA was performed as outlined in the manufacturer's instructions using a 96 well plate with the exception that anti-goat IgG standards were used instead of albumin standards; the standards' concentrations were as outlined per manufacturer despite use of anti-goat IgG. The 96 well plate was incubated at 37 °C for two hours; the final concentration was based on measured absorbance at 562 nm.

Denaturing Gel Electrophoresis

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was utilized to confirm the presence of PEI, PEG-PEI and IgG in each sample through gel migration. The following 11 samples were prepared: 3 μg samples of IgG, ct-DNA, and luciferase DNA; 5 μg samples of free PEI, free PEG-PEI, unmodified ct-DNA PEI polyplexes, unmodified ct-DNA PEG-PEI polyplexes, IgG modified ct-DNA PEG-PEI polyplexes, unmodified luciferase DNA PEI polyplexes, unmodified luciferase DNA PEG-PEI polyplexes and IgG modified luciferase DNA PEG-PEI polyplexes. Each sample was heated at 100 °C for 10 minutes. An electrophoresis tank with a 4–20 % Criterion Gel was filled with running buffer, 1X tris/glycine, which was prepared from 10X tris/glycine solution and DI water. Twenty microliters of each sample were loaded into the gel. Ten microliters of all blue standard were added as the ladder. The electrophoresis tank was allowed to run for 2 hours at 100 V and 50 mA. The finished gel was stained with coomassie for 1 hour on a rocker, washed with DI water three times and imaged.

Results and Discussion

PEG-PEI Conjugate

Utilizing an antibody-conjugated polyplex system allows for plasmid delivery to the brain through a receptor-mediated endocytosis. While antibody targeting permits the drug carrier to cross the BBB, the polymers used must facilitate antibody conjugation while maintaining structural stability. Branched PEI is a cationic polymer that can spontaneously

form polyion complex with the negatively charged phosphate backbone of plasmid DNA through electrostatic interactions. The positive charge, however, is limiting in terms of distribution out of the vasculature. The addition of nonionic maleimide PEG offers enhanced water-solubility and a "handle" for antibody modification. The reactive group on MAL-PEG-SCM, succinimidyl carboxyl methyl ester, reacts with a primary amine on the branched PEI to form an amide bond that links the two polymers.[8] A general schematic for this reaction is shown in Figure 1.

TNBS Assay

Structurally, PEI has several available amines per branched segment that could be targeted by SCM in the conjugation reaction. It has been shown, however, that roughly one-third of these amines are not available for conjugation.[9] For PEI with a molecular weight of 25,000 Da, it was assumed that each repeating unit of PEI is 43 g/mol and contains one amine. This would translate to roughly 580 amines per PEI molecule with 388 amines potentially

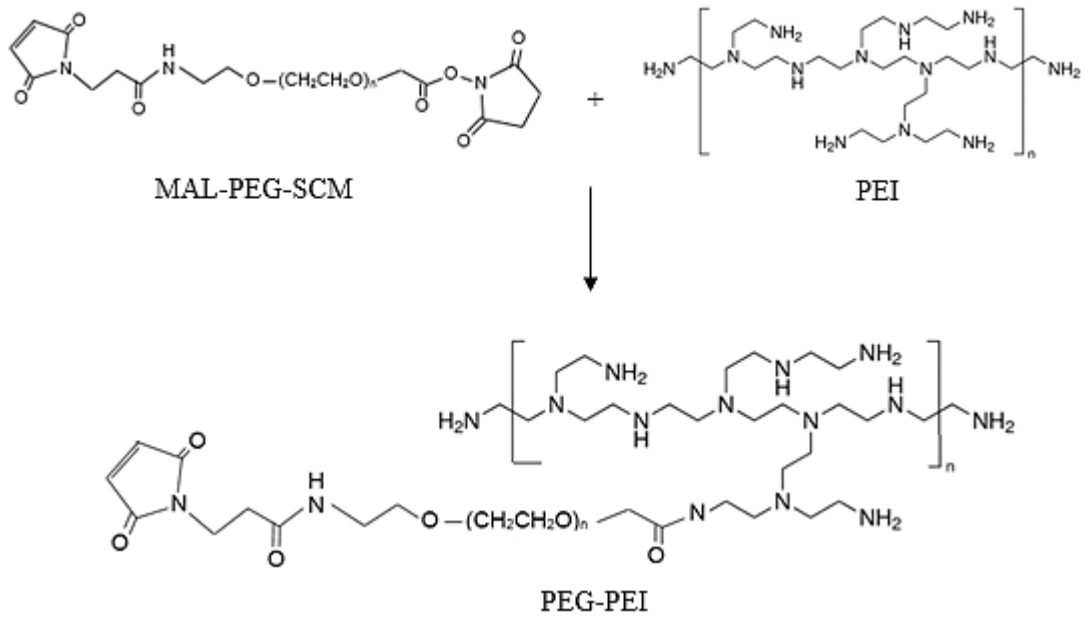
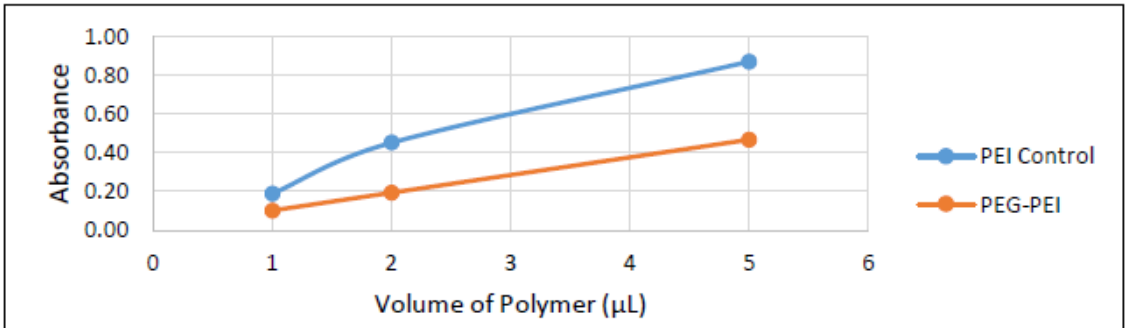


Figure 1. PEG-PEI Conjugation Reaction Scheme. The SCM ester on MAL-PEG-SCM reacts with a primary amine on the PEI molecule to form an amide bond. The resulting PEG-PEI conjugate has an available maleimide group for further coupling reactions.

available for conjugation. TNBS assay was performed to determine the amine content of the generated conjugate in comparison to a PEI control. TNBS was believed to be titrating the remaining amines and producing a signal that corresponded to the concentration of PEI in the samples. If less than 10% of the amines were being modified, then any loss in signal above the modification extent would have been attributed not to the modification of PEI by PEG, but to the discrepancy in the concentration of the overall PEI. The absorbance data plotted in Figure 2 shows about a 50 % reduction in absorbance between the PEI and PEG-PEI samples. This change, therefore, likely does not represent the extent of modification; the preparation technique used for the conjugate produced a PEG to PEI feed ratio of 20.83, which under ideal conditions would theoretically correspond to 5.15 % modification. Given intrinsic experimental error, this relatively low modification rate would present a challenge in determining amine content. Additionally, it is important to consider that to produce a purified PEG-PEI sample, the PEG-PEI solution was dialyzed, as described earlier. The collected data, given the expected modification rate, showed that the dialysis resulted in enough of a PEG-PEI concentration change that it was unclear how



PEG-PEI Volume (µL)	PEG Content (mol)	PEI Content (mol)	Molar Ratio of PEG/PEI	Amine Content (mol)
1	1.654E-07	3.892E-08	4.25	1.50859E-05
2	4.112E-07	6.176E-08	6.66	2.39379E-05
5	8.335E-07	1.933E-07	4.31	7.49219E-05

Figure 2. TNBS Assay Absorbance Data. Prior to dialysis of PEG-PEI, the PEI control and PEG-PEI solutions each contained 1.8 mg/mL of PEI. Equal volumes of PEI and PEG-PEI were reacted with TNBS buffer. The measured absorbance corresponded to the concentration of amine. Thus, the molar content of PEI was determined for each sample. The loss of signal was attributed to differences in the overall PEI concentration. Based on the lost PEI content, the molar content of PEG was determined.

much of the change in absorbance was due to dilution versus modification. Distinguishing between these two influences would have required further measurement of the total amine content by an independent method such as an elemental analysis, which was not performed in this study. Based on the data obtained, it was calculated that there were roughly four PEG per PEI molecule. Considering the expected amine content in each sample and the fact that one PEG conjugates to a single amine, this would translate to roughly 1 % modification, an extent of modification that TNBS is not sensitive enough to measure. TNBS assay measures the difference in modifiable groups, but to use this assay the starting amine content must be known. The belief that the dilutional effects of dialysis could be overcome by using a known concentration of PEI did not hold true in this experiment. Thus, it was found that TNBS assay was not a good technique for amine determination and NMR was needed to show successful modification.

NMR

NMR spectroscopy was performed to compare the structural differences between PEI and PEG-PEI. The spectrum of the buffer solution, 100 mM HEPES, in which these polymers were prepared was also obtained. Based on previous studies, it was expected that the peaks associated with PEI would be around 2.40-2.75 ppm for PEI[10] and roughly 2.00-2.60 ppm and 3.20-3.60 ppm for PEI and PEG, respectively, when conjugated.[11] As shown in Figure 3, the PEI spectrum contained a peak around 2.65-2.80 ppm, which would be an appropriate position for a PEI peak. While this area overlapped with a HEPES peak, the PEI and HEPES spectra showed that this peak was shifted in its position and differed in shape from that of HEPES; this supported the inference that this peak was associated with PEI. This was further confirmed in the PEG-PEI spectrum, which showed a similar overlapping in roughly the same area. The presence of a distinct peak around 3.51-3.61 ppm was believed to be due to the presence of PEG in the solution. Despite these conclusions, the overlapping HEPES peaks complicate interpretation of this data. A potential limitation was the presence of the HEPES peaks; only the PEG-PEI underwent dialysis and no further dialysis occurred prior to imaging. Future experiments would benefit from performing dialysis for both NMR samples prior to imaging. For the purposes of this study, the spectra data was sufficient to support successful conjugation.

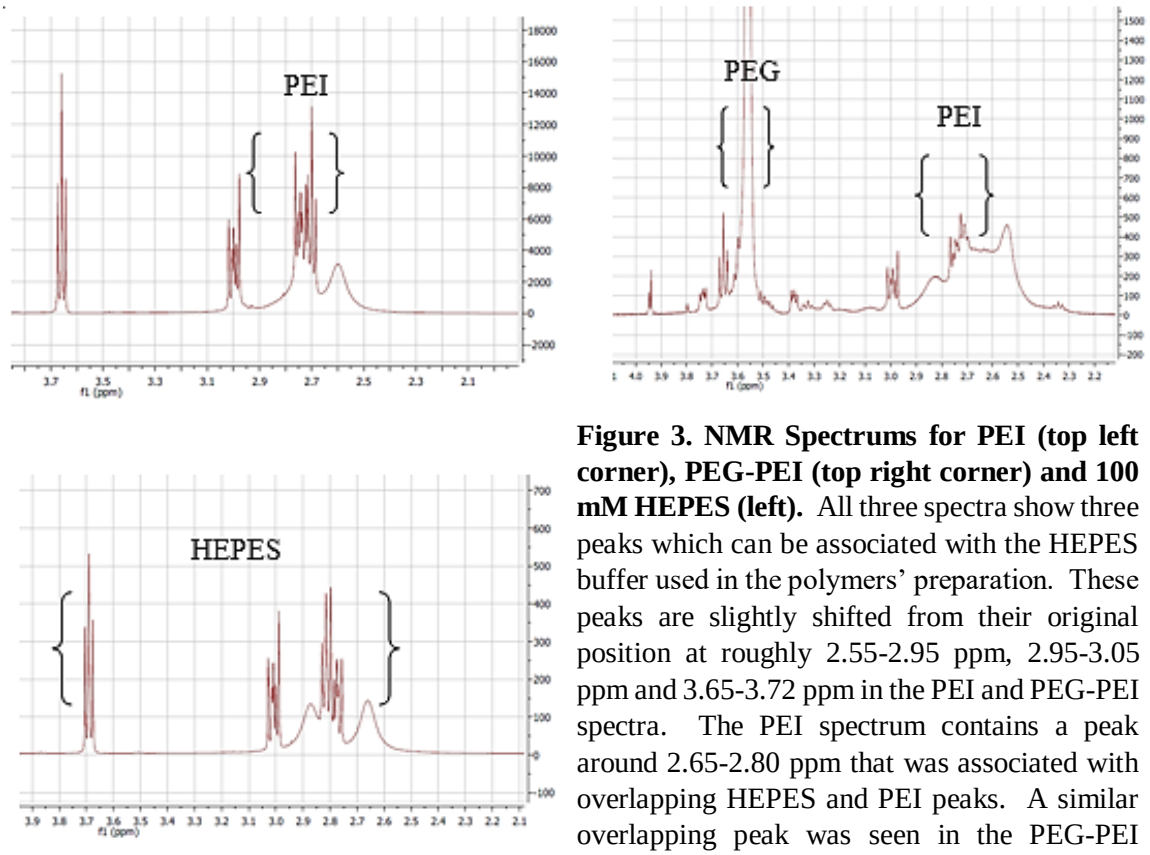


Figure 3. NMR Spectrums for PEI (top left corner), PEG-PEI (top right corner) and 100 mM HEPES (left). All three spectra show three peaks which can be associated with the HEPES buffer used in the polymers' preparation. These peaks are slightly shifted from their original position at roughly 2.55-2.95 ppm, 2.95-3.05 ppm and 3.65-3.72 ppm in the PEI and PEG-PEI spectra. The PEI spectrum contains a peak around 2.65-2.80 ppm that was associated with overlapping HEPES and PEI peaks. A similar overlapping peak was seen in the PEG-PEI spectrum, along with a distinct PEG peak at around 3.51-3.61 ppm.

Et-Br Exclusion Assay

Complete condensation of plasmid DNA by the carrier polymer is key to creating stable polyplexes that could successfully deliver the intended payload to the brain. Et-Br is an intercalating agent that is able to produce a fluorescent signal upon binding to plasmid DNA. This fluorescence is diminished in the presence of cationic species, such as PEI, which can displace the Et-Bt on the DNA. In measuring this change in fluorescence, it is possible to determine the molar ratio of polymer amines to DNA phosphates (N/P ratio) that results in full condensation of plasmid DNA.[12] A plateau in fluorescence denotes the equivalence point or the point at which full condensation has occurred. Et-Br exclusion assay was performed with both the PEI and PEG-PEI polymers. The ideal N/P ratio would be one that allowed for full condensation with both polymers. The expected amine

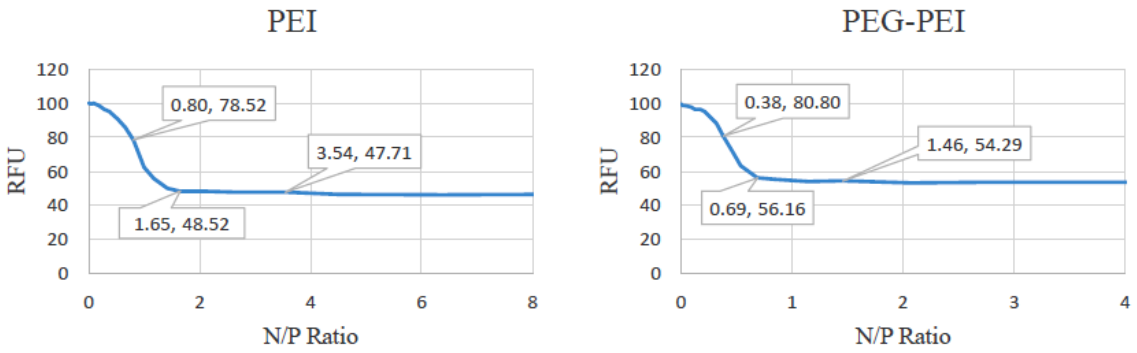


Figure 4. Graph of RFU Data Versus Calculated N/P Ratios. The plotted data shows the loss of fluorescence associated with each addition of polymer (0.5 mg/mL) to ct-DNA solution (20 µg/mL), which is represented as a N/P ratio. The point at which the loss of fluorescence plateaus is the equivalence point. This was shown to have occurred at roughly a N/P ratio of 1.65 for PEI and 0.69 for PEG-PEI.

content of each polymer and the volume of polymer added at each absorbance reading was used to calculate the N/P ratio. The equivalence point for PEG-PEI occurred after fewer additions of polymer in comparison to PEI, which was likely due to the stability offered by the neutral charge and steric contributions of PEG. Based on the data shown in Figure 4, it was determined that a N/P ratio of 1.7 would be sufficient to produce condensation in both polymers.

Agarose Gel Electrophoresis

Agarose gel electrophoresis allows for the visualization of plasmid DNA migration. At N/P ratios above the equivalence point, polyplexes should be stable and maintain plasmid DNA condensation. Below the equivalence point, free plasmid DNA is available and would be expected to migrate down the gel due to electrostatic attraction to the positively charged bottom of the electrophoresis tank. The absence of DNA migration indicates stable polyplex formation. Although the equivalence point for PEI was at a N/P ratio of 1.65, no ct-DNA migration was seen at a N/P ratio of 1 and higher. This may be attributed to the attractive forces between DNA phosphates and PEI amines, which may be enough at a N/P ratio of 1 to hold the plasmid DNA close to the PEI even without full condensation. The signal visible at the well for PEI ct-DNA polyplexes with N/P ratios of 1.7 and higher

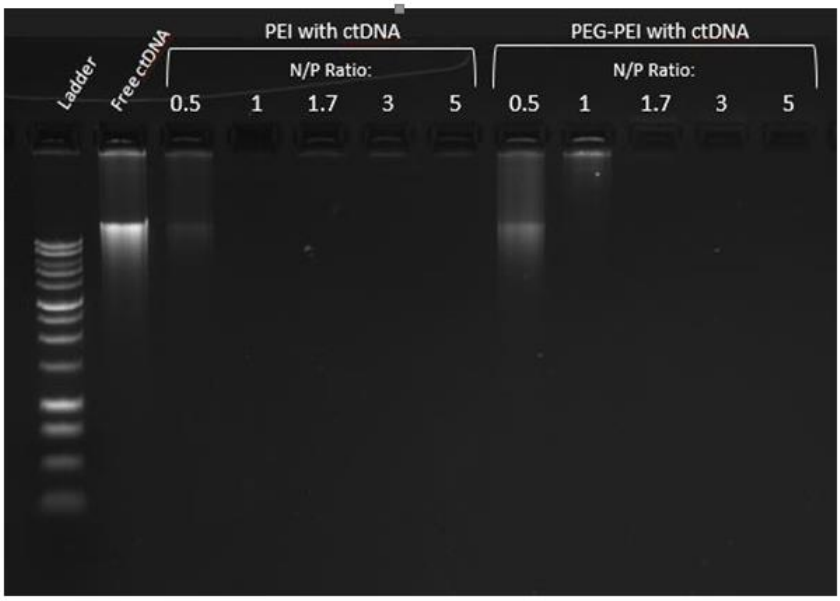


Figure 5. Agarose Gel of PEI and PEG-PEI ct-DNA Polyplexes at Variable N/P Ratios. Each well contains 20 μL of sample. For PEI ct-DNA polyplexes, stability was demonstrated before the equivalence point. For PEG-PEI ct-DNA polyplexes, stability was demonstrated at a N/P ratio slightly higher than the equivalence point. Both polymer polyplexes showed stability after the set N/P ratio of 1.7.

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was due to the presence of plasmid DNA in the polyplex. Since complex formation is based on electrostatic interactions, the polymers are not locked into place. The attraction of PEI to the negatively charged top of the electrophoresis tank, along with the polymer's motion, allows for plasmid DNA to produce a slight signal despite condensation. For PEG-PEI, although the equivalence point was determined to be at 0.69, polyplex stability was shown at a higher N/P ratio. This may imply that while a smaller number of PEG-PEI is needed for condensation, in the presence of an interacting force, such as the charge applied by the electrophoresis tank, alternative attractive forces may overcome the electrostatic interaction between PEG-PEI and plasmid DNA. Despite this, both polymer polyplexes showed stability at a N/P ratio equal to and greater than 1.7.

Particle Size and Zeta Potential Characterization

Dynamic light scattering (DLS) was used to characterize polyplex size and zeta potential. Given that PEI is a cationic polymer, PEI polyplexes were expected to have increasingly positive zeta potentials as the concentration of PEI increased. With PEG-PEI, it was

Polymer	N/P Ratio	Z-Ave (d.nm)	PdI	Zeta Potential (mV)
Control (Free ctDNA)	0	196.60	0.54	-15.97
PEI	0.5	144.00	0.26	-38.57
PEI	1.7	225.20	0.26	-17.38
PEI	5	210.40	0.18	-9.31
PEG-PEI	0.5	230.97	0.37	-17.23
PEG-PEI	1.7	183.93	0.30	-14.19
PEG-PEI	5	150.30	0.24	-1.64

Table 1. Size and Zeta Potential Data for PEI and PEG-PEI Polyplexes at Variable N/P Ratios. The average diameter of the polyplexes increased for PEI and decreased for PEG-PEI with higher N/P ratios. Overall, the zeta potential for PEI was negative rather than positive, but with increasing N/P ratios, the surface charge trended toward neutrality. The same was observed for PEG-PEI.

expected that the PEG component would contribute to a neutral surface charge; with the addition of more polymer, or higher N/P ratios, it was anticipated that the surface charge would move from a positive to a more neutral charge. The observed diameter for the measured polyplexes varied from 140 nm to 230 nm; these averages were likely skewed by aggregates, which were noted to be present for all samples. This was supported by the moderately polydisperse distribution noted in Table 1. Surprisingly, the overall zeta potential was negative. While both PEI and PEG-PEI showed a trend toward neutrality, it was unclear what contributed to the observed negative charge. Some literature indicates that pH may play a role in measured zeta potential, in addition to the N/P ratio used,[12] but overall it is accepted that PEI should produce positive zeta potentials at physiological pH.

Sulfhydryl Modification of IgG Antibody and Coupling to PEG-PEI Polyplexes

The addition of monoclonal antibodies to a polyplex system allows for receptor-mediated transport, but successful antibody targeting depends on an antibody modification process that retains antigen-binding activity. SATA modification of antibodies aids in the addition of a protected thiol group without loss of activity and offers the ability to store modified antibodies for prolonged periods of time.[8] The SATA modification chemistry is shown in Figure 6. Antibody thiolation generates a reactive group for conjugation with the PEG-PEI maleimide. Prior to deacetylation of the SATA modified antibodies, an Ellman's assay was performed to identify any free or unprotected sulfhydryl groups.

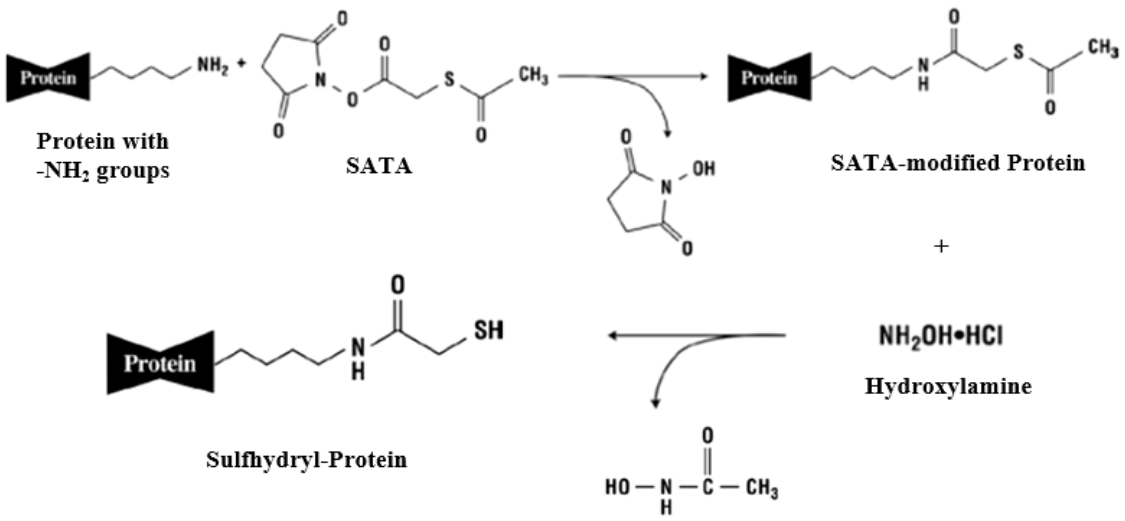

Figure 6. Protein Modification with SATA Reaction Scheme. SATA, containing a protected sulfhydryl group, reacts with the primary amines of the target protein to form an amide bond. The SATA-modified protein is then deacetylated with hydroxylamine to produce a free sulfhydryl group that can be used in a subsequent reaction.[13]

Ellman's reagent reacts with free sulfhydryl groups to form disulfide bonds and a reduced product that can be measured at 412 nm.[14] Any absorbance data generated from the assay should correlate to unprotected sulfhydryl groups. The calculated sulfhydryl concentration was 0.0614 nmol. This was deemed acceptable considering that some of the natural disulfide groups in the antibody may have contributed to the signal. After deacetylation, the thiolated antibody was combined with luciferase DNA containing polyplexes with a N/P ratio of 10 to produce IgG coated PEG-PEI polyplexes. The scheme for this reaction is shown in Figure 7. This process was performed with both ct-DNA and luciferase plasmid DNA, but the luciferase plasmid DNA was shown to have more consistent behavior. Agarose gel electrophoresis showed that both unmodified luciferase DNA containing PEG-PEI polyplexes and IgG modified luciferase DNA containing PEG-PEI polyplexes, both with a N/P of 10, retained their stability despite the described manipulation.

Transmission Electron Microscopy

Polyplex modification with IgG was expected to generate polyplexes with a different size and binding affinity when compared to unmodified polyplexes. The extent of gold particle

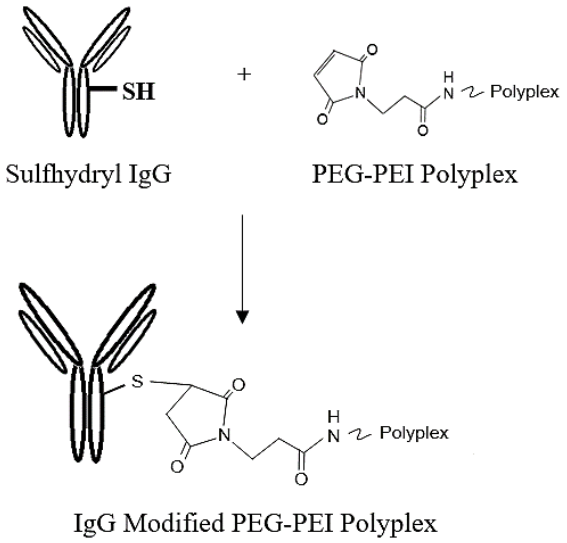

Figure 7. PEG-PEI Polyplex Modification with Sulfhydryl IgG Reaction Scheme. The generated sulfhydryl on IgG reacts with the maleimide of the PEG-PEI polyplex to form a thioether bond. The result is an IgG coated PEG-PEI polyplex.

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aggregation observed after combining polyplexes with anti-human IgG containing 5 nm gold particles was used as a surrogate to predicted target receptor binding affinity. The resultant TEM images can be seen in Figure 8. The gold particles in the anti-human IgG were in colloid form; imaging of this sample alone was completed to visualize gold particle and colloid distribution. TEM imaging of the anti-human IgG showed gold particles with a random distribution and colloids of up to 200 nm in diameter. The presence of colloid was seen in both the modified and unmodified polyplex images. For unmodified PEG-PEI polyplexes, random distribution of gold particles was seen in the background. Some gold particle aggregation was observed with unmodified polyplexes, but this was likely due to the mild affinity of the gold containing colloids to the PEG-PEI of the unmodified polyplexes. In comparison, the greater extent of gold particle aggregation at the IgG modified polyplexes, along with fewer background gold particles, indicated that the modified polyplexes demonstrated a greater affinity than the unmodified polyplexes. This was correlated to the presence of IgG in the modified samples. However, the observed aggregation was not consistent throughout the sample signifying that the degree of modification was not consistent from one polyplex to the next. The modified and unmodified polyplexes both appeared to be up to roughly 0.5 μm in diameter. Closer

analysis of the images showed that these polyplexes had been flattened out, possibly in the grid drying process, and were likely smaller than this in diameter when in their natural spherical or ovular shape. Based on these images, it was accepted that there was successful modification of polyplexes with IgG and that this IgG demonstrated a greater binding affinity than unmodified PEG-PEI polyplexes.

MicroBCA Protein Assay with IgG Standards

MicroBCA protein assay using anti-goat IgG standards was performed for modified and unmodified polyplexes. Previous versions of this experiment showed that using albumin standards resulted in absorbance data that included a signal from alternative protein sources other than IgG. Using IgG standards was believed to produce a more accurate measure of antibody concentration. The resultant IgG concentration in the modified polyplex sample was found to be 1.028 mg/mL. Given this value, the expected total concentration of IgG

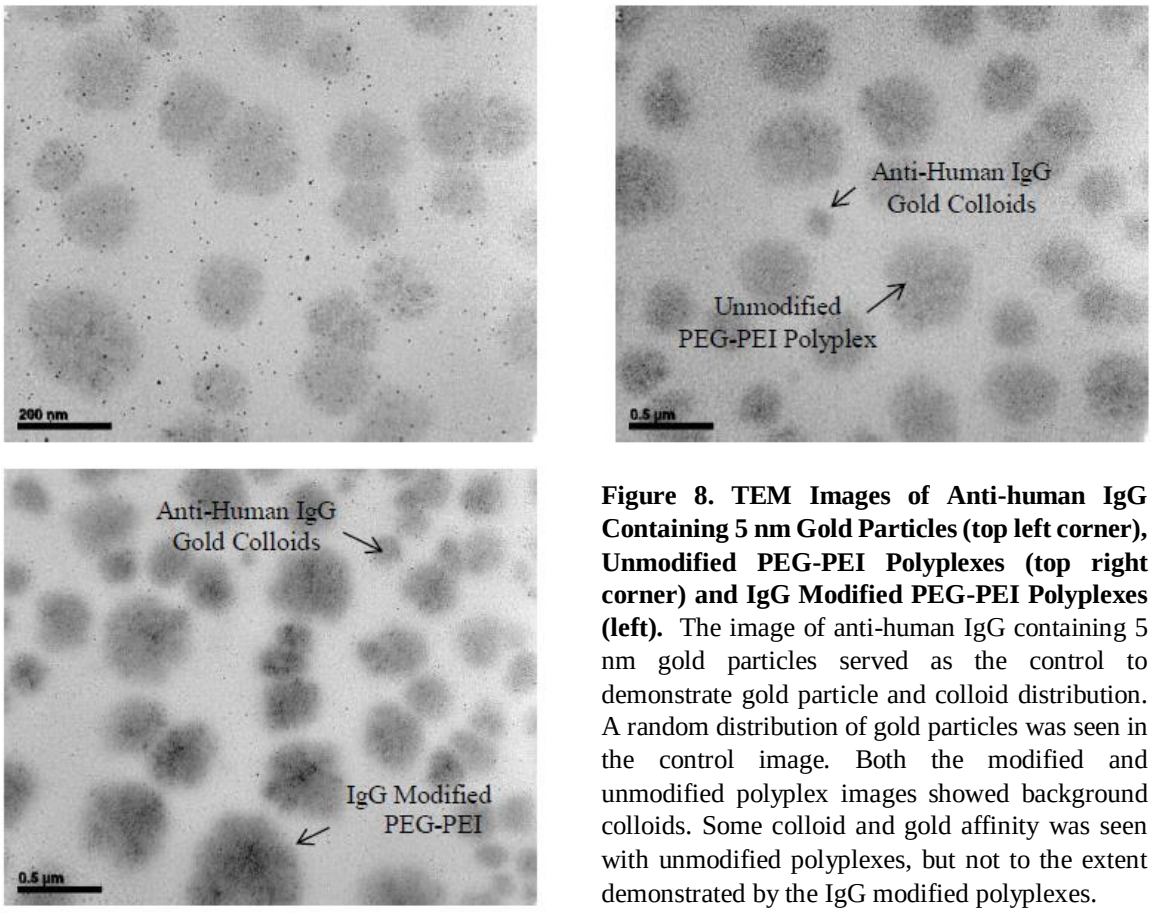

Figure 8. TEM Images of Anti-human IgG Containing 5 nm Gold Particles (top left corner), Unmodified PEG-PEI Polyplexes (top right corner) and IgG Modified PEG-PEI Polyplexes (left). The image of anti-human IgG containing 5 nm gold particles served as the control to demonstrate gold particle and colloid distribution. A random distribution of gold particles was seen in the control image. Both the modified and unmodified polyplex images showed background colloids. Some colloid and gold affinity was seen with unmodified polyplexes, but not to the extent demonstrated by the IgG modified polyplexes.

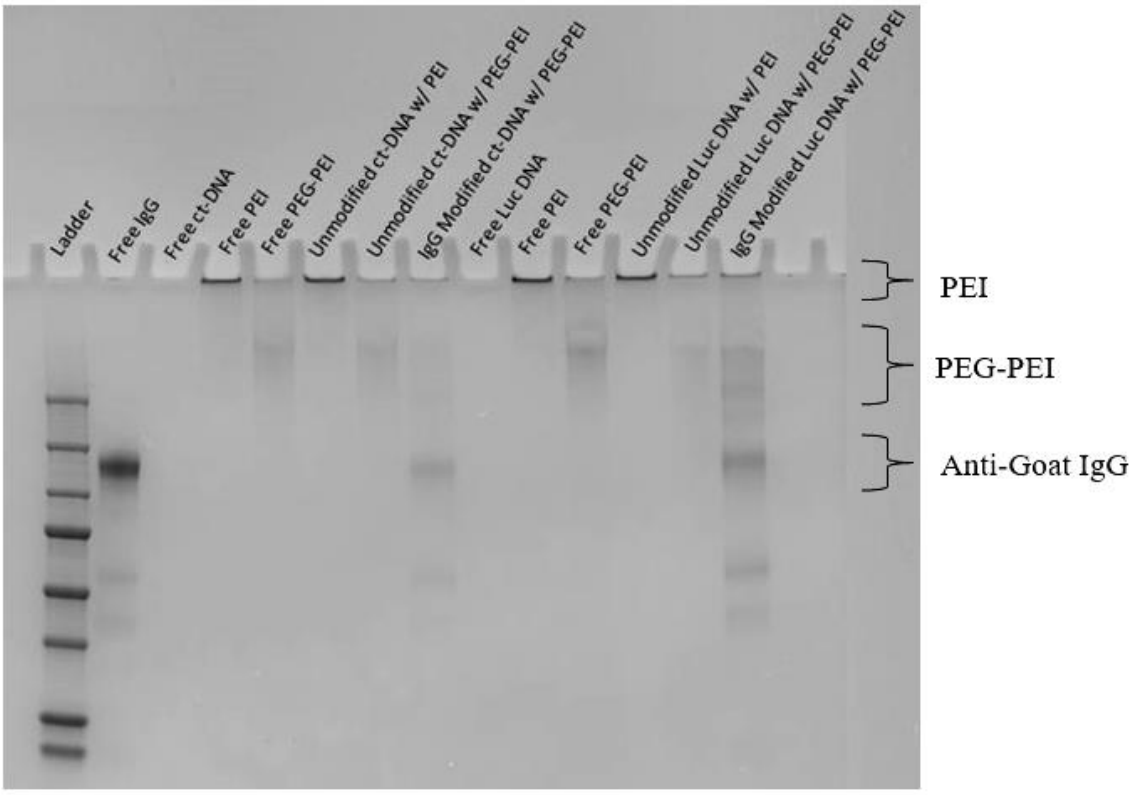

Figure 9. Denaturing Gel Comparison of Polymers and Polyplexes. The free samples corresponded to the plasmid DNA and polymer controls, while the modified and unmodified samples corresponded to the polyplexes. The migration pattern associated with each denatured sample revealed the varying components. Looking across the lanes, it is possible to see the presence of PEI, PEG-PEI and/or IgG in the samples.
*Luc DNA- Luciferase DNA

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would exceed the amount used to generate the modified PEG-PEI polyplexes, which was 2 mg. Therefore, this finding supported the belief that IgG modification was not consistent throughout the modified sample.

Denaturing Gel Electrophoresis

SDS-PAGE is an electrophoresis technique that can be used to visualize the migration patterns of denatured samples. By providing a comparison of variable samples, SDS-PAGE presented a method for identifying the presence of PEI, PEG-PEI and IgG in each sample. The imaged gel can be seen in Figure 9. As expected with unmodified PEI polyplexes, a band was seen at the well marking the presence of PEI which is drawn to the negatively charged top of the electrophoresis tank and does not migrate down the gel. In comparison, the unmodified PEG-PEI polyplexes only showed a faint band at the well and

had an additional segment indicating the presence of PEG. While some PEI may have broken apart from the PEG-PEI in the heating process to contribute to the band at the well, the weakened signal along with the additional smear confirmed the presence of the PEG-PEI conjugate. This same PEG-PEI smear was seen in the modified polyplex samples, but these sample also demonstrated a band in the region of IgG, indicating the presence of PEG-PEI conjugate and IgG. Given these observations, it was accepted that successful PEG-PEI conjugation and IgG modification were achieved in this study.

Closing Remarks

The goal of this study was to demonstrate that a stable formulation of an antibody-conjugated polyplex system composed of PEG-PEI copolymers complexed with plasmid DNA could be generated and that this system could have future utility for plasmid delivery to the brain. Through the experiments described in this paper, sufficient data has been generated in support of this goal. Future applications should seek to optimize the techniques described. Additionally, *in vitro* models examining the ability of these modified polyplexes to deliver a payload through a human endothelium would be needed to determine the required extent of IgG modification for successful delivery to the brain.

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