Demonstrated Transfection Using DNA and a Polymer with Low Cytotoxicity for Gene Therapies

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Senior Honors Thesis

Department of Biomedical Engineering, work completed in the Division of Pharmacoengineering and Molecular Pharmaceutics in the Eshelman School of Pharmacy

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Approved by:

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Introduction

The usefulness of gene therapies is largely dependent on the transfection efficiency of the therapy. Since gene therapy can treat some types of cancer, infectious diseases like AIDS, and several neurodegenerative diseases [2], there is a great need for effective carriers. Many carriers have been developed which can, to varying extents, deliver genes to several cell types. However, gene size limits, sustained expression [3], particle size, and net complex charge make delivery of therapies challenging [4, 5]. Transfection efficiency, or the ability to deliver chosen nucleic acids to cells and produce modified cells [6],

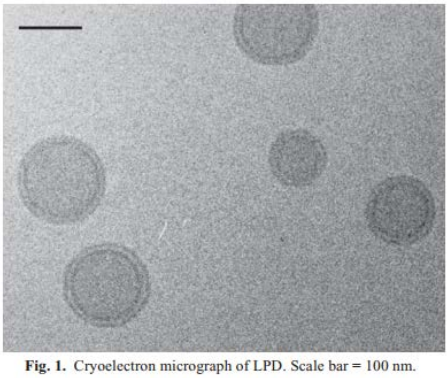

Fig. 1. Cryoelectron micrograph of LPD. Scale bar = 100 nm.

Figure 1. TEM of a Non-viral Particle for Drug Delivery [1]

must be maximized while minimizing immune response and damage to the host for a therapy to be clinically relevant [7].

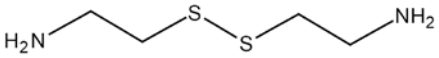

Figure 2. 2-[(2-aminoethyl)disulfanyl]ethan-1-amine hydrochloride structure (152.29 g/mol)

Gene delivery vehicles such as viral vectors can cause undesirable immune responses and be toxic. Non-viral systems hold more promise due to lower immunogenicity. Using liposomes, many formulations have been successful at entering the cell. Still, delivery systems are limited by their ability to succeed with endosomal escape. The carriers are frequently captured and destroyed by endosomes in the cytoplasm, reducing transfection efficiency [8]. Additionally, common non-viral systems such as covalent polyethyleneimine (PEI) conjugation are limited by cytotoxicity [9], and calcium phosphate models are limited by the size of the genetic material [10]. Complexes composed of liposomes, polycationic polymers, and DNA (LPD) have shown promise for a variety of applications, including cancer vaccines [11-13], peptide delivery to antigen-presenting cells[14], and other therapy possibilities. LPD Complexes are stable after storage for several months [15], are suitable for systemic therapy for intravenous delivery [16], and are modifiable for targeting [13, 17].

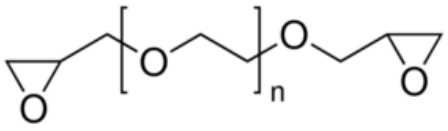

Figure 3. Poly(ethylene glycol) diglycidyl ether (MW by #: 500 g/mol)

Additionally, DNA has been used effectively as a therapeutic tool when combined with polycationic polymers alone. Degradable polymers can act as the polycationic polymer carrier [18], and charge ratio can be used to target delivery to specific organs and cell types [19]. Our project intends to overcome the challenge of endosomal escape by developing a polycationic polymer. A series of polymers were designed and synthesized. We have demonstrated that we can use a diamine to cure an epoxy-PEG (Polyethylene Glycol), as well as other reagents. In acidic environments, such as the interior of the endosome, the polymers degrade rapidly, and the byproducts increase the osmotic pressure within the endosome [20]. This increase caused the endosome to swell and burst, effectively allowing for endosomal escape. The new polymer was complexed to negatively charged DNA. Once the DNA has complexed to the novel polymer, a polar lipid was wrapped around the particle, allowing for entrance through the cell membrane (Figure 1).

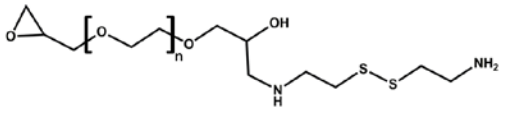

Figure 4. Polymer A Molecular Structure (652 g/mol)

Qualitative indicators of transfection were shown using a plasmid that encodes for Green Fluorescent Protein (GFP), and quantitative comparisons were performed using a plasmid that encodes for Firefly luciferase. This ongoing project works to end the search for a tunable and biocompatible carrier that delivers to targeted cells, succeeds with endosomal escape, and protects the genetic content, as well as adequately characterize the delivery vehicle.

Materials and Methods

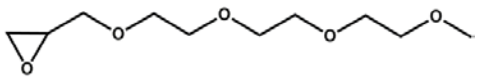

Figure 5. 2-((2-(2-(2-Methoxyethoxy)ethoxy)ethoxy)methyl) oxirane (MW 220 g/mol)

Polymer Synthesis: An acid-sensitive polymer was synthesized for use in developing a particle protocol and for particle characterization. A diepoxy was cured with a diamine using 4-(Dimethylamino)pyridine (DMAP from Sigma-Aldrich, St. Louis, MO) as a catalyst. 2-[(2-aminoethyl)disulfanyl]ethan-1-amine hydrochloride (Figure 2; from Enamine, Kiev, Ukraine) was dissolved in methanol with DMAP, and the solution was reacted with poly(ethylene glycol) diglycidyl ether (Figure 3, from Sigma-Aldrich, St. Louis, MO) at a 1:1 ratio while stirring at 50° C under nitrogen for 24 hours. The solvent was removed using rotary evaporation, and the polymer was purified using hexanes (Figure 4 shows expected structure). The product was characterized using nuclear magnetic resonance spectroscopy (NMR), and the new structure was named Polymer A.

A monoepoxy chemical with a structure similar to PEG and ending in a methyl group, 2-((2-(2-(2-Methoxyethoxy)ethoxy)ethoxy)methyl)oxirane (Figure 5; reagent from Sigma-Aldrich), was reacted at a 2:1 ratio with 2-[(2-aminoethyl)disulfanyl]ethan-1-amine hydrochloride to yield Polymer B (Figure 6). The reaction was allowed to continue for 24 hours in 2 mg/mL sodium borohydride (from Sigma-Aldrich) and 0.6 M NaOH (Thermo Fisher Scientific, Waltham, MA) in methanol while stirring at 50° C under nitrogen for 24 hours, and the solvent was removed using rotary evaporation. The product was characterized using NMR.

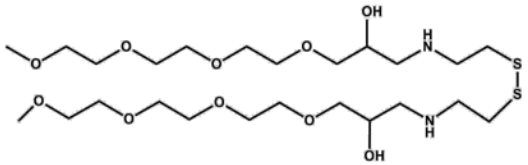

Figure 6. Polymer B Intended Structure (592.8 g/mol)

A simple diepoxy connected by a short carbon chain, 1,2,7,8 diepoxyoctane (Figure 7; reagent from Sigma-Aldrich), was reacted at a 1:1 ratio with 2-[(2-aminoethyl)disulfanyl]ethan-1-amine hydrochloride to yield Polymer C (Figure 8). The reaction was allowed to continue for 24 hours in 2 mg/mL sodium borohydride and 0.6 M NaOH in methanol while stirring at 50° C under nitrogen for 24 hours, and the solvent was removed using rotary evaporation. The product was characterized using NMR.

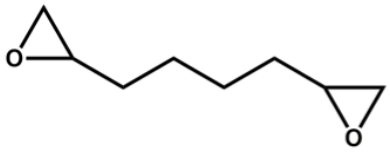

Figure 7. 1,2,7,8 diepoxyoctane structure

Polar Liposome Production: Liposomes were made from DOTAP (Avanti Polar Lipids, Alabaster, Alabama) and Cholesterol (Sigma-Aldrich, St. Louis, MO) using a 1:1

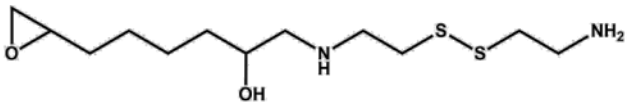

Figure 8. Molecular Structure of Polymer C

molar ratio. They were made using thin film hydration (R-215 Rotavapor, BÜCHI, New Castle, DE), and extrusion (Masterflex peristaltic pump model 77800-60, Cole-Parmer, Vernon Hills, IL) with 100 nanometer filters controlled for the size of the liposomes.

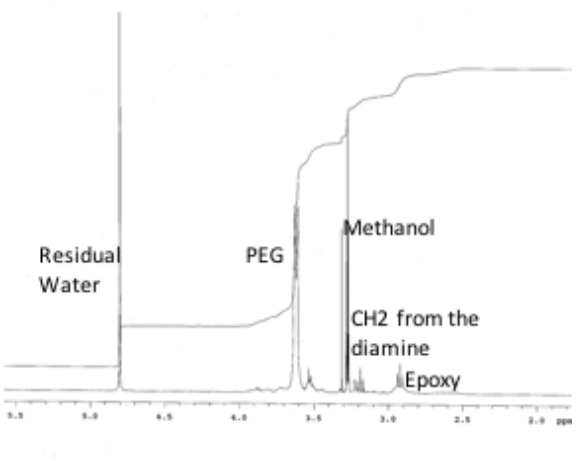

Figure 9. Polymer A NMR Spectrum

LPD complexing: Plasmid DNA (pDNA expressing GFP from InvivoGen, San Diego, California) was placed in solution in molecular grade water, and the polyacetal was placed in a second Eppendorf tube. Particle stocks were made by mixing the pDNA with the polyacetal solution at N/P ratios of 1:1, 3:1, and 5:1. The solution was allowed to shake for ten minutes, and liposomes were added at low, medium, and high mass ratios to the polymer. Polymer particles were made in the same manner without the addition of liposomes.

Cytotoxicity/ Cell Proliferation: Lactate dehydrogenase assay was conducted at 36 hours per manufacturer's protocol (Pierce from Thermo Fisher Scientific, Waltham, MA) to reveal if cells grow well with the particles, indicating if the particles are suitable for in vivo applications. MTT assays were conducted at 36 hours using 0.6 mg/mL Thiazolyl Blue Tetrazolium Bromide in complete media to determine cell viability and proliferation with particles present. PEI was used in assays for comparison.

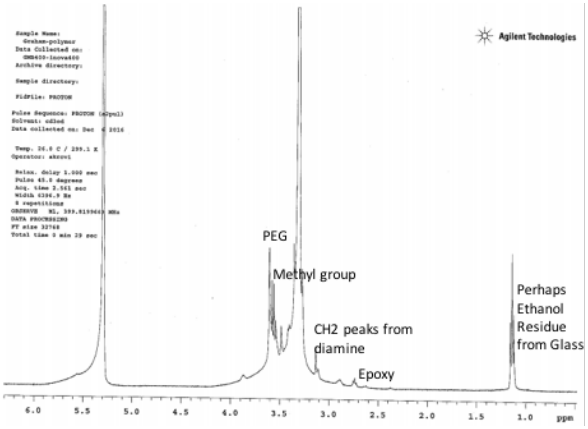

Figure 10. Polymer B NMR Spectrum

Qualitative Gene Expression: Gene Expression was verified using fluorescent microscopy to visualize the reporter protein GFP in NIH 3T3 fibroblast cells and RAW macrophages (ATCC, Manassas, VA). Cells were plated in a 96 well plated at a concentration of 1,500 cells per well. When they reached 70% confluency, media was removed and replaced by particle solutions suspended in DMEM Complete Media (Corning, Corning, NY). Expression was captured after 36 hours of incubation.

LUC Plasmid production: Plasmid containing luciferase gene (pLenti CMV Puro LUC (w168-1) from AddGene, Cambridge, MA) was transformed into competent bacteria, cultured overnight, and purified using a MaxiPrep Kit (Quiagen, Hilden, Germany). The sample concentration was found using NanoDrop (Thermo Fisher Scientific, Waltham, MA), and the plasmid was verified using restriction enzyme digest and gel electrophoreses.

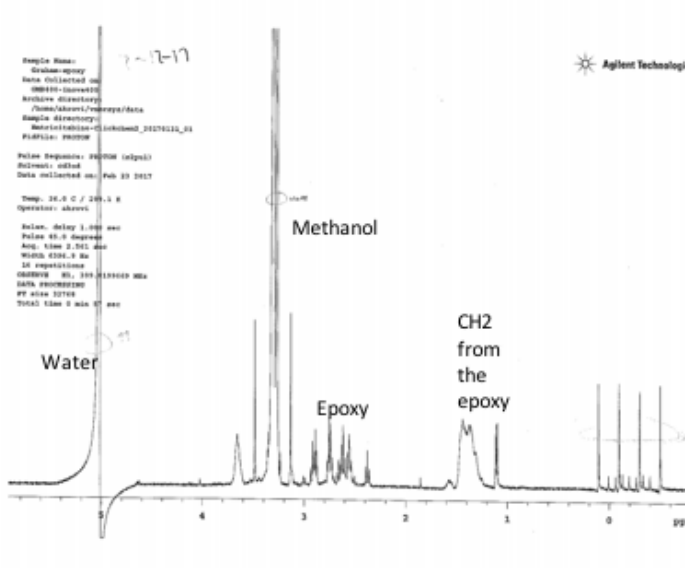

Figure 11. Polymer C NMR Spectrum

Quantify Gene Expression: Gene expression was compared with varying particle concentrations and charge ratios. Fibroblast cells were transfected with LUC pDNA particles for 36 hours. ATP and Luciferin were added, and luminescence was read on a plate reader (Molecular Devices Spectramax M5, Sunnyvale, California). PEI was used as a positive control.

Results/ Discussion

Polymer Synthesis: NMR confirmed the structure of Polymer A (Figure 9). NMR analysis revealed that Polymer B was simply a mixture of the reagents, and the synthesis did not occur as expected (Figure 10). Polymer C was characterized via NMR, but the findings were inconclusive. Peaks appear both in regions expected of the reagents and for the structure of the expected product (Figure 11).

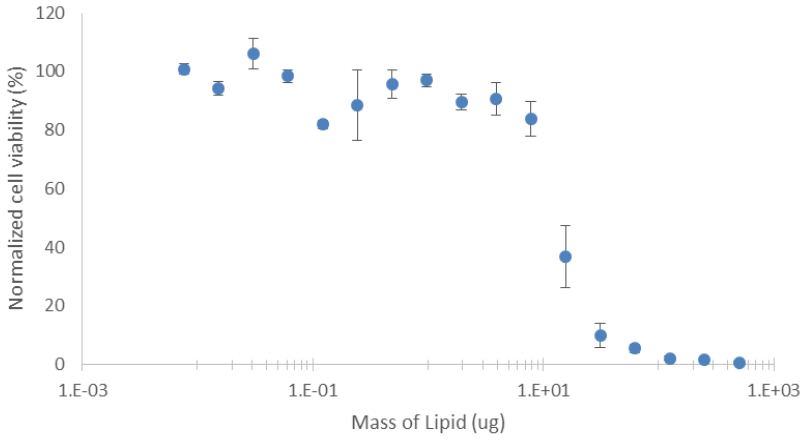

Figure 12. Cell Viability and Proliferation Assay (MTT) of LPD Particles with NIH 3T3 Fibroblast Cells: Data presented as average +/- standard deviation (n=3)

Cytotoxicity/ Cell Proliferation of LPD Complexes: Figure 12 shows that the LPD complexes using Polymer A allowed cell proliferation until very high concentrations, suggesting biocompatibility.

Qualitative Gene Expression of LPD Complexes: Figures 13 (left) shows a light microscopy image of a representative region of GFP transfected cells, and Figure 13 (right) shows green light indicative of green fluorescent protein expression using epiflourescent microscopy. While transfection was shown for many concentrations and charge ratios, the expression was inconsistent. Lipid was removed from the formulation for further tuning.

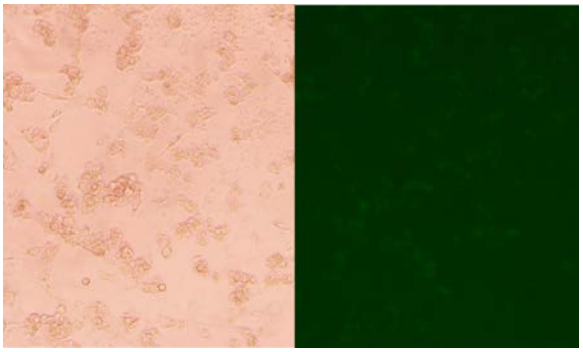

Figure 13. Light and Epifluorescent Microscopy of NIH 3T3 Fibroblasts Transfected with GFP from LPD Complexes (10x magnification)

Cytotoxicity/ Cell Proliferation of LPD Complexes: Figure 14 shows the LDH assay results of polymer/plasmid complexes. Very small amount of cell death was found when pDNA mass surpassed 1 µg, showing minimal cytotoxicity. Higher concentrations of particles resulted in greater cell death. Figure 15 shows the MTT results for Polymer A. Polymer A allowed for greater proliferation than PEI across the board, preferable by an order of magnitude. Decreasing n:p ratio (and thus polymer mass for a given amount of plasmid) resulted in greater proliferation for nearly all groups.

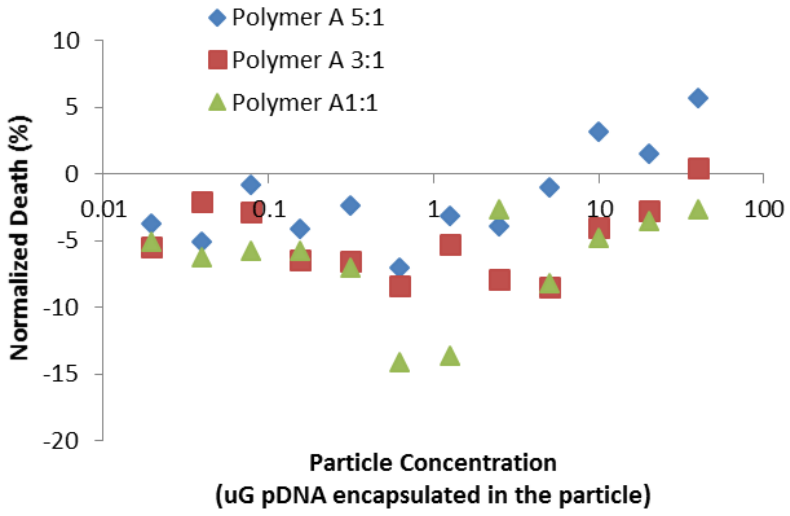

Figure 14. LDH Cytotoxicity Assay of Polymer/Plasmid Particles with NIH 3T3 Fibroblast Cells

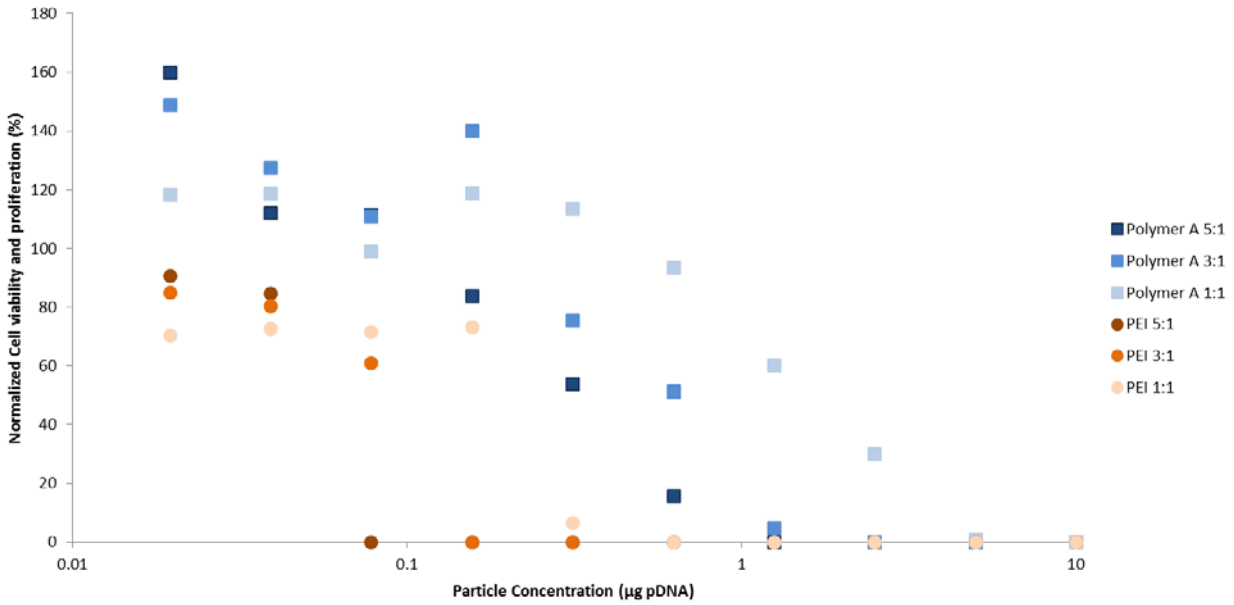

Figure 15. Cell Viability and Proliferation Assay (MTT) of Polymer/Plasmid Particles with NIH 3T3 Fibroblast Cells

Qualitative Gene Expression of Polymer A/pDNA Complex: Transfection with Polymer A complexed to plasmid resulted in brighter expression of GFP (Figure 16) in a more consistent way than the LPD complexes.

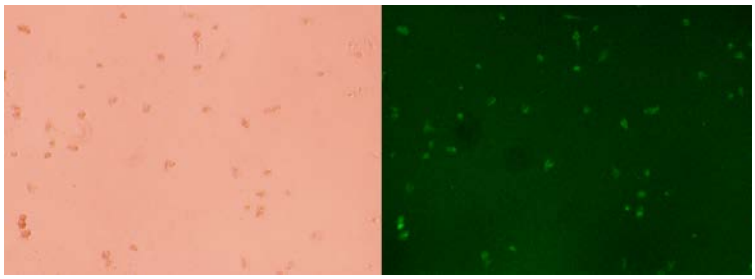

Figure 16. Light (Left) and Epiflourescent (Right) Microscopy of NIH 3T3 Fibroblasts Transfected with GFP from Polymer/Plasmid Complexes (10x magnification)

Quantified Gene Expression: Quantitative analysis of transfection using the LUC-encoding plasmid (Figure 17) resulted in very low levels of expression at all concentrations and N:P (nitrogen to phosphate) ratios of Polymer A (Figure 18). However, only one positive control showed bioluminescence. As such, the protocol for the assay needed to be developed further to allow observation of transfection. The lack of strong, consistent expression in the positive controls suggested that the assay could have a problem. While working

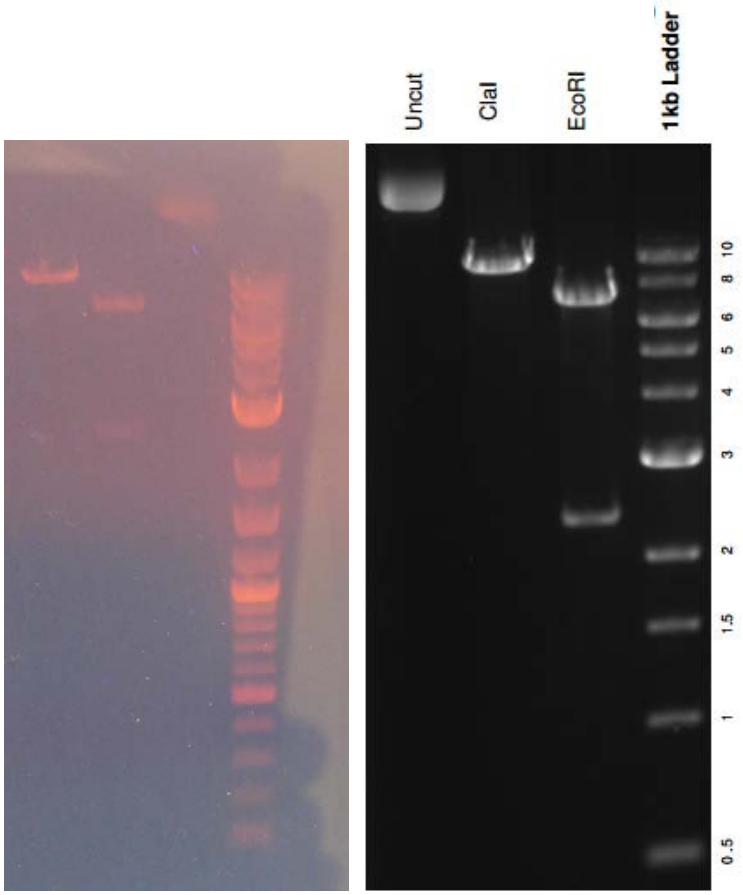

Figure 17. Gel Electrophoresis of Firefly LUC pDNA and Manufacturer's Digest

on this assay, a collaborator used her lab's resources and found no expression from Polymer A complexes, especially relative to the positive control (Figure 19).

Conclusions

Overall, a series of polymers were synthesized, and structure was confirmed for Polymer A, which was shown to be less toxic than PEI. Complexes made of the polycationic polymer and plasmid DNA were qualitatively shown to transfect fibroblast cells. The initial quantitative expression assay (luciferin assay) yielded inconclusive

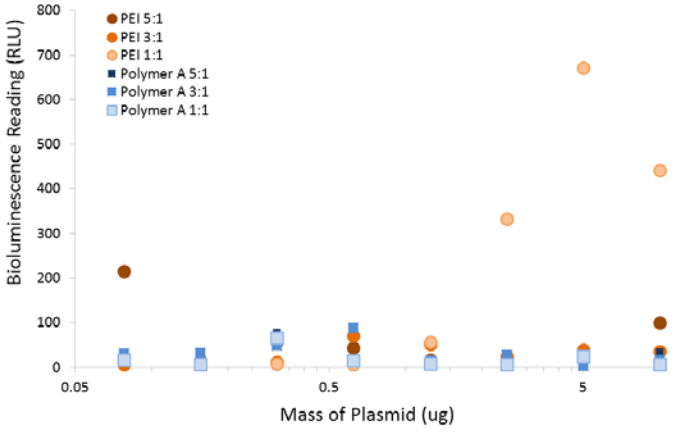

Figure 18. Bioluminescent Expression as an Indicator of Luciferase Concentration

data, and the luciferase assay was repeated by a collaborator. The new data supported the finding that Polymer A and pDNA complexes resulted in little to no transfection compared to positive controls. While qualitative transfection was shown with GFP pDNA, quantitative comparison of concentration and charge ratio was not possible due to lack of transfecting ability throughout. The lack of transfection in contrast with the GFP plasmid transfection could be due to different sizes of plasmids, charges interacting differently with the Firefly LUC plasmid, or other unexplored variables. Another likely issue was the size of the plasmids. The LUC plasmid had nearly an order of magnitude greater base pairs relative to the GFP plasmid, making the attachment and stabilization incomparable. Ideally, a quantitative assay using flow cytometry and GFP plasmid could be used.

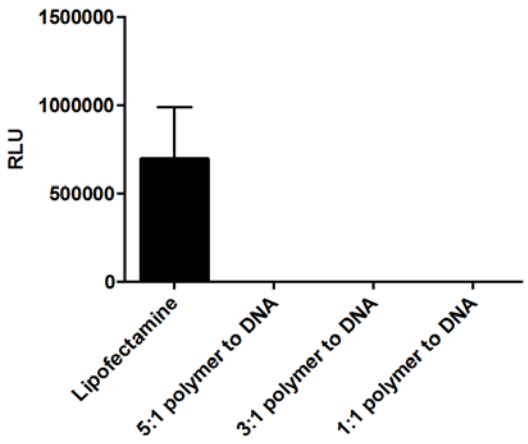

Figure 19. Bioluminescent Expression from Collaborator's Luciferase Assay: Bioluminescence measured as Relative Luminescence Unit (RLU) and Data presented as average +/- standard deviation (n=3)

The second polymer, Polymer B, could not be synthesized as planned. Polymer C may have been made, but further characterization and purification is necessary before it can be used.

Future Directions: To characterize the product from the reaction for Polymer C, Thin Layer Chromatography will be used to compare the polarity to the reagents' polarities. Then, column chromatography using silica gel will be used to filter the product by polarity to separate materials. Once structure is confirmed and the product is purified, biocompatibility experiments will be repeated, and transfection will be quantified using a luciferin assay to help determine the most effective formulation.

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