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The Characterization of AuNP-PEI Conjugates for siRNA Delivery to Cancer Cells”

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The University of Southern Mississippi

“The Characterization of AuNP-PEI Conjugates for siRNA Delivery to Cancer Cells”

by

Albert Joseph Nosser, Jr.

A Thesis
Submitted to the Honors College of
The University of Southern Mississippi
in Partial Fulfillment
of the Requirements for the Degree of
Bachelor of Science
in the Department of Chemistry and Biochemistry

May 2013

In Memory of My Beloved Aunt and Godmother

Lori Helen Nossner



Lori Helen Nossner graduated from the University of Southern Mississippi in 1976, earning a bachelor's degree in elementary education. For thirty-four years, she taught in the Vicksburg-Warren County School District, providing thousands of 1st grade students with a strong foundation for their future education. In the hearts of everyone she knew, Lori could be described as a selfless individual. She lived her life as a servant leader, always looking to fill the needs of others before her own. In many ways, I model my own life by how she lived her life. In my heart, I am truly grateful for my time with her. I will cherish the memories for the rest of my life. In January 2011, she was diagnosed with cancer, shortly before the beginning of my thesis research project. For nearly 18 months, she fought with a valiant effort through numerous rounds of chemotherapy treatments, never once complaining of any pain or discomfort. Throughout this project, she was my sole source of inspiration and motivation. During the difficult times, she provided me the strength to continue my research, in hope of finding a new chemotherapy method for treating cancer. Sadly, she lost her battle with cancer on July 8, 2012. While she is physically no longer with us, her spirit continues to thrive in the hearts of everyone she touched with her bright smile and contagious laugh.

“I’ve had many teachers who taught us soon forgotten things, / But only a few like her who created in me a new thing, a new attitude, a new hunger, / What deathless power lies in the hands of such a person.”

- John Steinbeck, “Captured Fireflies”

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Dr. Faqing Huang

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Abstract

Cancer is one of the leading causes of death for people in the United States. Despite the numerous cancer treatments available, many of these chemotherapy treatments cause negative side effects such as hair and weight loss. With this in mind, in the past 15 years, researchers have discovered a mechanism known as RNA interference, which can be used to control certain genes and their activity. This mechanism is based on the function of siRNA molecules, which can be inserted into cells.⁽¹²⁾ These siRNA molecules can be manipulated to target and eliminate specific cancer cells. However, these siRNA molecules are normally associated with inconvenient problems such as instability and a short half-life. These siRNA molecules also face an uphill task of entering these targeted cells. In most cases, cellular entryways are highly selective based on the size and type of cargo.⁽¹⁾⁽⁶⁾ With this in mind, researchers have discovered that gold nanoparticles (AuNPs) can be a suitable transport vehicle for siRNA molecules into targeted cells due to their easy synthesis, easy modification for specific pathways, and high biocompatibility with living tissue.⁽¹⁾⁽²⁾ Studies have shown that AuNPs can increase the half-life of siRNA molecules by six fold. Before assembling these gold nanoparticles with siRNA, they must be coated with a reagent of branched polyethyleneimine (C₁₂-PEI) chains in order to increase the stability of the gold nanoparticles, thus producing newly-formed AuPEI conjugates. Subsequently, the PEI will bind with siRNA based on their electrostatic interaction, forming a well-dispersed nanoparticle with uniform size and narrow size distribution.⁽⁸⁾

In Fall 2011, the Huang research group encountered several problems with the synthesis of these AuPEI conjugates. These experiments revealed data suggesting that aggregation occurred at certain concentrations of PEI. Experimental data also suggested that aggregation had occurred when loading these AuPEI conjugates with tRNA. With these problems in mind, the Spring 2012 semester of research has been geared towards determining the optimal PEI concentration for the synthesis of AuPEI conjugates. More specifically, the research effort was geared towards exploring how many PEI molecules are bound to each gold nanoparticle. The Huang research group designed a C₁₂PEI solution incorporated with fluorescein isothiocyanate (FITC) in order to gain a closer look into the synthesis of these AuPEI-FITC conjugates. By monitoring the fluorescence of these AuPEI-FITC conjugates, the results could provide information about optimal synthesis conditions. The Huang research group has conducted numerous experiments to study the effects of incubation time, temperature, and DTT (dithiothreitol) concentration on the amount of PEI-FITC on each gold nanoparticle. Based on the experimental results and its statistical analysis, the Huang research group has successfully determined that an estimated 1,000 PEI molecules are bound to each gold nanoparticle. Upon determining PEI density, the goal of the Summer 2012 semester was to optimize the AuPEI-tRNA conjugate assembly system. Based on extensive experimental data and analysis, the optimized conditions for assembly have been established for future research efforts.

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Chapter 1: Problem Statement

In today's world, cancer is one of the leading causes of death among the populations throughout the United States. Cancer is characterized as a type of disease in which cells grow exponentially, invade and kill healthy tissues in the body, and, in some cases, metastasize throughout the body. According to the American Cancer Society, nearly 1.6 million deaths were reported in the United States during 2011.⁽⁹⁾ For many years, medical researchers have worked countless hours in their laboratories, conducting experiments in order to discover new methods that can effectively treat patients with cancer. Currently, there are hundreds of chemotherapy treatments that can be used to inhibit the growth of cancer cells in the human body. While many of these cancer treatments have been proven effective in the clinical settings, there are many negative side effects associated with these chemotherapy treatments. On a positive note, most of these treatments effectively inhibit and potentially eliminate the growth of cancer cells in the human body. However, during this chemotherapeutic process, these treatments can also affect healthy tissues of the body. Most commonly, cancer patients can experience hair loss, weight loss, anemia, and fatigue when undergoing these chemotherapeutic treatments. Because of these negative side effects, medical researchers suggest that there still is much room for improvement in the clinical treatment of cancer.

In an effort to improve these current cancer treatments, medical researchers have worked tirelessly to devise alternative means of combating cancer with treatments with less negative side effects. After much research, scientists have finally pinpointed a viable option that could be exploited in order to effectively treat cancer patients. For the past fifteen years, scientists have studied the process of RNA interference for a better answer. More specifically, these biochemists have conducted numerous experiments in order to study the role of small interfering RNA

(siRNA) molecules in the process. Because RNA interference controls genes and their activities within living cells, this study has opened new doors for cancer research. By delivering these small interfering RNA molecules to a certain gene in a specific cell, medical researchers can observe, record, and analyze the effects on the cell due to the gene's suppression. With these effects of gene suppression in mind, these researchers can manipulate small interfering RNA to target specific genes in order to eliminate cancerous cells.

Originally, the research goal was to prepare gold nanoparticle-polyethyleneimine (AuPEI) conjugates that could be loaded with small interfering RNA molecules. These AuPEI-siRNA conjugates would have been specifically designed to target the CASP8AP2 gene linked to the inhibition of caspase 8 in apoptosis, or programmed cell death, in human nasopharyngeal epidermoid carcinoma cells (KB cells).⁽⁷⁾ With the principle of RNA interference in mind, these small interfering RNA molecules can suppress or “knock down” the expression of this CASP8AP2 gene. After analyzing the AuPEI-siRNA conjugate's ability to enter the KB cells and its suppression of the CASP8AP2 gene, these effects could be further studied in order to apply this concept to the expression of folate receptors in cancer cells. With the use of folate receptor expression, the process of apoptosis can be manipulated in order to efficiently reduce and/or effectively eliminate cancer cells.

During the Fall 2011 semester at the University of Southern Mississippi, the Huang research group successfully conducted experiments in order to characterize these gold nanoparticles (AuNPs) by its ultra-violet (UV) absorbance and its zeta (ζ) potential. However, towards the end of the semester, the research group experienced multiple incidences of aggregation during the steps of PEI coating and tRNA loading. These observations suggested that the PEI concentration used in the AuNP coating step could have reached its saturation point.

Therefore, with these problems in mind, the Spring 2012 semester of research was geared towards determining the optimal PEI concentration for the synthesis of AuPEI conjugates. More specifically, the research effort was designed to explore how many PEI molecules are bound to each gold nanoparticle. By designing a C₁₂PEI solution incorporated with fluorescein isothiocyanate (FITC), the Huang research group was able to gain a close look into the synthesis of the AuPEI-FITC conjugates. By monitoring the fluorescence of these conjugates, the results could provide information about optimal synthesis conditions. Throughout the semester, the Huang research group conducted experiments to study the effects of incubation time, temperature, and dithiothreitol (DTT) concentration on the amount of PEI-FITC on each gold nanoparticle. These series of experiments were conducted in order to determine the quantity of PEI molecules attached to the gold nanoparticle. Upon determining the amount of PEI molecules on each gold nanoparticle, the Huang research proceeded into Summer 2012 semester, seeking the goal of optimizing the system for adding tRNA molecules to the AuPEI conjugates. Once the optimized system for AuPEI-tRNA assembly has been established, future research efforts will be able to use siRNA molecules cost-effectively when preparing the final AuPEI-siRNA conjugates for the cell study phase of this project.

Chapter 2: Literature Review

RNA interference (RNAi) is a biological system within living cells that controls genes and their activities. The process of RNAi revolves around the functionality of two small RNA molecules: small interfering RNA (siRNA) and micro RNA (miRNA). It is known that siRNA and miRNA have the ability to bind with other types of RNA, while controlling the activity of specific genes. More specifically, siRNA can bind with specific messenger RNA (mRNA) molecules to prevent them from certain proteins that are harmful to living cells.⁽¹²⁾

The process of RNAi occurs in many eukaryotic organisms. During this process, the enzyme Dicer breaks down complex, double-stranded RNA molecules into smaller nucleotides. Next, these siRNA molecules are broken down into single-stranded RNA molecules. These two single-stranded RNA molecules are often referred to as the passenger (sense) strand and the guide (anti-sense) strand. While the passenger (sense) strand will break down, the guide (anti-sense) strand will enter the RNA-induced silencing complex (RISC). When the guide strand binds with a complementary fragment of an mRNA molecule, it can lead to post-transcriptional gene silencing. The RNAi mechanism, outlined

in Figure 1, has shown much promise related to research in various scientific fields of study such as biochemistry, genetics, molecular biology, and medicine. Based on previous research efforts, researchers determined that laboratory synthesized siRNA molecules can be introduced *in vitro* to a living cell. Upon insertion, these siRNA molecules will induce a partial or complete suppression of a specific cell. More importantly, the function of each gene in a particular organism can be determined by analyzing the effects caused by the suppression of the targeted gene expression. This process can also aid in pinpointing the specific components required for a certain process to occur within a living cell.⁽¹²⁾

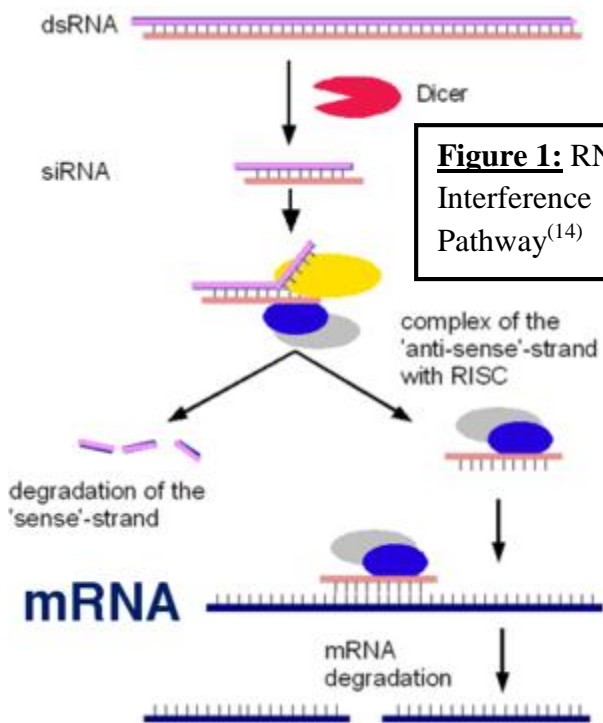


Figure 1: RNA Interference Pathway⁽¹⁴⁾

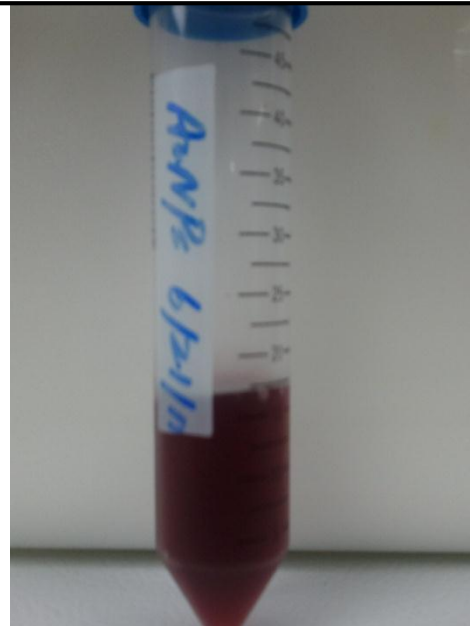
Despite the promise foreseen by the exploitation of the RNAi mechanism in cancer treatment research, there are several problems involving the delivery of siRNA molecules to a

particular gene in a living cell. First of all, these siRNA molecules are very unstable. From previous research conducted at the University of Regensburg, it was determined that siRNA molecules have a very short half-life, ranging from several seconds to one minute. With this problem in mind, researchers realize that there is a very small time frame to deliver these siRNA molecules to a specific destination within a living cell. Another problem with the delivery of these siRNA molecules involves the difficult task of delivering the proper segment of siRNA to its complementary segment of mRNA for the specific gene in a living cell. The key to an efficient delivery of these siRNA molecules is defined by the correct interaction with the cell membrane. In eukaryotic organisms, there are numerous entryways into each specific type of cell. With this in mind, these entryways are highly selective based what is being delivered into the cell. For the majority of these cellular entryways, they will vary in selectivity based on the size and the type of cargo. Due to the high selectivity observed in cellular entryways, they will control whether or not these siRNA molecules will be delivered to the specific site. They will also control the efficiency of the siRNA molecule delivery as well.⁽¹⁾⁽⁶⁾

With these problems in mind, researchers at the University of Regensburg discovered that small, mono-disperse nanoparticles with a certain zeta (ζ) potential and surface chemistry should be used in the delivery of siRNA molecules to a specific location with a living cell.⁽¹⁾ Before this discovery, researchers tested a side variety of carriers in hope that they would bind with siRNA molecules to form complexes.⁽¹¹⁾ For example, researchers used polyethyleneimines and nucleic acids to synthesize nanoparticles capable for efficiently delivering siRNA molecules. However, this system produced several problems for the delivery of siRNA molecules. First of all, this system produced nanoparticles that were too large for entry into a living cell. Often, these nanoparticles were measured to be larger than 100 nanometers. More importantly, this system

also produced a heterogeneous mixture of different nanoparticle species. Due to the wide variety of nanoparticle possibilities generated from this system, researchers were not able to determine which species caused these effects within the living cells. Finally, this system produced a small amount of nanoparticle species which left behind residual toxic effects within the living cells tested in these experiments.⁽¹⁾ Based on the problems observed in these previous systems, it has opened new doors, in terms of research, to find an effective transport vehicle for delivering siRNA molecules. Research-renowned scientists, Gregory Schneider and Gero Decher, have described nanoparticle research as a “boom topic” in the fields of colloid and material sciences.⁽⁴⁾

Figure 2: Gold Nanoparticle Solution⁽¹³⁾



In the past decade, researchers have conducted numerous experiments in order to study the characteristics and synthesis of gold nanoparticles, which have been pinpointed as a potential transport vehicle for delivering siRNA molecules to specific locations within a living cell.⁽²⁾ Before this important research discovery, gold nanoparticles were only used for transporting plasmid DNA and oligonucleotides into cells. When compared to free siRNA complexes, these conjugates produce a six-fold increase in the half-life of siRNA molecules. They have also provided experimental data, suggesting that these conjugates can increase the knockdown rates for gene expression. Shown in Figure 2, gold nanoparticles are considered to be the ideal transport vehicle for delivering siRNA molecules to specific destinations within a living cell. First of all, these gold nanoparticles can be easily synthesized and modified to fit certain requirements. Next, they are readily available in numerous sizes and shapes, which allow them to

fit into specific entryways of living cells. These gold nanoparticles are also highly biocompatible with living cells and tissues within eukaryotic organisms. More specifically, unlike previous nanoparticle synthesis systems, these small gold nanoparticles, ranging from fourteen to sixteen nanometers, are produced in a homogenous mixture that is free of aggregation. This eliminates problem that involves the inability to enter a living cell due to its size and cargo. It minimizes the inability to determine which nanoparticle species suppressed the specific gene in the experimental cell, which is caused by a heterogenous mixture of different nanoparticle species.⁽¹⁾

However, before conjugating these gold nanoparticles with siRNA molecules, the gold nanoparticles must be coated with a branched polyethyleneimine (PEI) solution in order to enhance the stability of the entire conjugate. AuPEI conjugates are commonly assembled through the layer-by-layer (LbL) approach. This process “uses two solutions of oppositely charged polymers into which the substrate (AuNP) surface can be dipped”.⁽³⁾

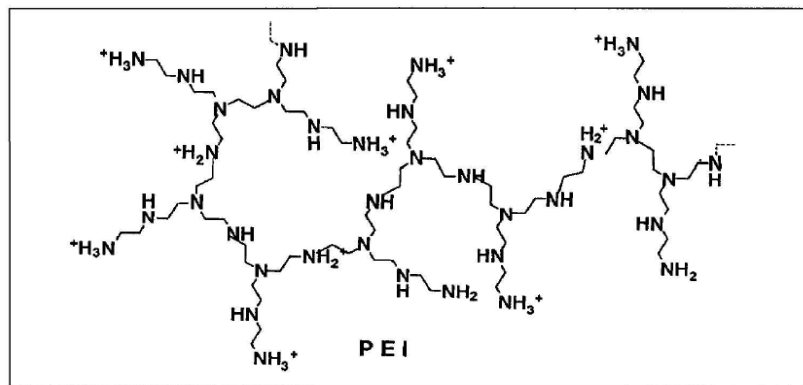


Figure 3: Branched Polyethyleneimine Structure⁽⁵⁾

“Sequential dipping causes material to be deposited on the surface because of electrostatic and hydrophobic interactions between the charged surface and polyelectrolyte. Once deposited, the

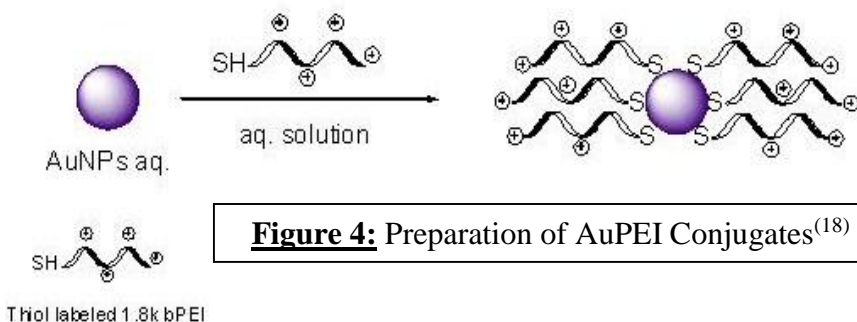


Figure 4: Preparation of AuPEI Conjugates⁽¹⁸⁾

layer of polyelectrolyte inverts the surface charge of the material it is adsorbed to, enabling a subsequent layer of

polymer to be deposited from the second solution”.⁽³⁾ More importantly, the layer-by-layer approach “can be repeated indefinitely to form a uniform multi-layered film of polymeric material”.⁽³⁾ As shown in Figure 3, PEI is a highly branched polymer with nearly twenty-five percent primary amine groups, fifty percent secondary amine groups, and twenty-five percent tertiary amine groups.⁽¹¹⁾ These branched PEI molecules also contain a C₁₂ linker which is manipulated by thiol chemistry to bind with these gold nanoparticles. These PEI molecules act as a “reductant and stabilizer”, which allows siRNA molecules to bind at the “appropriate weight ratio” based on their “electrostatic interaction” with each other. As shown in Figure 4, once the siRNA molecules are bound with the AuPEI conjugates, the product is formed into a “well-dispersed nanoparticle with uniform structure and narrow size distribution.” At the University of Science and Technology of China Hefei, the Song research group determined that these AuPEI-siRNA conjugates were responsible for increasing the gene knockdown of the green fluorescent protein found in MDA-MB-435s cells. This same research effort also discussed that these AuPEI-siRNA conjugates were responsible for increasing the gene knockdown of endogenous cell-cycle kinase, while also initiating cell apoptosis as well. More importantly, along with these impressive results, these AuPEI-siRNA conjugates proved to have no toxic effects on normal cells.⁽⁸⁾

In order to quantify the exact amount of PEI molecules on each gold nanoparticle, the Huang research group enlisted the aid of two common chemical solutions used in the laboratory, fluorescein isothiocyanate (FITC) and dithiothreitol (DTT), seen in Figure 5 and 6 respectively. FITC is a fluorescent dye incorporated in the PEI solution, which is used to coat the gold nanoparticles before assembling the conjugates with siRNA molecules. This dye absorbs ultraviolet light around the wavelength of 492 nanometers, while emitting visible light around

the wavelength of 518 nanometers.⁽¹⁰⁾ The use of the FITC dye is ideal because its absorption and emission wavelengths are not similar with the wavelengths at which the AuPEI conjugates will absorb and emit ultraviolet light.

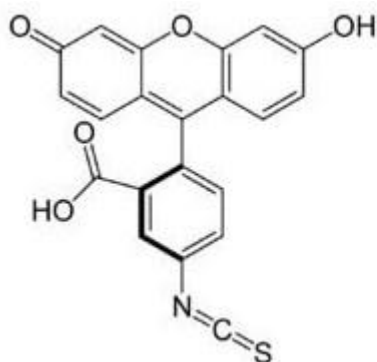
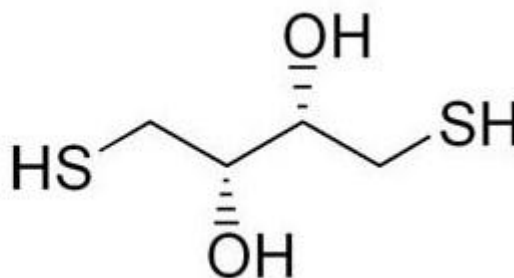
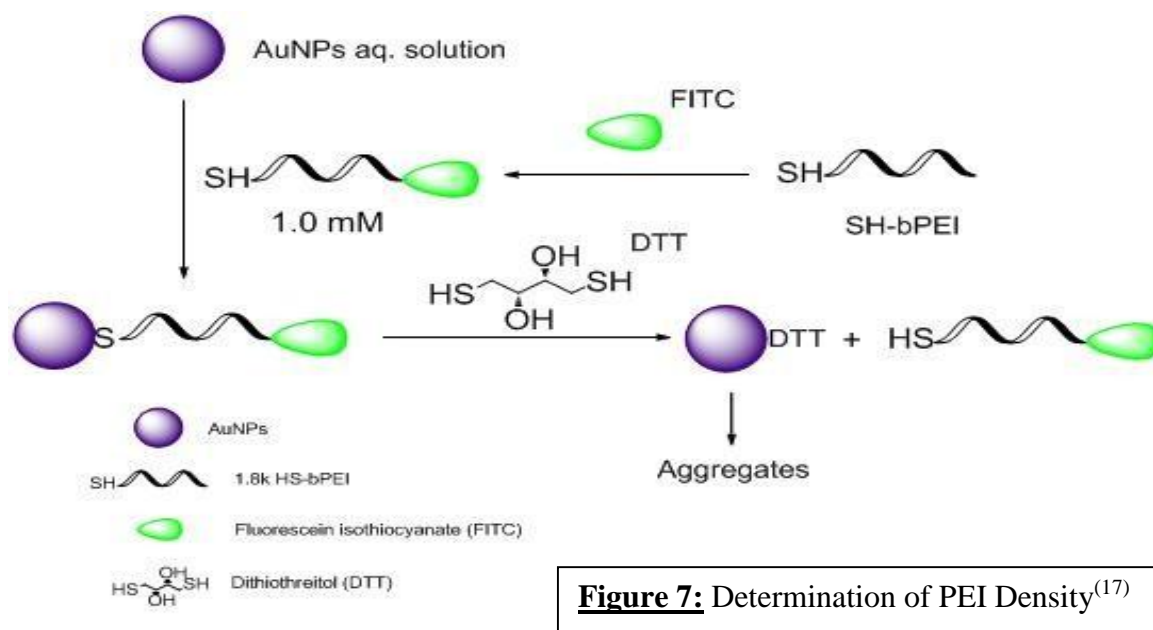


Figure 5: Chemical Structure of FITC⁽¹⁶⁾

More specifically, there should not be any interference in the graphs when these AuPEI-FITC conjugates are characterized by ultraviolet spectroscopy. By incorporating FITC into these AuPEI conjugates, this dye could be tracked by its fluorescent signal, which is measured and collected by a fluorometer. However, a thiol-exchange reaction must occur before measuring the fluorescent signal of an AuPEI-FITC solution sample. This thiol-exchange reaction mechanism is outlined below in Figure 7. For a given AuPEI-FITC sample, the solution is treated with a certain concentration of DTT, which is a strong reducing agent for breaking disulfide bonds. In the presence of DTT, this reagent will break the disulfide bonds between the gold nanoparticles and branched PEI-FITC molecules. The gold nanoparticles will bind with DTT, thus producing clearly visible signs of aggregation. On the other hand, the PEI-FITC molecules will release freely into the supernatant of the solution, which can be extracted and measured for its fluorescent signal. With this in mind, the Huang research group studied the DTT concentration effect on fluorescent signal, while devising different incubation temperatures, incubation time periods, and DTT concentrations in order to provide data parameters that were used to quantify the amount of PEI molecules on each gold nanoparticle.

Figure 6: Chemical Structure of DTT⁽¹⁵⁾





Chapter 3: Methodology

Preparation & Characterization of AuPEI-FITC Conjugates

Two parallel solution samples of AuPEI-FITC conjugates were prepared by adding 100 μL of 15 nM AuNP solution (synthesized by Dr. Huang)⁽¹⁹⁾ and 2.5 μL of 40 mM $\text{C}_{12}\text{PEI-FITC}$ solution (synthesized by Dr. Huang)⁽²⁰⁾ into a 1.5 mL cuvette. After preparing the solutions in their respective cuvettes, they were incubated at room temperature for ten minutes, followed by an incubation period of ten minutes in the 65°C heat block. After incubation, these two solutions of AuPEI-FITC were centrifuged at “full speed” (14,000 rpm) for thirty minutes in the Eppendorf Centrifuge 5415D. After centrifugation, the supernatant of each solution was removed, while maintaining the integrity of the pellet. Next, the pellet in each solution was re-suspended with 1,000 μL of pure water. It is important to note that the steps of centrifugation, washing, and re-suspension were repeated five times during this stage of the experiment. The lab group saved the supernatant of each washing in a separate cuvette for future procedures. Once each solution was re-suspended for the fifth time, both solutions were characterized by

ultraviolet (UV) spectroscopy with the use of the Hewlett Packard Agilent 8453 UV/VIS Spectrophotometer. During the UV characterization, a blank cuvette filled with 500 μL of water was tested by this instrument in order to balance the absorbance readings. Next, the sample cuvette filled with 500 μL of water and 10 μL of the AuPEI-FITC solution was tested by this instrument in order to determine its λ_{max} and absorbance reading. The data collected during the UV characterization was recorded into the Microsoft Excel software program for further data analysis.

Determination of PEI Density (DTT Concentration Effect on Fluorescent Signal)

In order to determine the DTT concentration effect on fluorescent signal, three control C₁₂PEI-FITC solution samples with no DTT concentration were prepared along with eight C₁₂PEI-FITC solutions with DTT concentrations of 20 mM, 50 mM, 100 mM, 200 mM, 500 mM, 1,000 mM, 1,200 mM, and 1,500 mM. These solution samples were prepared with stock solutions of 1.0 μM C₁₂PEI-FITC, 200 mM DTT, 2.0 M DTT, and pure water. The table below outlines the specific volumes of each solution required to prepare these samples.

	1.0 μM C ₁₂ PEI-FITC	200 mM DTT	Pure Water	[DTT]
Control 1	7.0 μL	0.0 μL	63.0 μL	0 mM
Control 2	7.0 μL	0.0 μL	63.0 μL	0 mM
Control 3	7.0 μL	0.0 μL	63.0 μL	0 mM
Sample 1	7.0 μL	7.0 μL	56.0 μL	20 mM
Sample 2	7.0 μL	17.5 μL	45.5 μL	50 mM
Sample 3	7.0 μL	35.0 μL	28.0 μL	100 mM
	1.0 μM C ₁₂ PEI-FITC	2.0 M DTT	Pure Water	[DTT]
Sample 4	7.0 μL	7.0 μL	56.0 μL	200 mM
Sample 5	7.0 μL	17.5 μL	45.5 μL	500 mM
Sample 6	7.0 μL	35.0 μL	28.0 μL	1,000 mM
Sample 7	7.0 μL	42.0 μL	21.0 μL	1,200 mM
Sample 8	7.0 μL	52.5 μL	10.5 μL	1,500 mM

After preparing these samples, each sample cuvette was spun by the Vortex Genie 2 for ten seconds in order to ensure a homogenous solution. After this step, a 60 μL aliquot of each sample was added into a cuvette in order to measure its fluorescent signal with the help of the PTI FRET Master Fluorometer. The fluorescent signal data collected during this stage in the experiment was recorded into the Microsoft Excel software program for future data analysis.

Determination of PEI Density (Temperature Effect)

Incubation Table			
	Sample #1	Sample #2	Sample #3
RT (Room Temperature)	30 minutes	1 hour	2 hours
Control Sample (70°C)	10 minutes	20 minutes	40 minutes
Test Sample (70°C)	10 minutes	20 minutes	40 minutes

First of all, a series of three control samples were prepared by adding 10 μL of 1.0 μM C₁₂PEI-FITC solution and 10 μL of 200 mM DTT solution into a 1.5 milliliter cuvette. These control samples were incubated in the 70°C heat block for the allotted times of 10, 20, and 40 minutes, which follows according to the previously outlined incubation table. After its respective incubation period, 80 μL of pure water was added into each cuvette in order to prepare an adequate amount of solution for future testing procedures. Next, each solution cuvette was centrifuged in the Eppendorf Centrifuge 5415D at “full speed” for thirty minutes. After centrifugation, a 70 μL sample of the supernatant was removed from the solution and saved in another cuvette for future test procedures. From this sample, a 60 μL aliquot was inserted into a cuvette in order to measure its fluorescent signal with the use of the PTI FRET Master Fluorometer. Finally, the lab group tested for aggregation by attempting to re-suspend the pellet and excess supernatant into a homogenous solution. The fluorescent signal data from these samples were recorded into the Microsoft Excel software program for future data analysis.

Secondly, a series of three 70°C test samples were prepared by adding 10 µL of AuPEI-FITC solution and 10 µL of 200 mM DTT solution into a 1.5 milliliter cuvette. These test samples were incubated in the 70°C heat block for the allotted times of 10, 20, and 40 minutes, which also follows the directions provided in the previously outlined incubation table. After its respective incubation period, the lab group added 80 µL of pure water into each cuvette in order to prepare enough solution for testing in the later procedures. Next, each solution cuvette was centrifuged at full speed for thirty minutes. After centrifugation, a 70 µL sample of the supernatant was removed from the solution and stored in another cuvette for testing in later procedures. Again, a 60 µL supernatant aliquot was inserted into a cuvette in order to measure its fluorescent signal with the aid of the PTI FRET Master Fluorometer. Next, an aggregation test was performed on the original cuvette by attempting to re-suspend the pellet and excess supernatant into a homogenous solution. Again, the fluorescent signal data from these samples were recorded into the Microsoft Excel software program for future data analysis.

Thirdly, a series of three room temperature test samples were prepared by adding 10 µL of AuPEI-FITC solution and 10 µL of 200 mM DTT solution into a 1.5 milliliter cuvette. These test samples were incubated at room temperature for time periods in the range of 30 minutes, 1 hour, and 2 hours. These time periods follow accordingly to the previously outlined incubation table. After each incubation period, 80 µL of pure water was added into each cuvette in order to secure enough solution for further testing procedures. Next, each solution cuvette was centrifuged in the Eppendorf Centrifuge 5415D at “full speed” for thirty minutes. After centrifugation, a 70 µL sample of the supernatant was removed from the solution and saved in another cuvette for future testing procedures. From this sample, a 60 µL supernatant aliquot was

added to a cuvette in order to measure its fluorescent signal by using the PTI FRET Master Fluorometer. Again, an aggregation test was performed on the original solution cuvette by attempting to re-suspend the pellet and excess supernatant into a homogenous mixture. The Microsoft Excel software program was used again in order to record and analyze the fluorescent signal data.

In addition to these samples, two additional control samples were prepared in order to provide more accurate data when determining the PEI density. For the first additional control, 10 μL of 1.0 μM $\text{C}_{12}\text{PEI-FITC}$ and 10 μL of pure water was added into a 1.5 milliliter cuvette. After spinning the control sample with the Vortex Genie 2 for ten seconds, 80 μL of pure water was added into the cuvette. After a second round of spinning, a 60 μL aliquot of the solution was inserted into a cuvette in order to measure its fluorescent signal with the use of the PTI FRET Master Fluorometer. For the second additional control, 10 μL of 1.0 μM $\text{C}_{12}\text{PEI-FITC}$ and 10 μL of 200 mM DTT were pipetted into a 1.5 milliliter cuvette. After spinning the control sample with the Vortex Genie 2 for ten seconds, an additional 80 μL of pure water was added into the cuvette to secure enough solution for future testing procedures. Next, a 60 μL aliquot of the solution was added into a cuvette in order to measure its fluorescent signal with the use of the PTI FRET Master Fluorometer. Along with the data from previously prepared samples, the fluorescent signal data was recorded into the Microsoft Excel software program for future data analysis.

Determination of PEI Density (DTT Concentration Effect)

In order to study the DTT concentration effect on the PEI density, stock solutions of 1.0 M DTT and 2.0 M DTT along with a AuPEI-FITC solution were used to prepare samples with the final DTT concentration set at 500 mM and 1.0 M. These samples were prepared by adding

10 μL of the AuPEI-FITC solution and 10 μL of the 1.0 M or 2.0 M DTT solution. These prepared samples were incubated under the time parameters outlined in the following table.

Incubation Table					
	Sample #1	Sample #2	Sample #3	Sample #4	Sample #5
500 mM at 70°C	10 minutes	20 minutes	30 minutes	40 minutes	60 minutes
1.0 M at 70°C	10 minutes	20 minutes	30 minutes	40 minutes	60 minutes

After these respective incubation periods, 80 μL of pure water was added into each sample cuvette. Each sample was spun by the vortex machine for ten seconds before centrifuging them in the Eppendorf Centrifuge 5415D at “full speed” for thirty minutes. After centrifugation, 70 μL of the supernatant was removed and stored in another cuvette for future testing procedures. From this sample, a 60 μL aliquot was inserted into the test cuvette in order to measure the fluorescent signal with the use of the PTI FRET Master Fluorometer. The fluorescent signal data collected by the fluorometer was recorded into the Microsoft Excel software program for future data analysis.

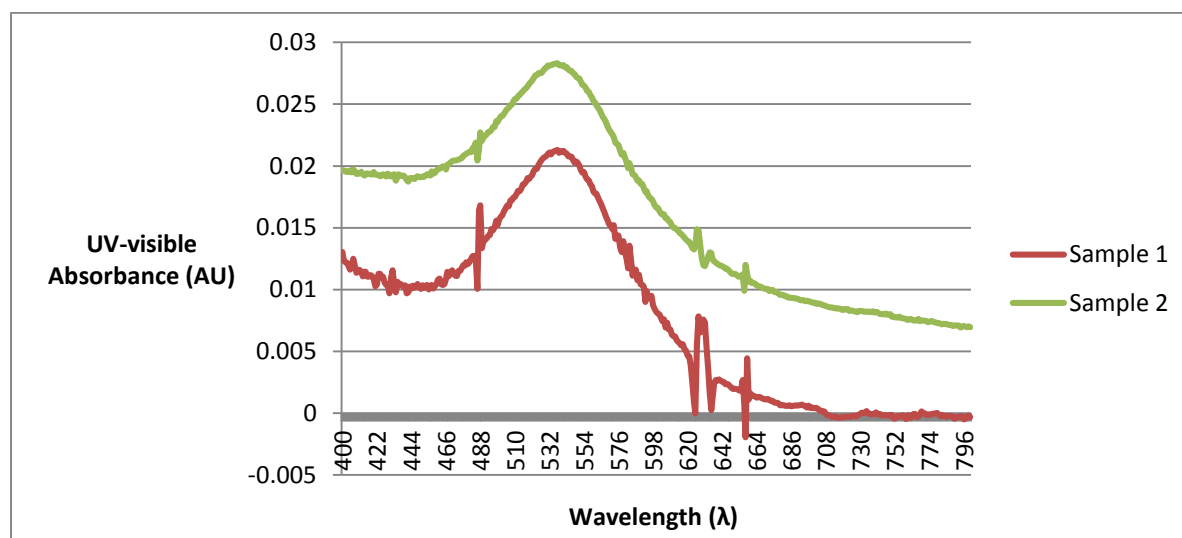
Optimization of the AuPEI-tRNA Conjugate Assembly System

The AuPEI-tRNA conjugates were assembled by adding 17.9 μL of 1.64 mM tRNA solution, 15.9 μL of AuPEI solution, and 821 μL of pure, de-ionized water into a 1-mL tube. Subsequently, the AuPEI-tRNA solution was equally divided amongst four solution tubes. Upon adding 250 μL of AuPEI-tRNA into each of the four tubes, each tube was marked at the 20 μL level for future instructions. Each of these solutions was centrifuged for 80 minutes at 7.0 rpm. After the first round of centrifugation, the supernatant of each solution sample was removed only to the level of the 20 μL mark. Upon removal of the supernatant, 180 μL of pure, de-ionized water was added to each of the sample before undergoing another round of centrifugation for 80

minutes at 7.0 rpm. Again, the supernatant of each solution sample was removed only to the level of the 20 μL mark. After removing the supernatant, another 180 μL of pure, de-ionized water was added to each solution sample in order to have a final volume of 200 μL . Finally, 10 μL of each solution sample and 500 μL of pure, de-ionized water were added into a cuvette in order to conduct UV absorbance experiments. It is important to note that three trials were conducted for each of the four solution samples.

Chapter 4: Results

Characterization of AuPEI-FITC Conjugates



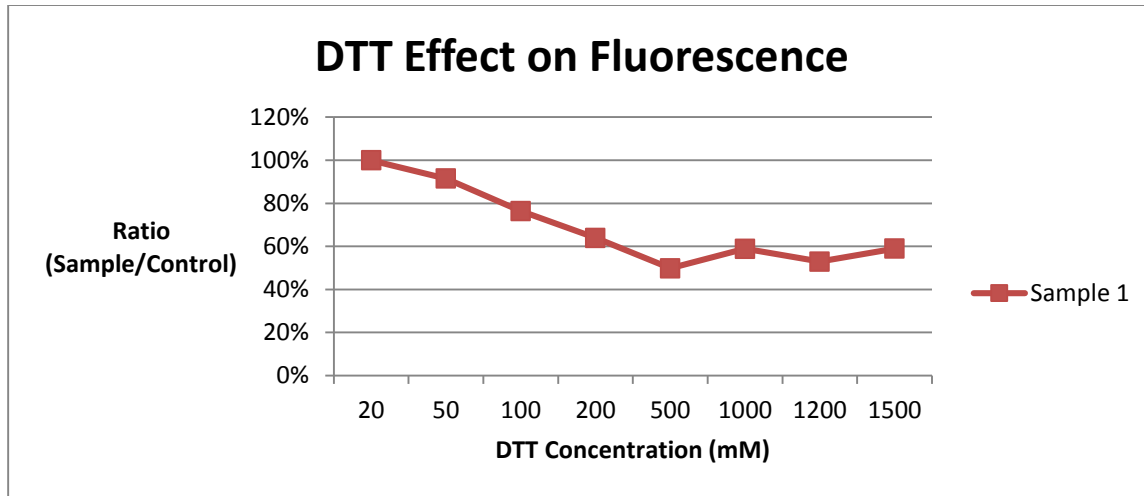
Samples	λ_{max}	Absorbance (AU)
# 1	537 nm	2.12913×10^{-2}
# 2	537 nm	2.83046×10^{-2}

Figure 8: AuPEI-FITC Conjugate UV/Vis Spectra

Table 1: AuPEI-FITC Conjugate Absorbance Data

DTT Concentration Effect on Fluorescent Signal

Figure 9: Graph displaying the DTT concentration effect on fluorescence



Determination of PEI Density (Temperature Effect)

Figure 10: Graph displaying sample PEI density while incubated at room temperature over 2 hours

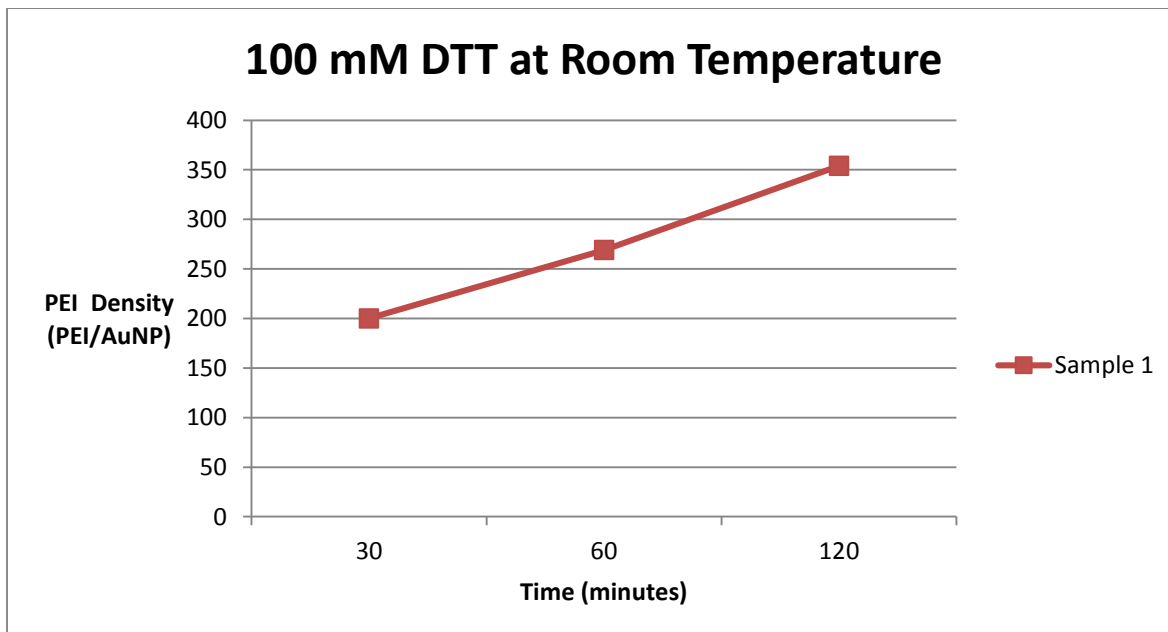
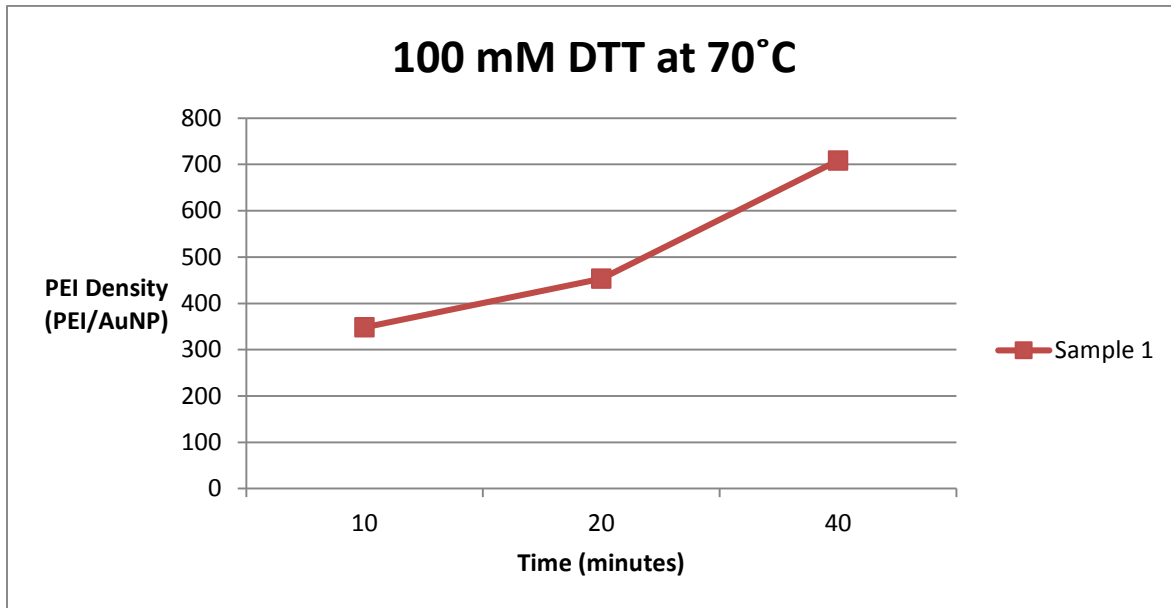


Figure 11: Graph displaying sample PEI density while incubated at 70°C over 40 minutes



Determination of PEI Density (DTT Concentration Effect)

Figure 12: Graph of sample PEI density in 100 mM DTT and 70°C incubation over 40 minutes

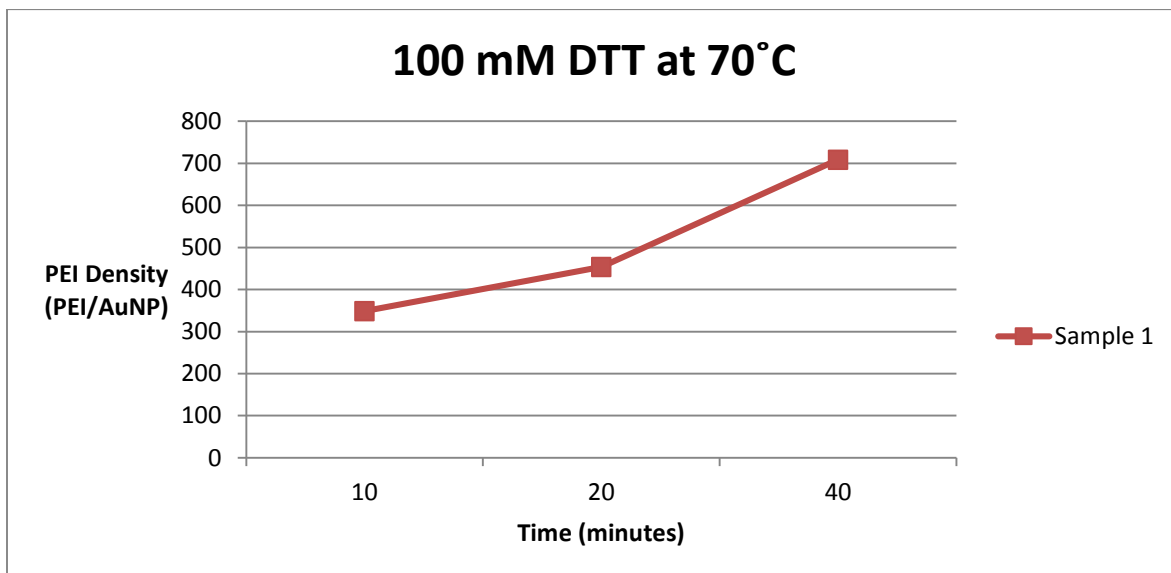
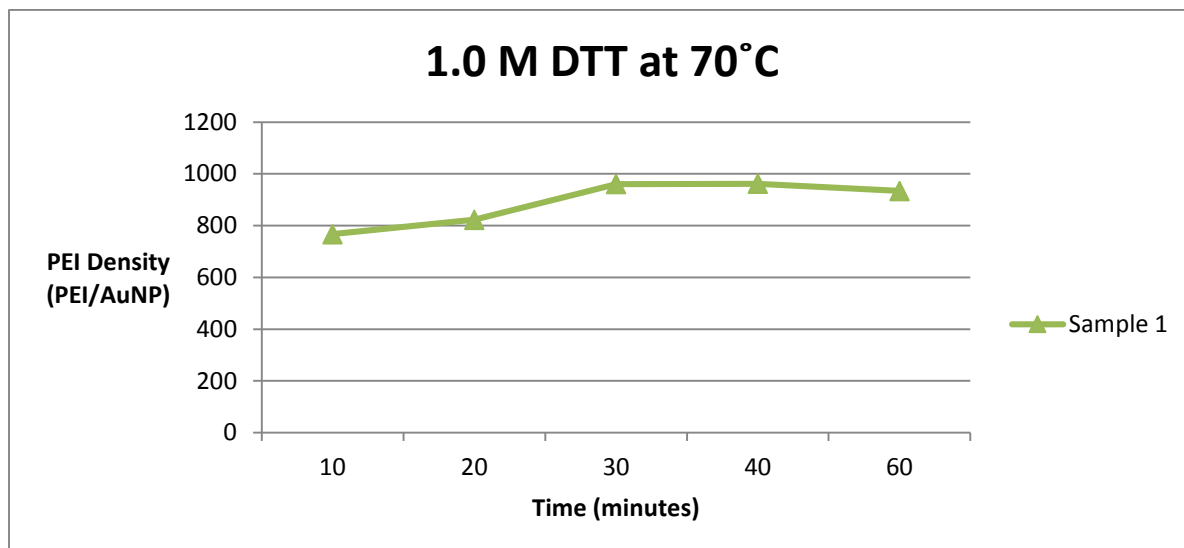
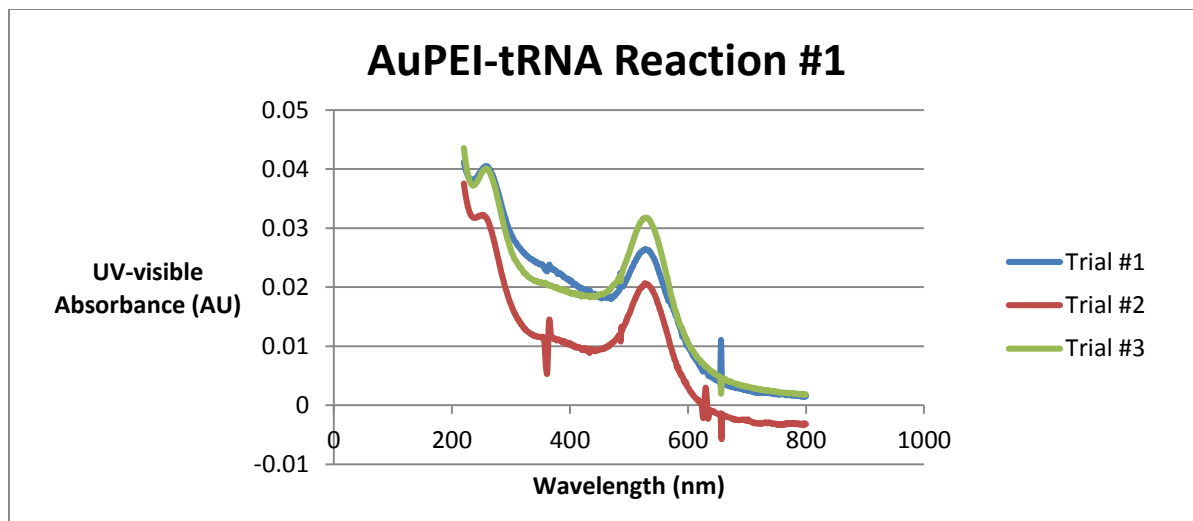


Figure 13: Graph of sample PEI density in 1.0 M DTT and 70°C incubation over 60 minutes



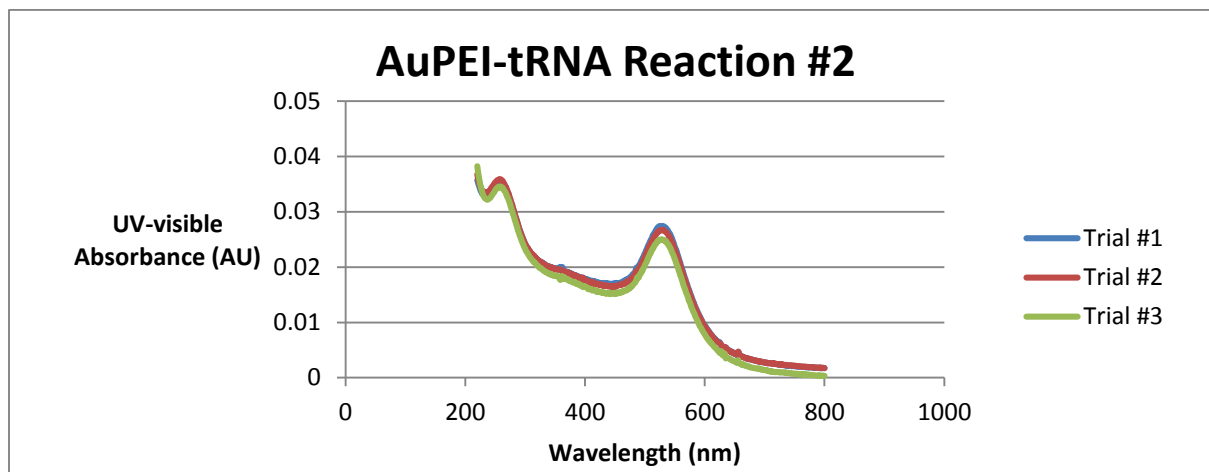
Optimization of the AuPEI-tRNA Conjugate Assembly System

Figure 14: UV Absorbance Graph and Table for AuPEI-tRNA Reaction #1



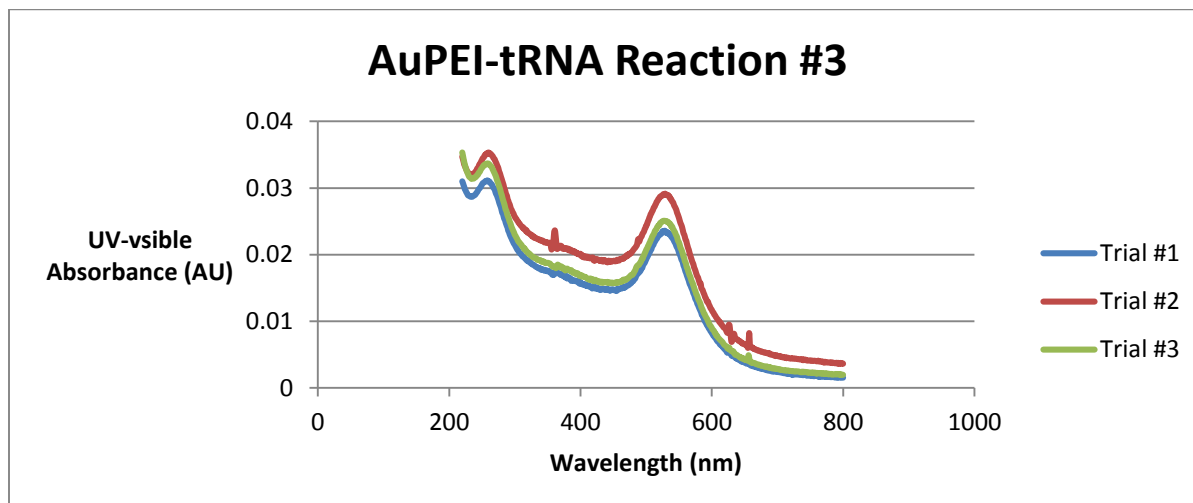
Trial	λ_{\max} (tRNA)	AU (tRNA)	λ_{\max} (AuPEI)	AU(AuPEI)	AU (800 nm)
1	259 nm	4.05188×10^{-2}	528 nm	2.64854×10^{-2}	1.54018×10^{-3}
2	252 nm	3.22275×10^{-2}	527 nm	2.06690×10^{-2}	-3.22819×10^{-3}
3	257 nm	4.00982×10^{-2}	528 nm	3.17550×10^{-2}	1.84917×10^{-3}

Figure 15: UV Absorbance Graph and Table for AuPEI-tRNA Reaction #2



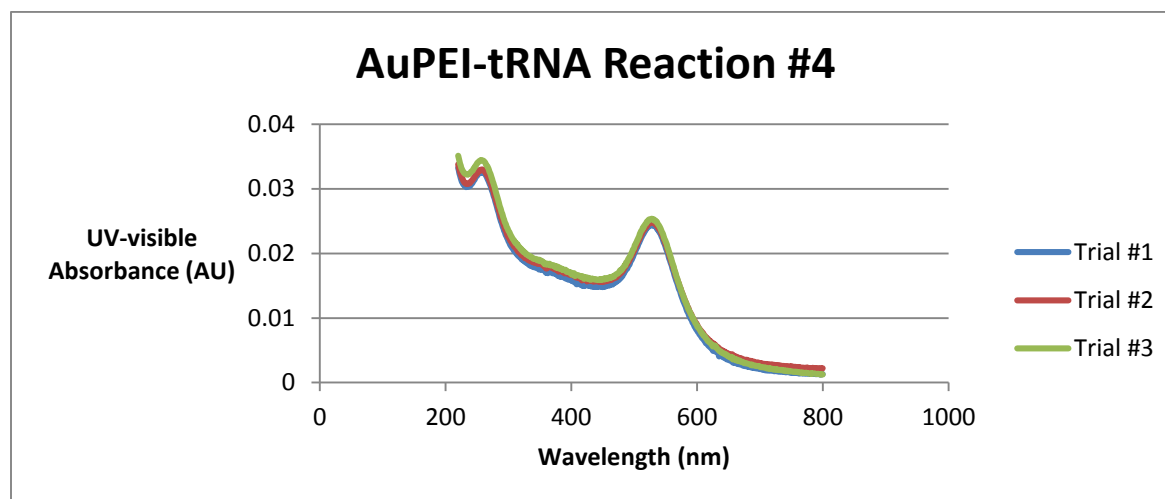
Trial	λ_{\max} (tRNA)	AU (tRNA)	λ_{\max} (AuPEI)	AU(AuPEI)	AU (800 nm)
1	258 nm	3.56808×10^{-2}	530 nm	2.74286×10^{-2}	1.75476×10^{-3}
2	258 nm	3.58863×10^{-2}	528 nm	2.67420×10^{-2}	1.72997×10^{-3}
3	259 nm	3.45387×10^{-2}	528 nm	2.50306×10^{-2}	3.43323×10^{-4}

Figure 16: UV Absorbance Graph and Table for AuPEI-tRNA Reaction #3



Trial	λ_{\max} (tRNA)	AU (tRNA)	λ_{\max} (AuPEI)	AU(AuPEI)	AU (800 nm)
1	258 nm	3.11470×10^{-2}	528 nm	2.35949×10^{-2}	1.54448×10^{-3}
2	260 nm	3.53165×10^{-2}	529 nm	2.91414×10^{-2}	3.63350×10^{-3}
3	258 nm	3.36494×10^{-2}	528 nm	2.50716×10^{-2}	1.97601×10^{-3}

Figure 17: UV Absorbance Graph and Table for AuPEI-tRNA Reaction #4



Trial	λ_{\max} (tRNA)	AU (tRNA)	λ_{\max} (AuPEI)	AU(AuPEI)	AU (800 nm)
1	260 nm	3.25904×10^{-2}	528 nm	2.44756×10^{-2}	1.31989×10^{-3}
2	257 nm	3.29518×10^{-2}	527 nm	2.48680×10^{-2}	2.22111×10^{-3}
3	257 nm	3.44729×10^{-2}	528 nm	2.53854×10^{-2}	1.27220×10^{-3}

Chapter 5: Discussion/Conclusion

Preparation and Characterization of AuPEI-FITC Conjugates

After successfully preparing two parallel solution samples, the Huang research group suggests that the following parameters are the optimal settings for synthesizing these AuPEI-FITC conjugates. In terms of incubation, they suggested that ten minutes at room temperature and ten minutes at 65°C ensured the binding of the gold nanoparticles and the PEI-FITC molecules. In terms of centrifugation, they suggested that centrifuging the samples at “full speed” for thirty minutes provided them with a well-intact pellet and no signs of the pellet diffusing into the supernatant of the centrifuged solution. In terms of washing, they suggested that removing nearly all of the supernatant without harming the pellet and adding 1,000 μL of pure water ensured the purification of the AuPEI-FITC solution. With no obvious signs of

aggregation observed during this stage, the Huang research group proceeded towards the characterization of these AuPEI-FITC conjugates.

During this stage, the Huang research group followed protocol by balancing the UV/VIS spectrophotometer with a cuvette filled only with distilled water. Once the UV/VIS instrument was prepared for sample testing, an aliquot of each sample was tested in order to determine the λ_{\max} and absorbance readings for the two parallel AuPEI-FITC solutions. As shown in Figure 8, both UV/VIS spectra for Sample #1 and #2 followed a similar graphical trend that portrays the same λ_{\max} value and similar absorbance readings. According to Table 1, both Sample #1 and #2 share a λ_{\max} value of 537 nanometers, which falls between the expected λ_{\max} range values of 534 and 540 nanometers. Also, Sample #1 solution was measured to have an absorbance reading of 2.12913×10^{-2} AU, while the absorbance reading of Sample #2 solution was measured to be 2.83046×10^{-2} AU. This slight change in absorbance between both samples suggests that there was a potential shift in the background absorbance when testing each sample. However, due to accurate λ_{\max} values and similar absorbance readings, the Huang research group decided that evidence given during the UV characterization of these AuPEI-FITC conjugates was sufficient enough to proceed to the steps involving the determination of the PEI density.

DTT Concentration Effect on Fluorescent Signal

Before determining the PEI density on each gold nanoparticle, the Huang research group decided to observe the effects that DTT concentration might have on the fluorescent signal of these AuPEI-FITC conjugates. With that in mind, they devised a procedure that would examine how the fluorescent signal would be affected when the DTT concentration was increased in a particular solution sample. During this procedure, they used stock solutions of AuPEI-FITC, 200 mM DTT, and 2.0 M DTT in order to prepare individual samples with a range of DTT

concentrations such as 20 mM, 50 mM, 100 mM, 200 mM, 500 mM, 1,000 mM, 1,200 mM, and 1,500 mM. An aliquot of the supernatant was removed from each sample and tested for any changes in fluorescent signal. After collecting the fluorescent signal data, it was analyzed with Microsoft Excel, which provided them with a graph that plotted DTT concentration (x-axis) versus the ratio of sample/control (y-axis). As shown in Figure 9, the graphical results suggested that the DTT concentration, indeed, did have an effect on the fluorescent signal of these AuPEI-FITC solutions. More specifically, the graphical results suggested that the fluorescent signal decreased as the DTT concentration increased in each solution. However, the fluorescent signal only decreased between the range of 20 mM and 500 mM. Beyond that point in the data, it seemed that there was no loss in fluorescent signal between the DTT concentrations of 500 mM and 1,500 mM. This observation suggests that these concentrations between 500 mM and 1,500 mM are likely to provide the DTT concentration parameters where the optimal PEI density will be determined. However, due to the decreasing fluorescent signal trend between 20 mM and 500 mM, it also suggests that PEI density in the following experiments must be corrected for the lost signal in order to accurately determine the amount of PEI molecules on each gold nanoparticle.

Determination of PEI Density (Temperature Effect)

In order to accurately determine the PEI density on each gold nanoparticle, the Huang research group devised a procedure that would pinpoint the optimal temperature at which the most PEI-FITC molecules would release into the supernatant solution when treated with a constant DTT concentration. During this procedure, they used stock solutions of AuPEI-FITC, 1.0 μ M PEI-FITC, and 200 mM DTT in order to prepare the control and test samples that would be incubated at room temperature and 70°C. After incubation and centrifugation, an aliquot of the supernatant was removed from each sample in order to test its fluorescent signal. After

collecting the fluorescent signal data, it was processed by Microsoft Excel in order to calculate the correct PEI density for each sample. Again, this software program organized the data into a graph which plotted incubation time (x-axis) versus PEI density (y-axis). As shown in Figure 10, the results suggest that a larger amount of time was required for the thiol-exchange reaction to fully complete in solution. At thirty minutes, the PEI density started around 200 PEI-FITC molecules per gold nanoparticle. After two hours, the PEI density had only increased to 350 PEI-FITC molecules per gold nanoparticles. This graph suggests that the PEI density only increased minimally over a two hour time span. On the other hand, Figure 11 suggests that a smaller amount of time was required for the thiol-exchange reaction to fully complete in solution. At ten minutes, the PEI density started around 350 PEI-FITC molecules per gold nanoparticle. At forty minutes, the PEI density had increased to 700 PEI-FITC molecules per gold nanoparticle. This graph suggests that the PEI density had increased more effectively in 70°C sample trials. Therefore, the Huang research group has determined that 70°C is the optimal incubation temperature at which the thiol-exchange reaction can occur more effectively.

Determination of PEI Density (DTT Concentration Effect)

In a final step towards determining the PEI density of these AuPEI-FITC conjugates, the Huang research group proposed another procedure that would identify the optimal DTT concentration at which the most PEI-FITC molecules will release into the supernatant solution during the thiol-exchange reaction. In this experiment, stock solutions of AuPEI-FITC, 1.0 M DTT, and 2.0 M DTT were used in order to prepare the test samples that would be incubated at the previously-determined optimal temperature of 70°C. After incubation and centrifugation, an aliquot of the supernatant was removed from each sample in order to test the fluorescent signal. With the fluorescent signal data on record, the Huang research group used Microsoft Excel to

calculate the correct PEI density for each sample. Again, this software program was used to organize this data into graphs that plotted incubation time (x-axis) versus PEI density (y-axis). After producing these graphs, the Huang research group compared the results in order to determine the optimal PEI density on each gold nanoparticle. As shown in Figure 12, the graph suggested that the optimal PEI density could not be clearly determined from its data. In this 100 mM DTT at 70°C graph, it appeared that the PEI density was continuing to increase through the measured incubation time range. This observation suggests that the thiol-exchange reaction needs more time to occur when using a 100 mM DTT solution. However, in Figure 13, the graph suggests that the optimal PEI density could be determined from its data. Based on the 1.0 M DTT at 70°C graph, it appeared that PEI density had reached a plateau mark around 1,000 PEI molecules per gold nanoparticle. This observation suggests that the thiol-exchange reaction has fully completed, and the optimal PEI density can be determined when using a 1.0 M DTT solution.

Optimization of the AuPEI-tRNA Conjugate Assembly System

After two rounds of centrifugation and purification, the Huang research group tested the UV absorbance and λ_{\max} readings for each of the four AuPEI-tRNA solution samples. Based on previous research data, it was expected to have a λ_{\max} for tRNA at 259 nanometers and another λ_{\max} for AuPEI between 528 and 530 nanometers. To compensate for the broad range between the two λ_{\max} points, the wavelength range was adjusted to cover the absorbance data points between 220 and 800 nanometers. For each of the four solution samples, three UV absorbance trials were conducted for accuracy and precision purposes. Based on the Reaction #1 data (see Figure 14), the λ_{\max} for tRNA exhibited a range of data points between 252, 257, and 259

nanometers, while the λ_{\max} for AuPEI was located at 527 and 528 nanometers. For Reaction #2 (see Figure 15), data from the trials suggested the λ_{\max} for tRNA to be in the range between 258 and 259 nanometers, while the λ_{\max} for AuPEI was located in the close proximity to 528 and 530 nanometers. Based on the Reaction #3 data (see Figure 16), the λ_{\max} for tRNA was suggested to be 258 and 260 nanometers, while the λ_{\max} for AuPEI was pinpointed to be 528 and 529 nanometers. For Reaction #4 (see Figure 17), the trial data suggested the λ_{\max} for tRNA to be 257 and 260 nanometers, while the λ_{\max} for AuPEI was identified to be at 527 and 528 nanometers. Overall, based on the UV data from these AuPEI-tRNA samples, the λ_{\max} values were similar to the expected λ_{\max} values for tRNA and AuPEI. Therefore, it can be concluded that the centrifugation and purification steps were efficient in producing an optimized system for assembling AuPEI-tRNA conjugates.

Conclusion

With the results from these procedures in mind, the Huang research group has calculated the optimal PEI density to be 1,000 PEI molecules per gold nanoparticle. The determination of PEI density is an important step towards developing these AuPEI-siRNA conjugates that can be delivered into cancer cells to induce apoptosis by targeting the expression of the CASP8AP2 gene. By knowing the exact amount of PEI on each gold nanoparticle, the Huang research group could theoretically calculate the amount of tRNA molecules that can be bound to these AuPEI conjugates. Upon establishing an optimized system for adding tRNA to the AuPEI conjugates, future research efforts can proceed towards the loading of siRNA molecules onto the AuPEI conjugates. Once the final AuPEI-siRNA product has been assembled, the Huang research group can continue into the cell study phase, where they can specifically target the CASP8AP2 gene in KB cells.

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