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Design of Gold Nanoparticle Mediated siRNA Anti-cancer Therapies

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by

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Abstract

As gene therapies continue to be developed, the role played by siRNA technologies in anti-cancer regimens seems likely to increase. And, as siRNA technologies continue to be studied, the use of gold nanoparticle conjugate systems to deliver siRNA in vivo will proliferate as well. In light of this trend in cancer therapeutics, this project aimed to design a AuNP carrier for siRNA. With the goal of creating an efficient base from which effective siRNA-AuNP conjugates could be synthesized, the development of C_{12}PEI coated AuNPs was closely investigated. Toward this end, AuNP-C_{12}PEI conjugates were synthesized with an average diameter of 25 nm, an average zeta potential of +58 mV, and an average of 258 C_{12}PEI units per AuNP. Additionally, C_{12}PEI-AuNPs were successfully conjugated with tRNA in preparation for siRNA binding studies.

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Chapter 1: Introduction

RNA interference, or RNAi, is a recently discovered mechanism by which specific genes are silenced in a target cell, tissue, or organism. The silencing effect has been attributed to the cleavage of mRNA by an enzymatic complex activated by the presence of small interfering RNA, or siRNA, that is complementary to the mRNA sequence. By cleaving the pre-translational mRNA, the protein encoded by the target gene will not be synthesized, essentially "silencing" the expression of the gene.

The interest in gene silencing is multifaceted; as the understanding of RNAi has progressed, multiple opportunities for application have become evident. In early studies, introduction of exogenous RNA was used to observe and document the function of genes in a variety of organisms.[1-3] Many contemporary projects are exploring the advantage of using RNAi as a mechanism for gene therapy. In this context, cancer therapeutics are being investigated intensely.[3-11]

Although much is known about the mechanism of the RNAi pathway, more research is needed to investigate both the delivery of siRNA to targeted cells, and the precision and efficiency of gene knockdown if steps toward therapeutic regimens are to be made. In the area of siRNA delivery, several promising methods of delivery are being developed. One method being explored by numerous research groups during the past decade is based on gold nanoparticles.[12] Regardless of the system being investigated,

optimizing the method used to target specific cells, the mode of conjugation of siRNA to the vehicle, and the release of siRNA once inside the target cell is crucial to progress toward clinical trials in cancer treatment. In the area of gene knockdown, further research must be done to isolate siRNA sequences that can negatively affect genes essential to tumor vitality without creating adverse effects in the rest of the genome. Also, additional studies may improve the understanding of factors that control the efficiency of gene knockdown *in vivo*.

As such, for the purposes of this project, the goal of utilizing RNAi was the development of novel cancer therapy agents, specifically AuNP-based nanoparticles, that display high efficiency and limited side effects. This project, in conjunction with the Huang research group, sought to develop an siRNA delivery vehicle that reduces the hindrances described above: non-specific delivery, inadequate potency, and low efficiency. Using AuNPs, a layered approach to synthesis was implemented, similar to the approaches of Elbakry et al. (2009) and J.S. Lee et al. (2009) described below. In anticipation of producing a therapeutic agent that could equal or surpass the benchmark of 80% gene knockdown achieved by Heidel et al. (2007), as reported below, significant attention was given to the first stage of nanoconjugate development involving the conjugation of C_{12}PEI to the AuNP surface in an efficient manner. Due to the exploratory nature of this design project, no definitive hypothesis was able to be tested. Rather, a consistent synthetic platform was attempted from which future, hypothesis-driven optimization could be performed.

Chapter 2. Literature Review

Various approaches have been used in the process of researching cancer therapeutics using RNAi. In general, each approach consists of two broad focal points: the gene target, and the mode of delivery. For example, in reference to gene targets, Kittler et al. (2004) suggest that genes associated with cell division are plausible targets for accomplishing tumor suppression. Screening for genes that, upon suppression, cause arrest of mitosis, defects in the spindle structure, or lead to cell death, they determined that at least 37 genes are essential for cell division. Six of these genes, one of which being RRM2, were determined to cause cell death at the entrance into mitosis.[4]

RRM2 refers to the second of two required subunits of the ribonucleotide reductase (RR) enzyme. RR is an important enzyme in the mitotic pathway because it catalyzes the reduction of ribonucleotides to their corresponding 2'-deoxyribonucleotides, which are essential for DNA synthesis and replication. Of the two subunits, RRM1 is expressed at a maintained level throughout the entire cell cycle, while RRM2 is only expressed leading up to mitosis. Two groups, Heidel et al. (2007) and Reid et al. (2009), have presented research concerning the potential effect of RR knockdown. Although each group reports RR as a useful target, the groups differ on which subunit is selected for suppression.[5,6]

Heidel et al. focus on the RRM2 subunit because it is over expressed in cancer cells and has been shown to adversely affect tumor cell growth. In summary, Heidel et al. screened computer databases for potential RRM2 target sequences and tested three preliminary siRNAs, eventually refining the siRNA sequences to determine the siRNA with greatest knockdown potential. After identifying the most successful siRNA through *in vitro* studies (siR2B+5, 80% knockdown), the team probed its knockdown potential *in vivo* and monitored the viability of infected organisms. The positive results yielded *in vivo* led to the conclusion that RRM2 knockdown by siR2B+5 is a potential candidate for further study. Importantly, Heidel et al. noted that RRM2 knockdown in tumor tissues showed no observable adverse effects in the organism.[5]

In contrast, Reid et al. focus on the RRM1 subunit, comparing RRM1 knockdown to RRM2 knockdown in equivalent cells. Reid's group demonstrates the efficiency of tumor growth suppression by siRNAs targeting either RRM1 or RRM2. However, their findings indicate that the siRNAs targeting RRM1 are somewhat more potent than equimolar concentrations of those targeting RRM2. A possible explanation given for the difference in potency is the apparently higher abundance at which RRM2 is expressed versus RRM1 in cells. Reid et al. also demonstrate that minor suppression, that is, suppression insufficient for cell death, of either RRM1 or RRM2 may cause tumor cells to become sensitive to other drugs, such as hydroxyurea.[6]

When the findings of these groups, Heidel et al. and Reid et al., are compared, a trade off is seen in the choice of which RR subunit to suppress. On one hand, RRM1

knockdown is more potent than RRM2 knockdown.[6] On the other hand, RRM2 is over expressed in cancer cells and knockdown has minimal adverse effects in other tissues.[5]

As mentioned above, the second major aspect of researching cancer therapeutics is the mode of delivery. In the context of RNAi, siRNA has been delivered virally, or in conjugation with polymers,[10,11] lipid based systems, and nanoparticles.[7-9] Although several options are currently available, the apparent defects associated with each delivery system are a hindrance to the advancement of RNAi therapy.[8] As such, research is being done in order to provide stability to the siRNA complex, reduce the lethality of carriers,[7-11] increase the specificity of carriers,[10,11] and improve the intracellular release of siRNA.[8,9,11]

One promising delivery method, developed by numerous groups throughout the past decade, seeks to achieve some of these goals through the implementation of gold nanoparticles (AuNPs),[12] which display a high degree of compatibility with living tissues.[8,9] As described by Giljohann et al. (2009), AuNPs are capable of significant functionalization with siRNA and effectively protect conjugated siRNA from degradation. The benefit of siRNA conjugation to AuNPs, as reported by Giljohann, is seen in the more persistent knockdown by siRNA-AuNPs when compared to siRNA alone.

Another group, Elbakry et al. (2009), implemented a “layer-by-layer” approach to forming siRNA-AuNP conjugates as a means to facilitate both the cellular uptake of the conjugated particles and the intracellular release of siRNA. Cellular uptake by

endocytosis was enhanced by incorporating polyethylene-imine (PEI) into the siRNA-AuNP conjugate particle, which decreased the net negative charge on the particle, and as a result decreased the repulsion from the cell membrane. Although Elbakry and coworkers report successful uptake of siRNA-PEI AuNPs, the enhanced stability of the nanoparticle was found to decrease the intracellular release of siRNA, necessitating the future implementation of biodegradable polymers capable of facilitating endosomal release.[8]

In regard to the incorporation of biodegradable polymers, authors J. S. Lee et al. (2009) sought to show that the addition of poly(beta-amino ester)s (PBAEs) and inclusion of disulfide linkages between siRNA and AuNPs would facilitate the uptake and intracellular release of the siRNA-AuNPs. The group focused on PBAEs that had been reported to successfully deliver DNA to cells, forming a library of polymers functionalized with several end groups. In addition to PBAEs, J. S. Lee et al. chose to attach siRNA to AuNPs via disulfide linkages, which are readily cleavable in the reducing environment of the cell. Two of the PBAEs tested demonstrated greater intracellular release (i.e. greater knockdown) than the commercial delivery vehicle Lipofectamine 2000. Importantly, these findings suggest the necessity of utilizing biodegradable polymers, as indicated by the absence of delivery when polymers are not used.[9]

Chapter 3: Experimental Methods

Because of the multifaceted nature of this project, significant attention has been given the development of successful and repeatable synthetic parameters for AuNP conjugates before attempting cell studies with siRNA for a gene target, such as RRM2.

As mentioned above, the layer-by-layer synthetic approach chosen closely resembles the work of other authors, especially Elbakry et al. (2009). Although the general scheme of coating AuNPs with a layer of cationic polymers, followed by a layer of nucleic acids, and a final layer of cationic polymers was retained, two important adjustments to Elbakry's method should be noted. First, rather than electrostatically binding the initial layer of PEI to AuNPs that have been pre-coated with 11-mercapto-undecanoic acid (MUA) via thiol chemistry, our group conjugated PEI to MUA prior to the coating step, allowing the entire molecule (named C_{12}PEI) to interact with the AuNP surface via thiol chemistry. Additionally, the outer layer of polymers we hope to implement will include a PEI-PEG copolymer that incorporates a folic-acid receptor targeting moiety, along with imaging agents needed for cell-studies, rather than PEI alone. It should also be noted that, due to the relative expense of siRNA when compared to tRNA, tRNA was selected as a model for siRNA behavior while nanoparticle synthesis is being optimized.

Beginning with the first layer, a replacement of citrate stabilizer with thiol-reactive C_{12}PEI, the goal was to develop a protocol that accomplished high PEI coverage, facile purification, and high recovery yield. To this end, several experiments were conducted to observe the effects, if any, of the final concentration of C_{12}PEI with respect to particle stability, size, and recovery yield.

The basic synthetic procedure used during C_{12}PEI coating is described as follows. First, a measured volume of C_{12}PEI was delivered to a 14.8 nM solution of citrate stabilized AuNPs (~15 nm diameter by DLS). After vortexed mixing, the solutions were allowed to incubate at room temperature for 30 min. Following incubation, a series of washes by centrifugation and resuspension were performed at 14,000 rpm. The washed samples were then characterized by UV-Vis spectrophotometry, as well as dynamic light scattering (DLS), including zeta potential measurements. Final concentrations of 0.5 mM, 1.0 mM, and 2.0 mM C_{12}PEI were examined, along with the method of addition, and the intensity of washing. With respect to washing, the variables manipulated were the length of time per wash, and the number of washes per sample. It should be noted that salt (NaCl) was not added at any point during the synthetic processes.

Along with experiments investigating the effect of the final concentration of C_{12}PEI, the number of C_{12}PEI units per AuNP was quantified by fluorescence spectrometry. For this portion of the project, a general fluorophore (fluorescein isothiocyanate, "FITC") was selected and incorporated into C_{12}PEI stock prior to beginning the nanoconjugate synthesis. After completing the final washing step as

described above, resuspended conjugates were treated with DTT (dithiothreitol) in order to reduce the sulfide bonds between the AuNP surface and C_{12}PEI. Following centrifugation of the DTT-treated solution to remove AuNPs, fluorescence measurements were taken and compared to the concentration of free C_{12}PEI in the last supernatant in reference to a standard curve. By relating the concentration of C_{12}PEI released from the particles to the recovered concentration of AuNPs from the washing procedure, the number of C_{12}PEI units per AuNP was ascertained.

In addition to the experiments concerning the initial layer of C_{12}PEI, similar work was done to design a useful protocol for RNA coverage onto AuNP-C_{12}PEI conjugates, using tRNA as a model system. Again, the goal was to develop a synthetic scheme that accomplished high RNA coverage (high payload), facile purification, and high recovery yield. For this step, a net anionic charge was sought as a result of tRNA binding in order to facilitate electrostatic binding of the next cationic layer. As before, overall size of the particles was being considered, but the major concerns were particle stability and monodispersity.

The approach to adding nucleic acids, in this case tRNA, to AuNP-C_{12}PEI conjugates was carried out in the following manner. First, the volume of tRNA stock (~26.4k MW, 1 μg/μl) required to achieve the desired final concentration was added to Ultrapure water. To this aqueous tRNA solution, a smaller volume of AuNP-C_{12}PEI was added dropwise while vortexing at low speed to ensure homogenous mixing. After mixing, the samples were allowed to incubate at room temperature for 30 min, and a

series of washes by centrifugation at 14,000 rpm was conducted as before. Samples were prepared with final concentrations of 1.0 μM, 2.0 μM, and 4.0 μM tRNA. And, as with C_{12}PEI, the washing steps were manipulated in an attempt to achieve high recovery yields with high purity while maintaining particle stability.

Chapter 4. Results

The following data shows results for both C_{12}PEI and tRNA layer experiments. AuNP- C_{12}PEI samples that were relatively monodisperse, stable in solution, and repeatable were chosen as candidates for tRNA trials.

The first two sets of graphs depict the effect of addition method of C_{12}PEI on particle size. The details of interest are the location of the plasmon resonance peak relative to that of the AuNP stock, along with the distribution dispersity of the size measurements. Figures 1a & 1b represent addition down the side of the tube, while Figures 2a & 2b show addition into the cap with immediate vortexing after cap closure. Similarly, Figure 3 depicts the UV-Vis results for a replication of the "cap-addition" method.

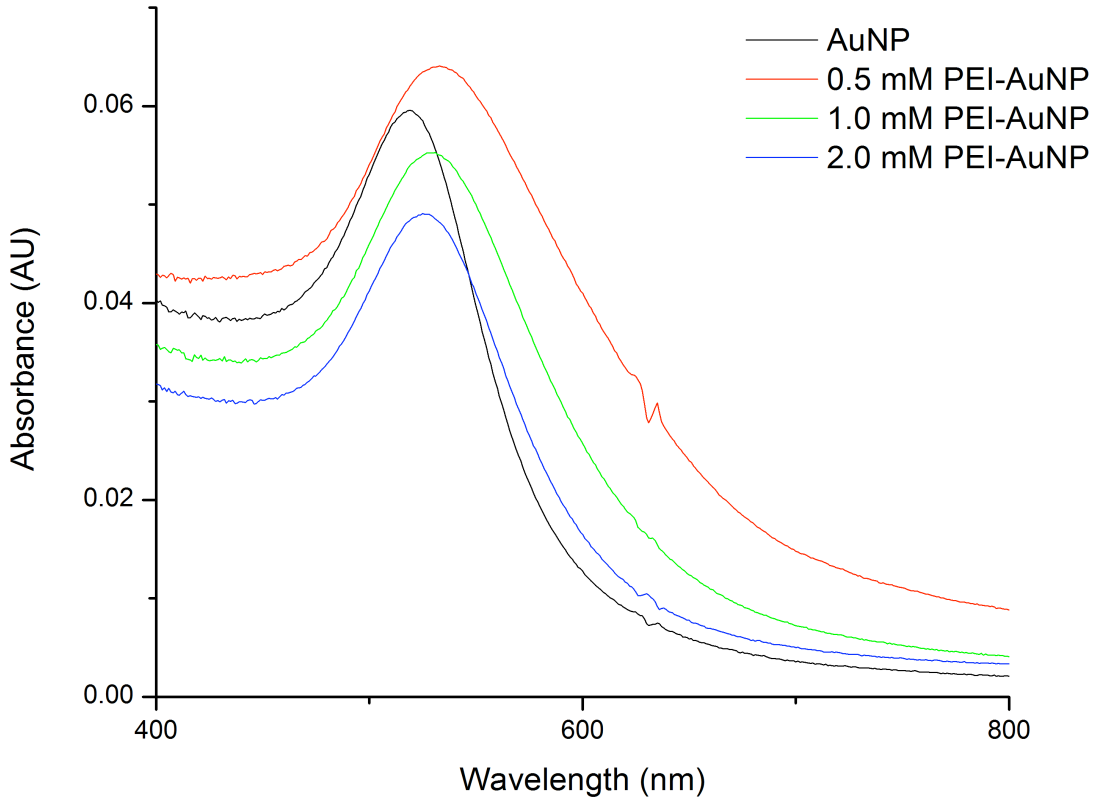

Figure 1a AuNP stock (black), shown with three samples of C_{12}PEI-AuNPs (red, green, blue) prepared by direct addition.

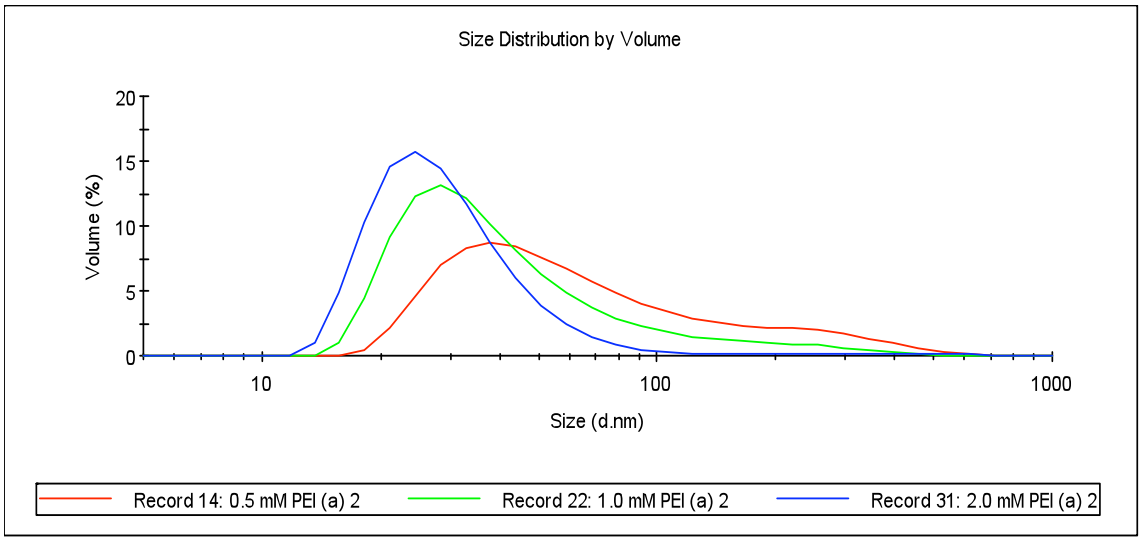

Figure 1b Representative size distributions for C_{12}PEI-AuNP samples prepared by direct addition.

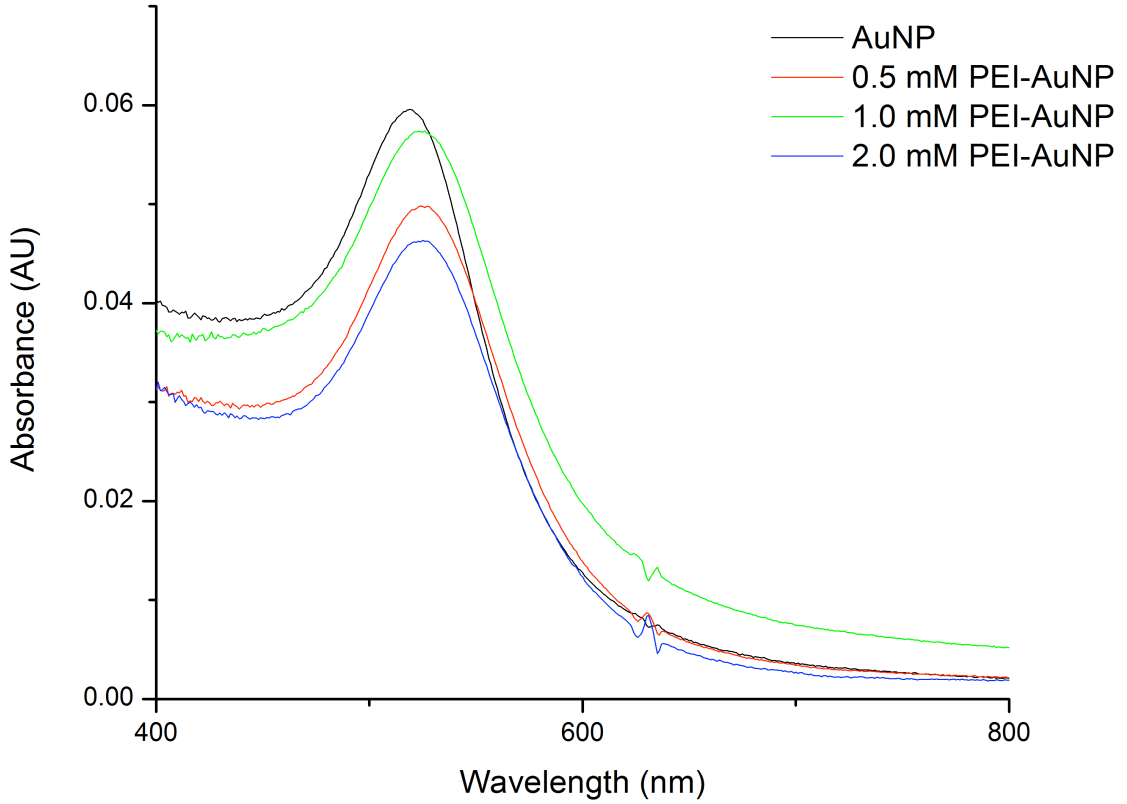

Figure 2a AuNP stock (black), shown with three samples of C_{12}PEI-AuNPs (red, green, blue) prepared by addition to the tube cap.

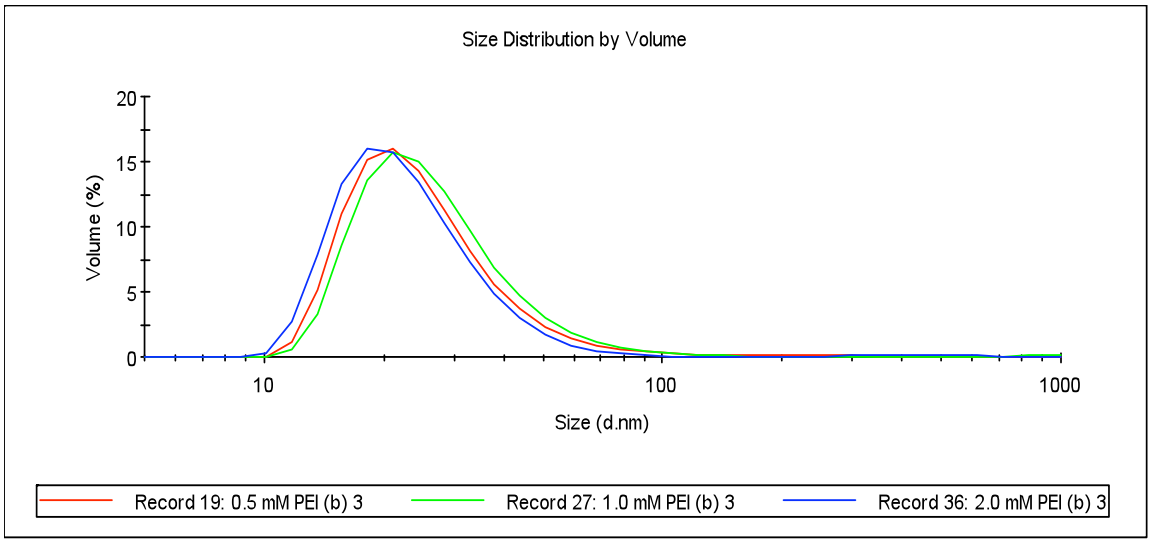

Figure 2b Representative size distributions for C_{12}PEI-AuNP samples prepared by addition to the tube cap.

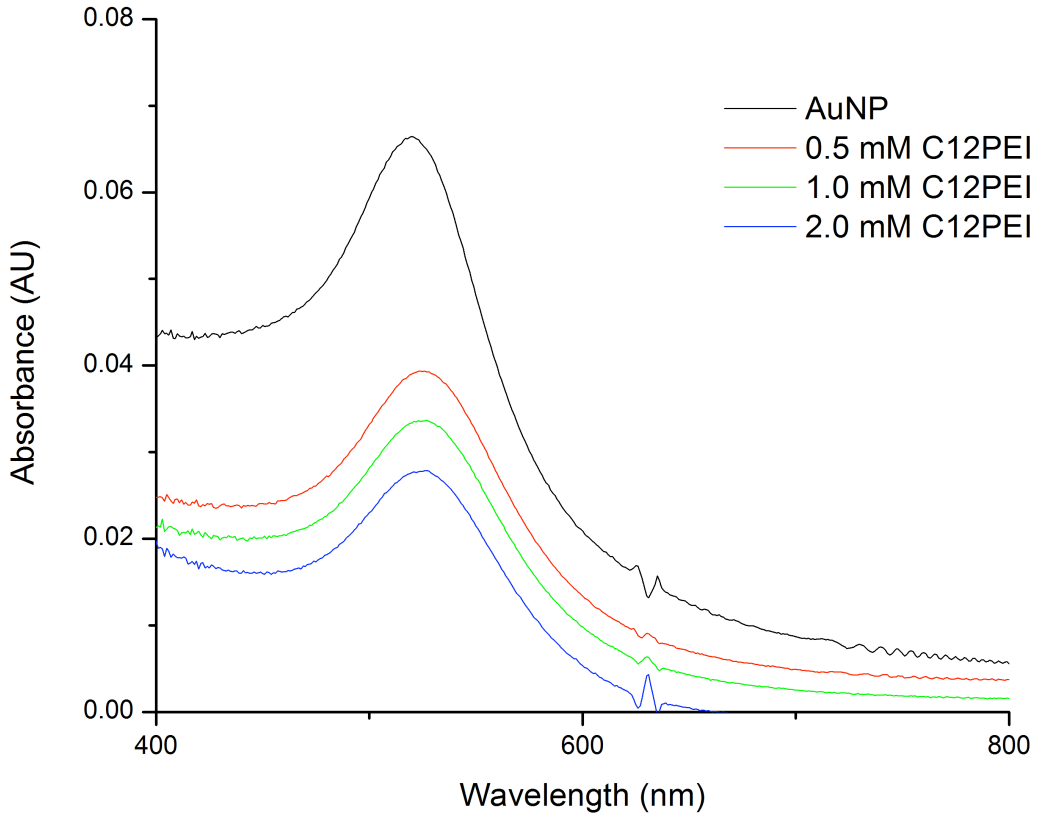

Figure 3 AuNP stock (black), shown with three samples of C_{12}PEI-AuNPs (red, green, blue). Replicated results prepared by addition to the tube cap.

The next set of graphs, Figures 4a – 4d, outline the process of fabricating tRNA-C_{12}PEI-AuNP conjugates, beginning with AuNP stock solution. First, Figures 4a & 4b show UV-Vis and DLS characterization after the first coating step. Figures 4c & 4d represent the subsequent tRNA coating step and the washing process implemented.

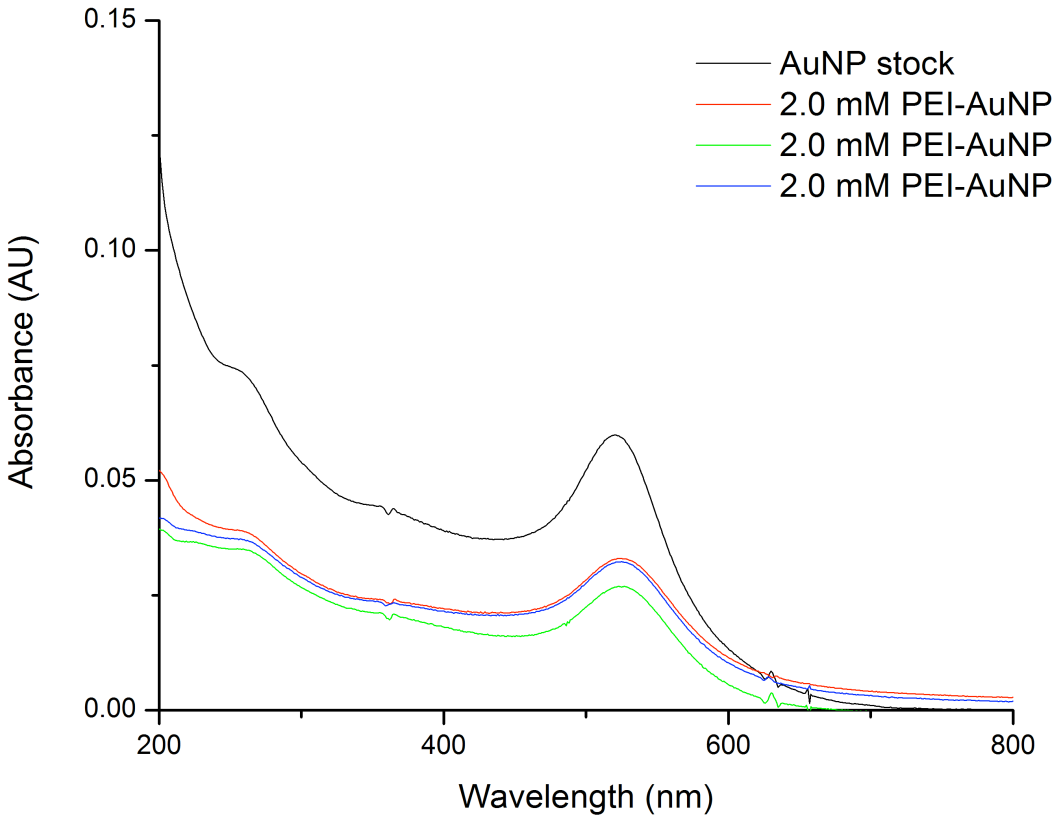

Figure 4a AuNP stock (black), shown with three samples of C_{12}PEI-AuNPs (red, green, blue). Replicated results prepared by addition to the tube cap, in preparation for tRNA binding.

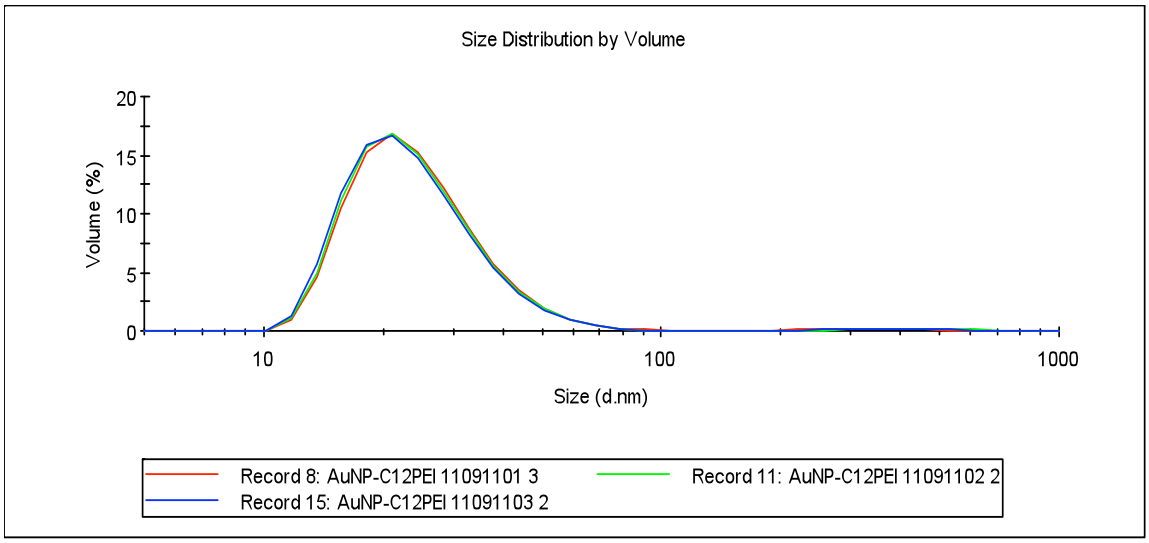

Figure 4b Representative size distributions for C_{12}PEI-AuNP samples prepared by addition to the tube cap. Same samples as shown in Figure 4a.

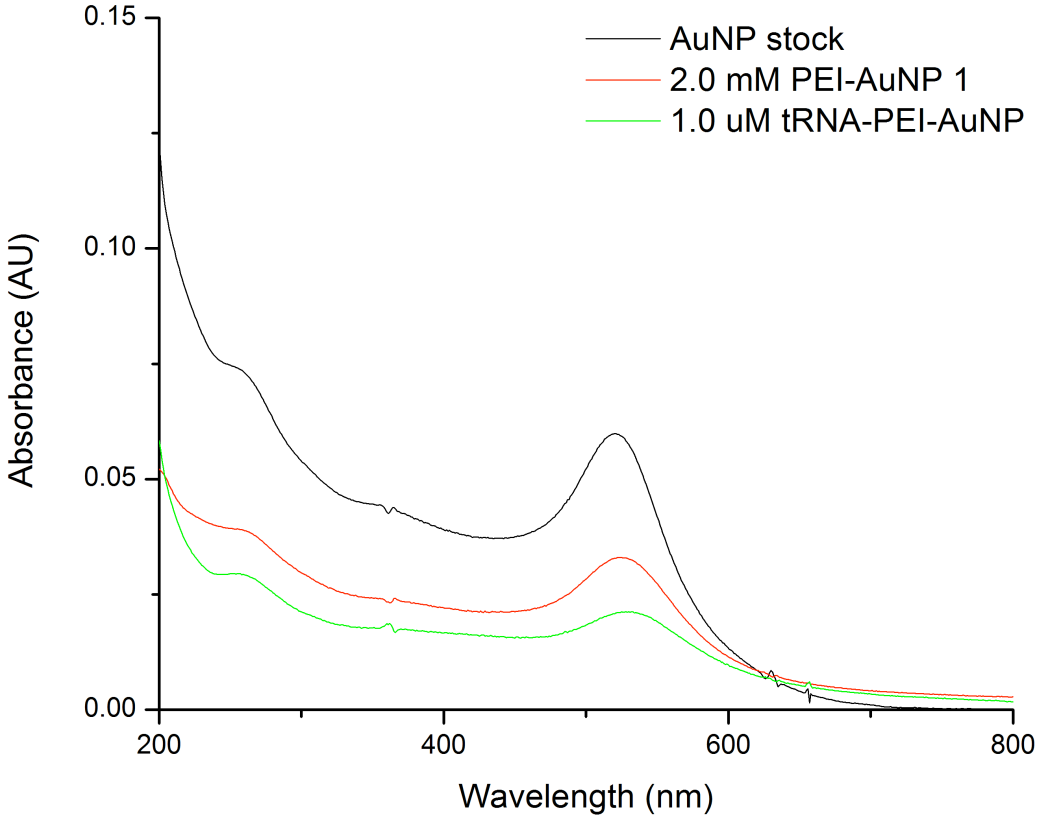

Figure 4c AuNP stock (black), shown with a representative sample of C_{12}PEI-AuNP (red), and the corresponding sample of tRNA-C_{12}PEI-AuNP (green).

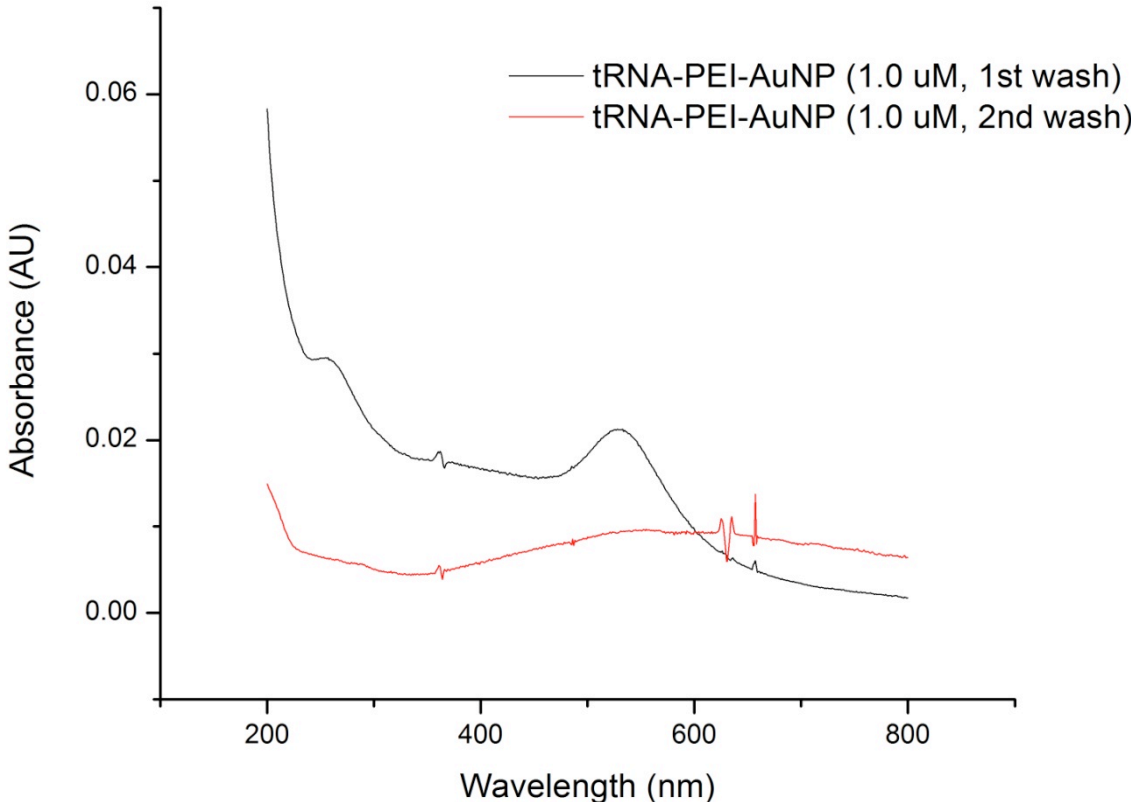

Figure 4d tRNA-C_{12}PEI-AuNP sample shown previously in Figure 4c, shown here with absorbance after a 2nd wash.

Table 1, shown below, summarizes the data found in Figures 1 – 4 above, along with zeta potential measurements, and AuNP recovery yield.

Sample Type	Original Sample Conc.	□□□□ (nm)	Δ □□□□ (nm)	Absorbance (AU)	Zeta Potential (mV)	Size DLS (d.nm)	% Recovery
AuNP	14.8 nM	519	0	0.05943	-47.4	14.39	n/a
AuNP-C12PEI	0.5 mM a	533	14	0.06407	44.1	87.55	107.5
	0.5 mM b	524	5	0.04982	39.5	28.09	83.6
	1.0 mM a	531	12	0.05526	46.8	54.48	92.8
	1.0 mM b	526	7	0.05740	41.3	28.67	96.3
	2.0 mM a	525	6	0.04908	44.1	30.13	82.4
	2.0 mM b	525	6	0.04632	43.2	26.71	77.7
AuNP-C12PEI	0.5 mM	526	7	0.03937	n/a	n/a	56.5
	1.0 mM	527	8	0.03366	n/a	n/a	48.3
	2.0 mM	527	8	0.02788	n/a	n/a	40.0
AuNP	14.8 nM	520	0	0.05992	n/a	n/a	n/a
AuNP-C12PEI	2.0 mM	523	3	0.03304	59.7	25.85	89.1
	2.0 mM	523	3	0.02691	55.9	25.46	71.6
	2.0 mM	523	3	0.03227	58.0	24.11	85.1
tRNA	1 µg/µL	259	n/a	0.48187	n/a	n/a	n/a
	1 µM	259	n/a	0.02095	n/a	n/a	n/a
	2 µM	258	n/a	0.04484	n/a	n/a	n/a
	4 µM	259	n/a	0.08882	n/a	n/a	n/a
AuNP-C12PEI-tRNA (crude)	1 µM	257, 528	8	0.04377, 0.02419	n/a	n/a	n/a
	2 µM	258, 529	9	0.05169, 0.02499	n/a	n/a	n/a
	4 µM	259, 529	9	0.07667, 0.02898	n/a	n/a	n/a
AuNP-C12PEI-tRNA (1^{st} wash)	1 µM	257, 528	8	0.02953, 0.02126	n/a	n/a	20.0
	2 µM	257, 529	9	0.04491, 0.02770	n/a	n/a	25.0
	4 µM	258, 529	9	0.05626, 0.03950	n/a	n/a	30.0

Table 1 UV-Vis, zeta potential, size, and AuNP recovery yield corresponding to particle description and concentration. AuNP-C_{12}PEI concentrations denoted by (a) were prepared by direct addition, and those denoted by (b) were prepared by addition to the tube cap. For AuNP-C_{12}PEI-tRNA samples, both □□□□ wavelengths and their corresponding absorbances are given, with the first corresponding to tRNA and the second corresponding to AuNP signature peaks.

Finally, based upon fluorescence measurements, the data leading to the number of C_{12}PEI units per AuNP for two parallel samples are shown below in Table 2, with an average of 258.1 C_{12}PEI units per AuNP.

Sample	[C_{12}PEI] Conjugated (nM)	[AuNP] Recovered (nM)	C_{12}PEI per AuNP
1	1155.68	5.25	220.1
2	1480.02	5.00	296.0

Table 2 Quantity of C_{12}PEI units per AuNP ([C_{12}PEI] during incubation was 1.0 mM) shown with recovery concentration of AuNP and concentration of conjugated C_{12}PEI from fluorescence measurements.

Chapter 5: Discussion

As reported above, successful preparation of AuNP-C_{12}PEI conjugates were achieved. Repeatable samples were synthesized at a final concentration of 2.0 mM, with a shift in peak absorbance of approximately 4 nm that corresponded to a particle size of approximately 26.5 nm. AuNP-C_{12}PEI conjugates were determined to be stable in aqueous solution with a zeta potential greater than +40.0 mV. Additionally, the number of C_{12}PEI units that can be conjugated to a single AuNP, following the protocol for a final C_{12}PEI concentration of 1.0 mM, was determined to be approximately 258. By adjusting the synthetic procedure to include addition of NaCl, the electrostatic repulsion between neighboring PEI branches could be diminished, potentially allowing for a ten-fold increase in C_{12}PEI units per AuNP.

Although unrepeated, AuNP-C_{12}PEI-tRNA conjugates were also achieved. Samples were synthesized at a final concentration of 1.0 μM, 2.0 μM, and 4.0 μM, corresponding to a shift in peak absorbance of approximately 5 nm from the previous layer of C_{12}PEI. tRNA coverage was confirmed by a zeta potential near -5.0 mV.

Despite preparation of several successful samples, implementing a successful centrifugation and washing procedure proved difficult and resulted in low repeatability. In an effort to minimize contamination by unconjugated reagent (i.e. C_{12}PEI, tRNA), samples were often subjected to long periods of centrifugation at maximum speed. Due to the strong covalent bond at the gold surface with C_{12}PEI, extended centrifugation showed minimal observed effects on AuNP-C_{12}PEI conjugates. However, the electrostatic

interactions between tRNA and C_{12}PEI, though relatively strong, were observed to be sensitive to the dense packing that occurs in the pellet during centrifugation. An example of this type of sample degradation due to washing can be seen above in Figure 4d.

In light of this observation, extensive washing may not be necessary in order to remove unconjugated reagent from crude samples to an acceptable degree. Elbakry et al. report adequate purification after 2 – 3 washings for PEI, and as few as 1 wash for RNA.[8] When compared with their results, the procedure above was found to eliminate the majority of excess reagent after a similar degree of washing. As such, it may be more effective to adopt a purification protocol that requires several washes of shorter time intervals, rather than fewer washes of longer time intervals. In the future, a centrifugation procedure such as this may allow for syntheses that are more repeatable and usable. Additionally, it is possible that centrifugation may be bypassed altogether by developing a dialysis-based purification method. Another alternative may be to develop two stable half-systems that could be integrated without the need to remove unreacted reagent.

In order to build upon the findings of this project, repeatable sample preparations that are accurately and quantitatively characterized must be obtained. After confirming the success of the tRNA conjugation protocol, the next step should involve establishing a procedure for addition of the final outer layer of polymer along with the targeting moiety. Upon satisfactory completion of the synthetic portion of the project, in vitro cell studies should be completed to observe the transfection and gene knockdown efficiency of AuNP-C_{12}PEI-siRNA conjugates against a gene target, like RRM2, in cancerous tissues.

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