Targeted Delivery of Ribonucleotide Reductase siRNA in Cancer Cells via Gold Nanoplexes

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Targeted Delivery of Ribonucleotide Reductase siRNA in Cancer Cells via Gold Nanoplexes

by

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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
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Abstract

Cancer treatment has much room for improvement, as therapies today cannot differentiate well between what cells are part of a tumor and which are healthy. Our research involves targeting cancer cells by their overexpressed folic acid receptors, and delivering small interfering RNAs (siRNAs) to silence genes crucial to cell survival by the RNA interference gene knockdown pathway. By using *Gaussia* luciferase siRNA (siGLuc) as a model for cell delivery, we have been able to test our ability to deliver siRNA via a gold nanoparticle delivery system, also developed in our lab. We also synthesized ribonucleotide reductase siRNA (siRRM2), which will become our lab’s gene of focus in the future, as this siRNA has potential to kill cancer cells.

Abbreviations

<table>
<thead>
<tr>
<th>Acronym</th>
<th>Description</th>
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<tbody>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>AuNP</td>
<td>gold nanoparticle</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>FA</td>
<td>folic acid</td>
</tr>
<tr>
<td>GLuc</td>
<td><em>Gaussia</em> luciferase</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger RNA</td>
</tr>
<tr>
<td>PEG</td>
<td>polyethylene glycol</td>
</tr>
<tr>
<td>PEI</td>
<td>polyethyleneimine</td>
</tr>
<tr>
<td>RISC</td>
<td>RNA-induced silencing complex</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>RNAi</td>
<td>RNA interference pathway</td>
</tr>
<tr>
<td>siGLuc</td>
<td><em>Gaussia</em> luciferase-specific siRNA</td>
</tr>
<tr>
<td>siRNA</td>
<td>small interfering RNA</td>
</tr>
<tr>
<td>siRRM2</td>
<td>ribonucleotide reductase subunit 2-specific siRNA</td>
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Chapter 1: Introduction

Cancer is a group of diseases distinguished by uninhibited growth and division of irregular cells. It is the second leading cause of death in the United States today, and it has no known cure. While there are hundreds of treatments that may or may not help improve a cancer patient’s lifestyle or longevity, they all have their own innate problems. While surgery is a common method of tumor removal, it is invasive and has many potential harmful side effects and results. Stem cell transplants are risky, as the body will reject them sometimes. Chemotherapy and radiation therapy both cause harm to normally functioning cells as well as cancer cells. Other therapies include hormonal treatments and biological treatments. While sometimes one or more of these treatments may be necessary for certain patients on a case-to-case basis, a new type of treatment is being developed called targeted therapy. The idea is that targeted therapy will only harm cancer cells while causing minimal damage to cells that are functioning properly.

Targeted treatment involves a few different approaches. First and foremost, there must be a way to target cancer cells specifically. Secondly, there has to be a treatment mechanism, or some molecule, to kill the cancer cell. Lastly, that molecule needs a delivery system in order to get to the cancer cell. Our form of targeting is by a folic acid molecule, our treatment mechanism is RNA interference (RNAi) with small interfering RNAs (siRNAs), and our delivery system is a gold nanoparticle (AuNP), polyethyleneimine (PEI), polyethylene glycol (PEG) conjugate nanoplex.

Folic acid (FA), or Vitamin B9, is crucial in maintaining many cell’s life cycles. It is believed to be important in DNA synthesis, but is known to be in high demand among
cells that divide rapidly, namely cancer cells. It has been shown that some types of cancers tend to overexpress folate receptors. It has been shown that cells overexpressing FA receptors intake more FA, and have the capacity to allow the intake of molecules conjugated to FA through endocytosis. It is believed that this endocytosis is via the caveolin receptor-mediated endocytotic pathway.

RNA interference (RNAi) is how this nanoplex will actually do damage to cancer cells. RNAi is an efficient regulatory method that cells use to silence genes and regulate gene expression via small interfering RNAs (siRNAs). These siRNAs are short RNAs, 18-21 nucleotides long, that complement specific portions of certain messenger RNAs (mRNA). An enzyme called Dicer will process siRNAs from a long double-stranded RNA, that then in turn is loaded onto an RNA-induced silencing complex, or RISC. RISC is a multiprotein complex that has an incorporated RNase, an enzyme that cuts RNA. With siRNA as its guide to a specific portion of an mRNA, RISC will cut an mRNA before it can be fully translated into protein. Figure 1 below illustrates this process:
Figure 1: The RNAi mechanism using siRNA to stop gene expression. The Dicer protein “dices” the specific double-stranded RNA into shorter fragments. A RISC (RNA-induced silencing complex) protein complex incorporates one strand of the dssRNA to the mRNA that it targets, and makes cuts in the mRNA, causing degradation. Therefore, there is no translation into the coded protein.
So, the goal of our research is to incorporate an siRNA into our delivery vehicle that will target an mRNA that is crucial for a cell to maintain its life cycle.

The siRNA I am using in my project is RRM2 siRNA, or the siRNA that targets ribonucleotide reductase subunit 2. Ribonucleotide reductase is an enzyme that catalyzes the reaction of forming deoxyribonucleotides from ribonucleotides.

Deoxyribonucleotides are important for DNA synthesis. Ribonucleotide reductase works via a free radical reaction, allowing for the removal of the 2’ hydroxyl group from the pentose sugar of a ribonucleotide. The siRNA I am using, which has sense and antisense strands, is made of the following sequence:

Table 1: Human Ribonucleotide Reductase siRNA Template DNA Sequence

<table>
<thead>
<tr>
<th>RRM2 Sense DNA (5’-3’)</th>
<th>CGTAATACGACTCACTATTAGGAGAGTAAGAGAAATCCGCATCT</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>AGATGCCGATTTTCCTCTCTCTCTCTAATAGTGAGTCGTATTACG</td>
</tr>
<tr>
<td>Antisense (5’-3’)</td>
<td>CGTAATACGACTCACTATTAGGAGATGCGGATTTCTCTTACTCTTT</td>
</tr>
<tr>
<td></td>
<td>AAAGGAGAGAGTAAGAGAAATCCGCATCTCTCTAATAGTGAGTCGTATTACG</td>
</tr>
</tbody>
</table>

Table 2: Human Ribonucleotide Reductase siRNA Sequence

<table>
<thead>
<tr>
<th>RRM2 siRNA</th>
<th>Sense (5’-3’)</th>
<th>AGGAGAGAGUAAGAGAAAUCGCAUCU</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Antisense (5’-3’)</td>
<td>AGGAGAUGCGGAAUUUCUCUUAUCUCUCUU</td>
</tr>
</tbody>
</table>

My work has involved this siRNA’s synthesis and the transfection of cells with siRRM2 bound to a gold nanoparticle drug delivery vehicle.

The main method of delivering the siRNA into the cells is by gold nanoparticles (AuNPs). Gold nanoparticles are a good delivery platform, mainly for its
biocompatibility, high load capacity, and lack of cytotoxicity. Overall, the methods of performing chemistry with AuNPs are not complicated, AuNPs can carry many siRNA molecules, and they show no harmful effects on cells themselves. The AuNPs were stabilized by polyethyleneimine (PEI) and polyethylene glycol (PEG), polymers that allow for linking of RNA and FA. A theoretical structure is shown below:

Figure 2: A Theoretical Structure of the Gold Nanoplex

![Figure 2: A Theoretical Structure of the Gold Nanoplex](image)

Figure 2: The gold nanoplex is synthesized by layer-by-layer technology, adding each layer as a sphere surrounding the last. My research experiments were mainly carried out with siRNA as the outer layer, as folic acid targeting is still a goal for future work.

The nanoplex was built with layer-by-layer technology, where we first linked PEI to AuNP, followed by RNA, followed by a PEI/PEG conjugate, while FA was added last.

Experiments were carried out in cultures of KB cells, a human nasopharyngeal carcinoma cell line, which are folate-receptor positive (FR+). Into these cells was
integrated the *Gaussia luciferase* gene. The *Gaussia luciferase* gene allows the cells to produce *Gaussia luciferase* (GLuc), an enzyme that reacts with the substrate luciferin to produce chemiluminescence, light produced as a product of this chemical reaction driven by ATP. The most important part about the KB cells having the ability to produce GLuc, however, is that they produce it and secrete the enzyme into the medium. This allows for us to measure the relative amount of the gene we have knocked down by measuring the amount of luminescence the medium can produce.
Chapter 2: Literature Review

Cancer as the Problem:

Theoretically, as described in the American Cancer Society’s “Cancer Facts and Figures 2012,” over half a million cancer deaths in 2012 will be caused by tobacco use, obesity, and poor nutrition—all areas that could easily be prevented. Some forms of cancer are caused by viruses like Hepatitis B and Human Papillomavirus. Regular screenings can help prevent all these kinds of cancer, which account for more than half of new cancer cases. However, still cancer remains a huge problem, and people who have it need to be rid of it. Treatments for cancer are being vehemently searched for around the world. It is estimated that 44.85% of men develop cancer in their lifetime, while 38.08% of women will do so as well. Typically, once cancer has manifested itself, it, in a sense, develops barriers to keep itself alive in the body. “The Hallmarks of Cancer” by Hanahan and Weinberg (2000) illustrates the six main qualities cancer demonstrates, which are “self-sufficiency in growth signals, insensitivity to anti-growth signals, tissue invasion and metastasis, limitless replicative potential, sustained angiogenesis and evasion of apoptosis.” They theorize that all cancers must maintain or acquire all six of these abilities at some point, in some way. Depending on how cancers develop these six qualities determine the best mode of action in treatment against that specific type of cancer.
Treating Cancer:

Treatments for cancer are a dime-a-dozen throughout the cancer treatment world, and targeted delivery is becoming the hope for modern and future cancer treatments. Up and rising targeted therapeutics use RNA interference mechanisms (RNAi) to stunt cancer cell growth. There are three known types of RNA utilized by RNAi: small interfering RNA (siRNA), short hairpin RNA (shRNA), and micro RNA (miRNA). Overall, of the three types, siRNA is the best to use in practice because of its small size (typically 21-25 nucleotides) and ability to interact with target mRNAs in the cytosol rather than the nucleus (the nuclear envelope presents another barrier).

The specific type of targeted treatment our lab is dealing with uses RNAi therapeutic technologies delivering siRNAs to gene targets. My research involves interfering with the translation of ribonucleotide reductase subunit 2 mRNA (siRRM2).

Problems with siRNA use in cancer therapeutics include its interference with other small RNA pathways which can be slightly cytotoxic if introduced in too high a dose. Also, there are barriers present in the delivery of siRNAs into the cell. Mainly, it is easier to deliver siRNA to all areas of the body than to specific loci, which is not incredibly useful in cancer treatment as the goal is to target a specific cluster of tumor cells. In addition, siRNA molecules are not stable in their single-stranded form, and must be coupled into a double-stranded structure containing a sense and anti-sense strand. These molecules then, are not easily taken up into cells on their own. So, the next research problem is finding a proper mechanism or platform to deliver siRNA molecules specifically to the cells they need to harm.
**Delivery of Targeted Treatment:**

Many delivery platforms have been utilized to transfer siRNA molecules into cells, some with decent success. These different particles include those like carbon nanotubes, gold nanorods, and gold nanoparticles.\(^5\) Gold nanoparticles are the most commonly used of these transport systems and are useful for delivery of many types of molecules, including DNA and other cancer therapeutics.\(^9\) Our laboratory utilizes the gold nanoparticle which binds to the siRNAs through PEI and PEG copolymers and electrostatic interactions, attaching spherically around the nanoparticle. In order to specifically target the cancerous cells, a molecule must be added that will attach only or mainly to cancer cells’ membranes. We designed and used a folate molecule-directed delivery method, utilizing folic acid as the valent structure on the nanoparticle, able to bind as a ligand to FRs.\(^{13, 14}\)

Folic acid is taken into rapidly dividing cells as it plays a role in cell division; cancer cells divide more rapidly than other cells, so they take in more folic acid. If the folic acid molecule they absorb so happens to be attached to a molecule with siRNAs that target a crucial gene’s expression, then at least one of the goals in this project will have been accomplished because siRNA will have successfully entered the cell’s cytosol where it has the potential to interact with mRNA.

**Delivery System – Gold Nanoparticle and the Nanoplex**

The gold nanoparticle is often used as a platform for molecular delivery. The AuNP’s used in this research project were 13-15 nm in diameter. The first layer bound to the AuNP was the polymer PEI, after which was bound the siRNA of interest. The fourth
layer then was a PEI-PEG copolymer to prevent degradation of the RNA from cytosolic nucleases. The fifth layer is folic acid, the targeting molecule theorized to allow receptor-mediated endocytosis of the entire nanoplex.6

**Small Interfering RNA, RNAi, and Ribonucleotide Reductase**

Small interfering RNAs are utilized by the RNA Interference pathway to regulate gene expression. In RNA interference, an siRNA will complement a corresponding mRNA and stop its translation. The siRNA is usually 21-25 nucleotides long and is double-stranded. The two strands, sense and antisense, have 3’ overhangs. Long stretches of RNAs are cut into these siRNAs by the Dicer enzyme. The antisense strand will associate with the RNA interference silencing complex (RISC), and will complement the mRNA to guide the RISC’s site-specific mRNA cleaving. In theory, for any known mRNA sequence, an siRNA could be synthesized and utilized by the RNAi pathway to knockdown a specific gene. For us, ribonucleotide reductase mRNA is the target of choice, as the enzyme ribonucleotide reductase is crucial for the synthesis of deoxyribonucleotides from ribonucleotides. Deoxyribonucleotides are essential in DNA synthesis. Therefore, the prevention of the formation of the ribonucleotide reductase enzyme would prevent a cell from synthesizing DNA, causing eventual apoptosis.

**Folate Receptor as the Target**

Folate receptors are overexpressed in cancer cells.12 This indicates a need for folic acid by cancer cells. It is thought that folic acid is important in cell division processes, ones that cancer cells undergo much more often than a normal cell. By using
folic acid in our gold nanoplex, we can potentially target these numerous folate receptors and cause receptor-mediated endocytosis of both the folic acid and the entire attached particle containing our protected siRNAs. Certain chemotherapies have also been designed to target folate receptors. One drug even killed 80% of a tumor cell population in one experiment.10
Chapter 3: Research Methods

This study involved the design of a gold nanoparticle based siRNA delivery system able to target the overexpressed folate receptors in cancer cells. The final siRNA and gene target of interest was siRRM2, the siRNA that targets the 2 subunit of ribonucleotide reductase. In this project, we sought to maximize efficiency of delivery of the nanoparticle to the cells, as well as the knockdown efficiency once delivered, while maintaining the lowest cellular toxicity possible.

Delivery Efficiency and the AuNP: Preliminary Studies Necessary for My Project

Much work has been done with the gold nanoparticle and its ability to be endocytosed into cancer cells. Experimentation involved using tRNA as a model while testing the proper amounts of PEI, RNA, PEI-PEG, and FA to add while maintaining proper surface charge and size of the nanoparticle. Surface charge and size of the nanoparticle were measured using Zeta Potential and Dynamic Light Scattering, respectively.

Cell Culture and Transfection

Cell culture involved the use of FR+ KB Cells, a folate receptor positive human nasopharyngeal carcinoma cell line. These cells were maintained in a folate free RPMI 1680 (GIBCO) medium supplemented with 10% fetal bovine serum (FBS) (Hyclone) with 100 units/mL penicillin and 100 μg/mL streptomycin (Millipore) at 37°C, 5% CO2 humidified air. Antibiotic additions will be referred to as “serum.”
Cells were transfected with siRNA using 0.4 μL/100 μL DharmaFECT Duo transfection reagent (Dharmacon). We counted cells via haemocytometer and plated specific numbers of cells into welled plates. Controls were only given fresh media while experimental wells were treated with DharmaFECT and siRNA or an AuPEI/siRNA complex.

**Gene Knockdown Efficiency: siGLuc Testing**

In order to test the delivery efficiency of our nanoparticle before synthesizing expensive siRRM2, we used siRNA against *Gaussia* luciferase as a luminescent model to test the relative gene knockdown ability once delivered into KB cells that had had *Gaussia* luciferase gene incorporated into their genome. These KB cells are also folate receptor positive, and important characteristic for other experiments performed.

siGLuc is able to target the mRNA corresponding with *Gaussia* luciferase, an enzyme capable of luminescence upon reacting with a corresponding substrate. We used the GLuc Assay Kit including rabbit anti-GLuc (coelenterazine) (New England BioLabs) and BioLux GLuc Assay Buffer #B3301A (New England BioLabs). Cells were treated with AuPEI/siGLuc complex and DharmaFECT transfection reagent. DharmaFECT was used as the control treatment as well. After 24, 48, and sometimes 72 hours, the cell medium was removed and analyzed by *Gaussia* Luciferase Assay Kit. The relative fluorescence against non-siGLuc-treated control cells was measured by Gen5 Luminometer. Results showed percentage of cells without knockdown compared to untreated cells, giving us an idea of how efficient our nanoplex is.
Synthesis and Preparation of siRRM2

We used the in vitro transcription (IVT) technique to synthesize our siRRM2. This method requires the use of T7 RNA Polymerase, a fast enzyme with a low error rate capable of polymerizing RNA from a DNA template with a specific promoter. The procedure and materials are available in the Ampliscribe T7-Flash Transcription Kit ASF3507 (Epicentre).

The reaction kit and procedure involved 1X Buffer, 10 mM DTT, 9 μM nucleotide triphosphates (NTPs), 0.4 μM DNA, 1 μL T7 Polymerase per 10 μL of reaction. The T7 Polymerase was kept on ice at all times. After adding the samples in the addition order described, they were incubated in 37°C for 2-4 hours. To perform the ethanol precipitation, I added 70 μL water and 10 μL 3 M NaAc to the 20 μL of mixture, 300 μL of EtOH. This was left to precipitate overnight. The solution was then centrifuged for 8 minutes on high speed, then the supernatant was removed. The pellet was resuspended in 20 μL of RNase-free water, then we began the purification process.

We annealed our strands after purification by adding them together in a 1:1 ratio, followed by heating and incubation at room temperature for 30 minutes. To analyze our annealing process, we ran a sample on a PAGE gel for 30 minutes, then used a BIORAD Molecular Imager FX to ensure there was only one band present.

RNA Purification

We added 20 μL of dye (2XBB and XC) to each sample. We loaded 20 μL samples onto the pre run PAGE gel, and allowed it to run for 30 minutes. After running,
we characterized the RNA by UV shadowing, then cut out only the RNA. The cut out gel was crushed to the consistency of mashed potatoes to increase surface area for heating and removal of the gel by sodium acetate and ethanol precipitation reactions.

**Cell Viability Tests**

We removed the medium from the cells in the 96 well plates, then washed with 100 μL of Phosphate Buffered Saline (PBS) (Hyclone) for 15 minutes. We removed the PBS and incubated in 50 μL of 4% Paraformaldehyde (PFA) (Hyclone) solution overnight.

**RT-PCR and qPCR**

To determine the percentage knockdown of the RRM2 gene by siRRM2, we used Reverse Transcription PCR to create cDNA from the lysed cells’ mRNA, then used Quantitative PCR to determine how much RRM2 mRNA remained by comparing a treated sample with a control sample, as well as comparing the concentrations to relative amounts of an abundant mRNA, β-actin.

We first transfected our annealed siRRM2 into two wells in a 24 well plate using DharmaFECT, while leaving two duplicate controls as only treated with DharmaFECT. We transfected 4.4 μL of DharmaFECT and 2.2 μL of 40 nM siRRM2 along with 220 μL RPMI medium and 880 μL RPMI/FBS/Serum into the two treatment wells, and used only DharmaFECT and RPMI + RPMI/FBS/Serum in the controls.
RNA Extraction

The cells were incubated for 48 hours, then treated with 125 μL Trizol reagent. The cells were mixed thoroughly by pipetting, then moved into a 1.5 mL tube. This was allowed to sit for 10 minutes.

Phase Separation

We then added 25 μL of chloroform and gently vortexed after 10 minutes. This allowed us to precipitate out the RNA in the top aqueous layer by centrifuging for 10 minutes at 4°C and 10,000 rpm in an Eppendorf Centrifuge 5804 R. The top aqueous layer was transferred to a new tube while the other layers were discarded.

RNA Precipitation

Then 62.5 μL of isopropanol was added to the solution, mixed well, then allowed to sit for 15 minutes. The solution was then centrifuged for 10 minutes at 4°C and 12,000 rpm. The RNA was observed as a small pellet in the tube. The supernatant was removed, then 150-200 μL of 70% ethanol solution was added, then incubated at -20°C for 30 minutes. This solution was then centrifuged for 10 minutes at 4°C and 10,000 rpm. The supernatant was removed and the RNA pellet was dissolved in 5 μL of diethylpyrocarbonate-treated water (DEPC water, used to treat for RNase). The RNA concentration was then measured by Genesys 10 Bio UV Spectrometer by using 1.5 μL of solution.

Reverse Transcription

To our remaining 3.5 μL of RNA solution, we added 8.0 μL DEPC water, 2 μL random RNA primers, and 1 μL of 10 mM dNTPs. These were heated in the Eppendorf Mastercycler PCR for 5 minutes on program “A7005ED.” The solution was removed
and quickly placed on ice with all other reagents. 0.5 μL of RNase inhibitor was added, followed by 4 μL of 5x RT Buffer and 1 μL of Reverse Transcriptase. After running the resulting solution for 2 hours in the PCR on program “A421H,” we added 200 μL of DEPC water and stored the solution in -20°C.

qPCR

We then, tested for our target gene, RRM2, comparing results to the gene for β-actin. We created a 2 uM primer stock for RRM2 by adding 1 μL of 200 uM RRM2 (F) primer and 1 μL of 200 uM RRM2 (R) primer to 98 μL of RNase-free water. We did the same with β-actin primers. To create our cDNA solution, we took 22 μL of our RT-stock and mixed with 44 μL of SYBR Green fluorescent dye (Invitrogen) and 13.2 μL RNase-free water. We then combined 18 μL of cDNA solution with 2 μL of primer solution.

We now had 8 duplicate experimental tubes for qPCR, containing control-treated cell cDNA solution being measured for each β-actin and RRM2 concentrations, as well as siRRM2/DharmaFECT treated cell cDNA solutions being measured for each β-actin and RRM2 concentrations.
Chapter 4: Results

Figure 3: Transfection of siGLuc (100 nM) by DharmaFECT and AuPEI

Figure 3: Transfection of siGLuc (100 nM) by DharmaFECT and AuPEI. This figure shows experimental expression of KB-GLuc cell media treated with siGLuc versus control cells (black) treated with only DharmaFECT. There was little knockdown after 24 hours and about 40% knockdown after 48 hours. DharmaFECT and AuPEI transfection showed similar results. Error bars indicate standard deviation across a triplicate study.

Figure 4: Comparing Synthetic siGLuc vs. IVT – siGLuc and DharmaFECT vs. Lipofectamine

Figure 4: Comparing Synthetic siGLuc vs. IVT – siGLuc and DharmaFECT vs. Lipofectamine. This experiment compared 100 nM synthetic (purchased) siGLuc effectiveness (blue) versus our IVT-synthesized AuPEI/siGLuc (purple). DharmaFECT transfection reagent was compared to Lipofectamine reagent (Invitrogen), and the two showed no difference. The error bars represent standard deviation across duplicate studies. Our siGLuc shows an apparent decrease in GLuc expression.
Figure 5: Relative Cellular Toxicity from Figure 2’s Experiment. Our IVT-synthesized AuPEI/siGLuc complex caused cell death more frequently than did other experimental trials. This accounts for much of the decreased siGLuc expression. The error bars represent standard deviation across duplicate studies.

Figure 6: UV-Vis Characterization of siGLuc. This figure shows the relative amount of sense and antisense strands acquired of purified siGLuc.
Figure 7: UV-Vis Characterization of siRRM2

This figure illustrates the relative yield of antisense and sense strands acquired after purification of siRRM2. The sense yield was very low on this first IVT/Purification, so more had to be synthesized in another batch.

Figure 8: RT-qPCR Amplification Plots

RT-qPCR comparing gene expression of β-actin and ribonucleotide reductase cDNA from mRNA transcripts. The number of cycles (x-axis) for fluorescence to reach the threshold (set at 1000 dR) indicates the relative amount of gene expression compared with a control. The more cycles, the less gene expression. This figure simply shows that our samples did have cDNA expressed. This was measured by tagging a fluorescent SYBR green dye.
Figure 9: RT-qPCR Dissociation Curve

The RT-qPCR dissociation curve allows us to visualize the two types of mRNA we targeted and the temperatures at which they dissociated via fluorescence. These two curves represent β-actin and ribonucleotide reductase cDNA. This figure only explains that we did indeed express two different cDNAs in our experiment.

Figure 10: Percentage RRM2 Gene Expression

Gene expression was determined by the reciprocal of two to the power of the difference between the cycle number of the control and the cycle number of the cells treated with siRRM2. Results show nearly 75% gene knockdown.
Figure 11: KB Cell Viability Test

In the first siGLuc experiments (Figure 3), where we used synthetic siGLuc (New Englad BioLabs) we seemed to achieve a lower gene knockdown percentage (40%) than expected. Since the AuPEI/siGLuc conjugate and DharmaFECT/siGLuc control had similar knockdown, we can assume the AuPEI/siGLuc at least gets inside cancer cells.

Figure 4 shows a repeat experiment after we had synthesized our own IVT – siGLuc. This data showed better knockdown, close to 60%, showing that our IVT-siGLuc caused less GLuc expression. However, Figure 5: Relative Cell Toxicity shows also that the cytotoxicity in our experimentation was high, meaning that perhaps the absence of GLuc expression was a result of cell death rather than gene knockdown.
Figure 6 shows the relative amount of sense strand to antisense siGLuc strands acquired after RNA purification. Figure 7 shows the sense strand of siRRM2 having a much lower purity than its antisense strand. Larger reactions were done to acquire enough sense strand to anneal with which to treat cells.

However, as Figure 10 shows, RT-qPCR told us our knockdown efficiency was nearly 75% with siRRM2 synthesized. Figures 8 and 9 allows us to visualize signatures of two distinct mRNAs selected for during qPCR. This shows us we indeed do have both β-actin and ribonucleotide reductase cDNA present. Figure 11 shows the relative cytotoxicity of our 20 nM siRRM2. While after 48 hours there was clearly apparent knockdown, it is not known exactly if the cells are dying from the knockdown of the gene or the activation of other cellular pathways16.
Chapter 5: Discussion and Conclusions

The main achievements in my research involved learning about the delivery efficiency of our nanoparticle and the knockdown efficiency of the siRNA we can synthesize. Using siGLuc, we were able to see a few things about both topics. Also, using RT-qPCR, we took a step toward an actual, potentially functioning cancer treatment by confirming our IVT-synthesized siRRM2’s gene knockdown ability.

Our RT-qPCR studies, showing 75% knockdown of the RRM2 mRNA level, compares hand-in-hand with the studies from Heidel et. al. and their research in studying different siRRM2 capabilities. Their gene expression after 48 hours seemed to be equivalent if not a little less than our gene knockdown\textsuperscript{15}. Their group used HeLa cells while we used KB, which may offer some explanation. Nonetheless, our siRNA capabilities align with other literature values for gene expression post-siRRM2 treatment.

The Heidel et. al. group also used a luminescent model similar to our GLuc assay, except they used an RRM2/luciferase hybrid plasmid that had been incorporated into HeLa cells to determine their knockdown efficiency and gene expression\textsuperscript{15}. I believe our method of determining gene expression is more accurate, as we measure our knockdown ability of siRRM2 with only the direct gene produced by cancer cells, not any gene that had been incorporated into the cells. We incorporated siGLuc and tested delivery efficiency separately in order to have better results in the future.

Problems with our results include the low yield of sense strand siRRM2 as described in Figure 7. This problem could arise for a number of reasons, including a less-specific promoter region for transcription or “old” materials that may have been
contaminated in some fashion over the years. If this issue were fixed and our sense siRRM2 IVT were more efficient, it would be possible to produce and purify more siRRM2 at a time, thus allowing for more experimentation and results involving the treatment of cancer cells.

Future studies will include the inclusion of siRRM2 in the gold nanoplex and treating KB cells again, eventually without DharmaFECT, to calculate the gene knockdown capability of the nanoplex by using RT-qPCR. First, there are other RNA binding considerations with the AuPEI complex that must be accomplished.

Future work may also involve the removal of the 5’ triphosphate group, a group known to trigger other pathways that could bring about cell death, like the RIG-I pathway\textsuperscript{16}. However, it is important to note that the siRNA we synthesize in our lab can effectively cause cell death in FR+ KB cancer cells, the first, most important step in synthesizing the nanoplex.
References


