

**CATIONIC BOVINE SERUM ALBUMIN NANOPARTICLES FOR DELIVERY OF  
SIRNA TO FIBROBLASTS**

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## ABSTRACT

Gene regulation through small interfering RNA (siRNA) is a useful way to improve therapeutics and treat diseases. However, since siRNA is rapidly degraded by nucleases in the bloodstream and is anionic and highly hydrophilic, it is not readily taken up by cells. Therefore, a variety of delivery systems that encapsulate siRNA are being developed to overcome these limitations. Protein nanoparticles have the potential to effectively deliver siRNA because siRNA can be encapsulated during the fabrication process. In this work, we have encapsulated siRNA inside 200 nm cationic bovine serum albumin (cBSA) nanoparticles. The positive charge on the cBSA protein enables the negative charge of the siRNA to electrostatically attract, creating a more stable nanoparticle. We measured nanoparticle uptake and intracellular delivery to GFP-3T3 cells using a combination of flow cytometry and fluorescence knockdown assays. cBSA protein nanoparticles are an innovative way to encapsulate siRNA, with the ability to adjust the amount of siRNA in the nanoparticle as needed and stable attraction of siRNA and cBSA protein.

## INTRODUCTION

### *RNA Interference and Small Interfering RNA*

RNA interference (RNAi) occurs when RNA regulates gene activity by “silencing” targeted genes. Small Interfering RNA (siRNA) are short strands of double-stranded nucleotides, consisting of a passenger strand and a guide strand. RNAi occurs when the siRNA binds to argonaute proteins and forms an RNA-Induced Silencing Complex (RISC). Then the guide strand directs this complex towards the target site on the messenger RNA (mRNA), where the mRNA is then cleaved by the enzyme, argonaute-2, thus “silencing” gene expression [1]. This

type of gene regulation is pivotal in creating a way to modify certain expressions of genes to help mitigate inflammation, drug delivery, combat cancer, and improve other therapeutics [2,3].

Since free siRNA is rapidly degraded in the bloodstream and cannot efficiently pass-through cell membranes, other methods of delivery need to be used. Nanoparticles have been found to be useful in drug delivery due to their small size and ability to encapsulate molecules, such as small molecule drugs, proteins, and nucleotides [4]. However, there are still some limitations to using nanoparticles for drug delivery including low encapsulation efficiency, inefficient cytosolic delivery, and biocompatibility. Therefore, there is still research that needs to be done to make nanoparticles more efficient at delivery and release of siRNA to targeted cells and continue the expansion of their use in therapeutics.

### ***Nanoparticles and the Blood Brain Barrier***

There is a need for treatments of many neurological diseases like brain tumors and Alzheimer's Disease that have no cure and few, if any, treatments for progression [5,6]. Although research in using nanoparticles to treat neurological diseases and injuries has increased, one of the hardest parts of drug delivery using nanoparticles is penetrating the blood brain barrier (BBB). The BBB separates the blood vessels from the extracellular fluid and cells in the brain to prevent dangerous materials such as pathogens from getting into the brain [7]. It is made of endothelial cells that directly surround the blood vessel and aid in the creation of tight junctions, which are areas between cells that are completely sealed and prevent most solutes and all particles from entering the brain. Outside of the endothelial cell layer are astrocytes, one of the most abundant glial cells in the brain, that help further seal the BBB and provide biochemical support to the endothelial cells [8].

More research is beginning to focus on how to get nanoparticles across the BBB to help suppress tumors, combat cancers, and reduce brain inflammation. One promising study showed menthol conjugated nanoparticles down regulated the tight junctions in the BBB enabling nanoparticles to penetrate and move to targeted areas [9]. However, it still remains difficult to test the efficiency of particles through the BBB *in vitro*. Researchers have found that in order to create the best representation of a true human BBB, cerebral cell lines from pigs or mice need to be used. These endothelial cell lines combined with astrocytes help create an *in vitro* cellular barrier that uses the typical cells seen in a BBB and creates tight junctions, which is what makes the BBB almost impenetrable [10].

### ***Types of Nanoparticles: Liposomes, Polymeric, and Protein***

There are different types of NPs used to encapsulate siRNA, including liposomes and polymeric nanoparticles. Liposomes are versatile due to having the ability to be altered at many different stages of the fabrication process. They also provide a protective property for their contents that prevents external degradation and have a biological membrane similar to cells, allowing for easier and more efficient delivery. However, liposomes can have high instability, especially when under 100 nm in size [11]. Polymeric nanoparticles have the potential for controlled release and their ability to protect their contents from degradation by storage in the inner core, which is protected by a polymeric shell. One of the biggest challenges for designing polymeric nanoparticles are loading sufficient cargo, biodegradability, and biocompatibility of polymers [12].

Protein nanoparticles, which are made entirely of proteins, are a new class of nanoparticles that could be used to encapsulate siRNA. They provide strong biocompatibility and

biodegradability [13]. One protein used for fabricating nanoparticles is Bovine Serum Albumin (BSA). BSA is known for its high biocompatibility, low toxicity, the ability for its properties to be easily manipulated, and its biodegradability making it a commonly used protein in biosciences. This protein is able to be crosslinked using a molecule such as 3,3'-Dithiobis(sulfosuccinimidylpropionate) (DTSSP), a crosslinker, to connect the lysine amino acids on each protein chain together to form a nanoparticle [14]. Since BSA NPs are anionic, they have low siRNA encapsulation efficiency, so cationic proteins are needed to efficiently encapsulate siRNA. The positive charge on a cationic bovine serum albumin (cBSA) protein allows for the negatively charged siRNA to electrostatically bind to the cBSA molecules, making it an ideal carrier for negatively charged cargo, like siRNA.

### ***cBSA Nanoparticles***

In this work, it will be determined if cBSA, is a good biomaterial to fabricate nanoparticles for use in siRNA delivery. The positive charge on the cBSA protein enables the negative charge of the siRNA to electrostatically attract, creating a more stable nanoparticle. A fabrication procedure was created for cBSA siRNA nanoparticles and measured their uptake and delivery in GFP-3T3 cells through flow cytometry, fluorescence knockdown assays, and MTT and LDH assays. A stable cBSA siRNA nanoparticle was able to be created that encapsulated siRNA. However, more work needs to be done to determine deliverability.

## METHODS

### *Making cBSA Protein*

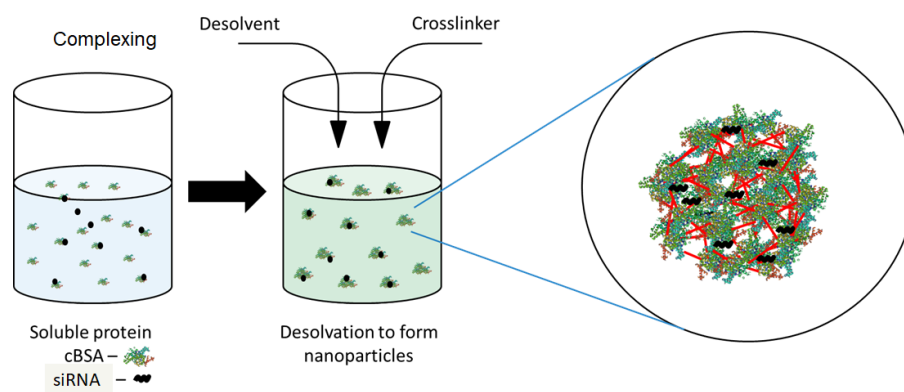
First, 50 mg of BSA is dissolved in 1 mL of 0.1 M 2-ethanesulfonic acid (MES) buffer and chilled on ice for 1 hr. Meanwhile, an ethylenediamine (EDA) solution is prepared by mixing 0.5 mL 99% EDA with 0.5 mL MES buffer. The pH of the solution is adjusted to 4.7 by adding 2.5 mL of 6N HCl. 30 to 40 mg of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) is then measured out and dissolved in 0.5 mL MES buffer. Next, the BSA solution is added to a 10 mL beaker and placed on a stir plate in the fume hood rotating at 800 rpm. Then, the EDC solution is added to the EDA solution, vortexed to mix, and added to the BSA solution. The solution is then covered and reacted for 2 hours. After the 2 hours, 0.4 mL of 4M acetate buffer is added to quench the reaction.

To collect the protein, the solution is added to a 10 kDa molecular weight cut off (MWCO) filter and spun at 5,000 x g for 20 minutes. The supernatant is then resuspended in 0.1x phosphate-buffered saline (PBS) solution. This process is repeated at least 6 more times, until there is no white residue left in the supernatant. Finally, the concentration of cBSA protein is measured using a bicinchoninic acid (BCA) assay. The surface charge is confirmed using zeta potential, and the size is confirmed using Dynamic Light Scattering (DLS).

### *cBSA siRNA Nanoparticle Fabrication*

cBSA nanoparticles were made by first creating a 1 mg/ml solution of DTSSP, a reversible crosslinker. Next, siRNA was prepared at both the 1x (10.52  $\mu$ M) and 2x (21.04  $\mu$ M) concentrations. 100 microliters ( $\mu$ l) of cBSA protein at a concentration of 10 mg/ml and 20 microliters ( $\mu$ l) of the prepared siRNA was pipetted into 2 mL tubes. Then, 400  $\mu$ l of the desolvent, acetone, was added

dropwise to the mixture. The desolvation process dehydrates the protein which results in the protein changing from a stretched conformation to a coiled conformation to create protein nanoclusters. Next, 25.2  $\mu\text{l}$  of the DTSSP solution was added in at 2 lysines (in cBSA) per 1 DTSSP molecule ratio to stabilize the nanoclusters, and the mixture reacted at 650 rpm for one hour. The entire process was performed at room temperature. The solution was then centrifuged at 10,000  $\times$  g for 10 minutes to obtain a pellet of nanoparticles. The supernatant was extracted, and the pellet of particles was resuspended in 300  $\mu\text{l}$  of 0.1x PBS. The newly suspended nanoparticles were then sonicated for 1 minute at 2 seconds on and 2 seconds off at 30 amps. After sonication, the nanoparticles were then centrifuged at 500  $\times$  g for 5 minutes to remove any large aggregates. The solution of nanoparticles was extracted using a pipette and put in a new 1.5 mL vial. Figure 1 shows a schematic of the cBSA siRNA nanoparticle fabrication procedure. Because ssDNA is less expensive than siRNA, ssDNA was used in replacement of siRNA in some initial experiments.



**Figure 1: A visual representation of siRNA cBSA Nanoparticle Fabrication.**

### *Measuring Nanoparticle Size*

A Zetasizer Nano ZS90 DLS instrument was used to measure the size of the nanoparticles after fabrication. DLS uses a 175-degree angled light signal to scatter off the molecules diffusing

throughout the solution, and this motion of randomness is measured through the constructive or destructive interactions of the molecules. The intensity of both constructive and destructive interactions is measured, and then a series of equations is used to compute the average size of the molecules in the solution [15]. Approximately 100  $\mu\text{l}$  of nanoparticles were pipetted into a cuvette and inserted into the instrument. The DLS took a total of 3 total size measurements consisting of an average of 10 measurements for each sample. We obtained PDI (polydispersity index, the distribution of size in the population), Z-Average (average particle size), and standard deviation for each average measurement. The protein setting was used for nanoparticle detection and PBS was used as the dispersion medium.

### ***Measuring Protein Concentration in Nanoparticles using BCA Protein Assay***

The protein concentration in the siRNA nanoparticles was measured using a BCA protein assay after fabrication. A 96-well plate was used for this procedure, and each nanoparticle sample had 3 separate wells so the final protein concentration could be averaged amongst the triplicates after measurement. 5  $\mu\text{l}$  of the siRNA nanoparticle sample and 20  $\mu\text{l}$  of milliQ water was pipetted into each well. The BCA assay solution was prepared according to the manufacturer's direction.

The two reagents from the BCA kit were combined, and 200  $\mu\text{l}$  of the combined reagent solution was added to each well. The plate was put in a 37  $^{\circ}\text{C}$  incubator for 30 minutes. The absorbance of the plate was then measured on a plate reader at a wavelength of 562 nm. The protein concentration in  $\mu\text{g}/\text{mL}$  for each nanoparticle solution was calculated using the following equations:

$$\text{Avg. Absorbance} = \frac{\text{well 1} + \text{well 2} + \text{well 3}}{3}$$

$$\text{Corrected Absorbance} = \text{Avg. Absorbance} - 0.092$$

$$\text{Concentration } (\mu\text{g/mL}) = \frac{\text{Corrected Absorbance}}{0.0011}$$

$$\text{Final Concentration } (\mu\text{g/mL}) = \text{Concentration } (\mu\text{g/mL}) * \frac{25}{5}$$

### ***Conformation ssDNA is in Nanoparticles***

#### *First Experiment*

In order to ensure that the ssDNA had been incorporated into the nanoparticle, the nanoparticle had to be broken apart and its contents run through gel electrophoresis. In one experiment, 30  $\mu\text{L}$  of nanoparticles were centrifuged at 10,000 x g for 10 minutes to separate the nanoparticles from the solution of PBS it was suspended in for storage. This nanoparticle pellet was then resuspended in 60  $\mu\text{L}$  of 50 mM DTT, a reagent that breaks apart the crosslinking of the protein on the nanoparticle to the ssDNA inside. The resuspended nanoparticle pellet was then incubated at 37 °C for 30 minutes. It was then put in a water bath sonicator for 15 minutes after incubation. Next, 5  $\mu\text{L}$  of SDS page buffer (Laemmli Buffer) was added to each nanoparticle sample to reduce the positive charge on the protein and allow the ssDNA to release. The samples were then put back in the water bath sonicator for 15 minutes. Lastly, DNA running dye was added to the samples so the solution could be seen when putting it into the gel.

Next, the samples were run on an agarose gel through gel electrophoresis. Gel electrophoresis uses an electrical current to push the ssDNA through the gel. ssDNA (and siRNA) is negatively charged, so it will be pushed from the negatively charged end to the positively charged end. Samples that have a higher molecular weight will not go as far in the gel as samples that have a lower molecular weight. 30  $\mu\text{L}$  of each sample was loaded and run through an agarose gel for 30 minutes at 150 V. Once the gel electrophoresis was completed, the gel was imaged, and then, the samples that

went through the gel were compared to a ladder that was put into the first well of the gel. The ladder provides molecular weight markings. From this, we were able to compare the marking of our sample to the marking of the ladder to ensure that the marking is from ssDNA since we know what the ssDNA molecular weight should be. The gel was analyzed using Image J.

### *Second Experiment*

A second experiment was conducted where we created our own Laemmli Buffer without blue dye (LD) using the following recipe: 7.88 g of Tris HCl at pH 6.8, 2.1 mL ddH<sub>2</sub>O, 4.7 mL glycerol, 1.2 g SDS, 0.93 g BME. Then, both ssDNA nanoparticles and cBSA nanoparticles were ran using the protocol outlined in experiment 1 with the following conditions:

1. ssDNA NP with blue dye Laemmli running buffer (BLD) + DNA dye
2. ssDNA NP with LD + DNA dye
3. ssDNA NP with LD + no DNA dye
4. cBSA NP with BLD + DNA dye
5. cBSA NP with LD + DNA dye
6. cBSA NP with LD + no DNA dye.

### ***GFP Knockdown in GFP-3T3 Cells***

GFP-3T3 cells are mouse fibroblast cells that are transfected with green fluorescent protein (GFP). Fetal Bovine Serum (FBS) cell media was used in both 1% and 10% concentrations. In some experiments, serum free media was also used. The cells were grown at 37 °C and passaged approximately every three days. GFP-3T3 cells were plated at 6000 cells per well in a 96 well plate. The cells were left to incubate overnight in a 37 °C incubator. Next, 2000 lipofectamine in serum free cell media was prepared at a ratio of 6 µl to 150 µl of serum free cell media. Then, a 10 µM anti-GFP siRNA working solution was prepared using 2 ul of 50 µM siRNA and 8 µl of serum free cell media. 1.5 µl of the siRNA solution was added to 150 µl of serum free cell media. Next, the lipofectamine solution was added to the siRNA solution. The mixture was then incubated at room temperature for

15 minutes. The media in the GFP-3T3 cells was aspirated out of each well using a 20  $\mu$ l pipette tip. Then, 100  $\mu$ l of the lipofectamine-siRNA solution was added to each well in the plate, and the plate was incubated for 30 minutes. Lastly, 100  $\mu$ l of 1% FBS Cell Media was added to each well.

The protein concentration in each batch of siRNA nanoparticles was normalized to get the amount of microliters of the solution needed to get 2  $\mu$ g, 1.5  $\mu$ g, 1.0  $\mu$ g, and 0.5  $\mu$ g of nanoparticles. In the 96 well plate, each treatment group was given 3 wells. First, the amount of nanoparticles were added to each well of the corresponding treatment group. It was assumed all siRNA was encapsulated into each nanoparticle. Next, 1% FBS Cell Media was added to each well so that the total amount of solution per well 100  $\mu$ l. The plates were then incubated overnight.

#### *Measuring Fluorescence of GFP-3T3 cells using a Plate Reader*

A plate reader was the first method used to measure the fluorescence of siRNA treated nanoparticles to determine if there was significant fluorescence knockdown in the nanoparticle treated cells. The plate was measured at 0 hr, 24 hr, 48 hr, and 72 hr post treatment. Each plate was measured at an excitation wavelength of 488 nm and an emission wavelength of 525 nm. The gain was set to 100. A one-way ANOVA was used to calculate significance of fluorescence at 24 hr, 48 hr, and 72 hr time points compared to the 0 hr time point.

#### *Measuring Fluorescence of GFP-3T3 cells using Flow Cytometry*

Flow Cytometry was a second method used to measure the fluorescence of siRNA treated nanoparticles. This method shows individual fluorescence by analyzing the light scatter a cell makes while passing them one by one through a laser. This fluorescence is then graphed and compared to the untreated control cells to evaluate significance of fluorescent knockdown using a one-way ANOVA. 3000 cells were passed through for each measurement at the 48- and 72-hour time points at

various concentrations of 1.0-7.5 ng/cell in both cells without siRNA and with siRNA. These time points were chosen because there was no expected knockdown to occur within the 24 hr mark.

### ***MTT and LDH Assays***

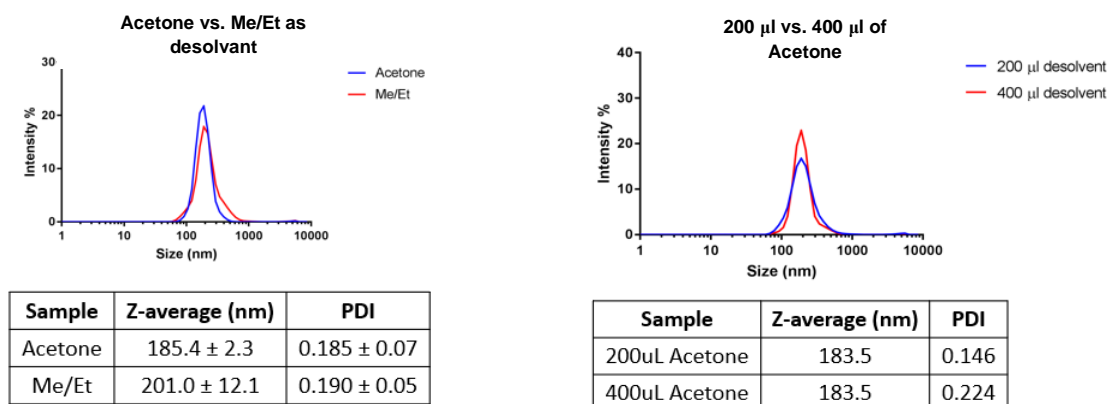
To measure the cytotoxic effects of siRNA cBSA nanoparticles on the GFP-3T3 cells, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) and lactate dehydrogenase (LDH) assays were used. An MTT assay measures the metabolic rate of cells while a LDH Assay measures the membrane integrity of cells. For the MTT assay, cells were first plated at 5,000-10,000 cells/well in a 96 well plate. Next, the cells were treated with various amounts of siRNA cBSA nanoparticles at 3 wells for each amount, except for 3 wells acting as the control. After 1 day of incubation, 10  $\mu$ l of MTT reagent was added to each well, and then the well was incubated for 4 hours. Next, 200  $\mu$ l of DMSO, organosulfur compound, was added to each well. In order to dissolve the formazan salt in the wells, the solution in the wells is pipetted up and down. Formazan salt changes color in the presence of active cells. To pop the bubbles from the pipetting, the plate is centrifuged at 1,000 for 1 minute. Lastly, the absorbance is measured on a plate reader at 570 nm and 630 nm. The absorbance of the siRNA treated cells is compared to the control cells.

An LDH Assay measures cell membrane integrity by converting pyruvate to lactate via the conversion of NAD to NADH. When the cells are in the presence of a toxic solution, they will release LDH, and the LDH reduces the NAD to NADH. In our experiments, cells were plated at 5,000-10,000 cells/well, and after a day of incubation, all wells except for the control were treated with various amounts of siRNA cBSA nanoparticles. The cells were then put back in the incubator for one more day. Next, 50  $\mu$ l of the LDH detection agent is added to each well, and the cells are incubated for another hour. The fluorescence of the cells is then measured and compared to the control cells.

## RESULTS AND DISCUSSION

### *Nanoparticle Characterization*

First, we determined the desolvent needed to form 200 nm nanoparticles. Two desolvents, acetone and a 2-part methanol:1-part ethanol mixture, were used. Figure 2 shows the size distribution results of using these two desolvents, where both desolvents produced approximately 200nm sized nanoparticles. However, using acetone as the desolvent resulted in a smaller Z-Average and PDI than Me-Et. 200uL and 400uL of acetone was used during the desolvation step to make cBSA nanoparticles and the size distribution of each batch was measured. From these results, we did not see a large difference in the reported Z-average, a measure of average particle size. We choose to move forward with 400  $\mu$ l of acetone because using this amount continuously produced a cloudier solution, which we correlated to a higher yield of nanoparticles and it consistently produced nanoparticles less than 200 nm in size.

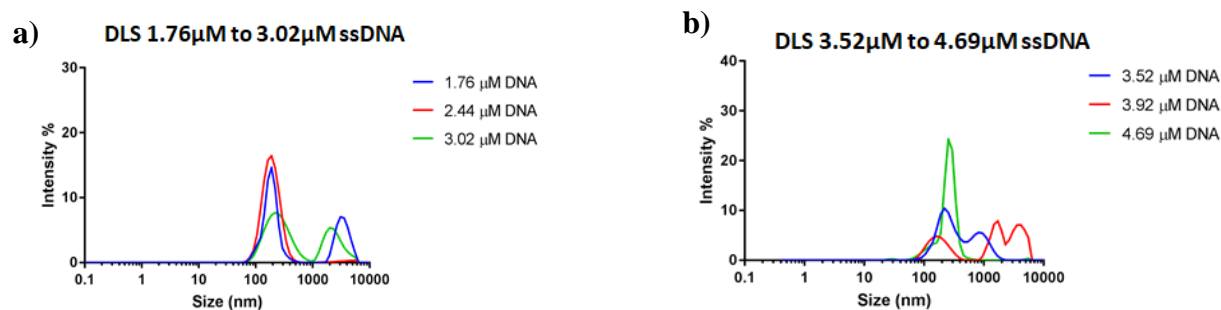


**Figure 2: Confirming Desolvent for Nanoparticle Fabrication** by a) comparing size distributions for acetone and MeOH/EtOH and 2) comparing size distributions for 200 ul of acetone and 400 ul of acetone.

When making our nanoparticles, it was important to determine how much siRNA could be loaded into the particle. We determined the nanoparticle loading efficiency by substituting ssDNA

primers for siRNA. siRNA is more expensive to use than ssDNA, but the ssDNA still has the same negative charge and length as siRNA. However, it may not be an exact replica because the ssDNA is single stranded while the siRNA is double stranded.

We made nanoparticles containing either 20  $\mu$ l, 30  $\mu$ l, 40  $\mu$ l, 50  $\mu$ l, 60  $\mu$ l, or 80  $\mu$ l ssDNA, corresponding to concentrations of 1.76  $\mu$ M, 2.44  $\mu$ M, 3.02  $\mu$ M, 3.52  $\mu$ M, 3.96  $\mu$ M, and 4.69  $\mu$ M ssDNA respectively. Each sample was then measured using dynamic light scattering (DLS) to get size distribution data, and zeta potential measurements were taken for all but the 3.02  $\mu$ M and 3.52  $\mu$ M samples (Table 1). Our goal nanoparticle size was less than 200 nm with little variation in size to ensure there are no aggregates. It has been found that 200 nm is the size threshold for drug delivery as nanoparticles over 200 nm in size are more difficult to internalize and are cleared out by the bloodstream more easily [16,17]. Both the nanoparticle samples with 1.76  $\mu$ M and 2.44  $\mu$ M DNA show sharp peaks around 200 nm (Figure 3a). The nanoparticles with 3.02  $\mu$ M DNA have a wider, shorter first peak around 200nm. However, the 1.76  $\mu$ M and 3.02  $\mu$ M samples have second peaks between 1000nm and 10000 nm which was not expected nor desired. Nanoparticles with ssDNA concentrations of 3.52  $\mu$ M, 3.96  $\mu$ M, and 4.69  $\mu$ M have average sizes that are much greater than 200 nm with very large standard deviations of the Z-average as seen in (Figure 3b, Table 1). The large standard deviation indicates that there is great variation in the size of each batch of nanoparticles meaning that batches are not reproducible. The PDI for 1.76  $\mu$ M, 3.96  $\mu$ M, and 4.69  $\mu$ M nanoparticles was high indicating that there was a wide size distribution. This high PDI was caused by the presence of large aggregates, which shifted the size distribution to the right. Our results indicated that with increasing amounts of ssDNA, the nanoparticles had a larger average size and produced aggregates.

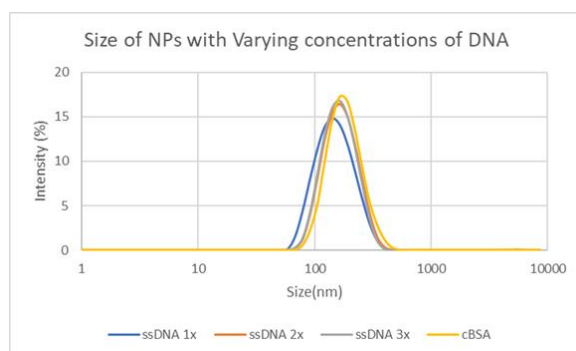


**Figure 3: Varying the amount of ssDNA in cBSA Nanoparticles by comparing the size distribution of nanoparticles for ssDNA concentrations of 1.76  $\mu\text{M}$ , 2.44  $\mu\text{M}$ , 3.02  $\mu\text{M}$ , 3.52  $\mu\text{M}$ , 3.96  $\mu\text{M}$ , and 4.69  $\mu\text{M}$ .**

**Table 1: Z-Average, PDI, and Zeta Potential for all ssDNA nanoparticles and soluble cBSA.**

Sample (amount of ssDNA added)	Z-average (nm)	PDI	Zeta Potential (mV)
Soluble cBSA			+ 15.2 $\pm$ 3.0
1.76 $\mu\text{M}$	178.9 $\pm$ 68.6	0.222 $\pm$ 0.04	- 22.3
2.44 $\mu\text{M}$	179.9 $\pm$ 9.6	0.164 $\pm$ 0.07	- 23.9 $\pm$ 2.8
3.02 $\mu\text{M}$	219.2 $\pm$ 8.8	0.194 $\pm$ 0.04	No Data
3.52 $\mu\text{M}$	409.5 $\pm$ 325.1	0.149 $\pm$ 0.03	No Data
3.96 $\mu\text{M}$	323.3 $\pm$ 170.1	0.356 $\pm$ 0.20	- 31.8 $\pm$ 2.3
4.69 $\mu\text{M}$	320.6 $\pm$ 124.3	0.359 $\pm$ 0.08	- 31.4 $\pm$ 0.6

Next, we wanted to determine if the change in volume of ssDNA supplied during nanoparticle fabrication caused aggregation since greater volumes in the previous experiment diluted the protein concentration. We know from previous work in the lab that lower protein concentration leads to larger particle sizes [18]. We varied the concentrations of ssDNA by creating 1x (10.5  $\mu\text{M}$ ), 2x (21.0  $\mu\text{M}$ ), and 3x (31.5  $\mu\text{M}$ ) solutions of DNA in a 20  $\mu\text{l}$  volume each. The nanoparticles were then measured by DLS to determine if the concentration of DNA affected the size of the nanoparticles when added in a constant, small volume. In Figure 4, ssDNA nanoparticles as well as cBSA nanoparticles, consistently peaked at only 200 nm and the Z-Average was under 200 nm for all samples (Table 2). This shows that reliable cBSA nanoparticles can be made with these concentrations of ssDNA. This means adding extra volume during nanoparticle fabrication when varying ssDNA concentration was the problem in the previous experiment.



**Figure 4: Varying the concentration of ssDNA in nanoparticles** by showing the size distribution of 1x (10.2  $\mu$ M), 2x (21.0  $\mu$ M), and 3x (31.5  $\mu$ M) ssDNA concentrations and the cBSA only nanoparticle.

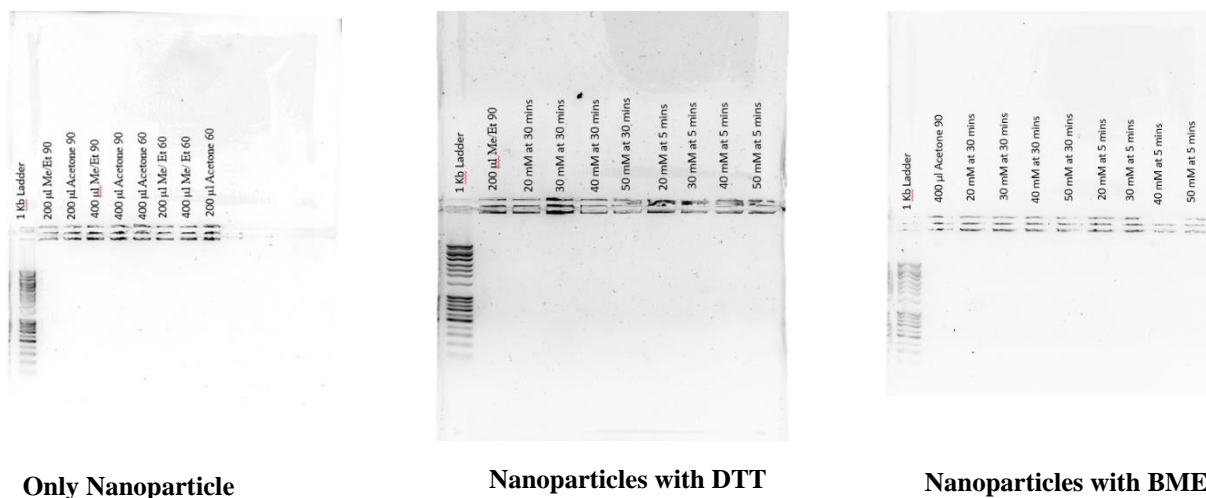
**Table 2: Z Average, PDI, and Standard Deviation for 1x ssDNA, 2x ssDNA, 3x ssDNA, and cBSA only nanoparticles.**

Sample	Z Average (nm)	PDI	SD (nm)
ssDNA 1x	139.2	0.115	9.15
ssDNA 2x	157.3	0.102	5.03
ssDNA 3x	154.3	0.096	7.30
cBSA	169.6	0.098	2.18

### ***Determining Nanoparticle Loading Ability***

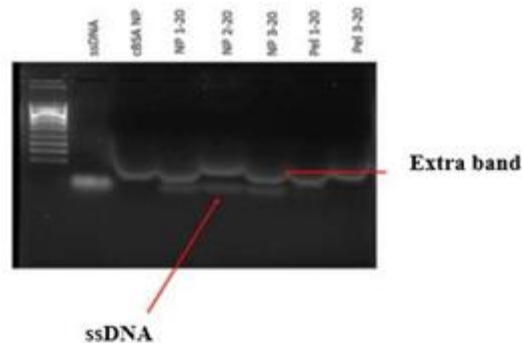
Next, we measured how much of the ssDNA added during cBSA nanoparticle fabrication is encapsulated in the nanoparticles. The cBSA nanoparticles are crosslinked using DTSSP, a cleavable crosslinker that contains a disulfide bond. This enables the particles to be stable outside of cells and break apart in the reducing conditions inside cells. To determine the overall amount of ssDNA encapsulated inside the nanoparticles, we first had to break the disulfide bond in DTSSP by incubating the nanoparticles in a concentrated solution of 20-50 mM DTT at various incubation times. DTT was chosen because it reduces the disulfide bonds in the DTSSP crosslinker. After incubation, an agarose gel was used to determine the amount of ssDNA in the nanoparticles by measuring any ssDNA bands present in the gel. No ssDNA bands were observed in the gel. This

experiment was repeated but using 2-Mercaptoethanol (BME) because BME is another agent used to reduce disulfide bonds. However, there were still no DNA bands observed. This indicates that DTT or BME alone are not strong enough to sufficiently cleave the nanoparticles and release ssDNA, or that there is no ssDNA in the particles (Figure 5).



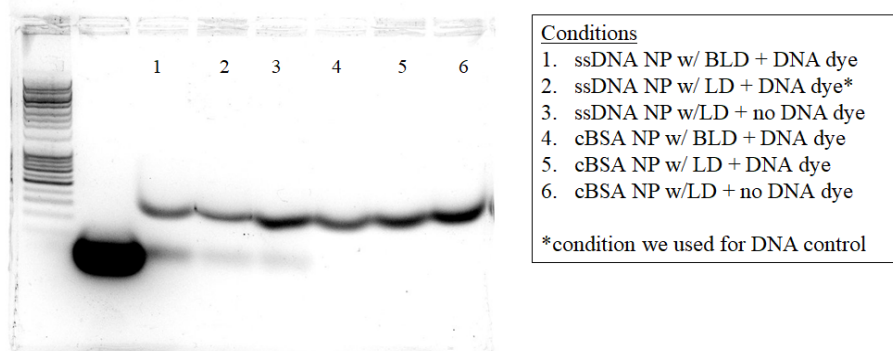
**Figure 5: Agarose Gels with only nanoparticles, nanoparticles with DTT, and nanoparticles with BME show no DNA bands indicating that DTT or BME alone are not suitable for breaking apart the nanoparticles.**

Since the nanoparticles were not able to be broken up with DTT or BME alone, our second experiment used a Laemmli buffer in combination with DTT. Laemmli buffer has a high concentration of BME and SDS. SDS can help further break up the nanoparticle by reducing the positive charge on the protein to further help release the DNA, respectively. After imaging the gel, we found that there were two bands in the gel (Figure 6). The lower band was the ssDNA, which was known based on previous gels and from the ssDNA positive control, but we did not know why there was an upper band forming. We hypothesized that the extra band was from the blue dye in the Laemmli running buffer because the ssDNA positive control sample was the only sample that did not contain the Laemmli running buffer.

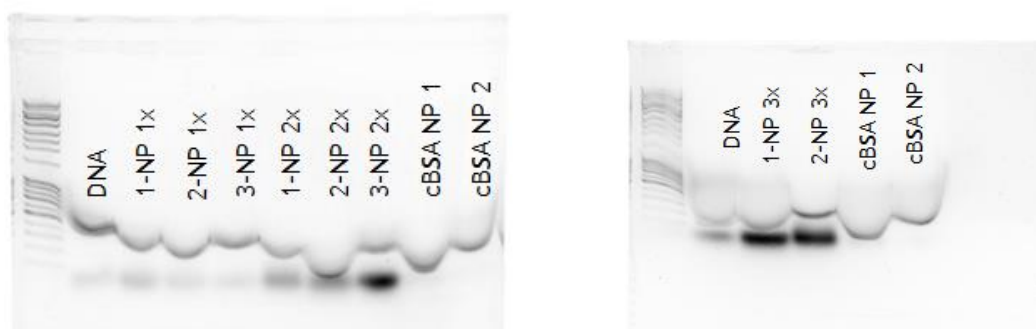


**Figure 6: The Nanoparticles are breaking up** when using Laemmli Buffer. The top band is hypothesized to be BME from the Laemmli Buffer, and the bottom band is DNA.

An experiment was then conducted using our own recipe of Laemmli Running Buffer. After imaging the gel, all conditions still showed the extra top band (Figure 7). Therefore, we could not conclude that the extra top band was from the blue dye in the Laemmli Running Buffer since samples without the dye still showed the band. From this, we hypothesized that the extra band was from the BME in the running buffer. Since this is essential to breaking the nanoparticles, we had to move forward even with the second band showing in the gel. We also concluded that since this band is seen in all samples, including nanoparticles without DNA, or cBSA nanoparticles, there is little to no interference in the lower, DNA band (Figure 8). In a future experiment, we could confirm that the extra band is from the Laemmli Running Buffer by running just the Laemmli Buffer in the gel to see if the band appears.



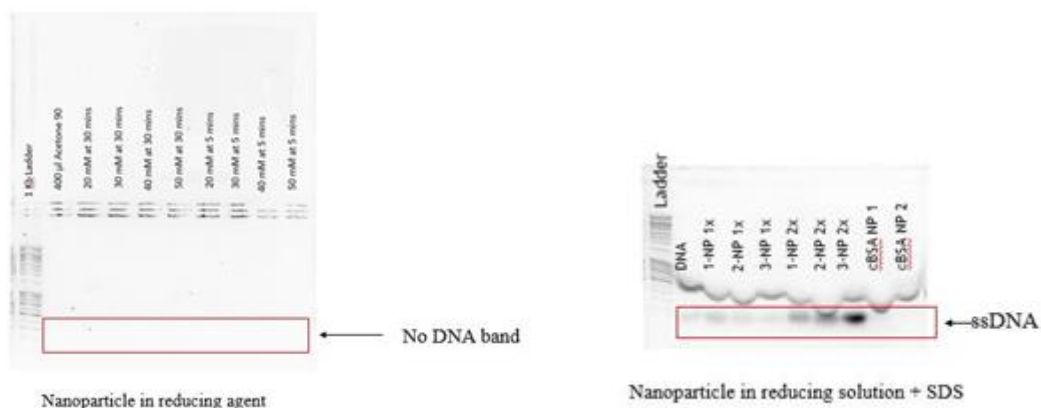
**Figure 7: Experiment using recipe of Laemmli Buffer without blue dye**, still shows top band in all conditions of nanoparticles showing that the top band is not due to the blue dye.



**Figure 8: All conditions of ssDNA NPs and cBSA NPs showed the extra top band.** The blue dye in the Laemmli running buffer did not cause the extra band. Upper band is shown in all conditions indicating that there is no DNA captured in the upper band.

Looking at Figure 8, it can be seen that some nanoparticles have ssDNA bands that are darker in intensity. The darker the ssDNA band, the more ssDNA has been incorporated into the nanoparticle. As expected, as the concentration of ssDNA used in the fabrication process increases, so does the darkness of the ssDNA band compared to the ssDNA control (1x concentration of ssDNA). In follow up experiments, it would be useful to use Image J to measure the intensity of each ssDNA band and compare it to bands from different kinds of nanoparticles. Then, the particle bands would be divided by ssDNA band to get the efficiency of ssDNA encapsulation by the nanoparticles.

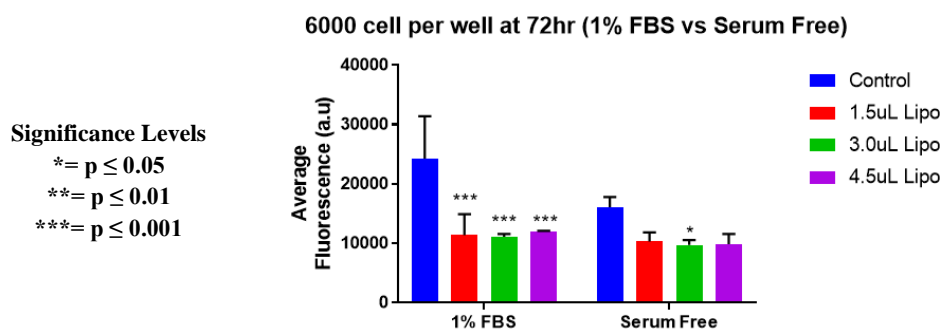
We know that DNA is incorporated inside the nanoparticle as opposed to being on the outside, because when we were not breaking apart nanoparticles, there was no DNA band (Figure 9). If the DNA was on the outside, it would have fallen off when the particles were exposed to the electric field in the gel. We will need to conduct a follow up experiment with a solution of SDS and no reducer (BME) to confirm that the NP does not break up with only SDS.



**Figure 9: DNA Gels showing that DNA is incorporated into the nanoparticle** because there is a) no DNA band when the nanoparticles are broken up and b) a DNA band when the nanoparticles are successfully broken up.

### *Lipofectamine Optimization for GFP Knockdown*

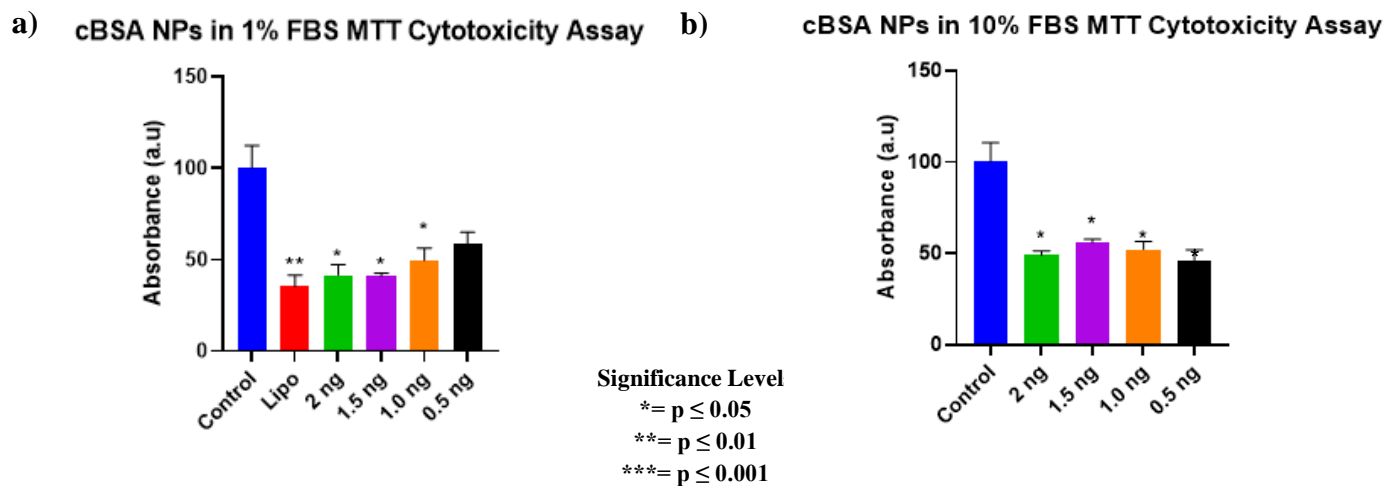
Before completing the entire GFP knockdown experiment, the protocol was optimized based on lipofectamine, which creates liposomes that encapsulate and deliver siRNA to cells, allowing us to see what conditions are ideal for the knockdown experiment. 1% FBS and Serum Free media were compared. 1% FBS showed more significant knockdown when looking at 6,000 cells/well. It was also concluded that the serum free media did not give enough nutrition to the cells causing them to die since the control cells decreased in fluorescence as well (Figure 10).



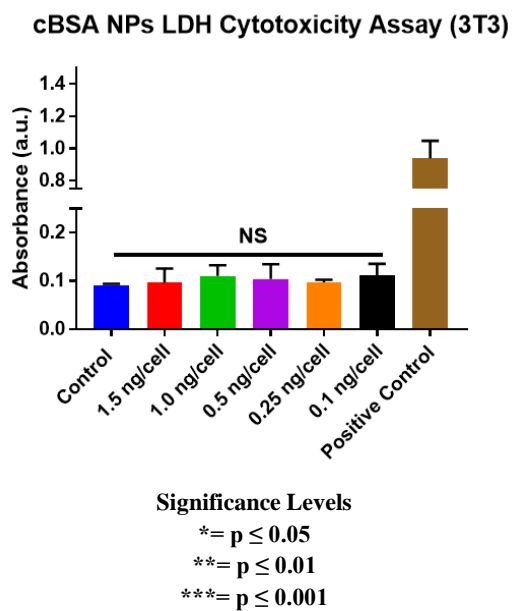
**Figure 10: 1% FBS shown to be best** due to higher significance at 6000 cell per well at 72 hr. Serum Free media might be leading to death of cells.

### ***Determining the Cytotoxicity of siRNA Nanoparticles in-vitro***

In the MTT Assay, both lipofectamine and the siRNA nanoparticles treatments showed a decrease in metabolic activity relative to the control. There were 6000 GFP-3T3 cells per well, and the control wells contained only cells. The treatments were 6  $\mu$ l of lipofectamine, 2 ng/cell of siRNA nanoparticles (5.4  $\mu$ l NPs per well), 1.5 ng/cell siRNA nanoparticles (4.05  $\mu$ l NPs per well), 1.0 ng/cell siRNA nanoparticles (2.7  $\mu$ l NPs per well), and 0.5 ng/cell (1.35  $\mu$ l NPs per well). When looking at Figure 11, all siRNA nanoparticle amounts, except 0.5 ng/cell of siRNA NP in 1% FBS Serum, show significant decrease in absorbance indicating that the metabolic activity of the cells decreased relative to the control. This trend was shown when the cells were in both 1% and 10% FBS supplemented cell media (Figure 11). We also conducted an LDH Assay, which measured membrane integrity. The results showed a non-significant difference between the control and siRNA nanoparticle amounts indicating that the membrane of the cells is remaining intact (Figure 12). Therefore, it is difficult to conclude whether or not the amount of siRNA nanoparticles we are treating cells with is truly cytotoxic. Since the metabolic activity of the cells is being decreased significantly, the siRNA cBSA nanoparticles need to be altered to maintain a sufficient level of metabolic activity.



**Figure 11: MTT Assay shows a significant decrease in absorbance** relative to control in both 10% and 1% FBS cell media indicating that all amounts of siRNA are cytotoxic to the GFP-3T3 cells.



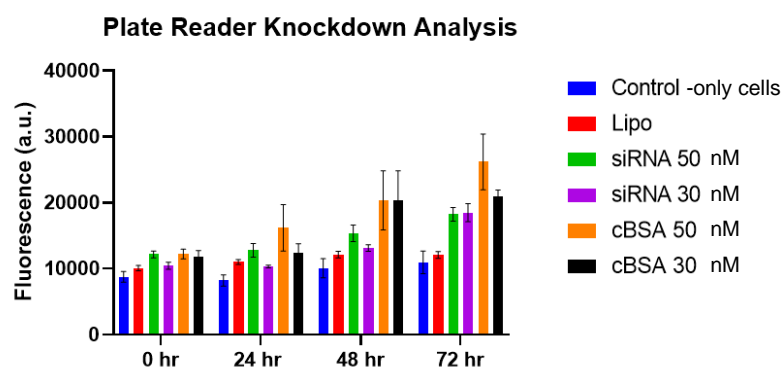
**Figure 12: LDH Assay shows non-significant decrease in absorbance** relative to control indicating that the membranes of the cells are maintaining their integrity.

## *GFP Knockdown using cBSA siRNA Nanoparticles*

### *Plate Reader Knockdown Analysis*

The siRNA that is encapsulated within our nanoparticles is an anti-GFP siRNA. The cells that are being transfected with the siRNA nanoparticles are GFP-3T3 cells. When the cells are transfected with the siRNA nanoparticles, we expect to see a decrease in fluorescence over time since the siRNA within the nanoparticle should escape and knockdown the green fluorescent properties of the GFP proteins in the cells.

We first used the fluorescence setting on the microplate reader to measure the fluorescence knockdown in our cells. However, we saw an increase in fluorescence with increasing time in both cells transfected with siRNA nanoparticles and cBSA only nanoparticles compared to the control of only cells, which was not expected (Figure 13). We concluded that this increase in fluorescence might be due to cells growing during the incubation period and creating a layer of new cells on top of the original layer, increasing the bulk fluorescence measured. This led us to use flow cytometry since it analyzes individual cell fluorescence as opposed to bulk fluorescence.

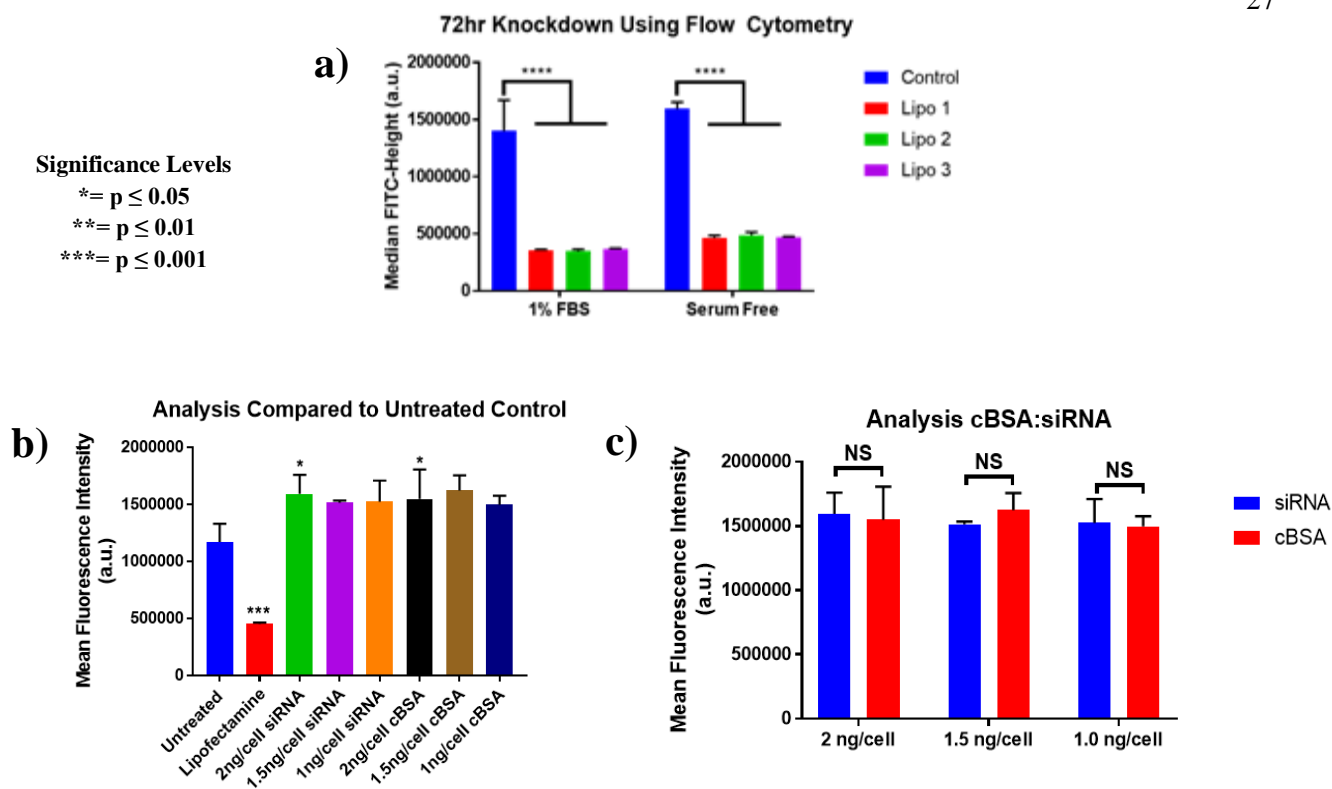


**Figure 13: Using the Plate Reader to analyze fluorescent knockdown of GFP-3T3 cells transfected with various concentrations of siRNA nanoparticles and cBSA nanoparticles.**

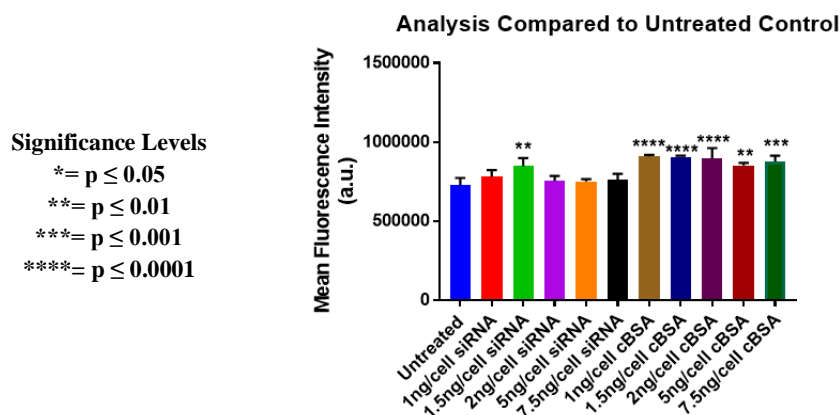
### *Flow Cytometry Knockdown Analysis*

In the first trial of GFP knockdown using flow cytometry, lipofectamine siRNA nanoparticles showed a significant decrease in fluorescence in all three samples compared to the untreated control which was expected (Figure 14a). However, all siRNA NP and cBSA NP amounts showed an increase in fluorescence compared to the control (Figure 14b). There was also no significant difference in knockdown between cBSA and siRNA NPs (Figure 14c). In subsequent trials of GFP knockdown using higher amounts of siRNA and cBSA NPs, there was still an increase in fluorescence compared to the control (Figure 15). The siRNA in the nanoparticle is supposed to be causing the knockdown in the cells, so the fact that siRNA and cBSA NPs showed no difference in knockdown could be due to the siRNA not leaving the nanoparticle. Because the uptake of the nanoparticles of the cells was not measured, another possibility is the cells did not internalize the nanoparticles.

These results disagree with the cytotoxicity assay results because if the treatment cells are fluorescing more than the control, there should be greater metabolic activity seen in the MTT assay. Both experiments will need to be replicated again in future studies.



**Figure 14: The fluorescence of GFP-3T3 cells treated with siRNA and cBSA nanoparticles increased compared to control and lipofectamine. There was no significant difference between the fluorescent knockdown of siRNA and cBSA nanoparticles.**



**Figure 15: The fluorescence increased relative to untreated control even with higher concentration of siRNA NPs. The difference between siRNA and cBSA NP fluorescence showed significance, but still not as high a significance as expected.**

## CONCLUSION AND FUTURE WORK

In conclusion, we were able to finalize a consistent fabrication protocol for our nanoparticles and determine the threshold for ssDNA that can be loaded into a cBSA protein nanoparticle. We were able to make nanoparticles that were under 200 nm with concentrations of 10.5  $\mu\text{M}$  (1x) and 21.0  $\mu\text{M}$  (2x). This procedure was also able to be used to make siRNA nanoparticles. We were also able to efficiently break up the nanoparticles using a combination of Laemmli Running Buffer and DTT. A protocol for using lipofectamine and siRNA nanoparticles in the knockdown of GFP in GFP-3T3 cells was created. We conducted several siRNA knockdown experiments. However, the results from these experiments showed that there was no knockdown of siRNA nanoparticles. From this work, we can conclude that cBSA nanoparticles are a promising biomaterial to use to encapsulate DNA and siRNA, but they do not seem to be a good material to deliver or release these cargos.

A follow up experiment needs to be conducted to show whether or not the siRNA nanoparticles are being taken up by the GFP-3T3 cells and delivering the siRNA. Although we showed that the nanoparticles have the ability to fall apart, it does not show that cells truly internalized them. This could be one reason why we are not seeing knockdown in cells. Another reason could be because the cBSA protein we are using to create our nanoparticles is too positive, which is not allowing the siRNA to escape from the nanoparticle and reach its target area. The MTT Assay is also showing a decrease in metabolic activity when treated with the cBSA nanoparticles. Therefore, we want to try to make our cBSA less positive for both the release of siRNA and to decrease cytotoxicity. One way of masking the positive charge of cBSA would be to functionalize it with histidines or imidazole groups which are only positive below a pH of 6.5. Typically, the cBSA is functionalized with amine groups which can be cytotoxic if there are too many. The cBSA siRNA

nanoparticles would be fabricated at a pH of 6.5 to ensure the siRNA attaches to the cBSA. In the endosomes, they would be positive and help with endosomal escape due to the proton sponge effect. The proton sponge effect states that the pH inside the nanoparticle would be low enough to cause an influx of water causing the nanoparticle to burst and release the siRNA inside. The siRNA would release once the DTSSP crosslink is broken, and the particle has escaped to the cytosol where the pH is 7.4 and the histidine/imidazole is less charged. Another way to mask the positive charge on the cBSA is to neutralize the amines after making the cBSA nanoparticles. This would be done through reacting the amine groups back to something uncharged or negative after crosslinking the nanoparticles.

This cBSA siRNA nanoparticle system has the potential to be used in delivery of siRNA to areas of the brain when conjugated with menthol. In the future, we would build an in-vitro BBB model out of endothelial cells sourced from a pig or mouse. Then, our cBSA siRNA nanoparticles would be conjugated with methanol. We would conduct experiments to evaluate whether or not our nanoparticles conjugated with menthol could cross the BBB. This would allow us to move into a novel area of therapeutics.

This research offers a new model for nanoparticle drug delivery that can encapsulate siRNA and if the delivery challenges are overcome, could be applied to a wide range of therapeutics.

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