#6 Biweekly Engineering Design Report
Project Title: Dual Antibody Conjugated Silk Nanoparticles as a Targeted Delivery System for Glioblastoma Multiforme (GBM) Therapies
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Things highlighted in this color are new!

Project Description
Glioblastoma Multiforme (GBM) is an aggressive tumor initiated by mutated astrocytes that can be found in the brain and spinal cord. As of now, the current treatment options for GBM are mainly surgery, radiation, and chemotherapy. These are all invasive or have severe side effects, making a targeted delivery system for chemotherapy using antibody-conjugated silk nanoparticles an important avenue to explore. The dual use of antibodies that target EGFRviii and IL-13Ra2 receptors are of interest. EGFRviii is a receptor expressed on the surface of around 30% of GBM cells, and not expressed in healthy brain tissue; IL-13Ra2 is expressed on 75% of GBM cells, but the low-level expression is found in the brain. The goals of this project are to determine the best receptors to target for GBM, determine the appropriate nanoparticle (NP) size for tumor uptake, and induce successful antibody conjugation to the silk nanoparticle surface.

Engineering Design Elements
What are the objectives of the project and the criteria for selecting them?
- The objective of the project is to evaluate an antibody-conjugated silk nanoparticle drug delivery platform as a treatment for Glioblastoma Multiforme (GBM). First, silk nanoparticles will be formulated to an appropriate size for tumor infiltration and cell uptake. Nanoparticles will then be conjugated with two antibodies of choice that target relevant cell receptors associated with GBM. Successful conjugation and preservation of antibody binding ability will be confirmed using fluorescence microscopy and a plate reader as a mechanism of visualizing fluorescence and measuring the concentration of the antibody. Targeted delivery will be evaluated in a U87 cell line to confirm efficacy in a 2D system. As of now, the current treatment options for GBM are mainly surgery, radiation, and chemotherapy. These are all invasive or have severe side effects; therefore, a targeted delivery system for chemotherapy is an important avenue to explore for this unmet need, limiting disease progression or recurrence while decreasing major side effects.

What system, component, or process is to be designed?
- We plan on formulating anti-IL-13Ra2 and anti-EGFRviii conjugated nanoparticles to target GBM tumor cells. These antibodies bind to IL-13Ra2 and EGFRviii respectively, which are both expressed on the surface of many mutated GBM cells and have little to no expression in healthy brain tissue. Since different receptors have been found to have varying levels of expression on healthy tissue and GBM cells, the dual targeting of two receptors is of interest. We determined the best receptor combination to target is IL-13Ra2 and EGFRviii. While IL-13Ra2 has one of the highest expression rates in patients, it is found in healthy brain tissues at low levels. EGFRviii, on the other hand, is only found on GBM cells, though it is only present in 20-30% of patients. The combined use

of receptors will reduce off-target interactions and increase the GBM cells targeted in patients. The nanoparticles will be fabricated to be within a 100-120 nm size range [1]. For our project, we will develop a protocol to conjugate antibodies to the silk nanoparticle using EDC/NHS, a crosslinking technique we have learned about through a literature review. EDC, in conjunction with NHS, allows for a 2-step coupling of two proteins without affecting the carboxyls of the second protein. Through literature review, past studies have used flow cytometry to calculate and validate the success of antibody-NP surface conjugation[2]. We plan to use fluorescence microscopy and a plate reader.

What need does it fulfill (clinical, research, etc)?
- As of now, the current treatment options for GBM are mainly surgery, radiation, and chemotherapy. These are all invasive or have severe side effects, so a targeted delivery system for chemotherapy would be an important avenue and unmet need to explore slowing disease progression and relapse while decreasing side effects.

What scientific, math, and/or engineering methods will be applied?
- Some of the scientific and engineering methods that need to be applied are silk processing, nanoparticle formation, antibody conjugation, imaging, and cell culturing. We will also likely employ fluorescent tagging to detect the presence of desired proteins and antibodies.

What realistic constraints (cost, safety, reliability, aesthetics, ethics, and social impact, etc) are to be considered?
- One constraint is the cost of antibodies, as we will be using two antibodies that are less common and therefore more expensive than typical antibody costs. Another constraint we must consider is the use of testing the conjugated nanoparticles on U87 cells; these cells have a highly heterogeneous population that may not be fully representative of our targeted receptors. As such, it is integral to test the nanoparticles on cells known to express the relevant binding sites before testing them on a heterogeneous population. This will allow us to confirm that any binding or lack of binding is due to the dual-antibody conjugated nanoparticles and not from a lack of the appropriate receptor. Since transfection has a huge time constraint, we may use flow cytometry to separate cells that do have IL-13Ra2 and EGFRviii receptors. Known limitations of targeted antibody therapies include off-target interactions. By researching and choosing antibodies with low-level expression outside of GBM tumors, we can decrease unnecessary exposure to chemotherapy. Furthermore, another constraint is time--there are few papers addressing dual-antibody conjugation, so the ability to successfully do so and develop techniques to evaluate our experiments may exceed our time limit of one school year.

What alternative solutions or changes to the plan will be considered?
- Our group decided that GBM was the best way to proceed onwards. Initially, we had only thought to conjugate just one antibody; our group's most recent alternative solution is to proceed with a dual targeting nanoparticle drug delivery approach and technique, targeting both IL-13Ra2 with EGFRviii. This will allow us to target a greater population of GBM cells while reducing off-target interactions. Since getting lentivirus certification or obtaining cells from City of Hope has been deemed unfeasible for this semester,

instead of transfecting U87 cells to get higher expression levels of targeted receptors, we could use cell sorting to isolate cells with IL-13Ra2 and/or EGFRviii expression and culture them from there. An alternative to EDC/NHS is coating the NPs with the antibodies, a process that would involve incubating the NPs in the antibodies diluted with PBS to induce tagging to the particle surface.

What are the planned tests and what are the quantitative milestones that will demonstrate achievement of the objectives?
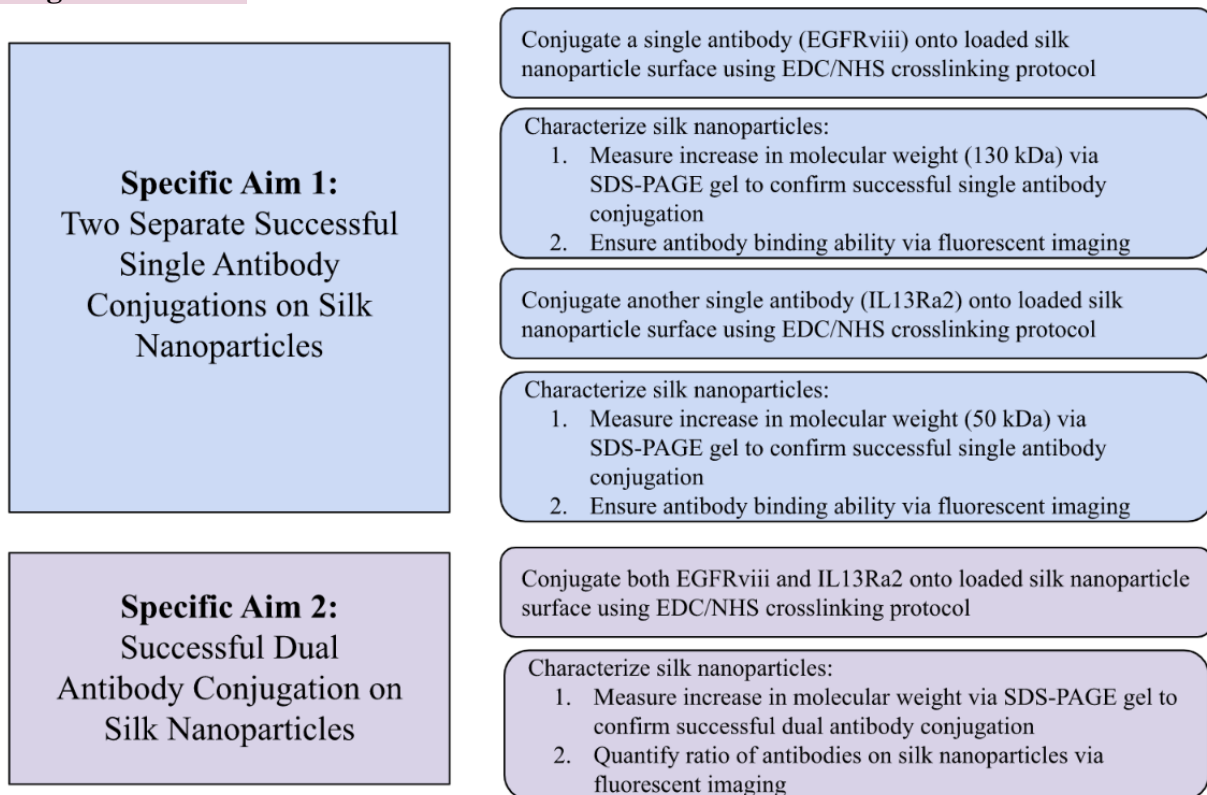- To start, silk processing in the Kaplan Lab will be conducted to formulate a batch of silk solution. The silk solution will be processed further in the Kaplan Lab to become silk nanoparticles. A DLS machine will be used to validate that the nanoparticle size is close to the project goal of 100-120 nm. For this report, we measured that our silk nanoparticles had an effective diameter of 96.86 nm to confirm that our formulations are progressing appropriately. Next, anti-EGFRviii and anti-IL13Ra2 have been ordered from Thermofisher, which will be conjugated using an EDC/NHS protocol. Antibody binding function will be confirmed using fluorescence microscopy to visualize secondary antibody-tagged nanoparticles as well as a plate reader to quantify antibody concentration. Since the antibodies are taking a bit of time to ship, we plan on practicing EDC/NHS conjugation and validation using IL-4 antibodies and secondary that are available in the lab.

Project Design Chart

Characteristic	Target Value	Why This Value	How We Will Test
Nanoparticle size	100-120 nm	Appropriate size for entering tumors via leaky vasculature and for tumor cell uptake	DLS particle sizing
Nanoparticle antibody expression	**TBD** → enough to have efficient uptake in GBM cells	Throughout various experiments, we will determine the target value for nanoparticle antibody expression based on which values optimize cellular uptake	Fluorescence microscopy with secondary antibody
Silk concentration	6%	6% silk has been determined by past studies to result in 100-120 nm particles	Concentration calculations by weighing 1000ul of silk solution, leaving overnight in a 60°C oven, and weighing the remaining silk
Uptake efficiency	**TBD** → enough to have efficient uptake in GBM cells	This value will be dependent on the various experiments we conduct to test nanoparticle antibody expression uptake efficiency (uptake is changed a	FITC and lysosomal fluorescent microscopy

		lot by cell line & nanoparticle size[3])	
Antibody conjugation validation	- 130kDa ↑ for anti-EGFR - 50kDa ↑ for anti-IL13Ra2 - Cells express fluorescence	This is important because we plan on conjugating antibodies using an EDC/NHS protocol. This will help us to validate that the conjugation was completed successfully.	Fluorescence microscopy and plate reader

Design Flow Chart

Specific Aim 1:
Two Separate Successful Single Antibody Conjugations on Silk Nanoparticles

Conjugate a single antibody (EGFRviii) onto loaded silk nanoparticle surface using EDC/NHS crosslinking protocol

Characterize silk nanoparticles:
1. Measure increase in molecular weight (130 kDa) via SDS-PAGE gel to confirm successful single antibody conjugation
2. Ensure antibody binding ability via fluorescent imaging

Conjugate another single antibody (IL13Ra2) onto loaded silk nanoparticle surface using EDC/NHS crosslinking protocol

Characterize silk nanoparticles:
1. Measure increase in molecular weight (50 kDa) via SDS-PAGE gel to confirm successful single antibody conjugation
2. Ensure antibody binding ability via fluorescent imaging

Specific Aim 2:
Successful Dual Antibody Conjugation on Silk Nanoparticles

Conjugate both EGFRviii and IL13Ra2 onto loaded silk nanoparticle surface using EDC/NHS crosslinking protocol

Characterize silk nanoparticles:
1. Measure increase in molecular weight via SDS-PAGE gel to confirm successful dual antibody conjugation
2. Quantify ratio of antibodies on silk nanoparticles via fluorescent imaging

- This flowchart describes the design of the proposed experiment. We will develop the dual antibody conjugated silk nanoparticles using established protocols used by the Kaplan Lab and EDC/NHS materials available, and we will characterize the nanoparticles produced. In the future, U87 cells will be sorted for EGFRviii and IL-13Ra2 receptor presence and cultured. The antibody-tagged nanoparticles will be loaded onto the cultured U87 cells and their uptake will be evaluated using FITC imaging. The efficacy of dual antibody and single antibody NPs will also be compared to determine the best antibody coating for GBM targeting. Eventually, the nanoparticles will be loaded with doxorubicin and live/dead assays will be conducted to evaluate the efficiency of the treatment model.

Introduction and Background

Glioblastoma Multiforme (GBM) is the most common tumor in the central nervous system (CNS) and accounts for 65% of all CNS malignancies[4]. GBM is one of the most deadly forms of cancer, with a median survival rate of just 12.6 months after diagnosis[5]. Attributing to this severe prognosis is the tumor's location in the brain or spinal cord, severely limiting the success of traditional chemotherapies, radiation therapies, and surgical removal. Nanoparticles, however, can mitigate many of the obstacles that currently available therapies cannot overcome. Their advantages include biocompatibility, reduced toxicity, excellent stability, enhanced permeability and retention effect, and precise targeting[6]. The unique targeting ability of these nanoparticles can be enhanced with antibodies that bind to proteins on the surface of the selected cancer cells and deliver the drug of interest.

While nanoparticles can be composed of various materials, silk was selected as the appropriate material due to its biocompatibility, availability, and ease of size optimization and loading[7]. Nanoparticles around 100 nm in the bloodstream are known to be too big to enter healthy tissue but can enter tumors due to their leaky vasculature. Once they have entered the tumor and bound to the cell receptors, they can be endocytosed to deliver the drug. Larger nanoparticles have been found to have longer rates of internalization; therefore, it may be advantageous to a NP large enough to only target cancerous tissue, yet small enough to be engulfed at an appropriate rate [8].

Epidermal growth factor receptors (EGFR) are transmembrane receptor tyrosine kinases (RTK) and are overexpressed in 50% of glioblastomas[9]. Epidermal growth factor variant three (EGFRviii) is a mutated wildtype EGFR expressed on the surface of GBM cells and commonly associated with GBM. This mutation can lead to continued expression of tyrosine kinases, and activate uncontrolled cell proliferation, growth, etc. EGFRviii is expressed in 25-33% of all GBM tumors in patients, and it is not expressed in normal brain tissue [10,11]. Some studies go so far as to claim that EGFRviii has never been detected in healthy tissue[11]. Its low expression in normal tissue makes it a suitable target for GBM therapies. Gliomas with EGFRviii have increased Ras activity, Akt/PI3k signaling, and increased expression of VEGF and IL-8[12]. EGFRviii CAR T cells are in Phase I studies and have shown low off-target toxicity[13].

Interleukin-13 receptor alpha2 (IL-13Rα2) was discovered as a glioma marker in 1995 by the Debinski laboratory[14], since then it has become one of the most studied tumor-specific antigens in glioblastoma research[15]. IL-13Rα2 is a high-affinity membrane receptor of IL-13 and is expressed in many tumors[16]. It has been found to be overexpressed in up to 75% of glioma patients[17,18]. Expression of IL-13Rα2 is high in the testis and placenta but has low expression in other organs[15]. A Phase III trial targeting IL-13Rα2 reported high levels of neurotoxicity due to off-target interactions with IL-13Rα1, a related receptor that is expressed in healthy brain tissue[19]. While this trial revealed the dangers of working with IL-13Rα2, it suggests that there is promise if an antibody more specific to IL-Rα2 is found and used. Currently, CAR T-cell therapy targeting IL-13Rα2 is now in Phase I clinical trials[20]. Dual combinations of IL-13Rα2 and EphA2 have been shown to be expressed in 90% of GBM patients indicating promising data for better targeting specificity[21]. EGFRviii and IL-13Rα2 targeted therapy have both been associated with recurrent antigen loss variants after initial treatment[22,23].

The combination of two receptor targets would allow for a greater number of GBM cell targets among its heterogeneous population, while also maintaining selectivity and reducing off-target interactions. Silk nanoparticles offer a unique opportunity to customize the drug, target, and dose of interest. In this project, dual-antibody conjugated nanoparticles will allow for more

direct targeting of GBM cells; compared to traditional therapies, a successful formulation will result in more efficacious treatment for better patient outcomes.

Preliminary Work

Before delving into our two Specific Aims, it is important to lay out the groundwork and foundation on which our project stands. While this is not something new that our project is developing, the first step involves formulating 100-120 nm diameter silk nanoparticles; therefore, it is important to establish a protocol for fabricating consistently sized silk nanoparticles. Silk will be made using a previously established protocol from the Kaplan lab. The target size distribution will be 100-120 nm, based on literature that linked the clinical efficacy of tumor vasculature penetration to this size range[1]. To achieve this size, we will produce a silk solution using 6% concentration, boiled for 30 min, and spun at 500 rpm for nanoparticle formulation. The nanoparticles will be fabricated by solvent emulsion techniques from the Kaplan Lab shown in the methods section. Solvent emulsion was chosen because it allows for precise control over nanoparticle size formation at this range. Nanoparticle size will be measured by dynamic light scattering (DLS), and a distribution of 100-120 nm will be acceptable for further processing.

Specific Aims

Specific Aim 1: Conjugate Anti-EGFRviii and Anti-IL13Ra2 Separately

In Specific Aim 1, antibodies anti-EGFRviii and anti-IL13Ra2 will be conjugated onto silk nanoparticles using EDC/NHS conjugation. EDC/NHS was chosen due to its prevalence in literature for analogous platforms. Using EDC/NHS protocols and materials available in the Kaplan Lab, antibodies will be conjugated onto silk nanoparticles in two separate experiments. It is expected that all nanoparticles will be conjugated with the antibodies. It is also not highly likely that only a portion of nanoparticles get conjugated to the antibodies; therefore, there is no need to isolate these nanoparticles successfully conjugated from the rest of the population. We will treat anti-EGFRviii and anti-IL13Ra2 nanoparticles with secondary mouse and rabbit antibodies respectively and use a Keyence fluorescence microscope to visualize fluorescence that would be present if conjugation to nanoparticles occurred. A plate reader will also be used to determine the concentration of antibody present by measuring the emission and excitation of the fluorescent secondary antibody. If time allows, additional experiments using ELISA can confirm specific binding. Conjugation will be deemed successful based on the visualization of secondary antibody-tagged nanoparticles compared to that of the control of nanoparticles coated in secondary and the standard curve. As of right now, there isn't a way to label some aspects of the population with a level of quantification without the use of flow cytometry. However, two potential options our group is looking at are (1) Finding a percentage of original concentration that was maintained overall (in our case, we calculated that 3.9% of original concentration was maintained in our IL-4 test run) or (2) Using DLS size difference to quantify the unconjugated nanoparticles with the conjugated nanoparticles.

Specific Aim 2: Dual Conjugation of Anti-EGFRviii and Anti-IL13Ra2

Specific Aim 2 will produce dually conjugated silk nanoparticles with anti-EGFRviii and anti-IL13Ra2. Dual conjugation was chosen due to previous literature linking it to enhanced therapeutic efficacy in tumor models[24]. We follow EDC/NHS and fluorescence protocols established in Specific Aim 1 to conjugate both anti-EGFRviii and anti-IL13Ra2 antibodies onto

silk nanoparticles as the protocol for dual conjugation is the same as the protocol for single conjugation. We are not expecting that conjugation of one antibody will interfere at all with the conjugation of another due to previous literature in the field. In terms of analysis, the means of our dual conjugated silk nanoparticles will be compared to that of our single conjugated silk nanoparticles. To confirm antibody binding and concentration, the nanoparticles will be treated with secondary mouse and rabbit antibodies for plate reading. It is known that silk nanoparticles experience some small amount of autofluorescence, as silk scaffolds do, but not by that much where it would interfere with plate reader measurements with our secondary antibodies. The fluorescence in each well plate will be qualitatively analyzed in comparison to all conditions. If time allows, anti-IL13Ra2 and anti-EGFRviii will be bound in different test ratios to determine nanoparticle surface coverage. To start, equal concentrations of each antibody will be tested and adjusted as the experiment progresses.

Methods

Silk Processing[25]
Cut cocoons and remove inside layers, weigh out 4.24g sodium carbonate, and add to 2L of boiling distilled water. Add 5g of cocoons to the solution to degum silk fibers so that sericin is washed away and only fibrin protein remains. Wash degummed silk three times in 1.5L of distilled water, changing the water each time, for 20 minutes each. Remove silk, pull by hand, and air dry inside a fume hood. Add silk into 9.3 M LiBr solution to remove beta sheets and let sit for at least 4 hours in a 60°C oven. Pour dissolved silk into dialysis tubing and place tubing into a 2L beaker of distilled water and spin for 3 days. Change dialysis water 3 times on the first day, twice on the second day, and once on the third day to wash out the LiBr solution. Collect silk solution on day 3 and centrifuge solution twice for 20 minutes at 5-10°C at 9000 rpm, then store in the fridge for up to two weeks.

Silk concentration calculation:
1. Weigh an empty weigh boat (W1)
2. Add 1 mL silk solution (measured accurately with a 1000uM micropipette) and record the weight (W2)
3. Leave the weigh boat in a 60°C oven overnight
4. Next day, weigh the weigh boat again (W3)
5. The concentration of the silk solution (w/v) is:
   % = (W3-W1/W2-W1) x 100

Silk Nanoparticles[26]
For 6% silk, 500 rpm, 30 min boil, on <u>day 1</u>, add 15-20 mL acetone to labeled small glass jar. Place a 3cm stir bar into the jar and secure the jar onto the center of the stir plate with polymer clay, set to 500 rpm. The vortex created should be centered in the jar and no sound should be coming from the stir bar. Measure out 4 mL silk & pour it into a specialized glass dropper. Using the small black knob on the left side of the glass dropper, twist slowly and carefully (following a titration technique). The solution should drop and fall into the side of the vortex which will help to create the desired nanoparticle size of choice. On <u>day 2</u>, add 2-5 mL of DI water as some of the acetone may have evaporated overnight. For <u>day 3</u>, the final liquid level should be around 4 mL. Move the solution to a 15 mL tube and add DI water up to 10 mL. Clean the sonicator tip

with ethanol and hold the 15 mL tube to the sonicator tip. Sonicate at 30% amplitude for 30 seconds 2x, cool down in between, and move the tube up and down (don't touch the sides or the bottom). Check the size with the particle solutions app on DLS/SEM.

Nanoparticle concentration calculation:
1. Weigh an empty weigh boat (W1)
2. Add 1 mL nanoparticle solution (measured accurately with a 1000uM micropipette) and record the weight (W2)
3. Leave the weigh boat in a 60°C oven for a few hours or on the bench at room temp overnight
4. Weigh the weigh boat again (W3)
5. The concentration of the nanoparticle solution (w/v) is:
   % = (W3-W1/W2-W1) x 100

EDC/NHS[27]

Antibody	
SF	500 mg
Antibody	166 mg
EDC	124 mg
NHS	40 mg

On day 1, reactants can be taken out from the fridge/freezer. EDC and NHS powders are weighed out into small individual jars, respectively. Based on calculations, the appropriate amount of 0.05 M MES buffer (pH 6) will be added to each to help dissolve the EDC and NHS separately. Calculations will occur to determine the amount of ultrapure water that will be added to the dissolving process (while accounting for the fact that the silk solution already has water in it). In a new jar with a 3cm stir bar, collect 2 mL of silk NPs, MES buffer, EDC +NHS, and 50 uL of the antibody. Set the jar on a stir plate at 200 rpm for 18 hours overnight. On day 2, two tubes with EDC/NHS nanosolution will be filled equally and weighed, using DI water to balance. The ultracentrifuge will be turned on and set to 60K rpm for 30 minutes at 4°C and run with balanced EDC/NHS nanosolutions. The supernatant will be taken out with a needle and resuspended with 3 mL DI water. Repeat two more times (three spins total). Soak the supernatant pellets in 1 mL of DI water and store them in the freezer.
EDC/NHS calculations:
1. EDC ratio is 500 silk fibroin (SF) to 124 EDC
2. NHS ratio is 500 SF to 40 NHS
   a. Use these ratios to determine how many mg each of EDC and NHS is needed to perform the protocol, respectively
3. Based on the silk nanoparticle concentration, 3.1 mL of buffer per 500 mg of silk is needed
   a. This ratio will help to determine the amount of MES in uL needed to be added in each respective EDC and NHS jar

Ultracentrifugation[27]

In order to remove antibodies that were not bound during EDC/NHS, ultracentrifugation of the nanoparticle solution is required. Ultracentrifugation will occur 3 times, and the supernatant from each spin will be collected to use when imaging to determine how much unbound antibody was left in the supernatant, to then help determine how much antibody is bound to the nanoparticles.

Spin 1:
1. Turn on the ultracentrifuge machine
2. Make sure the settings of the ultracentrifuge are 60K, 30 minutes, at 4°C
3. Grab two sealing tubes and fill them equally with the EDC/NHS nanoparticle solution
4. Fill the tubes with DI water to the lip of the tube, leaving the cylinder at the top empty for the stopper and cap
5. Weigh tubes and make sure they are within 0.01g to 0.02g of each other
6. Place tubes into centrifuge rotor balanced (e.g. one in hole 2 and one in hole 6)
7. Screw on the lid of the rotor and place onto the slit in a ultracentrifuge, pressing down on the silver center button to lock the two together (spin to make sure the rotor is calibrated)
8. Close the lid of the machine, and start the vacuum by pressing the vacuum button
9. Wait until the vacuum is 0 microns and the temperature is close to 4°C (around 6°C to 7°C) to start the machine

After Spin 1:
1. Before opening the ultracentrifuge, turn off the vacuum once it reaches 0 microns
2. Take out tubes from the rotor (if stuck, use a pipette tip and tweezers)
3. Remove supernatant around nanoparticle pellet using an 18G needle and syringe
4. Save supernatant in a 15 mL tube, label, and store in the freezer
5. Resuspend nanoparticle pellet in 3 mL of DI water using an 18G needle and syringe

Spin 2:
1. Repeat **Spin 1** steps 4-9

After Spin 2:
1. Repeat **After Spin 1** steps 1-5, label the supernatant tube

Spin 3:
1. Repeat **Spin 1** steps 4-9

After Spin 3:
1. Repeat **After Spin 1** steps 1-4
2. Instead of resuspending the pellet, soak the pellet in 1 mL of DI water
3. Store sealing tubes in the freezer

Secondary Antibody Tagging[28]
1. Thaw nanoparticles and supernatant at room temperature
2. Resuspend nanoparticles in a sealing tube, combine both tubes into a 15 mL tube and add DI water to make a 10 mL solution
3. Sonicate nanoparticle solution at 30% for 30 seconds twice
4. Obtain an arbitrary 4 mL solution containing:
    a. Antibody nanoparticles or supernatant
    b. Blocking butter (for nanoparticles only) → 0.2% BSA (blocking buffer solution)
    c. Secondary antibody (IL-4 has a concentration of 2 mg/mL)
5. Grab six 15 mL tubes
    a. Tube 1 (antibody nanoparticle with secondary):

          i.   4 mL solution in total
         ii.   Use 3 mL antibody-tagged nanoparticle solution
       iii.   Calculate the concentration of secondary antibody:
               1. $\frac{2\,mg/mL * 4mL}{2000\,microgram/mL} = 4$ µg/mL
       iv.   Calculate the concentration of the blocking buffer solution
               1. Want 0.2% of 4 mL to be blocking buffer: 8 µL
        v.   Fill the rest of the solution with DI water to make 4 mL
               1. In this case, 988 µL

    b. Tubes 2, 3, and 4 (supernatants with secondary)
          i.   3 mL of supernatant (each tube will be a different supernatant)
         ii.   4 µL of secondary antibody
       iii.   Fill will DI water until 4 mL solution
               1. In this case, 996 µL

    c. Tube 5 (blank nanoparticles with secondary)
          i.   4 mL solution in total
         ii.   Use 3 mL blank nanoparticle solution
       iii.   Calculate the concentration of secondary antibody:
               1. $\frac{2\,mg/mL * 4mL}{2000\,microgram/mL} = 4$ µg/mL
       iv.   Calculate the concentration of the blocking buffer solution
               1. 1 mL solution: 900µL of DI water, 100 µL BSA
               2. Want 0.2% of 4 mL to be blocking buffer: 8 µL
        v.   Fill the rest of the solution with DI water to make 4 mL
               1. In this case, 988 µL

    d. Tube 6 (blocking buffer)
          i.   1 mL solution: 900 µL of DI water, 100 µL BSA

6. Shake all tubes on a stir plate covered in tin foil (since the secondary is light sensitive) for 2 hours
7. Ultracentrifuge both nanoparticle tubes (Tube 1 and Tube 5) 3 times following the **Ultracentrifugation** protocol

Imaging
1. Retrieve a 96-well plate
2. Create a standard curve with 12 standards
    a. In this experimental case, wanted 8 µg/mL of antibody, so created a 1 mL solution with the secondary of a concentration of 2 mg/mL giving 4 µL secondary antibody out of the 1 mL solution
    b. Each standard is 200 µL of the first standard combined with 200 µL of DI water
3. Fill all 12 wells of the first row with 200 µL of each standard
4. Fill the first 3 wells of the second row with 200 µL of the 1° + 2° antibody NPs
5. Fill the first 3 wells of the third, fourth, and fifth rows with 200 µL of supernatants 1-3
6. Fill the first 3 wells of the sixth row with 200 µL of blank nanoparticles
7. Fill the first 3 wells of the seventh row with 200 µL of blank NPs + secondary antibody
8. Take the 96-well plate to the plate reader to quantify the fluorescence of the secondary antibody

a. Open the face of the machine, press the blue button in the top left corner to open the plate holder
b. Place plate in with NO lid
c. Press the blue button to close the plate holder and close the face of the machine
d. Log into the desktop and open the software
e. Set up a schematic of the plate reader on software by blocking out which wells have a solution in them
f. Run read
g. Save data to an excel file
9. Take the 96-well plate to the Keyence fluorescence microscope to visualize the secondary binding
   a. Turn on the desktop and log onto the Keyence software
   b. Open Keyence and orient the well plate to match the setup on the software
   c. Image well plate at an exposure of 1-6
   d. Save images

U87 Culturing

After taking 1mL U87 cell line out of the liquid nitrogen freezer and thawing in metal beads around 34°C, 100 µL of that cell suspension can be added to 9.9mL of cell culture media in a T75 to grow for 2+ days until 80-90% confluency is reached, which is about 2-3 million cells in a T75. U87 cell culture media contains MEM, FBS, penicillin, and streptomycin. U87 cells are non-adherent and naturally like to grow in aggregates, making spheroids. To prevent this, cells are cultured on plates that have been coated with Poly-L-ornithine (PLO), which promotes cell adherence and long-term culture of neuronal cells. After a few days, the cells will need to be processed, which involves suctioning out the old culture media, adding 2mL of trypsin (a digestive enzyme), and incubating for 5-10 minutes at 30°C until the cells have lifted from the PLO plate. After incubation, the flask is visualized under the microscope to determine if the cells have lifted. Once this has occurred, 8mL of cell culture media is added to the cell suspension, and the 10mL solution is transferred to a 15mL tube where it is centrifuged at a speed of 400G for 5 minutes. After centrifugation, the cells are left as a pellet at the bottom of the supernatant, which is removed to leave the pellet. The pellet is resuspended in 1mL of culture media. A PLO-seeded flask is taken from the incubator to plate the cells. To plate at a lower density, 100 µL of cell solution is added to 9.9mL of media for a 1:10 ratio, while a 1:5 ratio would be 200 µL of cell solution with 9.8mL of media. This is placed back into the incubator until processing.

Results

We were able to successfully obtain 13 mL of silk solution, which we then evaluated for concentration. We had created a 10% silk solution, and we wanted a 6% silk solution to obtain our properly sized nanoparticles. To do this, we wanted 30 mL of a 6% silk solution, so we took 18 mL of our 10% silk solution and added 12 mL of DI water to reduce the silk concentration to 6%. We used the 500 rpm, 6% silk, 30 min boil technique to formulate nanoparticles of 100-120 nm in diameter. Following the protocol listed above, we were able to formulate nanoparticles of a diameter of 74 nm, which was very small, suggesting that the DLS machine was broken and in need of service. We are planning on making more nanoparticles with new silk to test any other issues that might be causing the discrepancies in size.

We processed another batch of silk, following the same protocol with 6% silk, 30 min boil, and 500 rpm, and were able to get around 10mL of 96.86 nm silk nanoparticles, as seen in Figure 1 below. These nanoparticles will be effectively used in our experiments to test antibody conjugation during our IL-4 test run, but these will not be used for our final product as they are just a bit smaller than we would like to be efficacious enough for cellular uptake.
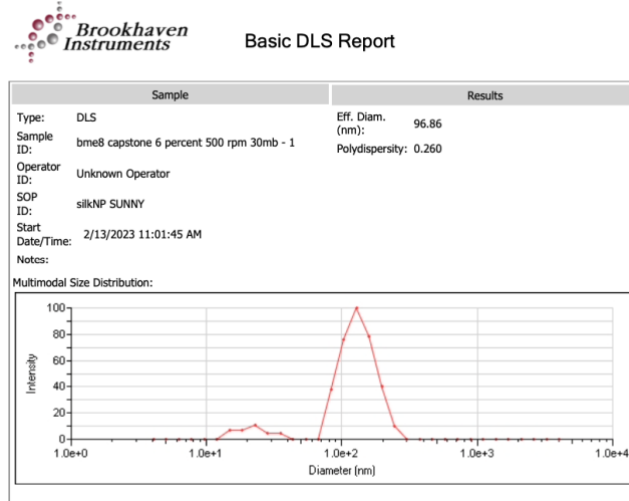
Figure 1. Basic DLS report of silk nanoparticles with an effective diameter of 96.86 nm.

The nanoparticles we obtained in this new batch of silk are consistent with not much batch-to-batch variation. We will be able to use this one batch and draw conclusions from that, given the use of the DLS machine to help with sizing. The 96.86 nm size is the effective diameter of an average particle measured by the DLS machine. This number is not extremely variable within the batch. A difference of ~3mm is not a big difference for nanoparticles, so much so that it would significantly affect nanoparticle behavior compared to 100 nm particles. That being said, for the next round of silk creation that we plan on completing in two weeks, to make sure that our nanoparticles are between 100-120 nm, we are planning on adjusting our protocol to a 200 rpm, 5% silk, 30 min boil technique. This will ensure that our particles will be 120 nm maximum, so if they end up being on the smaller side, which they normally are, we will likely end up in the proper range of 100-120 nm.

Since our ordered antibodies have not yet arrived, we ran through our methods using leftover IL4 from the lab to see how and if we needed to edit our protocol to allow for proper antibody conjugation. We started by measuring the concentration of our silk solution, which ended up being 21.9%. EDC-NHS was performed based on the protocol detailed above. After EDC-NHS was performed, the nanoparticle solution was ultracentrifuged three times to make sure the unbound antibody was washed off. To image IL-4 conjugation to the nanoparticles, the antibody nanoparticle solution, as well as the three ultracentrifugation supernatants and a solution of blank nanoparticles, were incubated with anti-rat Alexa Fluor 594, a secondary antibody. The fluorescence of this secondary is red and it has a maximum excitation of 590 nm and an emission maximum of 618 nm. To image, a 96 well plate was set up, as shown in Figure 2, with different solutions taking up the first six rows: a standard curve (developed using the Imaging protocol), IL4 conjugated nanoparticles tagged with secondary antibody (1º + 2º Ab NPs), three supernatants from three ultracentrifugation washes (SPN 1-3), and blank nanoparticles (SF NPs). A later experiment tested just blank nanoparticles tagged with secondary antibody (2º Ab SF

NPs) as a control to see if nanoparticle fluorescence was due to secondary binding to IL-4 conjugated to the nanoparticle surface or if the secondary was just coating the nanoparticle non-specifically. The information from the control experiment was included in the final data analytics. The antibody concentrations were extrapolated from the standard curve where conc = 0.0148(fluor) - 0.014 with R^2 = 0.99 (Figure 3). Using the plate reader, the fluorescence of the samples were quantified as seen in Table 1. Figure 4 shows that among the nanoparticles, antibody concentration was significantly different, suggesting the IL4 conjugation was successful. Furthermore, qualitative differences observed via fluorescence microscope (Keyence) support the quantitative findings of successful IL4 conjugation (Figure 5).
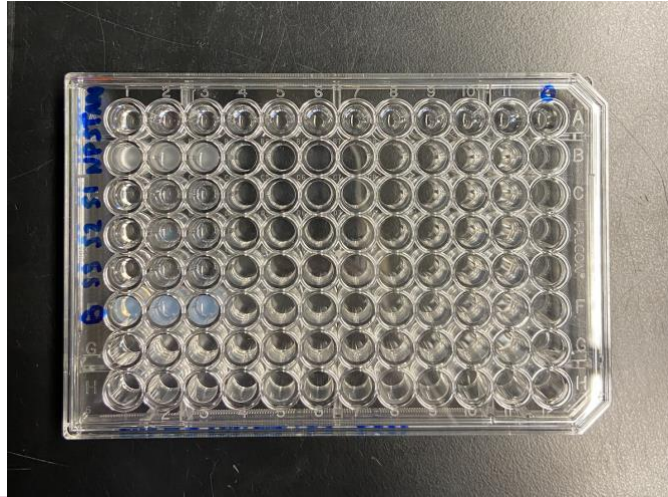
Figure 2. 96 well plate used for Keyence fluorescence microscope and fluorescent plate reader

Groups	Fluorescence			Concentration (ug/ml)			Avg Conc. (ug/ml)
A	17.13	16.92	17.48	0.239524	0.236416	0.244704	0.2402146667
B	4.439	4.735	4.942	0.0516972	0.056078	0.0591416	0.05563893333
C	22.74	22.65	22.78	0.322552	0.32122	0.323144	0.3223053333
D	24.75	25.52	24.75	0.3523	0.363696	0.3523	0.3560986667
E	0.1151	0.103	0.1023	-0.01229652	-0.0124756	-0.01248596	-0.01241936
F	11.85	9.602	8.986	0.16138	0.1281096	0.1189928	0.1361608

Table 1. Fluorescence and antibody concentration values of sample groups. (A) IL4 conjugated nanoparticles tagged with secondary antibody; (B-D) supernatant collected from ultracentrifugation washes 1-3 respectively; (E) blank nanoparticles; (F) blank nanoparticles incubated with secondary antibody.
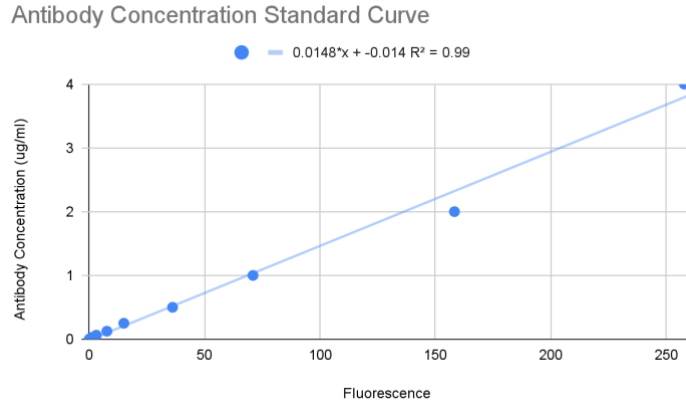
Figure 3. Standard curve of Goat anti-Rat IgG Alexa Fluor^{TM} 594 secondary antibody ranging from 0-4 ug/ml concentration. $Concentration = 0.0148 \cdot fluorescence - 0.014$.
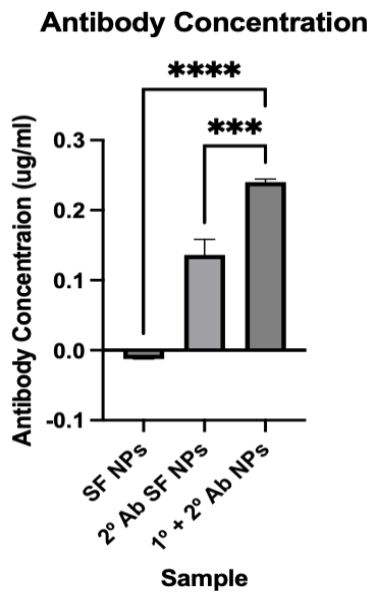
Figure 4. Antibody concentrations of blank nanoparticles, blank nanoparticles incubated with secondary antibody, and IL4 conjugated nanoparticles incubated with secondary antibody. (n=3; **** $p<0.0001$; *** $p = 0.0002$).
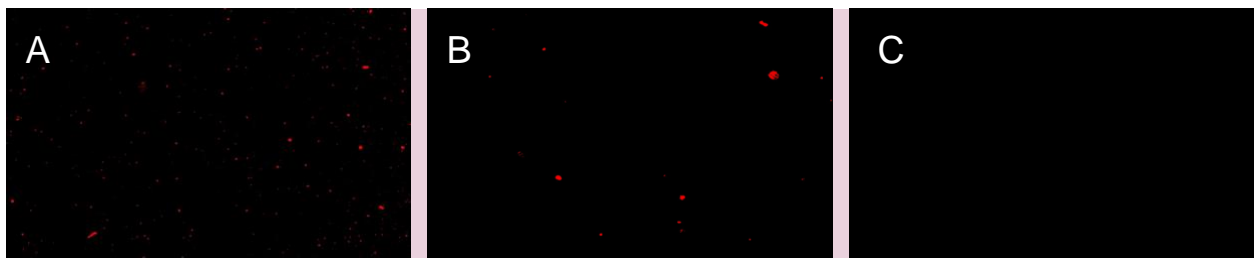
Figure 5. Fluorescent images of nanoparticles using the Keyence machine. (A) IL4 conjugated NPs tagged with Goat anti-Rat IgG Alexa Fluor^{TM} 594 secondary antibody; (B) blank NPs incubated with secondary antibody; (C) blank NPs with no secondary antibody. (1-6 exposure, 362 μm scale).

What else is going on in the field that would compete with the project plans?
Something interesting going on in the field that could compete with the project plans is that some researchers were able to test silk fibroin nanoparticles coated with Tween-80 in GBM cell lines and found that they were able to release doxorubicin for up to 72 hours. Being able to cross the blood-brain barrier is not necessarily something we must target in this capstone project, but it could be a future consideration to take into account if time permits. Our project also differs from this since ours would be more targeted due to antibody conjugation.

Discussion
We processed silk two times in the fall semester. The first time, the pH of the water was around 5, which affected the silk and resulted in unexpected visual cues after dissolving in LiBr. This prompted us to discard that batch and process the silk a second time, where the dissolving and dialysis of the silk were much more routine. As stated in the results, we were able to obtain a 13 mL 10% silk solution that we were able to change the silk concentration to 6% to create nanoparticles with about 74 nm diameter. These nanoparticles that were created will be used in our experiments to test conjugation, but 100-120 nm nanoparticles will be used to test efficacy. It was good we were able to have a trial round surrounding creation and were able to test out certain bugs, like the DLS machine not working properly, so that next time we make nanoparticles we can be more certain in our approach.

We discussed the possibility of receiving patient-derived U87 cells from the City of Hope that endogenously express both IL-13Ra2 and EphA2. Once we received the cells, we were going to determine if both receptors are expressed, and if so, we would have changed our project from targeting EGFRviii to EphA2 in order to bypass the viral transfection process. Unfortunately, the paperwork was taking too long to process to be useful this semester, so we decided to move forward with EGFRviii and IL-13Ra2 targeting, but to keep this information for future groups that may need those cells in the future.

After processing silk and formulating nanoparticles again this spring, we found that the nanoparticles had an effective diameter of 96.86 nm. While this is a little short of the target 100-120 nm range, it is close enough that we will use these nanoparticles for antibody conjugation testing. In the future, we will aim to have more appropriately sized particles when testing cellular uptake *in vitro*. While many changes have occurred to our original plans, we aim to end the year with a dually conjugated silk nanoparticle of 100-120 nm in size.

While we are in the process of waiting for our antibodies to arrive from Thermo Fisher, we decided to start our project with a test run using IL-4, provided by Olivia Foster from the Kaplan Lab. The IL-4 rat antibody that we used was two years expired, but to get a head start in the meantime, we decided to proceed with our investigations. That being said, our results should be taken lightly for now as we will replicate all of it in much more depth and precision when our official antibodies arrive in two weeks.

EDC/NHS was chosen due to its prevalence in literature for analogous platforms. Using EDC/NHS protocols and materials available in the Kaplan Lab, the IL-4 rat antibody was conjugated onto silk nanoparticles. The established EDC/NHS protocol was used to synthesize

chemically modified silk fibroin with amines of different sizes to produce cationic SF. The amine conjugation is accomplished via EDC/NHS coupling. For our project, we modified the protocol so that SF(D, E)-EDA numbers were substituted in the same ratios for the IL-4 rat antibody.

As a result of the secondary antibody imaging, Figure 5a presents the fluorescence of the IL4 conjugated NPs tagged with Goat anti-Rat IgG as compared to the blank NP with secondary and the blank NP with no secondary, Figure 5b and 5c respectively. Figure 5a shows the greatest amount of fluorescence with a more even distribution throughout the image, while figure 5b shows few aggregates of fluorescence. The fluorescence seen in 5b can be tied to nonspecific binding or coating of the nanoparticle. While it is present, it is visually still less fluorescent than that of 5a. It is important to note that the fluorescent image in Figure 5b was taken in an area with the highest fluorescence and was not representative of the fluorescent distribution of the whole sample. Figure 5c shows no fluorescence, indicating no fluorescence can be attributed to the silk. This visual data is further supported by the quantitative data provided via the plate reader in Figure 4.

The antibody concentration data in Table 1 was analyzed in Prism 9 using ordinary one-way ANOVA and Tukey's multiple comparisons tests. Compared to the two controls, the IL4 conjugated antibodies tagged with anti-rat secondary antibody quantitatively had significantly greater concentrations of antibody present. This suggests that the antibody found is not due to autofluorescence of the silk or non-specific binding, but rather due to the secondary antibody binding to the conjugated primary antibody on the surface of the nanoparticle. Initially, 6.25 ug/ml IL4 was added during the primary antibody conjugation. From Table 1, the average antibody concentration for IL4 conjugated NPs tagged with secondary was 0.24021 ug/ml. This suggests that 3.84% of the IL4 antibody was taken up and conjugated onto the nanoparticle surface during EDC/NHS. This percentage is likely low due to the expired nature of the primary IL4 antibody. It is also possible that some conjugated antibody was pulled off the nanoparticle surface during ultracentrifugation, as the concentration of antibodies in the supernatants increased with the number of washes. The number of washes will be optimized in the future to prevent the shedding of conjugated antibodies.

For our upcoming experiments, if our tumor-specific antibodies do not arrive promptly, we are considering using a tumor specific antibody from our mentor, Sunny Shaidani, or a general antibody from Olivia Foster to test dual conjugation. Although this would just be a proof of concept and not specific to GBM, it could provide valuable information we could carry on to our experiment with EGFRviii and IL-13Ra2, whenever they arrive. The experimental question that could be answered by practicing dual conjugation with the IL-4 we already have and a different antibody we source could be what concentrations are required of each to successfully have both stick to the nanoparticle. We are going in with the thought of equal concentrations of each antibody, but this could be disproven through experiments.

Future Direction
In the future, U87 cells with target receptors can be isolated using flow cytometry to be cultured and used for *in vitro* testing of uptake and eventually doxorubicin delivery efficiency. The dually conjugated nanoparticles will be fabricated with doxorubicin cores and live/dead assays will be

carried out to test for treatment efficiency and feasibility. Additionally, future experiments could use different antibodies, like EPHa2, or test the conjugation of more than two antibodies. Moreover, after testing the nanoparticles on U87s isolated to express IL13ra2 and EGFRviii, it would be beneficial to test the antibodies on a heterogeneous GBM cell line to establish the clinical relevance of the dual-antibody conjugated nanoparticles.

Project Website: Click Here!
Project Timeline: Click Here!

Participation: List individual contributions of each group member to the project
- **Maddie Yost**: GBM lit review research, antibody (EGFRviii) lit review research, lead silk processing and cell culture training for group, added to/edited Biweekly report and Midterm Technical Report/Presentation, Zoom meeting with Dr. Saul Priceman (PhD from City of Hope) who is an expert in the field for this type of research, sonification training with Sunny, silk nanoparticle creation & EDC/NHS protocol lab work, trained ultracentrifugation, performed secondary antibody conjugation and imaging for test IL-4 nanoparticles
- **Olivia Zeiden**: GBM lit review research, breast cancer lit review, silk processing, silk nano particle training, added to/edited Biweekly report, updated project timeline with relevant dates and aims, EphA2 antibody lit review, added to the midterm report and presentation, Zoom meeting with Dr. Saul Priceman (PhD from City of Hope) who is an expert in the field for this type of research, sonification training with Sunny, continued contact with Saul Priceman about cell lines, silk nanoparticle creation, performed secondary antibody conjugation and imaging for test IL-4 nanoparticles
- **Sabrina Zhang**: GBM lit review research, hepatocellular carcinoma lit review, silk processing and U87 cell culture training, silk nanoparticle training, edited project schedule, wrote brief blurb for Sunny on the need for our proposed GBM treatment, added to/edited Biweekly report, lit review for IL-13Ra2 as potential target, Midterm Mid Semester Technical Report, Zoom meeting with Dr. Saul Priceman (PhD from City of Hope), sonification training with Sunny, set up order form for antibodies, found 2 EDC/NHS kits for potential use, silk nanoparticle creation & EDC/NHS protocol lab work, performed secondary antibody conjugation and imaging for test IL-4 nanoparticles, data analysis.
- **Elysia Chang**: GBM lit review research, silk processing and cell culture, nanoparticle training, added to/edited Biweekly report, created the project timeline, HCC initial research, EGFRviii research to see if it is a good target, created Midterm Presentation, created/added to Midterm Mid Semester Technical Report, Zoom meeting with Dr. Saul Priceman (PhD from City of Hope) who is an expert in the field for this type of research, sonification training with Sunny, created/edited website, silk nanoparticle creation & EDC/NHS protocol lab work

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Appendix 1: Project Schedule

Aims	Sub-Aims	Completion	September	October	November	December	January	February	March	April	May
Define Project		100	🟩								
1st Biweekly Report		100	🟩								

Task	Description	Value										
Antibody Lit Review	Choose Antibody for Targeting Research	100		██								
Silk Processing		100		██								
Silk Nanoparticles		100		██								
2nd Biweekly Report		100		██								
Cell Culture Training		50		██								
Technical Proposal Report Draft		100		██								
Project Presentations		100		██								
Preparing Nanoparticles		100			██							
Start Website	Add in Home Page, People Sections, References, and Project Update	100			██							
3rd Biweekly Report		100			██							
Risk Assessment Analysis		100			██							
Update Website	Update Project Section and References	100			██							
4th Biweekly Report		100			██							
Update Website	Update Project Section and References	100			██							
Project Presentations		100				██						
Finalized Website		100				██						
Technical Report		100				██						
Order Materials Necessary for Antibody Conjugation		100					██					
Make silk		100					██					
Make nanoparticles		100						██				
Biweekly Report #5		100						██				

Start EDC-NHS of IL-4		100									
ELISAs of IL-4 conjugated antibody		100									
Biweekly Report #6		100									

Appendix 2: Antibody Decision Matrix

Consideration	Weight	IL-13Rα2	EGFRviii	EPHA2
Expression in healthy tissue	5	3	5	3
Presence in GBM cells	5	5	3	4
Relevance/available background info	1	5	5	3
		45	**45**	**38**

Appendix 3: Risk Analysis

Item Number	Process Function/Requirement	Risk Analysis					SEVERITY	OCCURRENCE	RPN	Risk Control	SEVERITY	OCCURRENCE	RPN	Risk reduced as far as possible (afap)?*	Risk/Benefit Analysis
		HAZARD (Potential cause of Hazard/Potential Failure Mode)	HARM (Potential adverse effect/Potential effect of failure)	Potential causes/mechanisms of failure	Currnet Process Controls - Prevention, Detection					RISK MITIGATION					Benefits Outweigh Risks? (Yes/No)*
1	*Conjugation*	*Poor conjugation efficacy of antibodies (anti-EGFRviii and anti-IL-13Ra2) to silk nanoparticles*	*Process: off target targeting due to lack of specificity*	*EDC/NHS failure*	*Flow cytometry*	4	3	12		*Ensure EDC/NHS protocol being followed is correct*	4	2	8	*afap*	*Yes*

						S	O	RPN		S	O	RPN		
2	*Reproducibility of silk batch*	*Inconsistent nanoparticle size and molecular weight*	*Process: cellular uptake ability and potential skewing of data*	*pH of deionized water too acidic, cross contamination of equipment in the silk processing room*	*Sterilization of equipment before use*	2	4	8	*Check pH of water before use*	2	2	4	*afap*	*Yes*
3	*Accuracy of testing*	*Poor accuracy of machine used during silk nanoparticle conjugation*	*Process: inconsistent silk size and MW across samples*	*DLS machine failure*	*Calibrating machine*	2	4	8	*Purchase a new DLS machine as this one may be broken*	2	2	4	*afap*	*Yes*
4	*Patient Receptor Expression*	*Patient doesn't express IL13Ra2 or EGFRviii receptors*	*Off-targeted binding due to lack of specific receptors*	*Patient genetics*	*Checking tumor cells first to see what is being expressed in the patient*	4	2	8	*Test only on patients that express both receptors through initial screenings*	4	1	4	*afap*	*Yes*
5	*Patient Targeting*	*IL13Ra2 and EGFRviii attack other areas expressing receptors of interest*	*Off-targeted binding due to expression on healthy tissue*	*Healthy tissue receptor expression*	*Choosing receptors that have low healthy tissue expression*	4	4	16	direct injection could decrease off target responses	4	2	8	*afap*	*Yes*
FINAL	Overall Residual Risk is Acceptable (Yes/No):													*Yes*

Scoring Metrics
Project Description: 2 points
Engineering Design Elements: 8 points (1 point per question)
Advice - Structure the design reports so they evolve into your mid semester and final technical reports, as a living document to make your writing and reporting easier