#5 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 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 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 via SDS-PAGE and fluorescence microscopy. 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 literature reviews. 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 SDS-PAGE and fluorescence microscopy.

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 SDS-PAGE and 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. Therefore, 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?

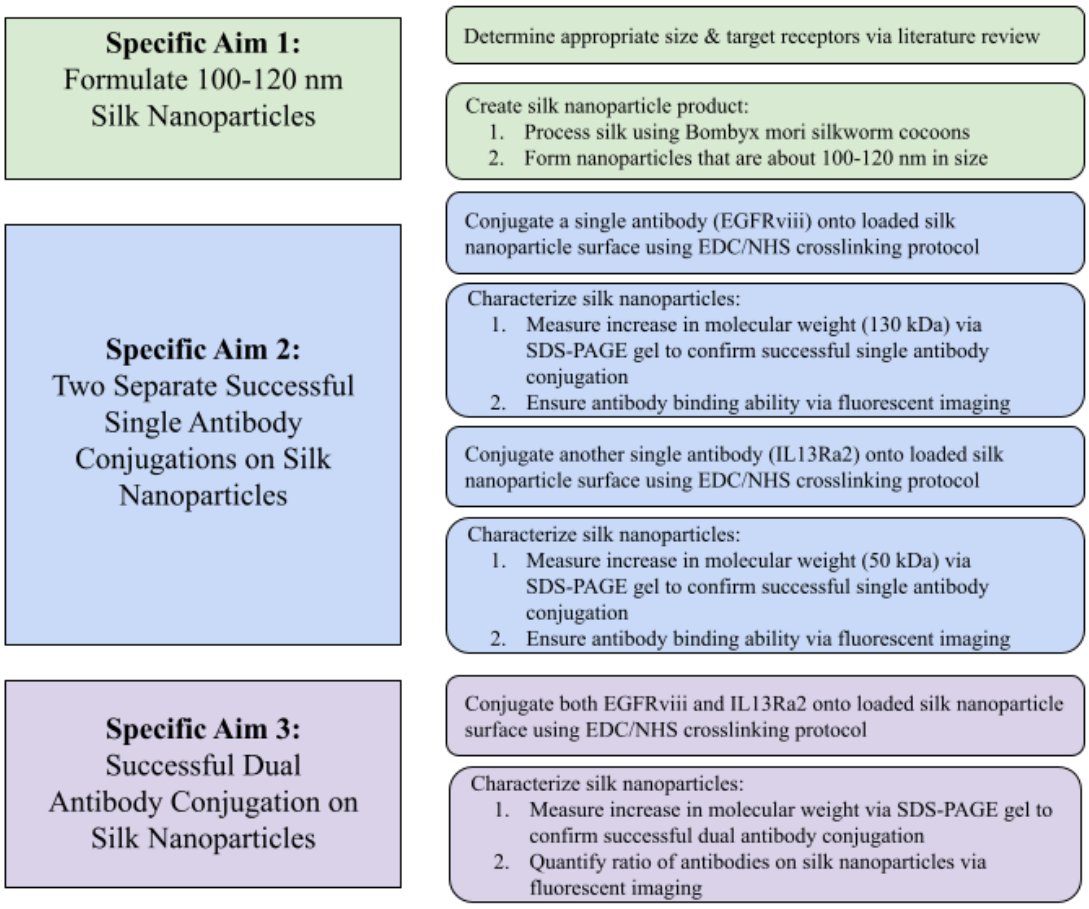
- To start, silk processing in the Kaplan Lab will be conducted to formulate a batch of silk solution. The silk solution will be processed further in the Kaplan Lab to become silk nanoparticles. A DLS machine will be used to validate that the nanoparticle size is close to the project goal of 100-120 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. Successful conjugation of these antibodies onto our nanoparticles will be validated using SDS-PAGE to see an increase in molecular weight. For the anti-EGFRviii, we are expecting a molecular weight increase of around 130 kDa and around 50 kDa for the anti-IL13Ra2 conjugation. Finally, antibody binding function will be confirmed using fluorescence plate reading to show that the fabrication is working appropriately. 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	FTIR Analysis, 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 60°C oven, and weighing 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 lot by cell line & nanoparticle size[3])	FITC and lysosomal fluorescent microscopy
Cell receptor	Cells express one	This is important to test the	SDS-PAGE and

expression	of each receptor	efficacy of dual antibody conjugation, making sure both biomarkers are expressed whether we transfect cells with both, or receive IL-13Ra2 cells and transfect with EGFRviii	Fluorescence microscopy

Design Flow Chart



- 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 use 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 has been found to 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 is 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 are 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.

PROJECT TIMELINE: Click Here!

SPECIFIC AIMS, METHODS, AND RESULTS:

Specific Aim 1: Formulate 100-120 nm diameter silk nanoparticles:

Specific aim 1 will establish a protocol for fabricating consistently sized silk nanoparticles. First, 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 Aim 2: Conjugate anti-EGFRviii and anti-IL13Ra2 separately

In specific aim 2, antibodies anti-EGFRviii and anti-IL13Ra2 will be successfully 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, we will conjugate the antibodies onto silk nanoparticles in two separate experiments. Binding efficacy will be quantified using SDS page against unconjugated nanoparticles. We expect to see an increase of ~130kDa and ~50kDa for anti-EGFRviii and anti-IL13Ra2 respectively in successfully conjugated nanoparticles. Next, we will treat anti-EGFRviii and anti-IL13Ra2 nanoparticles with secondary mouse and rabbit antibodies respectively for plate reading to check for maintained antibody structure and binding ability. If time allows, additional experiments using ELISA can confirm specific binding.

Specific Aim 3: Dual conjugation of anti-EGFRviii and anti-IL-13Ra2

Specific aim 3 will produce dually conjugated silk nanoparticles with anti-EGFRviii and anti-IL-13Ra2. Dual conjugation was chosen due to previous literature linking it to enhanced therapeutic efficacy in tumor models[24]. We follow EDC/NHS protocols established in specific aim 2 to conjugate both anti-EGFRviii and anti-IL-13Ra2 antibodies onto silk nanoparticles. Binding efficacy will be quantified using SDS page against unconjugated nanoparticles. We expect to see an increase of ~180 kDa for nanoparticles conjugated with both anti-EGFRviii and anti-IL13Ra2. To confirm antibody structure and bind ability, the nanoparticles will be treated with secondary mouse and rabbit antibodies for plate reading. If time allows, anti-IL-13Ra2 and anti-EGFRviii will be bound in different test ratios to determine nanoparticle surface coverage.

Methods

Silk processing and nanoparticle formation are protocols from previous studies, supplied by Nafis Hasan and Sunny Shaidani (Kaplan Lab). The EDC-NHS protocol was supplied by Shawn Cui (Kaplan Lab).

Silk Processing

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 on 9000rpm, 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. The 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

For 6% silk, 500 rpm, 30 min boil, on day 1, add 15-20 mL acetone to a graduated cylinder and transfer acetone to a labeled small glass jar. Place the correctly sized stir bar into a jar (small one, but not the smallest), and secure the jar onto the center of the stirrer 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 in a separate beaker & pour it into a specialized glass dropper. Using the small black knob on the left side of the glass dropper, twist slowly and carefully (think of a titration technique). The solution should drop and fall into the side of the vortex (not the center), which will help to create the desired nanoparticle size of choice. On day 2, add 2-5 mL of DI water (as some of the acetone may have evaporated overnight). For day 3, 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 Kim wipe and hold this 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 sides/bottom), and press stop. Check the size with the particle solutions app on DLS/SEM (on the button at the back of 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

SF(D, E)-EDA		SF(D, E)-Spermine		SF(D, E)-Poly-l-lysine		SF(D, E)-Poly-l-arginine	
SF	500 mg	SF	500 mg	SF	500 mg	SF	500 mg
EDA	166 mg	Spermine	133 mg	PLL	97 mg	PLA	116 mg
EDC	124 mg	EDC	126 mg	EDC	126 mg	EDC	126 mg
NHS	40 mg	NHS	76 mg	NHS	76 mg	NHS	76 mg

This protocol is used to synthesize chemically modified silk fibroin with amines of different sizes to produce cationic SF. The amine conjugation is done using EDC/NHS coupling. Instead of amines though, this protocol will be modified to conjugate antibodies. The SF(D, E)-EDA protocol will be used, substituting the proportions of EDA for the antibody instead. On day 1, reactants can be taken out from the fridge/freezer to allow them to get to room temperature. 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 into each jar 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 (slightly larger) jar with a stir bar, a pipette will be used to collect 2 mL of silk NPs, MES buffer, EDC +NHS, and 50 uL of the antibody. The jar will be set on a stir plate at 200 rpm for 18 hours overnight. On day 2, two tubes with EDC/NHS nano solution will be filled equally and weighed using DI water to balance. The ultracentrifuge will be turned on and set to 60K for 30 minutes at 4°C and run with balanced EDC/NHS nano solutions. The supernatant will be taken out with a needle and resuspended with 3 mL DI water. Each solution will be weighed out to be equal and balanced in the ultracentrifuge. Then, this will be spun again at 60K for 30 mins at 4°C, two more times (three spins total). The supernatant will be removed and stored. The pellets will be soaked in 1 mL of DI water and stored in the freezer.
EDC/NHS calculations:

1. EDC ratio is 500 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

U87 Culturing

After taking 1mL U87 cell line out of the liquid nitrogen freezer and thawing in metal beads around 34C, 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) to the T75, 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 been 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 using a P1000 pipette. A T75 flask with PLO seeded 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. The new T75 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 realized we had created a 10% silk solution, and we wanted a 6% silk solution to get the proper-sized nanoparticles. To do this, we determined 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, almost too small to the point where we think the DLS machine is 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 but 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.

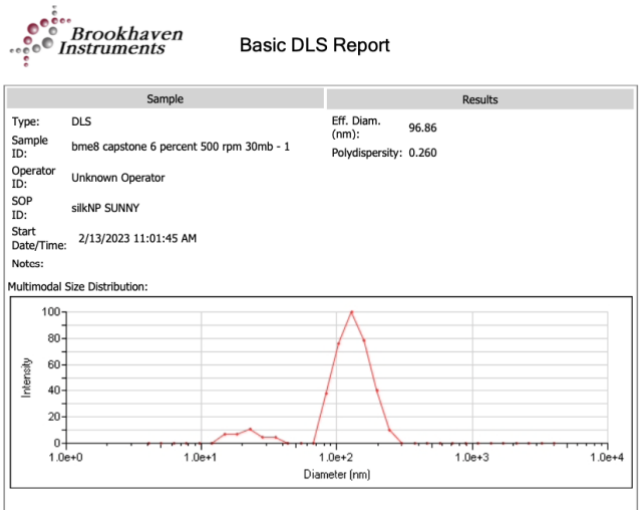


Figure 1. Basic DLS report of silk nanoparticles with an effective diameter of 96.86 nm.

For the next round of silk creation, to make sure 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 at a maximum of 120 nm, so if they end up being on the smaller side like they normally are, we will likely end up in the proper range of 100-120 nm.

To start the EDC-NHS process, we decided to do a test run using leftover IL-4 in 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 above and its efficacy will be detailed in the next report.

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 if time permits. Our project also differs from this since ours would be more targeted due to antibody conjugation.

PROJECT WEBSITE: Click Here!

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 will change our project from targeting EGFRviii to EphA2 to bypass the viral transfection process. Unfortunately, the paperwork was taking too long to process to be useful this semester--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.

Future Directions

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.

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 the group, added to/edited Biweekly report and Midterm Technical Report/Presentation, Zoom meeting with Dr. Saul Priceman (Ph.D. 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
- **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 (Ph.D. 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
- **Sabrina Zhang**: GBM lit review research, hepatocellular carcinoma lit review, silk processing and U87 cell culture training, silk nanoparticle training, edited project schedule, wrote a brief blurb for Sunny on the need for our proposed GBM treatment, added to/edited Biweekly report, lit review for IL-13Ra2 as a potential target, Midterm Mid Semester Technical Report, Zoom meeting with Dr. Saul Priceman (Ph.D. from City of Hope) who is an expert in the field for this type of research, sonification training with Sunny, set up an order form for antibodies, found 2 EDC/NHS kits for potential use, silk nanoparticle creation & EDC/NHS protocol lab work
- **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 (Ph.D. 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

Appendix 1: Project Schedule

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

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		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									
EDC/NHS of IL-4		100									

Singular EDC-NHS of IL13Ra2 & EGFRviii											
Fluorescence of IL13Ra2 & EGFRviii nanoparticles											
Dual Conjugation of EDC-NHS of IL13Ra2 & EGFRviii											
Fluorescence of dual conjugated IL13Ra2 & EGFRviii nanoparticles											

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		Risk Analysis							Risk Control					Risk/Benefit Analysis
	Process Function/Requirement	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	SEVERITY	OCCURRENCE	RPN	RISK MITIGATION	SEVERITY	OCCURRENCE	RPN	Risk reduced as far as possible (afap)?*	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*
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 screening s*	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*

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