BME 8 SEMESTER BIWEEKLY REPORT #3

Project Title: Dual Antibody Conjugated Silk Nanoparticles as a Targeted Delivery System for Glioblastoma Multiforme (GBM) Therapy

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Abstract: 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 is 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 dual antibody conjugation to the silk nanoparticle surface.

Keywords: glioblastoma, targeted therapies, silk nanoparticles, dual conjugation

ELEMENTS OF ENGINEERING DESIGN

What objectives were set?

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 for visualizing fluorescence and measuring the concentration of the antibody. Targeted delivery will be evaluated after this course using a U87 cell line to confirm efficacy in a 2D system.

What was 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 due to IL-13Ra2 having one of the highest expression rates in patients (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 referenced throughout our review of the literature. EDC, in conjunction with NHS, allows for a 2-step coupling of two proteins without affecting the carboxyls of the second protein. Past studies have used flow cytometry to calculate and validate the success of antibody-nanoparticle surface conjugation[2]. The current plan is 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; 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 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 using secondary antibodies 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?

The first 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. The second constraint is

testing the conjugated nanoparticles on U87 cells as 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. The last 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.

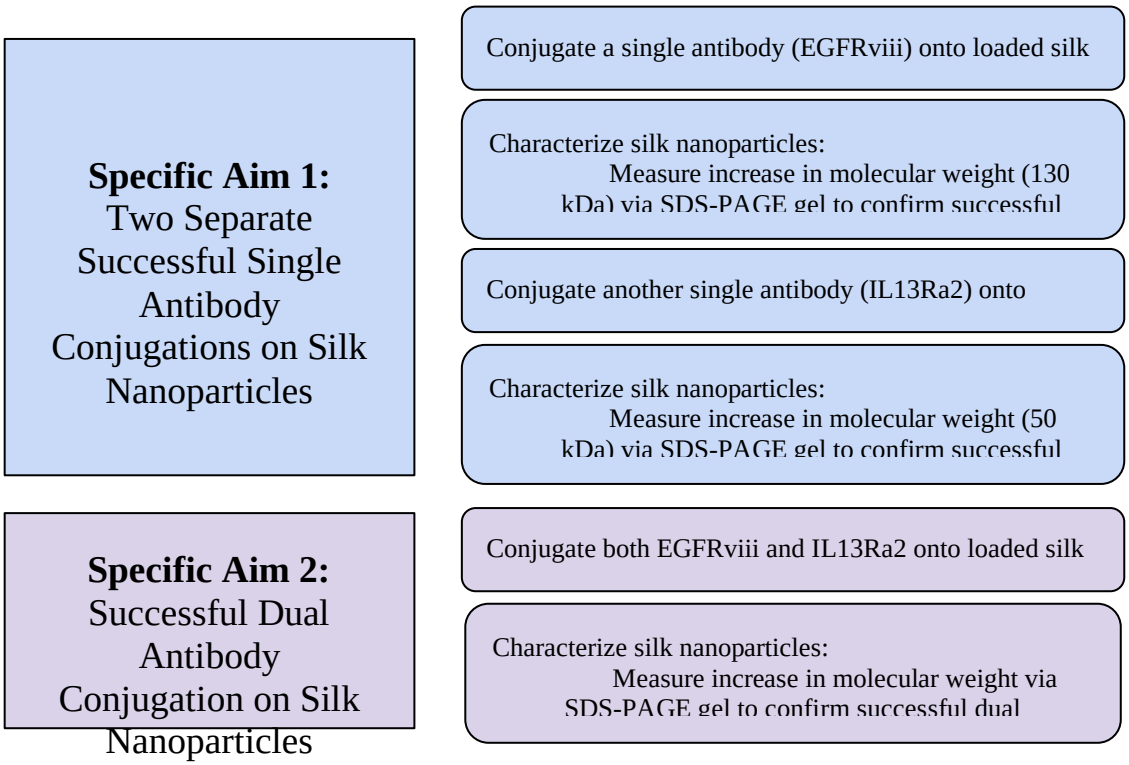
What alternative solutions or changes to the plan will be considered?

From our initial review of the literature, our group ruled out that GBM was the best way to proceed onwards. Initially, we thought to conjugate just one antibody. Our group's most recent 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 will be conducted to formulate a batch of silk solution. The silk solution will be processed further 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. Our initial silk nanoparticles had an effective diameter of 96.86 nm, confirming that our formulations are progressing appropriately. Subsequent batches of silk nanoparticles are currently being created and sizing will be checked appropriately. Anti-EGFRviii & 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 and 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 currently available in the lab.

DESIGN FLOW CHART



This flowchart describes the design of the proposed experiment. Dual antibody conjugated silk nanoparticles will be developed using established protocols by Kaplan et al. and EDC/NHS materials available. DLS 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[3]. GBM is one of the most deadly forms of cancer, with a median survival rate of just 12.6 months after diagnosis[4]. 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[5]. 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[6]. 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 nanoparticle large enough to only target cancerous tissue, yet small enough to be engulfed at an appropriate rate [7].

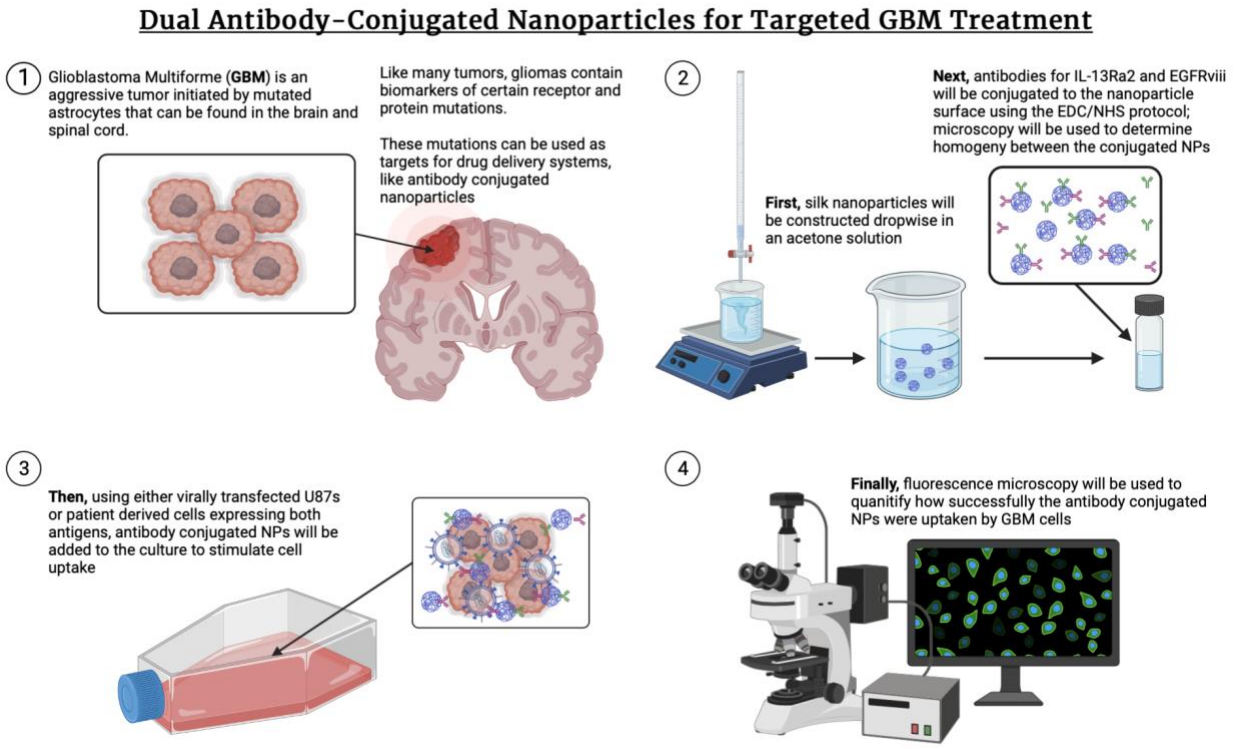
Epidermal growth factor receptors (EGFR) are transmembrane receptor tyrosine kinases (RTK) and are overexpressed in 50% of glioblastomas[8]. Epidermal growth factor variant three (EGFRviii) is a mutated wildtype EGFR expressed on the surface of GBM cells and commonly associated with GBM. This mutation 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 [9, 10]. Some studies go so far as to claim that EGFRviii has never been detected in healthy tissue[10]. 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 expression of VEGF and IL-8[11]. EGFRviii CAR T cells are in Phase I studies and have shown low off-target toxicity[12].

Interleukin-13 receptor alpha2 (IL-13Rα2) was discovered as a glioma marker in 1995 by the Debinski laboratory[13], since then it has become one of the most studied tumor-specific antigens in glioblastoma research[14]. IL-13Rα2 is a high-affinity membrane receptor of IL-13 and is expressed in many tumors[15]. It has been found to be overexpressed in up to 75% of glioma patients[16]. Expression of IL-13Rα2 is high in the testis and placenta but has low expression in other organs[14]. 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[17]. While this trial revealed the dangers of working with IL-13Rα2, it suggests a promise of an antibody more specific to IL-Rα2 to be found and used. Currently, CAR T-cell therapy targeting IL-13Rα2 is now in Phase I clinical trials[18]. 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[19]. EGFRviii and IL-13Rα2 targeted therapy have both been associated with recurrent antigen loss variants after initial treatment[20].

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. Silk will be made using a previously established protocol from Kaplan et al. 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, a silk solution will be produced using 6% concentration, boiled for 30 min, and spun at 500 rpm for nanoparticle formulation. The nanoparticles will be fabricated by solvent emulsion techniques shown in the methods section, chosen because it allows for precise control over nanoparticle size formation at this range. The size will be measured by dynamic light scattering (DLS), and a distribution of 100-120 nm will be acceptable for further processing. 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!

UNIFYING FIGURE FOR THE PROJECT



We were also able to create a unifying figure for our entire project, which is a simple figure that provides a visual of our project: the problem of interest, objectives, and end goals.

SPECIFIC AIMS, METHODS, AND RESULTS:

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 will successfully conjugate 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[21]. 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 our experiment progresses.

Methods

Silk Processing[22]

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. For silk concentration calculations, see Appendix 5.

Silk Nanoparticles[23]

For 6% silk, 500 rpm, 30 min boil, on day 1, 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 and no sound should be coming from the stir bar. Measure out 4 mL silk & pour it into a specialized glass dropper. Using the 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 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 hold the 15 mL tube to the sonicator tip. Sonicate at 30% amplitude for 30 seconds 2x and move the tube up and down (without touching the sides or the bottom). Check the size with the particle solutions app on DLS/SEM. For nanoparticle concentration calculations, see Appendix 5.

EDC/NHS[24]

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. For EDC/NHS calculations, see Appendix 5.

Ultracentrifugation[24]

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. For detailed directions on each of the 3 spins, refer to Appendix 5.

Secondary Antibody Tagging[25]

Throughout various experiments, the study will determine the target value for nanoparticle antibody expression based on which values optimize cellular uptake. This will be accomplished through secondary antibody tagging. Once nanoparticles are thawed at room temperature, they will be resuspended in a sealing tube and sonicated. A total of 5 tubes will be generated using nanoparticles, blocking buffer, and secondary antibodies. These 5 tubes will be shaken on a stir plate covered in tin foil for 2 hours before following the **Ultracentrifugation** protocol again. For detailed directions on the preparation of each tube, refer to Appendix 5.

Imaging

To validate the antibody conjugation, a 96-well plate reader and fluorescence microscopy was utilized. An expected size increase of 130 kDa is expected for anti-EGFRviii, and an expected size increase of 50 kDa is expected for anti-IL-13Ra2. Cells will express fluorescence, but it is important to note that some of it may also be attributed to the silk, which naturally exhibits some fluorescence. Imaging will help us validate whether the conjugation was completed successfully. For detailed directions and a step-by-step protocol, refer to Appendix 5.

Results

13 mL of silk solution was successfully obtained and evaluated for concentration. The original silk solution was 10% concentration, and we wanted a 6% silk solution to obtain our ideal sized nanoparticles. To do this, 18 mL of our 10% silk solution was taken and added to 12 mL of DI water to reduce the silk concentration to 6%. A 500 rpm, 6% silk, 30 min boil technique was utilized 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 during our first run through last semester, which was very small, suggesting that the DLS machine was broken and in need of service. New silk was created this semester to test any other issues that might be causing the discrepancies in size.

Another batch of silk was processed last month, following the same protocol with 6% silk, 30 min boil, and 500 rpm, and around 10mL of 96.86 nm silk nanoparticles was obtained, as seen in Figure 1 below. These nanoparticles were 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.

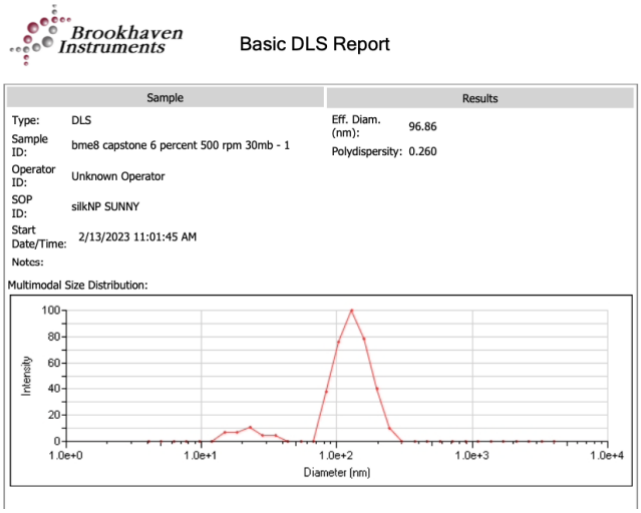


Figure 1. Basic DLS report of silk nanoparticles with an effective diameter of 96.86 nm.

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. For the next round of silk creation that we plan on completing during the last week of March, 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 and seem to be, 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 as a proof of concept using leftover IL-4 from the lab to see 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 below, 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 was quantified as seen in Table 1. Figure 4 shows that among the nanoparticles, antibody concentration was significantly different, suggesting that the IL4 conjugation was successful. Furthermore, qualitative differences observed via fluorescence microscope (Keyence) support the quantitative findings of successful IL4 conjugation (Figure 5).

We conducted the first trial of dual conjugation using rat anti-rVEGF and rabbit PSTAT3 as our selected antibodies. We followed the EDC/NHS protocol above and used the same concentration of each antibody in a 1:1 ratio where 166 mg of each was added to the reaction. Following the same protocol as single antibody conjugation, goat anti-rat AlexaFluor 594 and goat anti-rabbit AlexaFluor 488 secondary antibodies were used. We created a standard curve for the new secondary antibody, blue goat anti-rabbit AlexaFluor 488. Fluorescence imaging using the Keyence microscope and ImageJ analysis showed evidence of overlap between the two antibodies, suggesting dual conjugation of two antibodies on a single nanoparticle is possible (Figure 6). ImageJ found that 19.909% of the total antibodies conjugated overlapped.

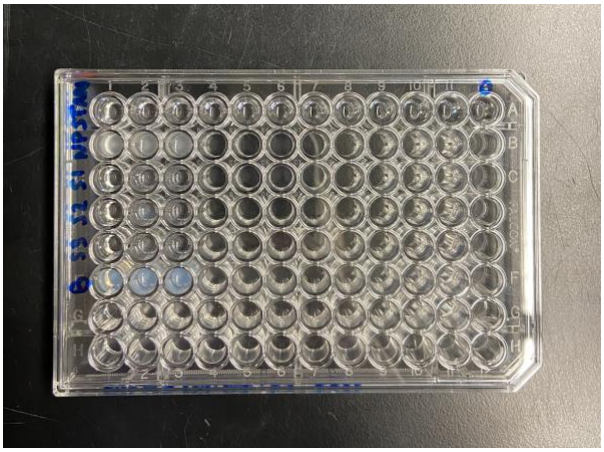


Figure 2. 96 well plate used for Keyence fluorescence microscope and fluorescent plate reader

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.

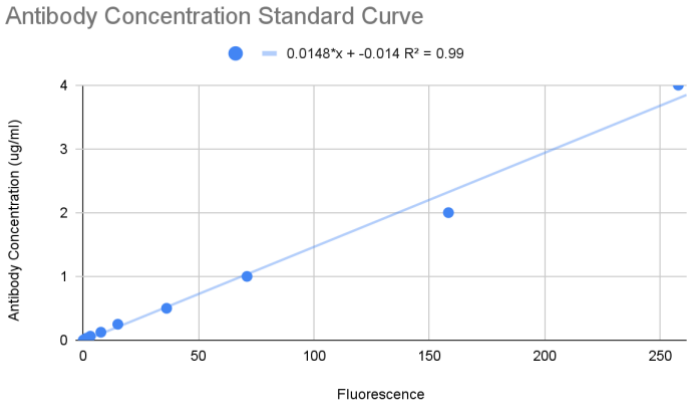


Figure 3. Standard curve of Goat anti-Rat IgG Alexa Fluor™ 594 secondary antibody ranging from 0-4 ug/ml concentration. $Concentration = 0.0148 \bullet fluorescence - 0.014$.

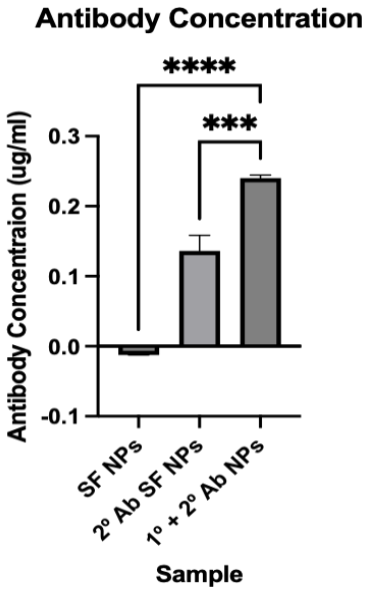


Figure 4. Antibody concentrations of blank nanoparticles, blank nanoparticles incubated with secondary antibody, and IL4 conjugated nanoparticles incubated with secondary antibody. (n=3; **** $p<0.0001$; *** $p = 0.0002$).

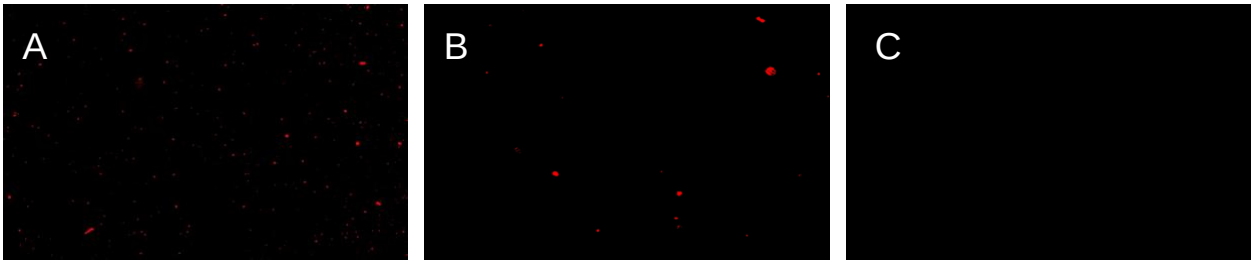


Figure 5. Fluorescent images of nanoparticles using the Keyence machine. (A) IL4 conjugated NPs tagged with Goat anti-Rat IgG Alexa Fluor™ 594 secondary antibody; (B) blank NPs incubated with secondary antibody; (C) blank NPs with no secondary antibody. (1-6 exposure, 362 µm scale).

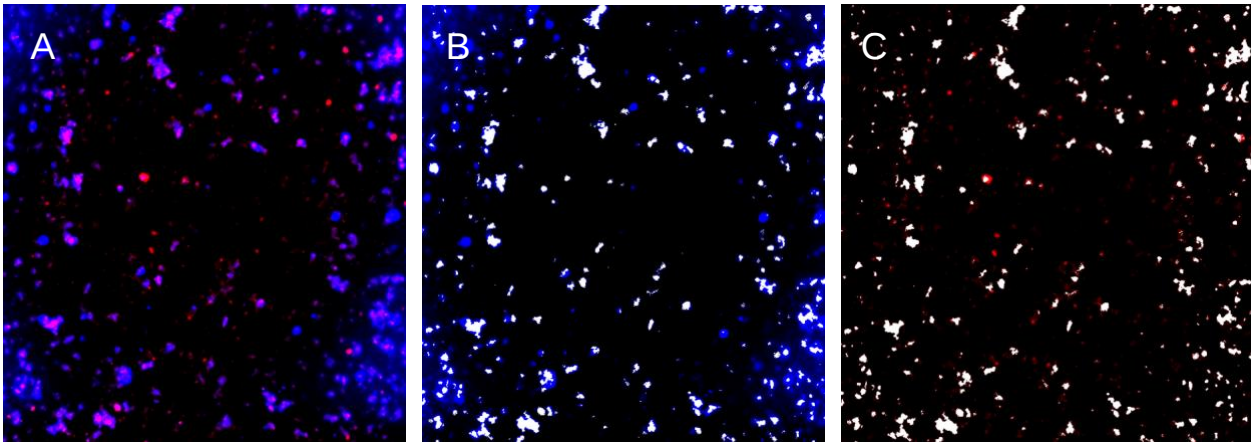


Figure 6. Fluorescence images of nanoparticles conjugated with PSTAT3 and anti-rVEGF. Red signifies the presence of anti-rVEGF, and blue signifies the presence of PSTAT3. White signifies the area where the two signals overlap. (A) Fluorescence signals of both antibodies. (B) PSTAT3 signal with the area of overlap shown. (C) anti-rVEGF signal with the area of overlap shown.

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.

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, a 13 mL 10% silk solution was obtained that we were able to change the silk concentration to 6% to create nanoparticles with about 74 nm diameter. These nanoparticles will be used in our experiments to test conjugation, but 100-120 nm nanoparticles will be used to test efficacy. It was good that we were able to have a trial run surrounding creation and were able to test out certain bugs like the DLS machine not working properly so that next time 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 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 kept 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. 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. 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. IL-4 rat antibody was conjugated onto silk nanoparticles, and 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.

Figure 5 displays the resulting fluorescence of the secondary antibody imaging. Figure 5a presents the fluorescence of the IL4 conjugated nanoparticles tagged with Goat anti-Rat IgG as compared to the blank nanoparticles with secondary and the blank nanoparticles 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 dual conjugation experiments, fluorescence imaging of the secondary antibodies showed that not only were both antibodies able to be conjugated, but they were able to do so in the same area. We were further able to quantify the overlap: 19.909% of the total signal from both secondary antibodies had overlapping areas. This can be seen in Figure 6B and 6C, where the white area is 19.909% of the blue and red areas summed together. While the nanoparticles were sonicated prior to imaging, the nanoparticles seen were still aggregates. Previous literature has shown that X Ray photon spectrometry as well as direct protein assays have been used to determine if the antibodies are conjugated to the same individual nanoparticle. We will be doing further research to understand other feasible mechanisms for us to determine the presence of both antibodies on the surface of the nanoparticle. However, preliminary findings show that the two antibodies are able to colocalize and conjugate in the same specific areas. This is very promising

as dual conjugation has not been widely studied, and these findings show we are getting closer to successfully dual conjugating two antibodies onto the same silk nanoparticle.

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 the experiments we conducted are using PSTAT3 and anti-rVEGF, not our tumor-specific antibodies, the information we are collecting is proof of concept. This could provide valuable information we could carry on to our experiment with EGFRviii and IL-13Ra2, whenever they arrive.

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.

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
- **Olivia Zeiden**: GBM lit review research, Breast cancer lit review (ultimately ruled out), met with maddie to learn silk processing, met with Sunny for silk nano particle training, added and edited Biweekly report, updated project timeline with relevant dates and aims, EphA2 Antibody lit review, added to the midterm report and presentation,added to midterm report #3, 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, edited to biweekly report, animated K-12 poster
- **Sabrina Zhang**: GBM lit review research, hepatocellular carcinoma (ruled out target) lit review, met with Maddie for silk processing and U87 cell culture training, met with Sunny for silk nanoparticle training, edited project schedule, wrote brief blurb for Sunny on the need for our proposed GBM treatment, added to/edited Biweekly report #1, lit review for IL-13Ra2 as potential target, added to Biweekly report #2, 3, 4 and 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, set up order form for antibodies, found 2 EDC/NHS kits for potential use
- **Elysia Chang**: GBM lit review research, met with Maddie and the group to learn silk processing and cell culture, met with Sunny to conduct silk nanoparticle training, added to/edited Biweekly report, created the project timeline, HCC initial research (ruled out target), EGFRv3 research to see if it is a good target, created Midterm Presentation file

(because unable to present in-person/on Zoom due to the Society of Women Engineering 2022 Conference in Texas), 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, animated K-12 poster.

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	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 Web Site		**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**									
K-12 Poster		**100**									
Mid Semester Presentation		**100**									
Biweekly Report #6		**100**									
Dual Conjugation of EDC-NHS of anti-pStat3 and anti-rVEGF		**100**									
Fluorescence of dual conjugated anti-pStat3 and anti-rVEGF nanoparticles		**100**									
Create new batch of silk NPs		**100**									
Biweekly Report #7		**100**									
Singular EDC-NHS of IL13Ra2 & EGFRviii	*waiting on antibody delivery										
Fluorescence of IL13Ra2 & EGFRviii nanoparticles	*waiting on antibody delivery										
Dual Conjugation of EDC-NHS of IL13Ra2 & EGFRviii	*waiting on antibody delivery										
Fluorescence of dual conjugated IL13Ra2 & EGFRviii nanoparticles	*waiting on antibody delivery										
Biweekly Report #8											
Biweekly Report #9											

Final technical poster presentations											
Final web site											
Final technical report											

Appendix 2: Antibody Decision Matrix

Consideration	**Weigh t**	**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: 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/SEM imaging
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[26])	FITC and lysosomal fluorescent microscopy or flow cytometry
Cell receptor expression	Cells express one of each receptor	This is important to test the 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	Flow cytometry and/or Western blot

Appendix 4: 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 (EPHa2 and IL13Ra2) 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 EphA2 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 EphA2 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*

Appendix 5: Methods (Extended)

Silk Processing[22]

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[23]

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[24]

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[24]

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[25]

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 1º + 2º 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

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