BME SENIOR CAPSTONE PROJECT

Project Title: Antibody Conjugated Silk Nanoparticles for Targeted Doxorubicin Delivery in Glioblastoma Multiforme (GBM)

Names: Maddie Yost, Sabrina Zhang, Olivia Zeiden, Elysia Chang

Faculty Advisor: Dr. David Kaplan

PI/Mentor: Sunny Shaidani

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, so a targeted delivery system for chemotherapy using antibody-conjugated silk nanoparticles would be 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 dosing and tumor uptake, induce successful antibody conjugation to the silk nanoparticle surface, and determine the proper antibody quantity required to have the nanoparticle be attracted to targeted receptors expressed by U87 cells.

Keywords: glioblastoma, targeted therapies, silk nanoparticles

ELEMENTS OF ENGINEERING DESIGN

What was designed?

We plan on formulating a dual-antibody conjugated nanoparticle loaded with doxorubicin to target GBM tumor cells. Our target of interest is an antibody for a receptor expressed on the surface of many mutated GBM cells and not expressed 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 will determine what is the best antigen combination to target in this situation based on striking a balance between the expression levels in GBM versus expression levels in surrounding healthy brain tissue. Currently, we are focusing on EphA2, EphA3, MGMT, EGFRviii, and IL-13Rα2 as potential targets. For our project, we will design a protocol to conjugate antibodies to the silk nanoparticle. It will likely be done using EDC/NHS, a crosslinking technique we have learned about through literature review. EDC, in conjunction with NHS, allows for a 2-step coupling of two proteins without affecting the carboxyls of the second protein. The silk nanoparticles will be loaded with doxorubicin so that when the nanoparticles are bound and endocytosed by the cells, the drug will be released within, killing them. Additionally, we will conduct a literature search to determine the appropriate nanoparticle size for proper dosing and the ability for tumor uptake.

What objectives were set?

The objective of the project is to use antibody-conjugated silk nanoparticles as a potential method of targeted delivery for the treatment of Glioblastoma Multiforme (GBM). We aim to develop a 3D GBM model by seeding silk sponges with U87 cells that are transfected with a mutation of choice to carry the receptor we plan to target. We also plan to formulate silk nanoparticles of an appropriate size, loaded with doxorubicin, and conjugated with our antibodies of choice. We will add these nanoparticles to the 3D model and conduct imaging techniques such as live dead assays to quantify and analyze the efficacy of the drug delivery system. We also plan on validating antibody attachment to the particle surface using western blots, ELISAs, and fluorescent tagging. These objectives were chosen as they are crucial steps for developing and testing the nanoparticles to evaluate their potential and efficacy as a future treatment. 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 address an unmet need to explore slowing down disease progression or relapse while decreasing major side effects.

How were basic science, math, and/or engineering sciences applied?

Some of the scientific and engineering methods that need to be applied are silk processing, nanoparticle formation, antibody conjugation, doxorubicin loading of the nanoparticles, silk sponge formation for 3D brain model testing, and cell culturing. We will also likely employ western blots, ELISAs, fluorescent tagging, or mass spectrometry in order to detect the presence of desired proteins and antibodies.

How were the objectives tested and evaluated?

The objectives will be tested and evaluated by three specific aims. Firstly, we need to define the best antibody and receptor for targeting GBM through extensive literature reviews on the subject matter. This aim has already been partially achieved by identifying a dual targeting nanoparticle

drug delivery approach and technique, focusing on IL-13Ra2 combined with either EphA2 or EGFRviii. Secondly, we need to culture and seed target-receptor transfected U87 cells on 3D silk sponges, and be able to test for efficacious antibody-receptor binding. This aim will be tested and evaluated through one of the following methods: western blots, ELISAs, and/or fluorescent tagging of receptor to antibody to secondary fluorescently tagged antibody. Finally, we need to determine to control and evaluate the ratio of the two antibodies for the best specific targeting and uptake, as well as the nanoparticle conjugation method. This will be evaluated by more literature review (particularly with regards to EDC/NHS technique) to help determine how much we need, how much will stick, and how to document this.

What realistic constraints were considered?

One of the realistic constraints is understanding the right receptor to target; currently, our group is leaning towards a dual-targeting nanoparticle drug delivery technique, combining IL-13Ra2 with either EGFRviii or EphA2. 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. Finally, the U87 cells we are planning on using require transfection for the expression of biomarkers commonly found in patient tumors. None of us have ever performed viral transfection before, so this is a new area requiring training and certificates from the Biosafety Office. If this is needed for our project this year, we will need GFP/RFP expressing cell lines, for example. 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. The lab we are working in has experience with doxorubicin, so we were also constrained by only targeting tumors for which doxorubicin is an effective therapy.

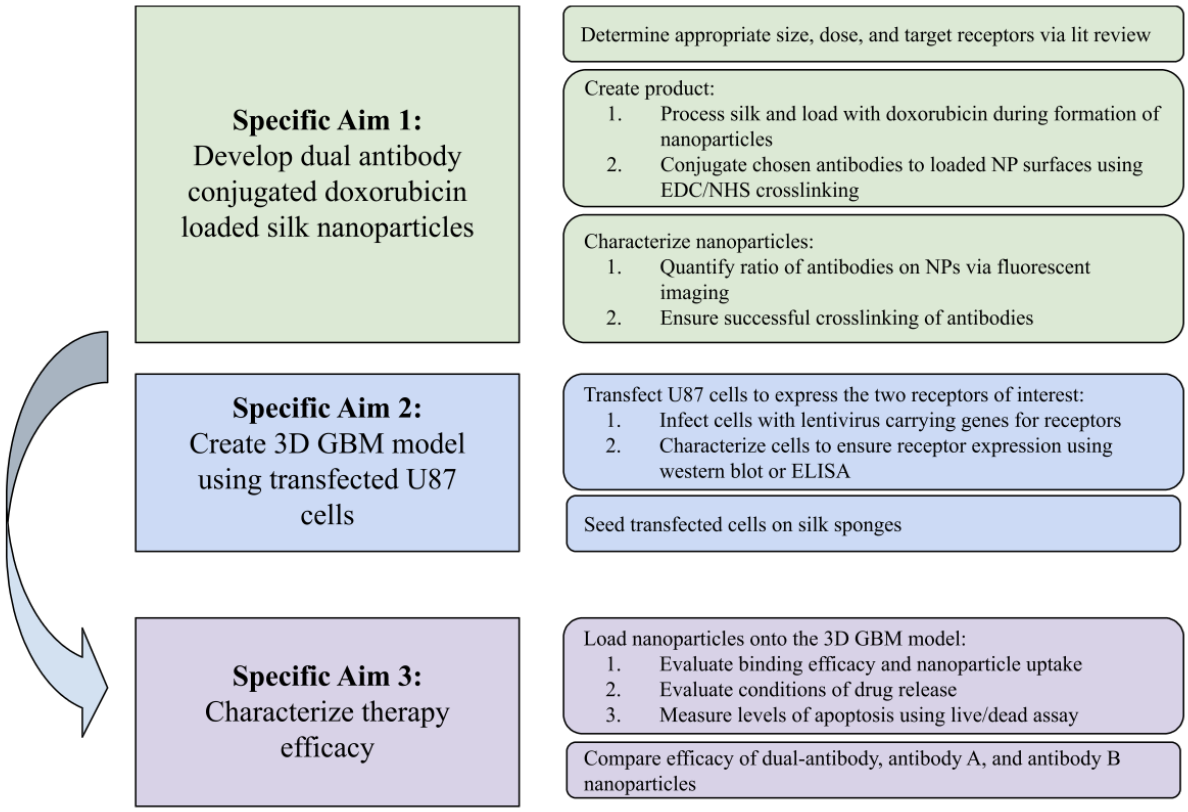
What alternative solutions were considered?

One alternative solution to the plan that we were considering, initially, was to focus on using antibody-conjugated silk nanoparticles as a potential method of targeted delivery for the treatment of Hepatocellular Carcinoma (HCC) since nanoparticles commonly cluster in the liver due its role in the body and the hepatic portal vein. Ultimately, we determined that HCC was not a great target for these nanoparticles as we wanted to use doxorubicin as our loaded drug, which is not a common treatment for HCC. As a result, our group circled back and decided that GBM was the best way to proceed onwards for now. 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, combining IL-13Ra2 with either EphA2 or EGFRviii. This will allow us to target a greater population of GBM cells while reducing off-target interactions.

To what extent did the final result meet the set objectives?

Some of the milestones and results that we have achieved so far include learning how to process silk and culture U87 cells in the Kaplan Lab (led by one of our group members Maddie) as well as learning how to make silk nanoparticles from Sunny (our lab mentor). A future quantitative milestone would include a protocol that has reproducible significant efficacy in producing either IL-13Ra2 and EGFRviii OR IL-13Ra2 and EphA2 antibody-conjugated silk nanoparticles.

DESIGN FLOW CHART



This flowchart describes the design of the proposed experiment. We will develop the dual antibody conjugated doxorubicin loaded silk nanoparticles using established protocols used by the Kaplan Lab and EDC/NHS protocols published online, and we will characterize the nanoparticles produced. We will also transfect U87 cells to express our target receptors. These cells will be cultured, then later seeded onto silk sponge scaffolds to create a 3D GBM model. Together, specific aims 1 and 2 will be used in conducting specific aim 3. The loaded nanoparticles will be loaded onto the 3D GBM model, and its performance will be evaluated. The efficacy of dual antibody and single antibody NPs will be compared to determine the best antibody coating for GBM targeting.

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[1]. GBM is one of the most deadly forms of cancer, with a median survival rate of just 12.6 months after diagnosis[2]. 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, are able to 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[3]. 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[4]. Further research will be done to determine the suitable size and loading dosage for the doxorubicin-loaded silk nanoparticles, for which we will follow an established protocol. Nanoparticles around 100 nm in the bloodstream are known to be too big to enter healthy tissue, but are able to 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 [5].

Epidermal growth factor receptors (EGFR) are transmembrane receptor tyrosine kinases (RTK) and are overexpressed in 50% of glioblastomas[6]. 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, 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 [7, 8]. Some studies go so far to claim that EGFRviii has never been detected in healthy tissue[8]. 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[9]. EGFRviii CAR T cells are in Phase I studies and have shown low off-target toxicity[10].

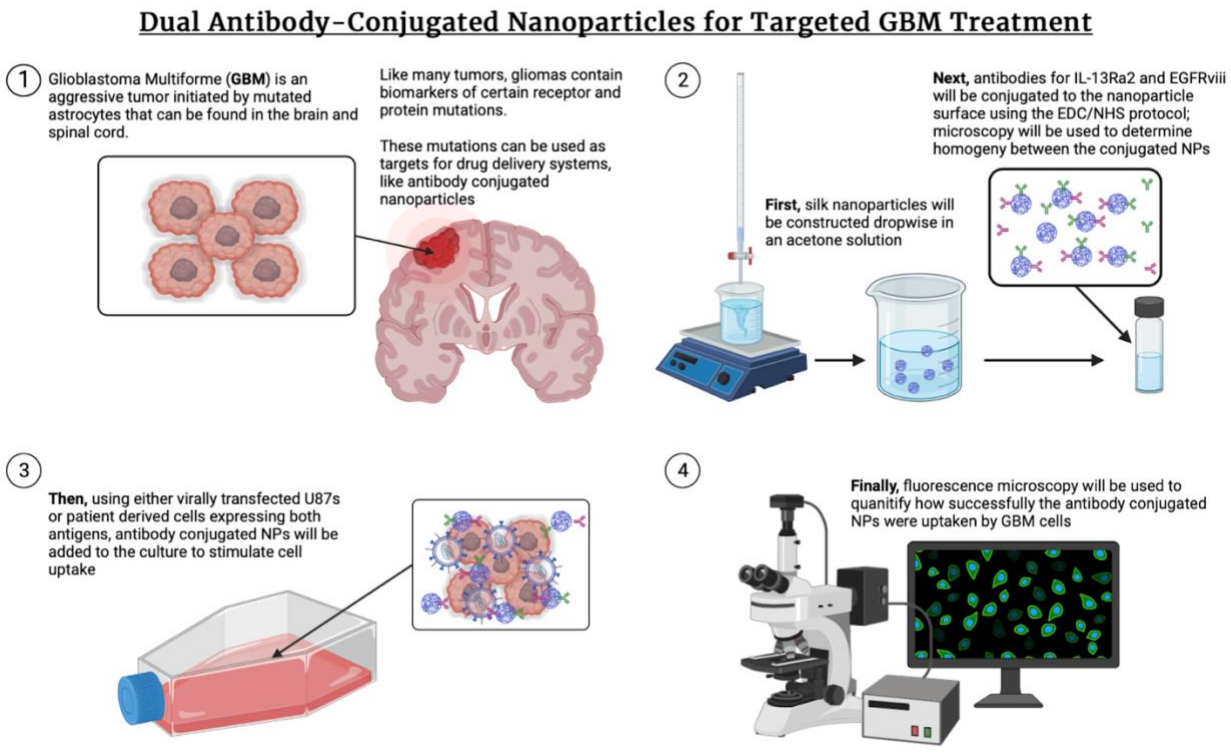
Eph receptors are a class of tyrosine kinase receptors, and they are divided into A and B categories to indicate their extracellular domains. Subclass A indicates they are anchored to the membrane through glycosylphosphatidylinositol (GPI) linkage[11]. EphaA2 receptor was first discovered in 1990 and has since become a highly relevant receptor due to its abundance in several solid tumors[11, 12]. EphA2 is over-expressed in ~60% of GBM tumors and is present in 98% of cells at moderate and or strong levels[13]. It is found to have a low-level expression in normal brain tissue. EphA2 is an attractive target since its associated with poor prognosis and survival due to its role in tumor cell proliferation, growth, and neovascularization [14]. Functionally, EphA2 is a transcriptional target of the Ras-MAPK pathway[15]. Additionally, it has been found that in cancer cells, EphA2 receptors have ligand-independent kinase activity[16].

Interleukin-13 receptor alpha2 (IL-13Rα2) was discovered as a glioma marker in 1995 by the Debinski laboratory[17], since then it has become one of the most studied tumor-specific antigens in glioblastoma research [18]. IL-13Rα2 is a high-affinity membrane receptor of IL-13 and is expressed in many tumors[19]. It has been found to be overexpressed in up to 75% of glioma patients[20]. Expression of IL-13Rα2 is high in the testis and placenta but has low expression in other organs[18]. 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[21]. 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[22]. Dual combinations of IL-13Rα2 and EphA2 have shown to be expressed in 90% of GBM patients} indicating promising data for better targeting specificity[23]. EGFRviii and IL-13Rα2 targeted therapy has both been associated with recurrent antigen loss variants after initial treatment[24].

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, doxorubicin loaded dual-antibody conjugated nanoparticles will allow for the targeted delivery of chemotherapy to GBM cells; compared to traditional therapies, the successful formulation of this therapy will result in more efficacious treatment for better patient outcomes.

UNIFYING FIGURE FOR THE ENTIRE PROJECT



This simple figure provides a visual of our project: the problem of interest, objectives, and end goals.

SPECIFIC AIMS, METHODS, AND RESULTS

Specific Aim 1: Develop dual-antibody conjugated silk nanoparticles loaded with doxorubicin

To test for the feasibility and efficacy of the project, the therapy must first be developed. Through literature reviews, we will determine the ideal NP size, doxorubicin dose, and antibody combination. Established protocols are already in use at the Kaplan Lab for processing silk, formulating doxorubicin nanoparticles, and measuring the size of the nanoparticles for characterization. Doxorubicin loading success should be visible under microscope due to its red color; we will aim to have up to 80% of nanoparticles to be loaded with the drug. Chosen antibodies will be conjugated onto the surface of the NPs using EDC/NHS protocols from past studies, and further testing will be conducted to determine the best way to conjugate two different types of antibodies at a constant, optimizable ratio. We hope to fabricate nanoparticles with an antibody A:B ratio in three groups--1:1, 2:1, and 1:2. High resolution fluorescent imaging will likely be used to quantify antibody conjugation on individual NPs and quantified using ImageJ. This is necessary to ensure reproducible targeting effects and to better understand

the formation of this therapy of interest. Successful completion of this aim will conclude in an established protocol for consistent dual-antibody conjugated nanoparticles.

Specific Aim 2: Create a 3D GBM model using transfected U87 cells

We hypothesize that a 3D model of GBM will allow us to get the best *in vitro* observations of treatment efficacy and behavior. U87 cells are malignant glioblastoma cells; however, because they are a heterogenous population, it is best to transfect the cells to express the receptors of choice so that more cells can be targeted by the antibodies. The U87 cells will be infected with a viral vector carrying the DNA sequence for either receptor or cultured. After cells are cultured and passaged, cells can be tested for receptor protein expression using western blots, ELISA, or fluorescent imaging. We hope to obtain a significant concentration of receptor protein when testing with western blots and find that at least 60% of the U87s express one or more of the transfected receptors. Binding affinity of receptors to the purchased antibodies can be evaluated using ELISA, which will suggest the ideal receptor to antibody (therapy) ratio for best binding and interaction. Once they proliferate to a certain number, the transfected U87s will be seeded onto a silk sponge scaffold to form a 3D model and used for further testing. The expected outcome of this aim is a 3D GBM model with U87 cells that express target receptors that can bind to the purchased antiboies. Completion of this aim will produce a U87 culture transfected with receptors of interest to use in later experiments

Specific Aim 3: Characterize nanoparticle efficacy

Characterization of nanoparticle efficacy is extremely important as it validates the feasibility of the product as a potential therapy for GBM. Nanoparticles of three groups would be loaded onto the 3D GBM models developed in Specific Aim 2: antibody A + B, antibody A, and antibody B. The efficacy of the three groups would be compared in terms of nanoparticle uptake and cytotoxicity. Nanoparticle uptake will be evaluated based on fluorescently imaging nanoparticles (using FITC) and lysosomes, then superimposing the images for overlap. The uptake of the three groups will be compared to the uptake of nanoparticles with no antibody coating (control), and their success will be measured with an uptake efficiency of 50% or above. Apoptosis will be quantified via live/dead assay. The three groups will be compared to the administration of free-doxorubicin; a marker of success is to have greater cytotoxicity compared to the non-encapsulated group. These are all important markers of nanoparticle success for targeting, drug delivery, and most importantly, apoptosis of GBM cells. Successful completion of this aim will establish the efficacy of the dual-conjugated antibodies and confirm successful binding.

Methods

Silk processing, nanoparticles formation, and doxorubicin loading are protocols from previous studies[4].

***Silk Processing*[4]**

Cut cocoons and remove inside layers, weigh out sodium carbonate to create a 0.02M sodium carbonate solution and add to distilled water. Add cocoon to boiling solution to degum silk fibers so that sericin is washed away and only fibrin protein remains. Wash degummed silk three times 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 4 hours at 60C. Inject silk LiBr solution into a dialysis cassette and remove air bubbles. Change dialysis water 3 times on the first day, twice

on the second day, and once on the third day to wash out LiBr solution. Collect silk solution on day 3 and centrifuge solution twice, then store in the fridge for up to two weeks.

Silk Nanoparticles [4]

5% silk solution will be added dropwise to acetone while maintaining a 75% acetone solution with doxorubicin in solution. The shear force of the droplets in the wall of the vortex will result in the formation of nanoparticles, and doxorubicin will be encapsulated within in the process. Precipitant will then be centrifuged at 48,000 g for 2 hours. Supernatant is aspirated and pellet is resuspended in 20mL of distilled water. Vortex and apply two sonication cycles at 30% amplitude for 30 seconds. Repeat centrifuging and resuspension 2> times. Resuspend pellet in distilled water and store for later use. The values mentioned in the above protocol can be optimized to produce the desired nanoparticle size as determined from literature reviews.

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 30C 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 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 will be evaluating for concentration and using to formulate doxorubicin loaded nanoparticles of a selected size. The group also knows how to culture U87s, so transfected versions of U87 cells expressing biomarkers IL-13Ra2 and EGFRviii will be used to demonstrate cellular uptake of the silk NPs.

DISCUSSION AND FUTURE WORK

We processed silk two times this 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 silk a second time, where the dissolving and dialysis of the silk was much more routine. As stated in the results, we were able to obtain a 13 mL silk solution that is stored in the fridge and will soon be used for nanoparticle fabrication.

One competing study found that silk fibroin nanoparticles coated in Tween-80 were able to release doxorubicin for up to 72 hours in GBM cells lines[25]. The Tween-80 is a surfactant that helps the nanoparticle cross the blood brain border, which addresses a huge issue in targeting brain/spinal cord cancers. While crossing the blood-brain barrier is not necessarily something we must target in this capstone project, it could be a future consideration to consider if time permits. By combining both receptor-mediated targeted delivery and ability to cross the blood brain barrier, the project could address yet another need for the treatment of glioblastomas.

CONCLUSION

The conclusions we have made so far as a group through various literature reviews and speaking to advisors is that glioblastoma multiforme is the best target for antibody-conjugated nanoparticles as a mode for chemotherapy delivery. GBM tumors express many cancer-related antigens, specifically IL-13Ra2, EGFRviii, and EphA2, all three individually present in 24-75% of high-grade gliomas[18]. IL-13Ra2 has been used as an antigen target for drug delivery in a clinical trial due to its 75% expression rate but exhibited neurotoxicity as it was found to also target some elements of healthy tissue[22]. Our thought is that combining an antibody for IL-13Ra2 with another antibody would decrease neurotoxicity as it would increase specificity of the nanoparticle. Being able to control how much of one antibody is present versus the other could also combat neurotoxicity as having more of the secondary antibody present on the surface compared to the IL-13Ra2 antibody would prevent uptake in healthy cells. The current thought is that an antibody for EGFRviii would be the best dual targeting agent as EGFRviii targets downstream IL-13Ra2, and when both are present, the two interact to promote GBM growth through upregulation of RTKs and RAS/RAF/MEK/ERK and STAT3 pathways[26].

INDIVIDUAL CONTRIBUTIONS

- **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
- **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 #1 and #2, updated project timeline with relevant dates and aims, EphA2 Antibody lit review, added to the midterm report and presentation
- **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 and Midterm Mid Semester Technical Report
- **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

ACKNOWLEDGEMENTS

- **Sunny Shaidani**: Thank you for taking us under your wing and helping us throughout the course of this project. We cannot express our gratitude to you for all that you do!

REFERENCES

(1) Ellsworth, S.; Ye, X.; Grossman, S. A. Clinical, Radiographic, and Pathologic Findings in Patients Undergoing Reoperation Following Radiation Therapy and Temozolomide for Newly Diagnosed Glioblastoma. *Am J Clin Oncol* **2017**, *40* (3), 219-222. DOI: 10.1097/coc.0000000000000136 From NLM.
(2) Carlsson, S. K.; Brothers, S. P.; Wahlestedt, C. Emerging treatment strategies for glioblastoma multiforme. *EMBO Mol Med* **2014**, *6* (11), 1359-1370. DOI: 10.15252/emmm.201302627 From NLM.
(3) Gavas, S.; Quazi, S.; Karpiński, T. M. Nanoparticles for Cancer Therapy: Current Progress and Challenges. *Nanoscale Res Lett* **2021**, *16* (1), 173. DOI: 10.1186/s11671-021-03628-6 From NLM.
(4) Wongpinyochit, T.; Johnston, B. F.; Seib, F. P. Manufacture and Drug Delivery Applications of Silk Nanoparticles. *J Vis Exp* **2016**, (116). DOI: 10.3791/54669 From NLM.
(5) Zhang, M.; Gao, S.; Yang, D.; Fang, Y.; Lin, X.; Jin, X.; Liu, Y.; Liu, X.; Su, K.; Shi, K. Influencing factors and strategies of enhancing nanoparticles into tumors in vivo. *Acta Pharmaceutica Sinica B* **2021**, *11* (8), 2265-2285. DOI: https://doi.org/10.1016/j.apsb.2021.03.033.
(6) Aldape, K. D.; Ballman, K.; Furth, A.; Buckner, J. C.; Giannini, C.; Burger, P. C.; Scheithauer, B. W.; Jenkins, R. B.; James, C. D. Immunohistochemical Detection of EGFRvIII in High Malignancy Grade Astrocytomas and Evaluation of Prognostic Significance. *Journal of Neuropathology & Experimental Neurology* **2004**, *63* (7), 700-707. DOI: 10.1093/jnen/63.7.700 (acccessed 10/21/2022).
(7) Pelloski, C. E.; Ballman, K. V.; Furth, A. F.; Zhang, L.; Lin, E.; Sulman, E. P.; Bhat, K.; McDonald, J. M.; Yung, W. K. A.; Colman, H.; et al. Epidermal Growth Factor Receptor Variant III Status Defines Clinically Distinct Subtypes of Glioblastoma. *Journal of Clinical Oncology* **2007**, *25* (16), 2288-2294. DOI: 10.1200/JCO.2006.08.0705 (acccessed 2022/10/20).
(8) Johnson, H.; Del Rosario, A. M.; Bryson, B. D.; Schroeder, M. A.; Sarkaria, J. N.; White, F. M. Molecular characterization of EGFR and EGFRvIII signaling networks in human glioblastoma tumor xenografts. *Mol Cell Proteomics* **2012**, *11* (12), 1724-1740. DOI: 10.1074/mcp.M112.019984 From NLM.
(9) Bonavia, R.; Inda, M. M.; Vandenberg, S.; Cheng, S. Y.; Nagane, M.; Hadwiger, P.; Tan, P.; Sah, D. W.; Cavenee, W. K.; Furnari, F. B. EGFRvIII promotes glioma angiogenesis and growth through the NF-κB, interleukin-8 pathway. *Oncogene* **2012**, *31* (36), 4054-4066. DOI: 10.1038/onc.2011.563 From NLM.
(10) Choi, B. D.; O'Rourke, D. M.; Maus, M. V. Engineering Chimeric Antigen Receptor T cells to Treat Glioblastoma. *J Target Ther Cancer* **2017**, *6* (4), 22-25. From NLM.
(11) Wykosky, J.; Debinski, W. The EphA2 receptor and ephrinA1 ligand in solid tumors: function and therapeutic targeting. *Mol Cancer Res* **2008**, *6* (12), 1795-1806. DOI: 10.1158/1541-7786.Mcr-08-0244 From NLM.
(12) Lindberg, R. A.; Hunter, T. cDNA cloning and characterization of eck, an epithelial cell receptor protein-tyrosine kinase in the eph/elk family of protein kinases. *Mol Cell Biol* **1990**, *10* (12), 6316-6324. DOI: 10.1128/mcb.10.12.6316-6324.1990 From NLM.

(13) Wykosky, J.; Gibo, D. M.; Stanton, C.; Debinski, W. Interleukin-13 receptor alpha 2, EphA2, and Fos-related antigen 1 as molecular denominators of high-grade astrocytomas and specific targets for combinatorial therapy. *Clin Cancer Res* **2008**, *14* (1), 199-208. DOI: 10.1158/1078-0432.Ccr-07-1990 From NLM.
(14) Ogawa, K.; Pasqualini, R.; Lindberg, R. A.; Kain, R.; Freeman, A. L.; Pasquale, E. B. The ephrin-A1 ligand and its receptor, EphA2, are expressed during tumor neovascularization. *Oncogene* **2000**, *19* (52), 6043-6052. DOI: 10.1038/sj.onc.1204004 From NLM. Liu, F.; Park, P. J.; Lai, W.; Maher, E.; Chakravarti, A.; Durso, L.; Jiang, X.; Yu, Y.; Brosius, A.; Thomas, M.; et al. A genome-wide screen reveals functional gene clusters in the cancer genome and identifies EphA2 as a mitogen in glioblastoma. *Cancer Res* **2006**, *66* (22), 10815-10823. DOI: 10.1158/0008-5472.Can-06-1408 From NLM.
(15) Macrae, M.; Neve, R. M.; Rodriguez-Viciana, P.; Haqq, C.; Yeh, J.; Chen, C.; Gray, J. W.; McCormick, F. A conditional feedback loop regulates Ras activity through EphA2. *Cancer Cell* **2005**, *8* (2), 111-118. DOI: 10.1016/j.ccr.2005.07.005 From NLM.
(16) Miao, H.; Li, D. Q.; Mukherjee, A.; Guo, H.; Petty, A.; Cutter, J.; Basilion, J. P.; Sedor, J.; Wu, J.; Danielpour, D.; et al. EphA2 mediates ligand-dependent inhibition and ligand-independent promotion of cell migration and invasion via a reciprocal regulatory loop with Akt. *Cancer Cell* **2009**, *16* (1), 9-20. DOI: 10.1016/j.ccr.2009.04.009 From NLM.
(17) Debinski, W.; Obiri, N. I.; Powers, S. K.; Pastan, I.; Puri, R. K. Human glioma cells overexpress receptors for interleukin 13 and are extremely sensitive to a novel chimeric protein composed of interleukin 13 and pseudomonas exotoxin. *Clin Cancer Res* **1995**, *1* (11), 1253-1258. From NLM.
(18) Sharma, P.; Debinski, W. Receptor-Targeted Glial Brain Tumor Therapies. *Int J Mol Sci* **2018**, *19* (11). DOI: 10.3390/ijms19113326 From NLM.
(19) Zeng, J.; Zhang, J.; Yang, Y. Z.; Wang, F.; Jiang, H.; Chen, H. D.; Wu, H. Y.; Sai, K.; Hu, W. M. IL13RA2 is overexpressed in malignant gliomas and related to clinical outcome of patients. *Am J Transl Res* **2020**, *12* (8), 4702-4714. From NLM.
(20) Sattiraju, A.; Solingapuram Sai, K. K.; Xuan, A.; Pandya, D. N.; Almaguel, F. G.; Wadas, T. J.; Herpai, D. M.; Debinski, W.; Mintz, A. IL13RA2 targeted alpha particle therapy against glioblastomas. *Oncotarget* **2017**, *8* (26), 42997-43007. DOI: 10.18632/oncotarget.17792 From NLM. Brown, C. E.; Warden, C. D.; Starr, R.; Deng, X.; Badie, B.; Yuan, Y. C.; Forman, S. J.; Barish, M. E. Glioma IL13Rα2 is associated with mesenchymal signature gene expression and poor patient prognosis. *PLoS One* **2013**, *8* (10), e77769. DOI: 10.1371/journal.pone.0077769 From NLM.
(21) Thaci, B.; Brown, C. E.; Binello, E.; Werbaneth, K.; Sampath, P.; Sengupta, S. Significance of interleukin-13 receptor alpha 2-targeted glioblastoma therapy. *Neuro Oncol* **2014**, *16* (10), 1304-1312. DOI: 10.1093/neuonc/nou045 From NLM.
(22) Brown, C. E.; Alizadeh, D.; Starr, R.; Weng, L.; Wagner, J. R.; Naranjo, A.; Ostberg, J. R.; Blanchard, M. S.; Kilpatrick, J.; Simpson, J.; et al. Regression of Glioblastoma after Chimeric Antigen Receptor T-Cell Therapy. *N Engl J Med* **2016**, *375* (26), 2561-2569. DOI: 10.1056/NEJMoa1610497 From NLM.
(23) Wykosky, J.; Gibo, D. M.; Stanton, C.; Debinski, W. Interleukin-13 Receptor α2, EphA2, and Fos-Related Antigen 1 as Molecular Denominators of High-Grade Astrocytomas and Specific Targets for Combinatorial Therapy. *Clinical Cancer Research* **2008**, *14* (1), 199-208. DOI: 10.1158/1078-0432.Ccr-07-1990 (acccessed 10/21/2022).

(24) Sampson, J. H.; Heimberger, A. B.; Archer, G. E.; Aldape, K. D.; Friedman, A. H.; Friedman, H. S.; Gilbert, M. R.; Herndon, J. E., 2nd; McLendon, R. E.; Mitchell, D. A.; et al. Immunologic escape after prolonged progression-free survival with epidermal growth factor receptor variant III peptide vaccination in patients with newly diagnosed glioblastoma. *J Clin Oncol* **2010**, *28* (31), 4722-4729. DOI: 10.1200/jco.2010.28.6963 From NLM. Brown, C.; Starr, R.; Naranjo, A.; Wright, C.; Bading, J.; Ressler, J. Adoptive transfer of autologous IL13-zetakine+ engineered T cell clones for the treatment of recurrent glioblastoma: lessons from the clinic. *Mol Ther* **2011**, *19* (suppl 1), S136-S137.
(25) Pandey, V.; Haider, T.; Chandak, A. R.; Chakraborty, A.; Banerjee, S.; Soni, V. Surface modified silk fibroin nanoparticles for improved delivery of doxorubicin: Development, characterization, in-vitro studies. *International Journal of Biological Macromolecules* **2020**, *164*, 2018-2027. DOI: https://doi.org/10.1016/j.ijbiomac.2020.07.326.
(26) Newman, J. P.; Wang, G. Y.; Arima, K.; Guan, S. P.; Waters, M. R.; Cavenee, W. K.; Pan, E.; Aliwarga, E.; Chong, S. T.; Kok, C. Y. L.; et al. Interleukin-13 receptor alpha 2 cooperates with EGFRvIII signaling to promote glioblastoma multiforme. *Nature Communications* **2017**, *8* (1), 1913. DOI: 10.1038/s41467-017-01392-9.

APPENDIX

Project Timeline

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**									
Culturing HUVECS		**25**									
Technical Proposal Report Draft		**100**									
Project Presentations		**100**									
Preparing Nanoparticles	**Load Doxorubicin**	**0**									

Start Website	**Add in Home Page, People Sections, References, and Project Update**	**0**									
3rd Biweekly Report		**0**									
Risk Assesment Analysis		**0**									
Update Website	**Update Project Section and References**	**0**									
Order Materials Necessary for Antibody Conjugation		**0**									
4th Biweekly Report		**0**									
Update Website	**Update Project Section and References**	**0**									
5th Biweekly Report		**0**									
Project Presentations		**0**									
Finalized Website		**0**									
Technical Report		**0**									

Short Term Specific Aims

There are three specific aims of this project. Firstly, we needed to define the best antibody and receptor for targeting GBM through extensive literature reviews on the subject matter. This aim has already been partially achieved by us selecting a dual targeting nanoparticle drug delivery approach and technique, focusing on either IL-13Ra2 combined with either EphA2 or EGFRviii. Secondly, we needed to produce U87 culture in a way where we can test if the antibody receptor binding is efficacious. This aim will be tested and evaluated through one of the following methods: western blots, ELISAs, and/or fluorescent tagging of receptor to antibody to secondary fluorescently tagged antibody. Finally, we need to determine the adequate number of antibodies there are in comparison to the others as well as the nanoparticle conjugation method. This will be evaluated by more literature review (particularly with regards to EDC/NHS technique) to help determine how much we need, how much will stick, and how to document this.

Specific Aim 1: Define the best antibody receptor combination to target GBM

We hypothesize that the best combination of antibodies will include a combination of IL-13Ra2 and either EphA2 or EGFRviii. Initial antibodies will be determined using literature reviews. Next, we will test whether these antibodies properly target GBM cells using transfected U87

cells or patient-derived cells. The expected outcome of this study is qualitative and quantitative data on the efficacy of antibody binding and the prevalence of each receptor in GBM patients. Successful completion of this aim will characterize the prevalence of each antibody in GBM patients and determine which antibody combination will be most relevant.

Specific Aim 2: Produce and transfect U87 cells to test antibody efficacy
We hypothesize that proper transfection of U87 cells will produce cultures that adequately bind with the antibodies of choice. To test transfected U87 cells, we will use western blot and ELISA. Moreover, to determine antibody binding, we will use fluorescent tagging of the receptor to antibody to the secondary fluorescently tagged antibody. We will determine if our cell cultures produce antibody binding efficacy in dual combination and single combination in transfected cells. Completion of this aim will elucidate the proper transfection of U87 cell lines.

Specific Aim 3: Determine antibody conjugation technique for adequate targeting and binding
We hypothesize that antibody quantity will have a large effect on binding efficacy and targeting. We will first determine an antibody conjugation technique through literature reviews to establish the best method of attachment to control the amount. We will then use fluorescent tagging to study the effects of different antibody combination ratios and load. The expected outcome will be a standardized technique of antibody conjugation that regulates antibody conjugation quantity. Successful completion of this aim will aid in consistent nanoparticles for use throughout our study.

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