Pratt Fellows Undergraduate Research Semester 2 Update

Jason Liu and Simone Costa
Dr. Ashutosh Chilkoti Laboratory, Pratt School of Engineering, Duke University, Spring 2016

Introduction and Motivation

Glioblastoma Multiforme (GBM) is the most common and aggressive form of brain cancer that upon diagnosis, has a median patient survival of only 14.6 months [1]. The current standard of care for GBM patients involves surgical resection, radiation, and chemotherapy. Despite research efforts to treat GBM, none have appreciably improved survival rates, but there have been several promising leads [2]. In the past decade, there has been a rise of clinical trials involving the use of monoclonal antibodies or modified antibody fragments conjugated with potent exotoxins, known as immunotoxins. One notable example of an immunotoxin currently in a phase I clinical trial is in the form of a single-chain variable fragment (scFv)-toxin fusion protein. This first-generation treatment is specific to the epidermal growth factor receptor (EGFR) and epidermal growth factor receptor vIII (EGFRvIII), both highly expressed in GBM, with EGFRvIII exclusive to the disease. The engineered toxin, derived from Pseudomonas aeruginosa exotoxin A (PE), is highly potent and has had immunogenic epitopes mutated and natural targeting domain deleted [3]. This combination was found to be successful in pre-clinical studies, with improvement in survival within a murine GBM model [4]. However, this treatment is limited by a short *in-vivo* half-life and non-uniform distribution within the target tumor site.

We plan to adapt this immunotoxin design with the addition of elastin-like polypeptides (ELP). ELPs exhibit lower critical solution temperature transition behavior, aggregating when heated above their characteristic transition temperature, and solubilizing upon cooling. These characteristics can be used to extend a fusion protein drug's bioavailability by utilizing this phase transition behavior to create depots to modify that drug's molecular weight. Importantly, these depots, when locally injected intratumorally, also allow for site-specific and prolonged drug release.

Our approach is to construct an antibody-ELP-toxin recombinant fusion protein that combines the site-specificity of a full length monoclonal antibody (anti-EGFR/EGFRvIII), the inverse temperature transition behavior mechanics of ELP, and the cytotoxicity of the toxin in order to create a longer lasting and more efficacious GBM drug.

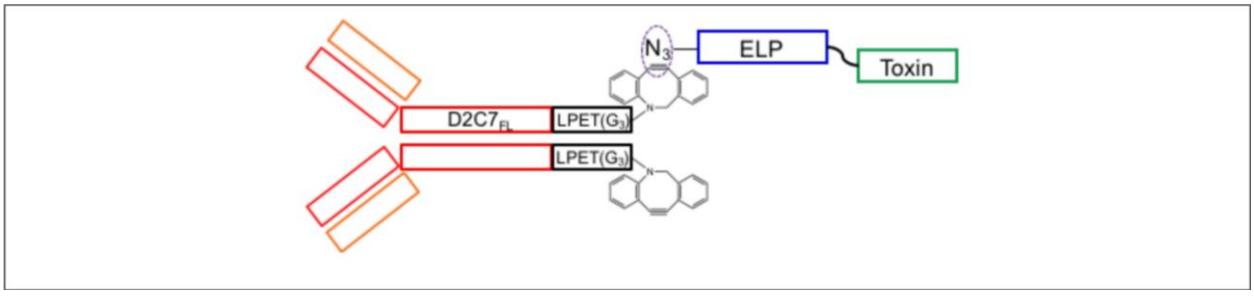

Figure 1: The Antibody-ELP-Toxin, with two components: (1) The antibody is a fully humanized anti-EGFR/EGFRvIII ($D2C7_{FL}$) antibody. This antibody will be modified to include a C-terminal $LPET(G)_3$ sequence for SrtA modification with a dibenzocyclooctyl (DBCO) moiety. (2) Azide-$(VPGVG)_{60}$-(PE toxin) recombinant fusion protein, connected by a flexible linker. These two components will be conjugated via copper-free click chemistry, is a highly specific and spontaneous reaction in which the DBCO of the antibody will covalently and irreversibly bond with the azide functional group of the AzF-$(VPGVG)_{60}$-(PE toxin).

Specifics

The sub-goals will be to express, purify, and characterize the components of this planned immunotoxin drug. HEK293 human embryonic kidney cells will be used to express fully humanized anti-EGFR/EGFRvIII antibody with a C-terminal LPET$(G)_3$ sequence. This sequence is required for Sortase A conjugation. Triglycine-dibenzocyclooctyl (DBCO) small molecules will be synthesized by an amide coupling reaction of triglycine and amine-DBCO. The anti-EGFR/EGFRvIII antibody will be modified with the triglycine-DBCO molecule via Sortase A reaction to yield anti-EGFR/EGFRvIII antibodies with DBCO functionality. The ELP and toxin will be expressed in *Escherichia coli* as a fusion protein and purified via inverse transition cycling, a purification technique used for ELP and ELP fusion proteins [5]. The ELP will have the sequence $(VPGVG)_{60}$, will have an azide-containing unnatural N-terminal amino acid, azidophenylalanine (AzF), and will have the toxin fused to the ELP at its C-terminus. This AzF-$(VPGVG)_{60}$-toxin will be genetically encoded to be expressed as one protein.

For construction of the final conjugate, the purified AzF-ELP-toxin will then be conjugated to the antibody by copper-free click chemistry. This is a highly specific and spontaneous reaction in which the DBCO of the antibody will covalently and irreversibly bond with the azide functional group of the AzF-ELP-toxin.

Completed Steps

The $(VPGVG)_{60}$-toxin gene has been successfully constructed in a pET24 vector backbone. A gBlock (IDT) containing the toxin gene was inserted into the vector using Gibson assembly. This gene was then ligated to an existing ELP gene of desired length ($(VPGVG)_{60}$) using PRe-RDL (Plasmid Reconstruction by Recursive Directional Ligation) [6]. Once ligated and the desired construct was successfully cloned, the purification process was optimized for the production of ELP-toxin. We attempted purification by using either heat or salt (NaCl) or both to trigger phase transition, changing the buffer used to resuspend cell lysate and subsequent protein pellets, changing the growth and induction temperature, changing the expression cell line (SHuffle T7 cells rather than BL21 DE3 cells), and isolating and purifying only the insoluble fraction. Inverse transition cycling was unsuccessful as a purification technique, yielding undesired truncation products which can be seen in **Figure 2.** However, a previously established refolding protocol for protein expression and purification was successful in producing higher purity protein product. Refolding was required because our protein product seems to be found within insoluble inclusion bodies after *E. coli* expression. A TAG leader sequence, a stop codon that encodes for AzF incorporation when the appropriate AzF-specific translational machinery is provided, was cloned into the plasmid construct and added to the N-terminus of the ELP-toxin sequence. With this, we were able to express and purify the azido-functionalized ELP-toxin portion of our immunotoxin, with a yield of about 110 mg per L of culture. AzF functionality of our product was confirmed in the fluorescent SDS-PAGE gel shown in **Figure 3**.

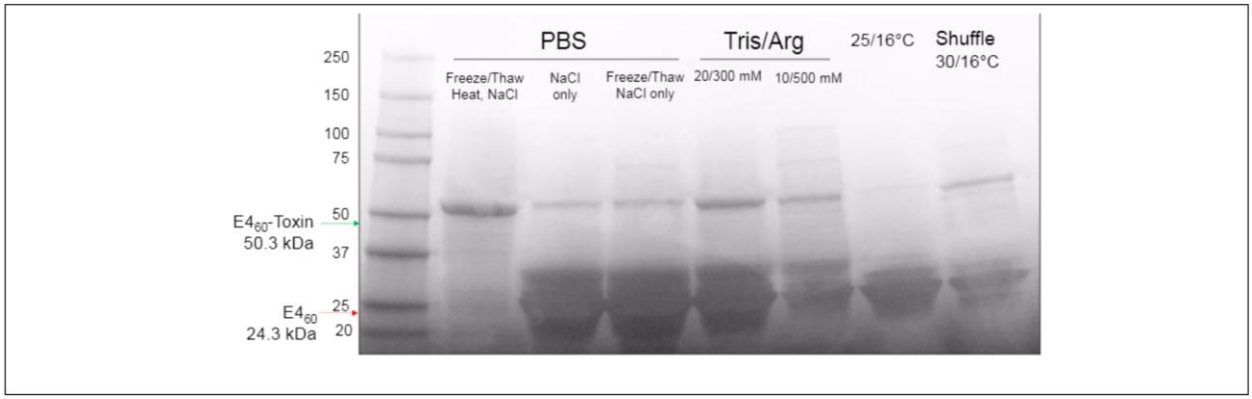

Figure 2: SDS-PAGE of optimization of purification of $(VPGVG)_{60}$-toxin product. All samples showed signs of aggregation or truncation products, which can be seen as the lower molecular weight products, indicated by the $E4_{60}$ arrow. The lanes from left to right are: protein ladder, heat/salt transitioning after a freeze/thaw cycle of the cell lysate in PBS, salt only transitioning in PBS, salt only transitioning after freeze/thaw cycle of the cell lysate in PBS, 20/300 mM Tris/Arg resuspension buffer, 10/500 mM Tris/Arg resuspension buffer, BL21 growth at 25 C with protein expression at 16 C after induction, and alternatively using SHuffle T7 cells for expression.

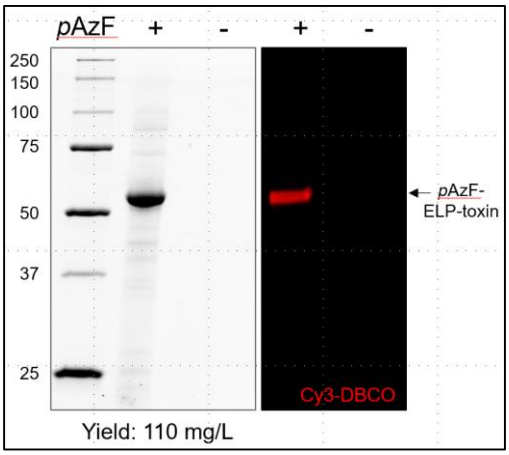

Figure 3: Left: SDS-PAGE gel of the optimized expression of AzF-$(VPGVG)_{60}$-toxin, shown with the correct molecular weight. Right: SDS-PAGE fluorescent gel confirming the AzF-functionality of our product. The Cy3-DBCO fluorescent dye labels via copper-free click chemistry, conjugating the Cy3 fluorescent signal to AzF-containing product. Because the lower band is shown to be fluorescently active at the correct molecular weight, we can confirm AzF functionality and molecular weight of our product.

In order to observe the effect of conjugating ELP into the antibody-toxin, we also cloned a sequence that codes for AzF followed by 6 histidine residues for purification via nickel column chromatography and the toxin (AzF-His_6-toxin). Using nickel column chromatography, we successfully expressed and purified this AzF-His_6-toxin construct, which we will later click onto a DBCO-functionalized anti-EGFR/EGFRvIII antibody. **Figure 4** confirms the success of this purification. The approximate yield with this process was around 10 mg per liter of culture. The molecular weight and AzF functionality of the AzF-His_6-toxin product is shown in **Figure 5**.

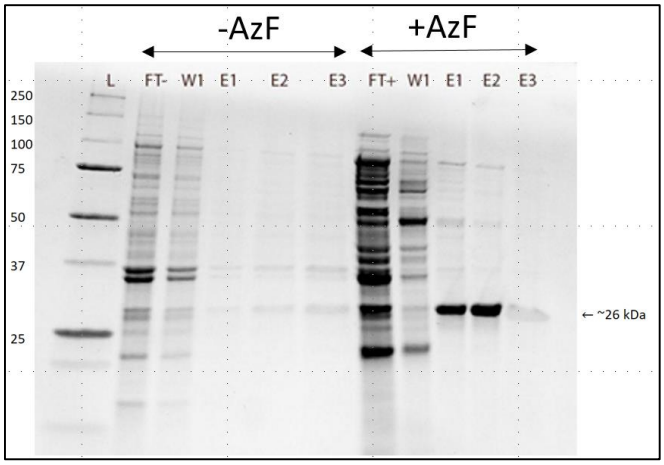

Figure 4: The nickel column purification products. "-AzF" product was expressed without azidophenylalanine (AzF) as a control, and "+AzF" product was expressed with AzF. The large bands in +AzF lanes E1 and E2 are where AzF-His_6-toxin is expected to be found (~26 kDa). The lack of significant bands around this molecular weight in the flow-through lanes (FT+/-) indicate that most of our product was isolated from the cell lysate. Proper nickel-histidine binding between the AzF-His_6-toxin histidine tag and the nickel column was confirmed as the wash lanes that have contaminant bands and no significant band the expected length (W1 in both ±AzF).

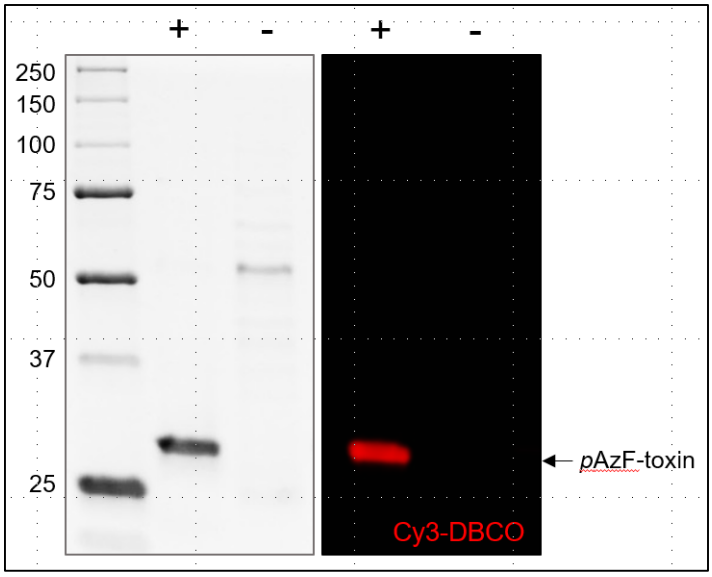

Figure 5: Left: SDS-PAGE showing the AzF-toxin expression product. +/- indicates the addition of azidophenylalanine (AzF) during expression. Right: fluorescent SDS-PAGE of the +/- AzF-toxin product labeled with a fluorescent dye, Cy3-DBCO. The fluorescent dye labels via copper-free click chemistry, conjugating the Cy3 fluorescent signal to AzF-containing product. Because the lower band is shown to be fluorescently active at the correct molecular weight (~26 kDa), we can confirm AzF functionality and molecular weight of our product.

Fully humanized anti-EGFR/EGFRvIII with the C-terminal LPET(G_3) recognition sequence on the heavy chain was successfully expressed in HEK293 embryonic kidney cells. We employed two different signal peptides (IL-2 and azurocidin) for antibody expression to observe whether the cleavage of those signal peptides affected our yield. We found that the IL-2 signal peptide was most well suited for expression of anti-EGFR/EGFRvIII. We also optimized the Sortase A conjugation conditions for modifying the antibody with the G_3DBCO small molecule. Preliminary experiments showed successful Sortase A-mediated conjugation to form DBCO-functionalized antibody. This was verified through conjugating azide-functionalized fluorescent dye (Cy5-N_3) to anti-EGFR/EGFRvIII-DBCO via copper-free click chemistry and running the product on a polyacrylamide gel, as shown in **Figure 6.**

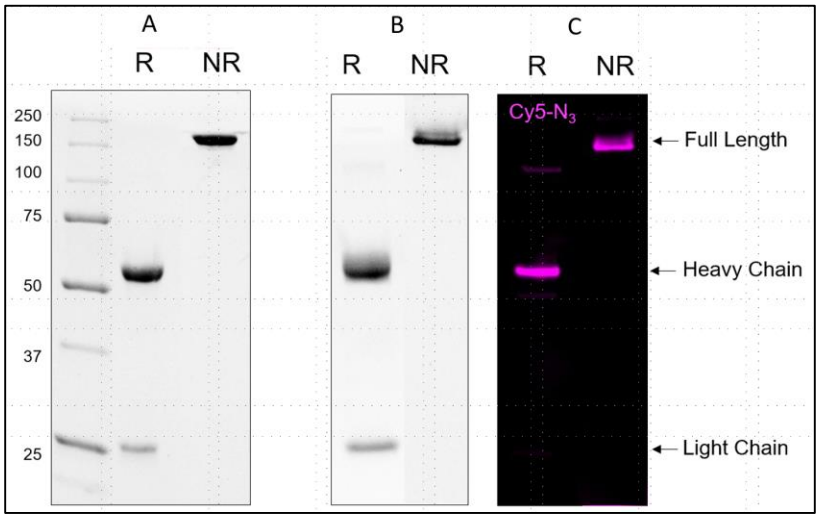

Figure 6: A: SDS-PAGE showing the pre-Sortase A reduced and non-reduced molecular mass of the anti-EGFR/EGFRvIII antibody expression product. B: SDS-PAGE showing the post-Sortase A reduced and non-reduced molecular mass of the anti-EGFR/EGFRvIII antibody conjugated with DBCO. C: fluorescent SDS-PAGE after the Sortase A reaction between G_3-DBCO and antibody with a C-terminal LPET$(G)_3$ and labeling with a fluorescent dye, Cy5-N_3. The gel shows similar molecular weights to that of the pre-Sortase A reaction because the conjugated molecule is small (~500 Da). The fluorescent dye labels only the heavy chain of the anti-EGFR/EGFRvIII.

When attempting to recreate this final conjugation step between anti-EGFR/EGFRvIII-DBCO and AzF-ELP-toxin, we found that the linker molecule G_3-DBCO was not completely purified after its synthesis, and had degraded over time. Because small-molecule purification was outside of the scope of this project, we sourced pure G_3-DBCO from the Duke University Small Molecule Synthesis Facility.

Although the linker molecule was impure, we could conduct binding affinity studies of fluorescently tagged anti-EGFR/EGFRvIII antibody for their target epidermal growth factor receptor (EGFR) and variant EGFRvIII, using flow cytometry. We received three mouse fibroblast (3T3) cell lines that expressed only EGFR, only EGFRvIII, or a negative control, on their cell membranes. I was responsible for culturing these cell lines and performing the flow cytometry experiments. This

included incubating the cells with the fluorescently tagged (Cy5) anti-EGFR/EGFRvIII antibody, and performing the flow cytometry to measure the relative fluorescence of the labeled cells.

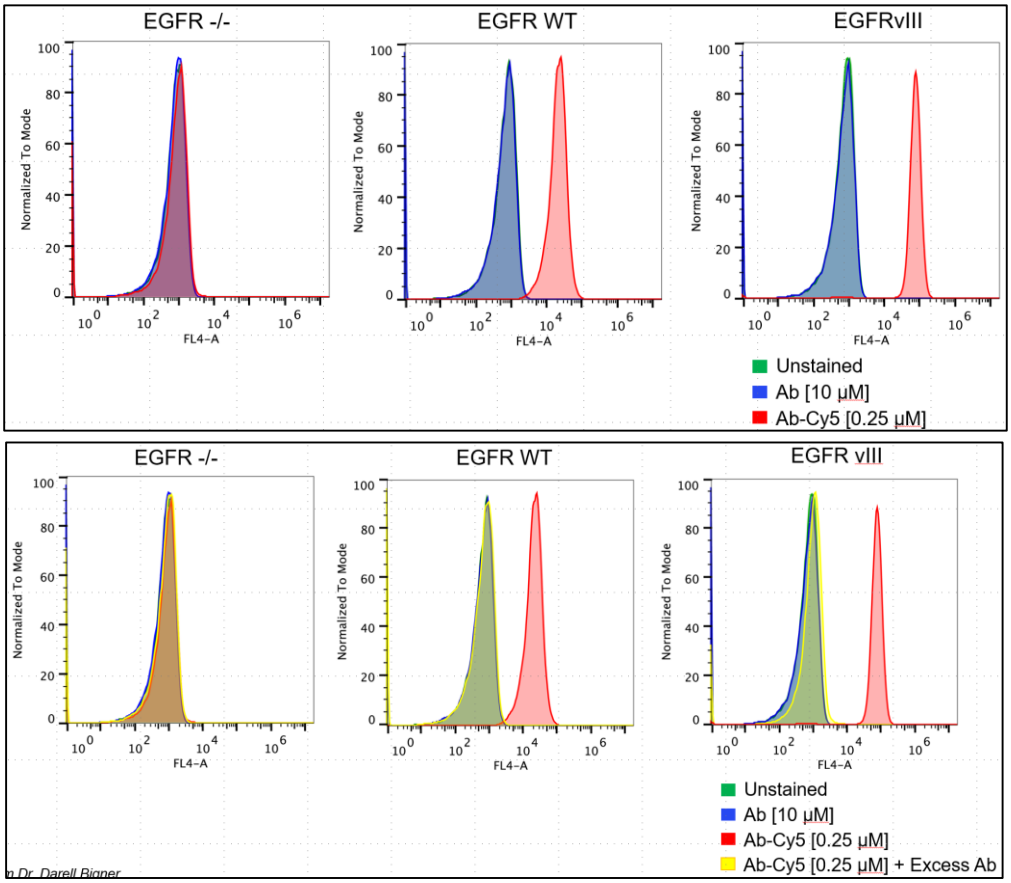

Figure 7: Flow cytometry results of fluorescently labeled anti-EGFR/EGFRvIII antibody on mouse fibroblast (3T3) cells that express no EGFR (EGFR -/-), EGFR wild type (EGFR WT), and mutant EGFR (EGFRvIII). There was no significant shift in the fluorescence when the labeled antibody was mixed with -/- cells (green), no significant shift when unlabeled antibody was incubated with both EGFR and EGFRvIII-expressing cells (blue), but a significant shift when labeled antibody was incubated with EGFR and EGFRvIII-expressing cells (red). To verify that fluorescence is related to binding events, we were able to observe competitive binding between labeled antibody and excess unlabeled antibody on both EGFR- and EGFRvIII-expressing cells (yellow).

To confirm that conjugating an ELP to the toxin does not interfere with the toxin's cytotoxicity and that our expression/purification protocol was viable, we used a cell-free assay for translation inhibition of luciferase. The ELP-toxin construct lacks a targeting domain, so its cytotoxicity cannot be tested *in vitro*. The cell-free assay contains all the components required for transcription and translation. Upon transcription and translation of luciferase, and addition of the luciferin reagent, luminescence is produced; this luminescent signal can be quantified using a plate reader. The toxin acts by targeting and inactivating elongation factor 2, preventing protein translation; this process requires the presence of a co-factor nicotinamide adenine dinucleotide (NAD). We tested ELP only, a single chain variable fragment-toxin (scFv-toxin, provided by Dr. Bigner) as a positive

control, our purified AzF-ELP-toxin (with and without NAD introduced), and AzF-toxin. Our results were normalized by a negative control with no treatment. Our results indicate that the toxin's activity is maintained even when conjugated to ELP, with more than 97% inhibition of protein translation compared to the negative control. This inhibition was found to be similar to that of scFv-toxin, which was previously established to have this effect.

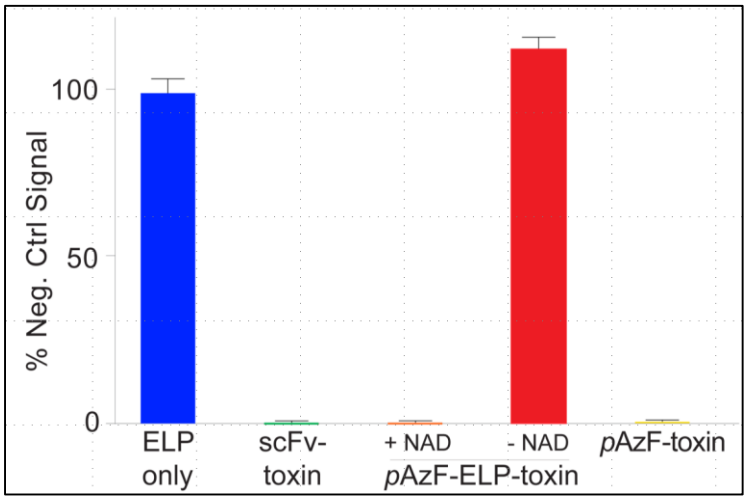

Figure 8: Cell-free expression assay with luciferase. ELP only did not appreciably inhibit expression of luciferase. This is consistent because ELP has no previously known effect on protein translation. The scFv-toxin and purified AzF-ELP-toxin with NAD showed significant luciferase translation inhibition. The treatment without NAD did not inhibit luciferase, because NAD is a required substrate for this toxin's activity, demonstrating specificity of the toxin activity. The AzF-Toxin also significantly inhibited luciferase translation.

Current/future Steps

With the pure G_3-DBCO now available, with anti-EGFR/EGFRvIII-LPET$(G)_3$ antibody, AzF-ELP-toxin, and AzF-toxin on hand, we are able to construct the full-length immunotoxin. We will characterize and verify its molecular weight using SDS-PAGE, thermal responsivity and behavior (though thermal turbidity scanning and Dynamic Light Scattering), and its activity through *in vitro* cytotoxicity assays.

Additional Completed Tasks

I helped clone, express, and purify PCE (photocrosslinkable ELP), PCD (diblock of ELP for micelle formation), and PCM (proteolytic sites so that mass spectroscopy can characterize incorporation efficiency of AzF). This served as extra practice for cloning and ELP expression and purification.

I also assisted with another project to improve drug delivery of the chemokine CCL3, a known adjuvant for GBM tumor suppression by dendritic cell (DC) vaccines [7]. This new technology involves antigen conditioning of *ex vivo* dendritic cells to a protein found in 90% of glioblastoma

specimens: dendritic cells are preconditioned the cells with a potent recall antigen (diphtheria/tetanus toxoid), and reintroduced into the patient. This method was effective in suppressing tumors within mice, with dose-dependent CCL3 chemokine introduction. We seek to improve the drug delivery mechanics of CCL3 by genetically fusing $(VPGVG)_{60}$ to both the N- and C- termini to see if this can improve circulation time or promote sustained release via ELP-drug or drug-ELP depots. We have successfully expressed $(VPGVG)_{60}$-CCL3 but plan to further optimize expression. I also plan to clone, express, and purify CCL3-$(VPGVG)_{60}$, with the ELP on the C-terminus of CCL3 to test whether fusion order has an effect on activity.

Skills Learned

- General Chilkoti Lab standards: lab upkeep, locations of reagents, general etiquette, autoclaving, restoring old media stocks, making new agarose plates, refilling pipette tip boxes.
- DNA cloning techniques: bacterial plasmid DNA extraction, restriction enzyme cutting, ligation, PCR, PRe-RDL.
- DNA and protein characterization techniques: Agarose/SDS-PAGE/stain-free/labeled gels, Nano-drop spectrophotometry, Typhoon gel imager, Cary Transition Temperature Scanning
- Basic bacterial gene cloning with EB5α cell line, bacterial protein expression with BL21 cell line/Shuffle Cells, expression of photosensitive ELP products
- General ELP Inverse Transition Cycling protocol and dialysis technique
- Antibody expression techniques in cell culture hood and protein G column purification
- Flow Cytometry for binding affinity assays

References

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