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Click Chemistry Protein Immobilization

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Dr. Nic Leipzig's Lab

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Executive Summary

Protein engineering is the major topic of discussion for this project report, more specifically, the use of click chemistry in order to test a new way to enable azide-tagging and to measure how efficient this tag is in a protein immobilization process. Two major proteins that were used in the lab were interferon-gamma (IFN-γ) and stromal cell derived factor 1α (SDF-1α).

The main purpose of the first part in the lab was to try and determine a way to measure the moles of dye per mole of protein in order to detect an azide tag on a recombinantly produced azide-IFN-γ protein. This was done by using click chemistry to bind the azide tagged protein to a dye and then measured the azide tag efficiency. There is no commercial kit that allows measurement of the amount of azide that is tagged to a protein, so the goal was to develop a solution to this issue.

The main purpose of the second part of the project was to make a protein for the lab, SDF-1α, and tag it with an azide group. From here, an immobilization experiment was performed and a measure of the processes efficiency was recorded. This was completed first by creating the azide protein via processes of expression, isolation, and purification of the protein and, finally, immobilizing and quantifying the process.

Some limitations that came into play with this project is the amount of protein that could be used due to effort and time needed to create it, the amount of dye that could be used, and the amount of time to complete the project. Research can always be done more accurately when having more data, unlimited time to test different scenarios, and a better design of experiment.

The results that were obtained from performing the first part of the project were very inaccurate and made no sense. The method was tried three different times and each time was unsuccessful. The first results showed moles dye per mole protein values between 1.9 and 3.2. This isn't possible because the protein was tagged with only one azide group, thus only one dye molecular can react with it. The second results were very low and contained negative values, which is impossible. Finally, the third trial results made the most sense of the three runs but still had values that were very low. It was not a proper method to move forward with when trying to measure the amount of azide that was tagged to the protein. If there was more time to perform this experiment, other methods could be tested to try and come up with a better solution to the issue of not having a commercial kit that allows the measurement of the amount of azide tagged to a protein.

The second part of the project presented a few different results along the way to the goal of determining the immobilization efficiency. First, the sodium dodecyl sulfate poly-acrylamide gel electrophoresis (SDS-PAGE) analysis of test expression results showed that the soluble and insoluble for the SDF-1 plasmid and SDF-1 plasmid plus CaNMT enzyme could both work for the experiment. It was decided to move forward with the soluble because it is better to not have to renature if it isn't necessary. Second, the fast protein liquid chromatography (FPLC) results for both the SDF-1 protein and SDF-1 with an azide tag protein allowed the determination of the proper range of the protein (based on molecular weight then comparison with FPLC results to the standards) in order to calculate the protein concentrations of both. The range of SDF-1 protein that was collected, based off the FPLC results and graph, was from 1E10 to 1G7, which

resulted in a protein concentration of 15.79 μg/mL. The range of azide tagged SDF-1 protein that was collected, based off the FPLC results and graph, was from 1F3 to 1G4, which resulted in a protein concentration of 12.52 μg/mL.

An ELISA was performed three different times in order to quantify the immobilization process. The first trial results obtained a standard curve but the data didn't make sense because the concentrations obtained for the SDF-1 protein were much higher than the SDF-1 with an azide tag concentrations. It is believed that some of the hydrogels might have attached to the plates and didn't totally dissolve, which could be why the SDF-1 protein concentrations were higher. It was discovered later on that the lysozyme in the lab was bad and might have been the reason for the results being thrown off. The second and third results that were obtained from the supernatant, which was saved from the solution the gels were in, had results that could not be converted due to the standard curves not working out. It was later discovered that the ELISA kit used had standards that were messed up and it was not a performance issue on my part.

A definite conclusion can be drawn that engineering a protein, expressing it, and purifying it can be completed. There are no definite conclusions that may be drawn from the results stated above for the first part of the project other than knowing the quantification method tested in the lab will not work and a new method must be discovered. The second part of the project had a confirmed definite conclusion that either the soluble or insoluble, from the SDS-PAGE results, could be used to move forward with the experiment. The ranges from the FPLC results were an estimate based off the standard so no definite conclusions could be drawn from that. No definite conclusions could be drawn from the immobilization process due to an error in the kit used in the lab.

This project gave a great experience in a lab setting with a biotechnology focus, really helped expand my knowledge of protein engineering. Specifically, it helped me gain firsthand exposure to recombinant methods of protein tagging and better ways to do so. It gave a better understanding of the proteins IFN- γ, SDF-1, and protein functions overall. The impacts the proteins have on benefiting people in society were also a great thing to learn when doing background research on them. It was very helpful to get lab experience with a topic that was very interesting especially that relates to possibilities of future career work. The project definitely allowed my confidence level to work in a lab go up and feel a lot more comfortable with interviewing in the biotech field. Some of the skills that were obtained in the lab experience was sectioning, pipetting, performing different protein assays, and expressing, isolating, and purifying proteins. Lastly, I feel like I had some impact and helped benefit Dr. Leipzig's research lab along with the graduate student, Trevor, whose main focus is on this subject.

Some recommendations for future work would be to improve on a method in order to determine the tagging ratio for azide-tagged proteins and help develop a protocol to do so. Other work that could be done is to express, isolate, and purify more proteins with multiple experiments and do more sandwich ELISAs to try and get the best results possible. The increase in sample size would definitely be a help if there was no budget on lab equipment, kits, chemicals, etc as well.

Introduction

The focus and purpose of this honors project deals with protein engineering in Dr. Nic Leipzig's research lab. The two main proteins that were used in the lab were IFN-γ and SDF-1α.

The goal of the project was broken down into two main parts. The first part was to use click chemistry to bind azide-tagged INF- γ to a dye and determine the tagging ratio (moles of dye per mole protein). The dye was used as a way to measure the azide-tagging efficiency. Previously, biotin-streptavidin was used to immobilize proteins, tagging them with biotin using a commercial kit to measure the amount of biotin per mole of protein to confirm that the protein was tagged. However, with the new method using azide tagged proteins, there is no commercial kit that can be bought. Another way of measuring the moles of dye per mole of protein, in order to detect the azide tag, needed to be tested. This was worthwhile in order to try and accomplish an effective and cheaper way to tag proteins with an azide group.

The second part of the project was to make a protein called SDF-1, tag it with an azide group, immobilize, and finally measure how efficient the immobilization process was. A very critical concept throughout completing the second part of the project was the purpose of the enzyme CaNMT. This enzyme is what allowed the azide group to tag to the N-terminal end of the SDF-1 protein by click chemistry when working through the SDS-PAGE protocol. Also, tagging the proteins with an N-terminal azide tag can only be done when making it recombinantly.

The first part of the project was important in trying to accomplish an effective and cheaper way to tag proteins containing an azide group but wasn't successful in the short

time that was given. The second part gave an appropriate experience in the lab with a biotechnology focus with the expressing, isolating, and purifying of the SDF-1 protein. A better understanding of protein engineering, the IFN- γ and SDF-1 proteins, azide-tagged proteins, protein functions overall, impacts proteins have on benefiting people, and the appropriate methods to use in the lab was also accomplished.

This project was worth doing because it gave a great experience in a lab setting and also helped increase the knowledge needed to pursue a career in a biotechnology setting. This project will also help benefit Dr. Leipzig's research lab along with the graduate student, Trevor, whose main focus is on this subject.

This report will present an overview of what was attempted and accomplished over the span of one year working in the lab. A brief background section that describes the two main proteins in depth, along with certain methods used along the way to the completion of the project, will be given with a comparison with prior research. Finally, the results found from the project will be shown followed by a discussion section to summarize the results.

Background

Proteins are macromolecules in living systems that play very important roles in the body's every day processes. They are required for the regulation, structure, and function of the body's organs and tissues and also do most of their work in cells. Proteins are made up of organic compounds called amino acids (20 different types) that are attached in long chains and, both together, are known as the building blocks of life. Two examples of protein functions are enzymes and antibodies, which were both used in this project. Enzymes are what carry out the chemical reactions that occur in cells and also help out with the formation of new molecules. Antibodies help protect the body by attaching to specific foreign particles, such as bacteria and viruses [7].

Recombinant proteins are created from the recombination of genes that forms DNA through genetic engineering. They are formed from cloned DNA sequences that usually encode a protein or enzyme with a known function. The recombinant DNA technology allows for the altering of human or mammalian proteins at larger quantities. By placing genes from humans or animals into bacteria, yeast cells, or mammalian genetic material, these microorganisms can make proteins, by being used as producers, for research, medical, or academic purposes [8].

A hexa-His sequence, also known as a His-Tag sequence, is a combination of six or more consistent histidine residues that act as a binding site for recombinant protein expression and purification. A target protein usually contains the placement of the His-Tag on the N-terminal and the Tag contains a cleavage site for a specific protease. A purified His-Tag protein cleaves off the His-Tag, with the specific protease that treated the His-Tag protein, or not if the tag isn't affecting the active site of the protein [8].

Interferon-gamma (IFN-γ), also known as Type II Interferon, is a small protein used for many different applications including testing for detection of mycobacterium tuberculosis (TB) infection, it modulates intestinal epithelial cell function, has been originally identified by its anti-viral activity, and acts as a potent modulator of the immune system [1 & 2]. INF-γ was used to differentiate neural stem cells into neurons in the lab. In the past, immobilized INF-γ was shown to be very effective in differentiating neural stem cells into neurons and the focus now was to improve on the method of immobilization.

Stromal cell derived factor-1α, also known as C-X-C motif chemokine 12, is a chemokine protein used for many different applications including the regulation of migration of hematopoietic progenitor cells, stimulates tyrosine phosphorylation of multiple focal adhesion proteins [10], recruits and alters the function of plasmacytoid precursor dendritic cells in human tumors [11], and is a highly efficacious lymphocyte chemoattractant [12]. SDF-1 was the main protein used in the lab for the second part of the project to test a method of immobilization.

Fast protein liquid chromatography is a protein technique that is a high-performance chromatography due to small-diameter stationary phases that allow high resolution. It features fast flow rates, high loading capacity, biocompatible aqueous buffer systems, and stationary phases in many different chromatography modes like gel filtration, affinity, ion exchange, and reversed phase. It was a technique developed for proteins originally and it allows reproducible separation by incorporating a high level of automation, which includes peak collection. This system also allows the application of biological samples like plasmids in addition to proteins [9].

An enzyme-linked immunosorbent assay (ELISA) is a plate-based assay that is designed for detecting and quantifying substances such as antibodies, peptides, proteins, and hormones [4]. There are four different types of ELISAs including direct, indirect, competitive, and sandwich, the type of ELISA that was used for this project. A sandwich ELISA is the least common type used but it is a highly sensitive and efficient way to detect antigens [6]. In a sandwich ELISA, an antigen is quantified between capture and detecting antibodies. A quick overview of the different stages of a sandwich ELISA consist of coating the ELISA plates, adding a blocking buffer, adding the sample solution, adding the antibody enzyme conjugate, adding substrate, and finally, reading the results in a plate reader. Figure 1 below shows a quick diagram of the sandwich ELISA [5].

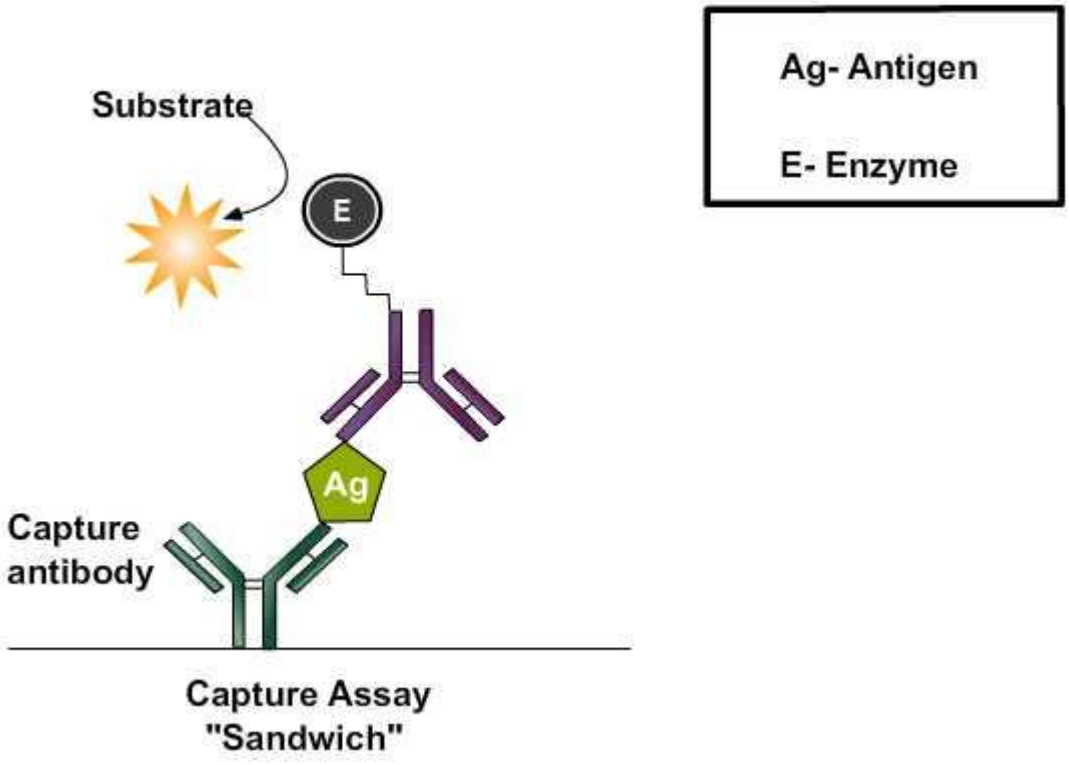


Figure 1 shows an easy visual of a sandwich ELISA.

Immobilization is the technique used for the chemical or physical fixation of enzymes, cells, organelles, or other proteins onto a solid support to increase stability and make sure to sustain their continued use. Methods for protein-biomaterial immobilization of different molecules are all similar but there is no universal applicable method of certain molecular immobilization. Some of the most common methods to use include adsorption (simplest method that involves reversible surface interactions between the supporting material and enzyme), crosslinking (enzyme molecules form three-dimensional structures), covalent bonding (between supporting material and enzyme), entrapment (free flowing enzymes in solution/restricted by the lattice structure of a gel), and encapsulation (enveloping enzymes within semipermeable membranes/very similar to entrapment) [14].

Experimental Methods

The following methods were used to complete the project. For the first part of the project a solid red dye, tetramethylrhodamine- dibenzylcyclooctyne (TAMRA-DBCO) was dissolved in dimethylsulfoxide (DMSO) at a ratio of 2 mg : 1 mL. A calculated amount of dye to react with a given amount of the made IFN-γ protein (or azide-tagged IFN-γ protein) was added to the protein. The vial was covered in foil to keep it out of light and was set aside to occur overnight. The next day, size-exclusion chromatography was used to remove the unbound dye. A ratio of 2:1 dilutions were made with phosphate buffered saline (PBS) and protein and two samples from each dilution were added to a plate reader. Measured absorbance values were taken using a microplate UV/vis spectrometer at wavelengths of 280 nm and 553 nm. These results were used to calculate protein concentrations and the degree of labeling (moles of dye per mole protein). Figure 2 below shows the multifunctional protein labeling via enzymatic N-terminal tagging and elaboration by click chemistry protocol, which was the approach/concept used for this first part of the project [15].

PROTOCOL

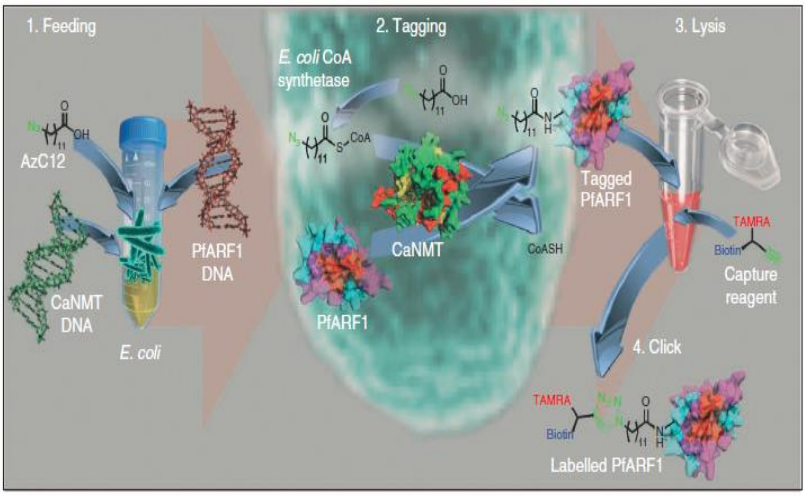


Figure 1 | Illustrative overview of the protocol. In the feeding stage (1), *E. coli* co-transformed with constructs for the expression of transferase (CaNMT) and target protein *Plasmodium falciparum* ADP ribosylation factor 1 (PfARF1) are incubated with tagged myristate (AzC12). *E. coli* acyl-CoA synthetase catalyzes the formation of AzC12-CoA (the substrate for NMT) *in vivo*. Tagging (2) is carried out *in vivo* by the newly synthesized CaNMT, attaching tagged myristate onto the N-terminal glycine of the target PfARF1. After lysis (3), the tagged protein is further elaborated by attachment of biotin (affinity) and TAMRA (fluorescence) labels[14] using 'click' chemistry (4).

Figure 2 shows an illustration with a description of the protocol for the click reaction that enables us to immobilize proteins [15].

The second main part of the project followed a long protocol in order to achieve the final results. A short summary of the protocol that was followed for this part of the project is explained. Protein production was done by transforming *E. coli* with plasmid (trying to get the highest yield of plasmid possible) to produce the desired azide-tagged SDF-1 protein and used a sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis of test expression. Next, a scale-up procedure came into play before the isolation and purification of recombinant protein took place. The isolation and purification step was done by cell lysis and Ni-NTA affinity chromatography. Finally, a dialysis was performed and the protein concentrations were determined for SDF-1 and azide-tagged SDF-1 after a fast protein liquid chromatography (FPLC) was used to further purify the proteins. Figure 3 below shows the summary of the recombinant *E. coli* protein production [3].

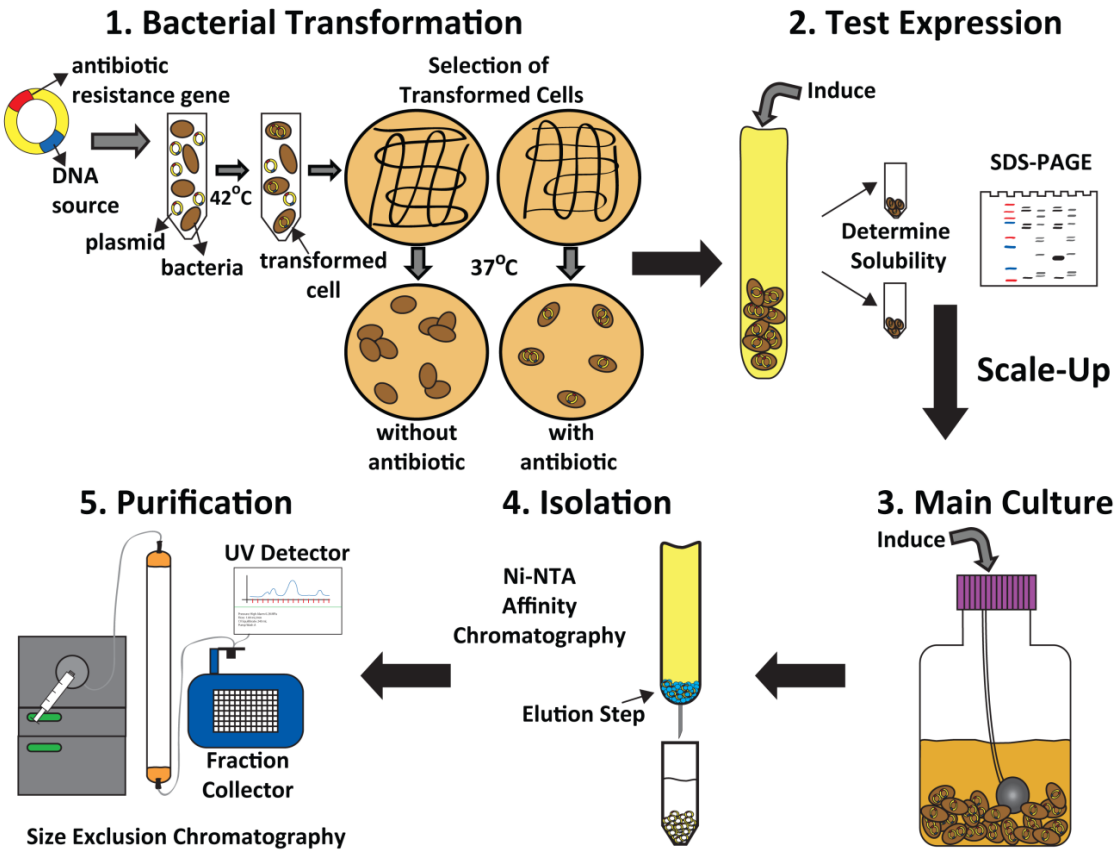


Figure 3 shows the entire expression, isolation, and purification procedure [3]

The full procedure that was followed for the expression, isolation, and purification of the azide-tagged SDF-1 protein can be found in [3] but also included some changes that can be found in the Appendix.

The FPLC results helped determine the range for the SDF-1 protein when compared with the standard. The protein was collected and a bicinchoninic acid (BCA) assay was completed in order to determine the protein concentrations of SDF-1 and SDF-1 with an azide tag.

Finally, an immobilization process began with reacting the SDF-1 azide tagged protein with dibenzocyclooctyne that is attached to methacrylamide chitosan (MAC) material. There was 100 ng of SDF-1 azide tagged protein per one gram of MAC material. The volume of SDF-1 azide was calculated using a 1:100 dilution (10 µL protein: 990 µL PBS) and knowing the concentration of SDF-1 azide obtained from the BCA assay. There was 24.3 µL of SDF-1 azide added to 0.0289 grams of MAC. The solution was vortexed and was placed on a shaker overnight at 4˚C. This same procedure was completed the next day for SDF-1 without an azide tag except 0.8398 µL SDF-1 was added to non-reacted MAC (no cyclic alkyne).

The next step was to make the photoinitiator, which generates free radicals to cross-link our material. The compound used was 1-hydroxycyclohexyl phenyl (135.9 mg) and the solvent used was 1-vinyl-2-pyrrolidinone (453 µL). Together, 5 µL photoinitiator was added for every 1 mL of material. The photoinitiator plus material solutions are immiscible so speed mixer was used for 3 minutes at 3000 RPM. Next, 100 µL of solution was added to 3 different slots on a microplate and put under a UV light in order to cross-link. This was done for both SDF-1 azide and SDF-1. After exposure to the light, the solutions turned into hydrogels due to the crosslinking that took place with the reaction between the acrylic groups on the MAC material, which was started by the photoinitiator. After the crosslinking process was completed, the hydrogels were put into three separate 2 mL tubes for each protein. PBS was then added to each tube (500 µL) so the material that didn't react would diffuse out into the PBS. All six tubes were placed in a 37˚C incubator for one week.

After one week, lysozyme was dissolved in a 50 mL conical tube at 200 mg/4mL in PBS. The pH was then adjusted to around 5.0. Each gel was centrifuged at 13,000 x g for 8 minutes. The supernatant was removed and saved in the -80˚C freezer. 500 µL of the dissolved lysozyme was added per gel and was vortexed then stored in a 37˚C incubator prior to further analysis.

The final part to complete the ELISA was to follow a protocol from the company PeproTech [13]. A mini ELISA kit was provided with the protocol in order to complete it. This protocol was followed with the need for 39 plate wells (three for each of the six solutions plus three for each of the seven standards). Once the plate reader gave data in Excel, a standard curve was plotted and based off this graph, the protein concentrations were calculated for the six samples.

Data and Results

Tables 1-3 are the results from the first part of the project when trying to detect an azide tag on a recombinantly produced azide- IFN-γ protein.

Moles Dye per Mole Protein			
<>	1	2	Avg
A	3.1927	4.3546	3.7736
B	2.3541	3.2104	2.7823
C	2.4384	2.2057	2.3220
D	2.1769	1.9710	2.0739
E	1.8936	1.8816	1.8876
F	1.9621	1.9764	1.9693
G	1.9474	2.0355	1.9914
H	1.8847	2.1420	2.0133

Table 1 shows the moles of dye per mole of protein for the first trial of experiments. The values range from about 1.9 to 3.2, which means the data doesn't make sense. Only one dye molecular can react with the tagged protein because it was tagged with only one azide group. This data set is not useful.

Moles Dye per Mole Protein			
<>	1	2	Avg
A	0.1630	0.1809	0.1720
B	0.1851	0.1820	0.1836
C	1.8084	0.1908	0.9996
D	-3.2586	0.1531	-1.5528
E	-1.2084	0.1788	-0.5148
F	0.2059	0.2136	0.2098

Table 2 shows the moles of dye per mole of protein for the second trial of experiments. The values range from about -1.6 to 0.2, which means the data doesn't make sense. Only one dye molecular can react with the tagged protein because it was tagged with only one azide group. It is impossible to obtain negative values for this experiment and this data set is not useful.

Moles Dye per Mole Protein			
<>	1	2	Avg
A	0.5052	0.5601	0.5326
B	0.2649	0.2890	0.2770
C	0.2001	0.2001	0.2001
D	0.1560	0.1474	0.1517
E	0.1590	0.1798	0.1694
F	0.1740	0.1690	0.1715

Table 3 shows the moles of dye per mole of protein for the third trial of experiments. The values range from about 0.15 to 0.53, which has the best data of the three trials but still not good enough with values being too low. Only one dye molecular can react with the tagged protein because it was tagged with only one azide group.

Table 4 below shows the protein code (amino acid sequence) that was sent to the GenScript company in order to obtain the proper azide tagged SDF-1 protein needed for the second part of the project.

Stromal cell derived factor 1 (SDF-1) Azide Tagged protein (N-terminus)

Myr-Sequence **Spacer**
MGLYVS EFPKPSTPPGSSGGAP

SDF-1 (AA23 to AA156)
MDAKVVAVLA LVLAALCISD GKPVSLSYRC

PCRFFESHVA RANVKHLKIL NTPNCALQIV

ARLKSNNRQV CIDPKLKWIQ EYLDKALNK

TEV Cut **His tag**
ENLYFQG HHHHHH

Table 4 shows the protein code that was created and sent to GenScript.

Figures 4 (a) and 4 (b) below dealt with the part of the procedure with obtaining the SDS-PAGE results. Figure 4 (a) shows the standard ladder that shows the molecular weights in kilodaltons (1 Dalton = 1 gram per mole) to compare with the actual SDS-PAGE results and is also shown in lane 3 in Figure 4 (b). Figure 4 (b) shows the actual results that were obtained in the lab when running the experiment and it is easily seen how well the SDF-1 plus CaNMT enzyme results match up with the standard ladder. The results show the molecular weight estimation of the SDF-1 plus enzyme lanes match up

around the 50 kDa ladder mark. The results for the SDF-1 protein by itself wasn't as easy to see but the results show very lightly colored bands around the 10 kDa ladder mark. Therefore, a band was present for both soluble and insoluble and allowed us to choose which procedure to move forward with.

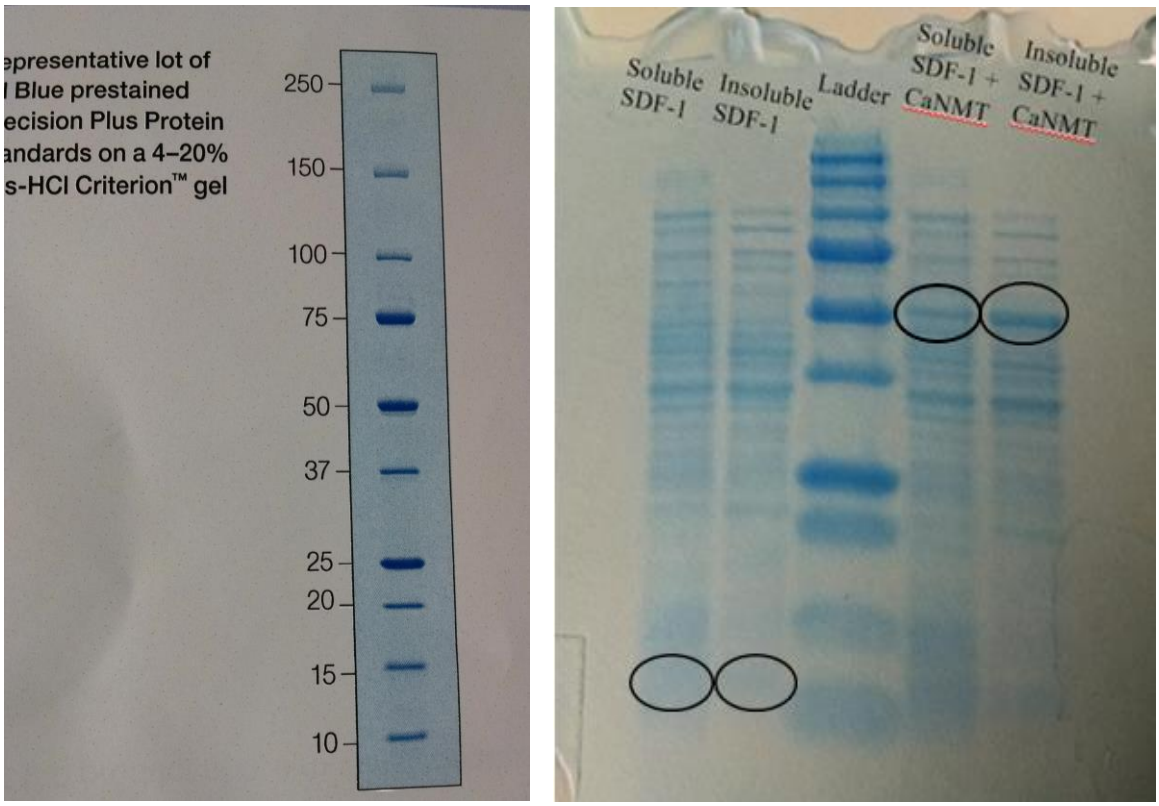


Figure 4 (a) shows the scale to compare the results of the SDS-PAGE analysis of test expression with in order to determine whether the protein expresses in the soluble and/or insoluble fractions. Figure 4 (b) shows the results of the SDS-PAGE analysis of test expression in the lab.

The next figure (Figure 5) shows the gel filtration standard components used to relate the proteins used (SDF-1 and SDF-1 azide) to determine the proper letter they follow on the gel filtration standard curves (Figure 6). The letters are based on the molecular weight ranges in which the SDF-1 and SDF-1 azide proteins have similar molecular weights to the letter “E." The standard curve from Figure 6 (specifically the latter “E” curved area) is then used to compare with Figures 7 and 8 in order to obtain the proper ranges to collect the SDF-1 and SDF-1 azide proteins. Each range that corresponds to the letter “E” curve in Figures 7 and 8 were clearly marked on each figure.

Table 1. Gel Filtration Standard Components

Component	Molecular Weight*	Amount per Vial (mg)
Thyroglobulin (bovine)	670,000	5.0
γ-globulin (bovine)	158,000	5.0
Ovalbumin (chicken)	44,000	5.0
Myoglobin (horse)	17,000	2.5
Vitamin B_{12}	1,350	0.5
		18

Figure 5 shows the gel filtration standard components with their corresponding letters and molecular weights. The component/letter that one would want to choose relates with the molecular weight of the protein that is being used for the experiment.

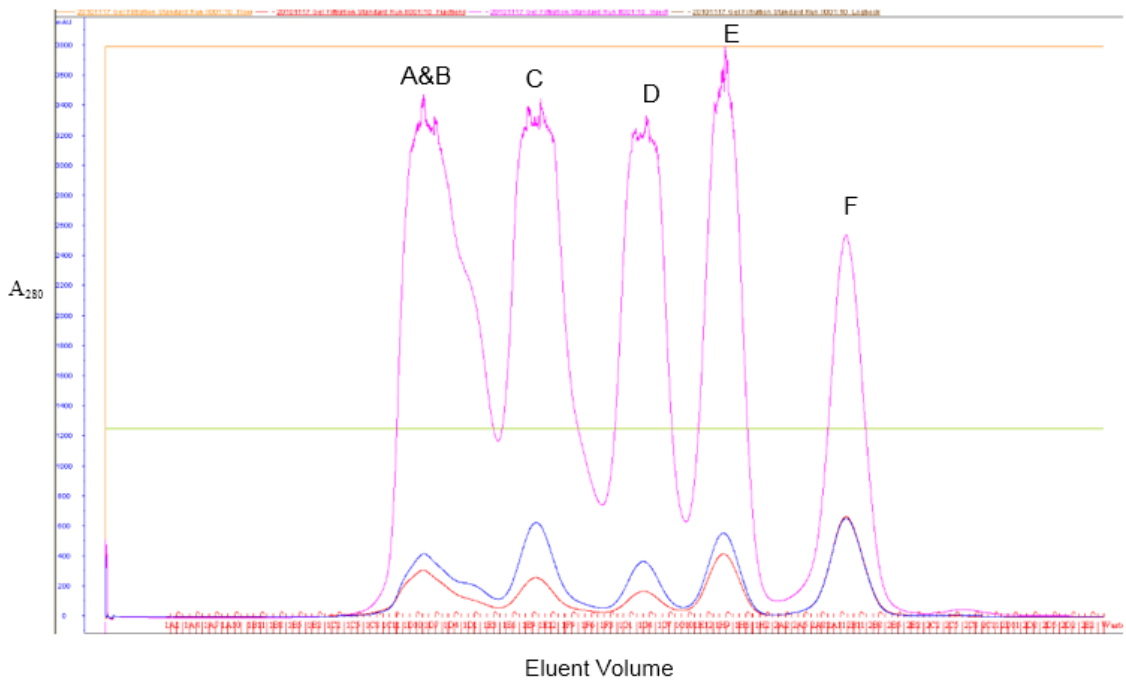


Figure 6 shows the gel filtration standard peaks from the FPLC. The letter we determined from Figure 5 (Letter “E” for SDF-1 proteins) based on molecular weight allows us to take a look at this figure and determine the peaks we need to relate with in Figures 7 and 8, which contain the actual results that were obtained from the FPLC.

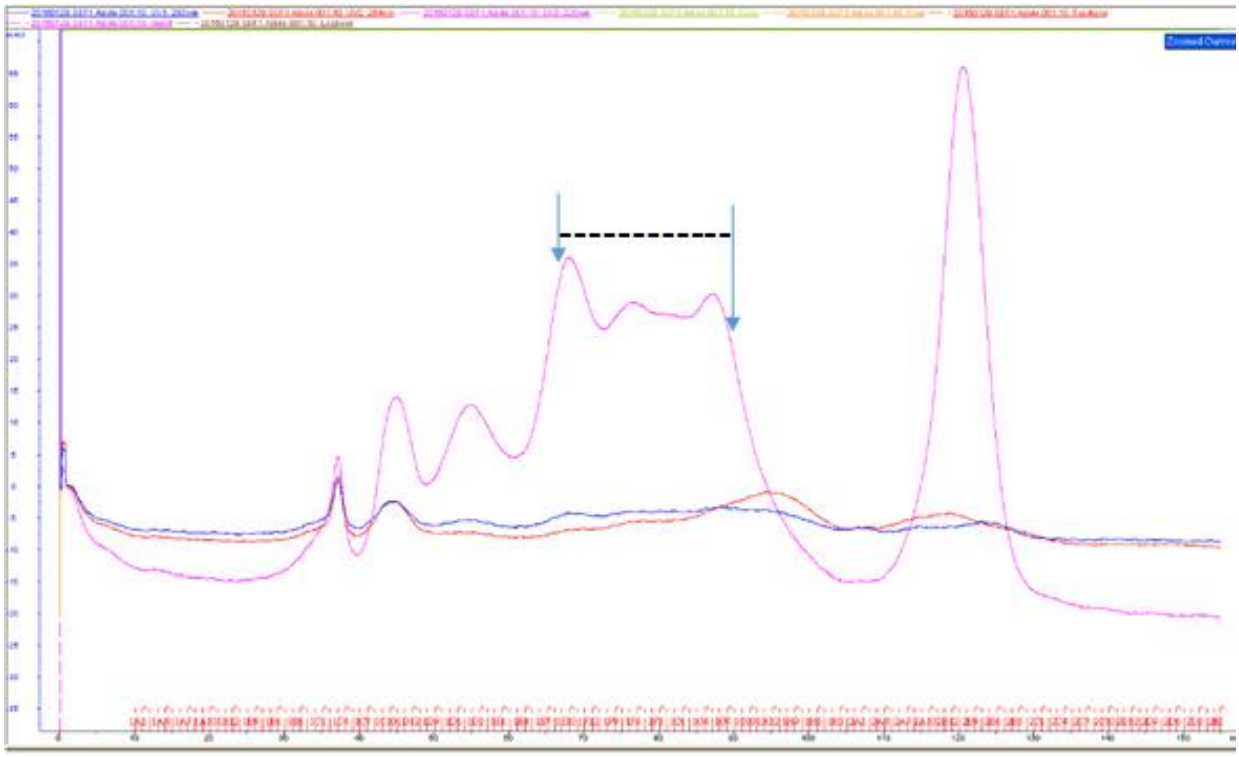


Figure 7 shows the FPLC results for the azide-tagged SDF-1 protein. The range that was determined and labeled on the figure above (1E10 – 1G7) was based on the previous Figure 6. The figure allowed us to collect our protein from the determined range since it corresponds with the proper molecular weight for SDF-1 azide. The other peaks represent the other letters/components that would relate with different molecular weights and not our protein of interest.

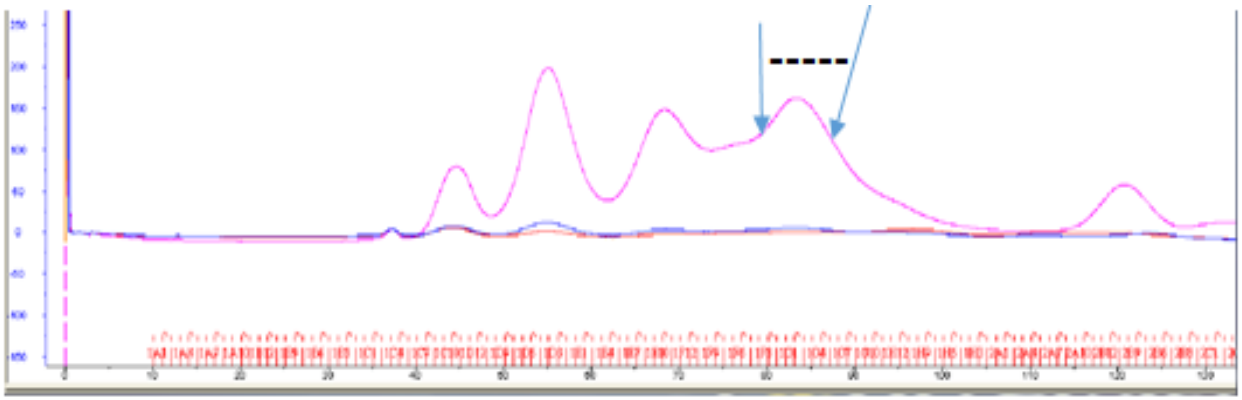


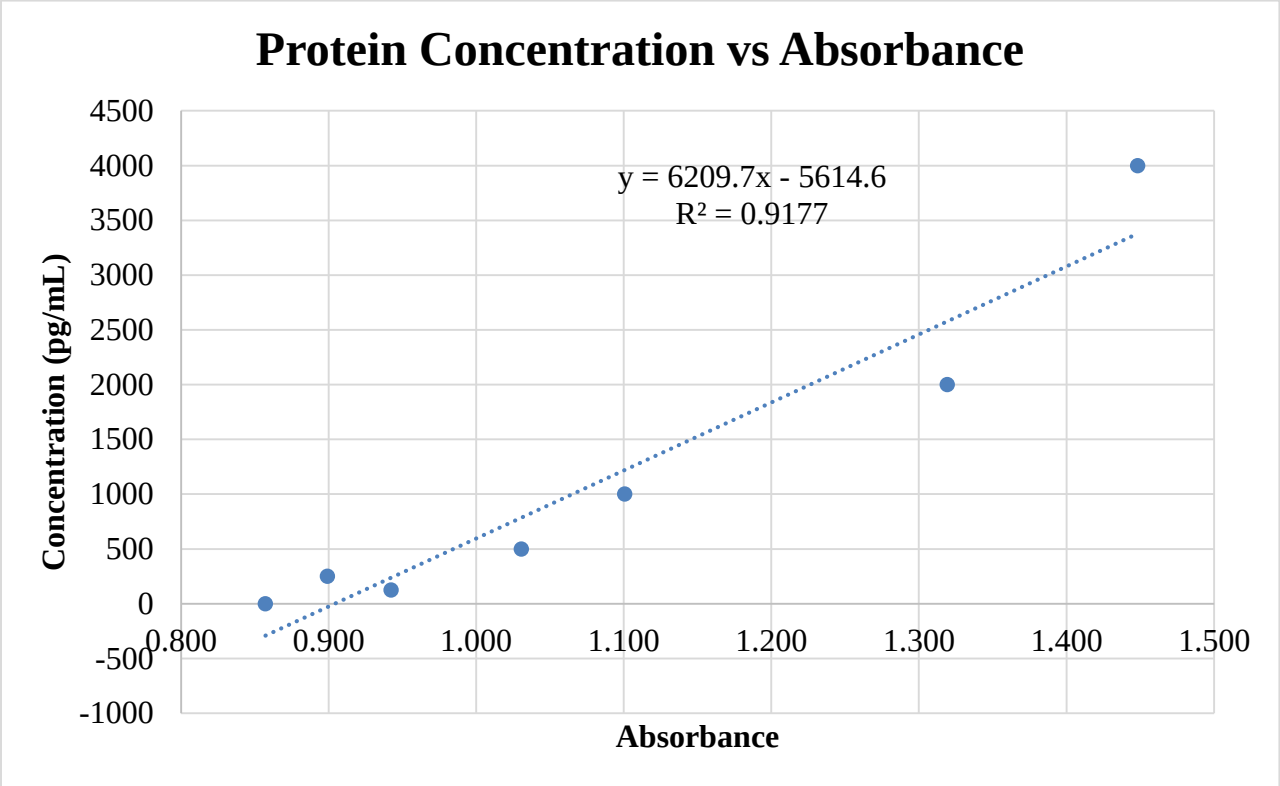
Figure 8 shows the FPLC results for SDF-1 protein. The range that was determined and labeled on the figure above (1F3 – 1G4) was based on the previous Figure 6. The figure allowed us to collect our protein from the determined range since it corresponds with the proper molecular weight for SDF-1. The other peaks represent the other letters/components that would relate with different molecular weights and not our protein of interest.

After the ranges were determined from Figures 7 and 8, the proteins were collected and a BCA assay was completed. The assay allowed for the determination of the proteins' concentrations. The protein concentrations for SDF-1 and SDF-1 with an azide tag can be seen in Table 5 below.

	SDF-1	SDF-1 Azide
Protein Concentration (µg/mL)	15.79	12.52
Standard Deviation	0.3991	5.335

Table 5 shows the average protein concentrations recovered for the SDF-1 and SDF-1 azide proteins. It also shows the average standard deviation of the protein concentrations found.

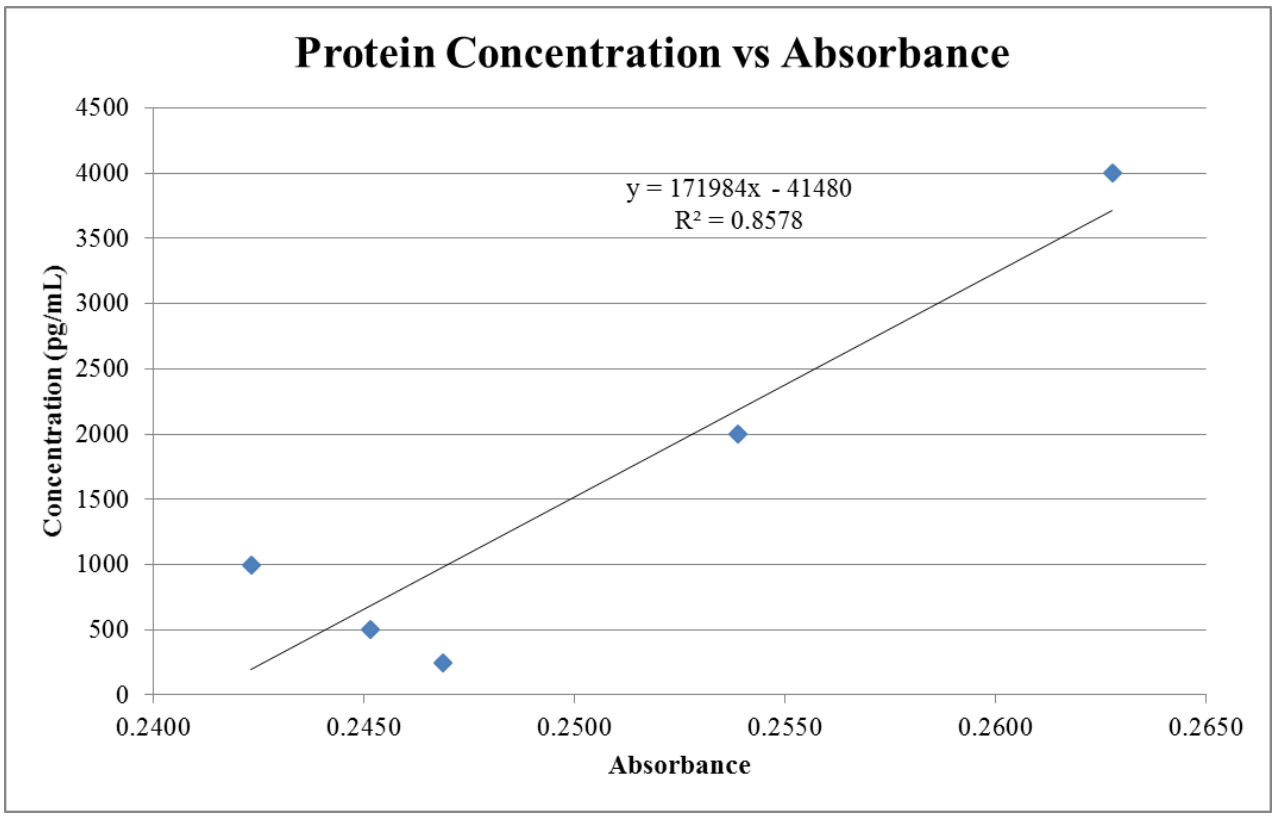
The following begins the results for the release study from performing three different trials for the sandwich ELISA. Graphs 1-3 and Table 6 are presented below.



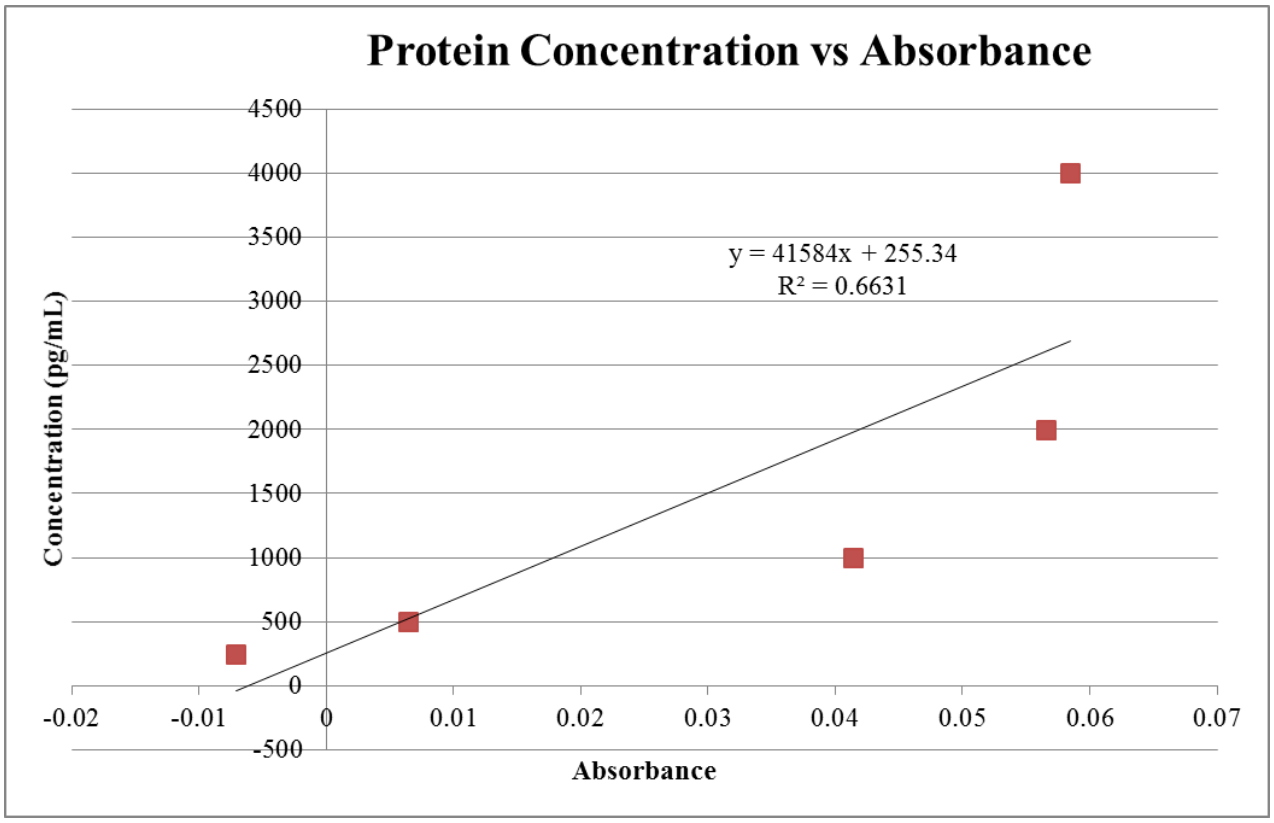
Graph 1 shows the standard curve obtained from the first trial of the ELISA. The y-axis is the protein concentration and the x-axis is the absorbance values obtained from the plate reader in the lab. The absorbance values that were obtained from the plate reader for our SDF-1 and SDF-1 azide samples were put in for the value of "x" in the "y equals" equation. This allowed for the determination of the protein concentrations that are in Table 6.

Protein	Concentrations (pg/ml)
Azide	5106
Azide	6008
Azide	5661
SDF1	9909
SDF1	9799
SDF1	11169

Table 6 shows the protein concentrations that were obtained from the absorbance values for the samples that were put into the "y equals" equation in Graph 1 for the first trial of the ELISA. The SDF-1 concentrations are higher than the SDF-1 azide concentrations, which is not what we would have expected. Experimental error most likely was the reason for this occurrence.



Graph 2 shows the standard curve obtained from the second trial of the ELISA. The y-axis is the protein concentration and the x-axis is the absorbance values obtained from the plate reader in the lab. The absorbance values would be obtained from the plate reader for our SDF-1 and SDF-1 azide samples and put in for the value of "x" in the "y equals" equation for the determination of the protein concentrations. This didn't happen because this standard curve being very messed up due to the kit having issues with the standards.



Graph 3 shows the standard curve obtained from the third trial of the ELISA. The y-axis is the protein concentration and the x-axis is the absorbance values obtained from the plate reader in the lab. The absorbance values would be obtained from the plate reader for our SDF-1 and SDF-1 azide samples and put in for the value of "x" in the "y equals" equation for the determination of the protein concentrations. This didn't happen because this standard curve being very messed up due to the kit having issues with the standards.

Discussion/Analysis

The data that was obtained from trying to perform a new method to tag proteins with an azide group and determine the tagging ratio in part one of the project was very inaccurate and didn't make any sense. This was attempted three different times in the lab and it never worked out the way that was expected. The first trial of results made no sense due to the values being too high. The maximum value could only be one because the protein was tagged with only one azide group, thus only one dye molecular could react with it. The second trial results were either too low or negative which makes no sense, and the third trial results were all low values. The data can be seen in Tables 1-3 and was neglected due to the procedure not being a method that worked with meaningful results.

The second part (main part) of the project was then completed after the lack of time for the first part. The first objective that was completed, in order for the main part to be accomplished, was to make a protein code and send it to the company GenScript. The plasmid that was made used pET-28a as a "virus" to help attack the *E.coli* cells with the proper gene (amino acid) sequence. The company had to play around with DNA base pairs in order to find the best optimal sequence according to the code in Table 4.

The protocol for the expression, isolation, and purification of the azide-tagged SDF-1 protein began once the plasmid was received from GenScript. The first part within the protocol that presented results was the SDS-PAGE step. Lane 1 in Figure 4 (b) shows the soluble single results, lane 2 shows insoluble single results, lane 3 shows the ladder to compare with, lane 4 shows soluble double results, and lane 5 shows insoluble double results. The single transformation consists of SDF-1 plasmid and the double

transformation consists of SDF-1 plasmid plus enzyme. The difference between the soluble and insoluble is that the soluble bacteria pellets are used to determine if the recombinant protein is in the cytoplasm regions of the bacteria cells and the insoluble pellets will undergo denaturation procedures in order to release proteins found in inclusion bodies of bacteria cells. The goal is to try and achieve the highest yield of recombinant protein when deciding between the soluble or insoluble. After analyzing the SDS-PAGE results in Figure 4 (b), it was determined that both soluble and insoluble could work for the experiment when comparing the results to the Figure 4 (a) scale and the lane 3 ladder. It was decided to move forward with the soluble or native procedure for the isolation and purification of recombinant protein in step 6 due to the native procedure being quicker versus the nonnative procedure.

The FPLC results in Figures 7 and 8 were compared with Figure 5, showing the molecular weight distribution of components, and with Figure 6, which shows the gel filtration standard curves for the separations. The range that was determined, from Figure 7, for the SDF-1 azide protein was from 1E10 to 1G7. The range determined from Figure 8 for the SDF-1 protein was 1F3 to 1G4. The protein concentrations for SDF-1 and SDF-1 with an azide tag were calculated from here by doing a BCA assay. The protein concentration for SDF-1 was found to be 15.79 μg/mL and the protein concentration for SDF-1 azide was found to be 12.52 μg/mL.

An ELISA was performed after obtaining and calculating the SDF-1 and SDF-1 azide protein concentrations. This was done in order to quantify the immobilization efficiency. After the first ELISA was performed, a standard curve was obtained (Graph 1) but the results (Table 6) didn't make sense because the SDF-1 protein concentrations

were higher than the SDF-1 azide concentrations. Some experimental error with not having the hydrogels totally dissolve in solution or have some of the hydrogels remain stuck to the plate may have occurred and caused this. Also, it was later determined that the lysozyme used in the experiment was bad and could have messed with the results. Second and third trials were performed with the supernatant that was saved from the solution the gels were in. These results were not useful due to the standards not producing good data (Graph 2 and Graph 3). It was discovered that the kit purchased from the PeproTech company had been messed up and, therefore, was making our standard curves not turn out properly.

Some experimental errors that may have occurred throughout the experiments that were performed in the lab include pipetting issues, measuring issues, contamination issues, human error, following the procedure the exact same way, equipment errors, or environmental impacts. One specific experimental error that occurred while doing the second part of the project in the lab was found in step 5.3.3 of the scale-up and main culture procedure. The inducing with IPTG was supposed to occur after the OD_{600nm} reached between 0.7-0.8. This was not the case in the lab due to the numbers not reaching between the 0.7-0.8 range. The single reached a value of 0.162 and the double reached a value of 0.174. Both samples were induced with IPTG with these values anyway and the experiment proceeded on.

For future work, if I had more time to work on this project I would try new methods to determine the azide-tagging efficiency and try to create a new kit to detect the amount of azide-tagging. For the 2^{nd} part, I would run the experiment through a few more times in full and do more ELISAs to get the best results. Also, I would take more time to

make sure all equipment/compounds/etc. are up to date and increase the sample size for testing.

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Appendix

The following changes were made to the full procedure that was followed for the expression, isolation, and purification of the azide-tagged SDF-1 protein:

1. Designing of the target protein – azide-tagged SDF-1 with the specific amino acid sequence sent to the company can be found in the data and results section of this report.
2. Making agar plates – antibiotics used in step 2.3 were ampicillin (AMP), kanamycin (KAN), and a double with tetracycline (TET) + KAN
3. In step 4.1, add 2 μL of the SDF-1 plasmid to the single transformation and add 4 μL of the SDF-1 plasmid plus 4 μL CaNMT enzyme to the double transformation.
4. In step 4.2, add 5 mL broth plus 5 μL KAN to the single and add 5 mL broth plus 5 μL KAN plus 5 μL Amp to the double.
5. In step 4.7, add 5.25 μL of IPTG.
6. In step 4.8, shake for 3 hours at 250 RPM.
7. After step 4.9.3, make sure samples are cooled to room temperature before proceeding to step 4.9.4.
8. In step 5.2, the single flask should contain 20 μL KAN and the double flash should contain 20 μL KAN plus 20 μL AMP.
9. In step 5.3.1, add 1800 μL KAN to the single and 1800 μL plus 1800 μL AMP to the double.
10. In step 5.3.2, set the pressure of air between 5-10 psi.

11. In step 5.3.3, induce the single with 1800 μL IPTG nd the double with 1800 μL IPTG plus fatty acid (dissolved in DMSO) with an azide tag.
12. In step 5.4.2, make sure to save the supernatant in case it is needed.
13. In step 6.2.2.2, add 5 mL of elution buffer seven different times for both the single and double or until no protein is detected (no more blue color).
14. In step 6.2.2.2.2, use two cuvets instead and add 250 μL of Bradford reagent to each cuvet.
15. In step 6.3.1, the pH wanted for the SDF-1 protein is 7.4
16. In step 6.3.2, place protein sample in dialysis buffer 1 containing 3600 μL nano water, 400 μL PBS, and dithiothreitol (DTT). After, place the protein sample in dialysis buffer 2 containing 3600 μL nano water plus 400 μL PBS.
17. In step 6.3.3, put SDF-1 and SDF-1 azide into two separate 6 mL dialysis tubes. Centrifuge at 4,000 g at 4°C. Make sure to further purify the proteins by using FPLC.
18. Stop after step 6 is complete. Do not follow step 7.