

Hunt for a Genetically Engineered, Rationally Designed, Stealth Peptide
to Prevent Non-Specific Protein Interactions

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Abstract

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Chemical Engineering

The objective of this study is to identify the best zwitterionic peptide as a tail of a fusion protein to preserve protein stability and bioactivity without immunogenicity. Two major strategies currently available to achieve this protective affect are (a) chemical conjugation and (b) genetic engineering. This study pursues the later approach. A major obstacle to the genetic engineering approach as identified by previous studies is related to expression yields which is unique to fusion proteins. For this study, Enhanced Green Fluorescent Protein (EGFP) was used as a model protein. The four amino acid chains (all 10kDa in length) were examined. They were fused to EGFP as a vehicle to study expression, purification, structure, and activity. The best candidate has the best expression yield and protein activity. The results of this study will be used as the starting point of ongoing research efforts aimed at increasing circulation time and decreasing the foreign body response for protective and therapeutic proteins; replacing PEGylation with these highly hydrated and bio-degradable peptides.

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INTRODUCTION

Protective and Therapeutic Proteins

With the rise of genetic engineering protective and therapeutic proteins are becoming increasingly important in our society. Miracle drugs like organophosphate hydrolyze (OPH), a bio scavenger, which one day hopes eliminate the threat of nerve agents on the battle field. OPH has an almost catalytic effect treating lethal nerve agent exposure making it a better candidate than the human bodies internal protective protein, human butyrylcholinesterase (hBCE) which only works on a stoichiometric basis making it ineffective against lethal doses of organophosphates. Other animals such as cows have a similar protective protein, butyrylcholinesterase (BCE), making them a possible donor candidate, but this would require impractically large quantities of animal plasma and there would still be immunogenic issues as well as Unfortunately, OPH has similar immunogenicity issues as animal blood synthesized BCE. These immunogenic issues could be solved with a genetically engineered, zwitterionic stealth peptide.

Therapeutic proteins represent a large and growing field of drug research. Recombinant produced insulin and monoclonal antibodies account for the majority of revenue generated by this class of pharmaceutical and combined they accounted for ~\$150 billion in sales in 2013, an increase of approximately 50% from 2008.¹⁻³ Therapeutic proteins operate via many varied paths; however, they can be generally grouped into five different mechanisms: resupply a needed protein, create a new function, enhance a pre-existing pathway, inhibit a molecule or organism, or act as a vehicle for another therapeutic.⁴ Unfortunately, developing therapeutic proteins is very challenging. Prior to the discovery and use of cloning and genetic engineering scientist first utilized proteins such as human Growth Hormone (hGH) found in human corpses, but sourcing

and concentration were just a few of many obstacles.⁵ With the discovery of genetic engineering it became possible for researchers to express these human proteins in many ways resolving these first obstacles.

By identifying the specific gene that expresses the desired protein in the human body, researchers were now able to place that gene inside a cell line and allow the cell to produce the protein. There are several options as far as what bacterial cell line to utilize, but *E. coli* is one of the most common. Unfortunately, this new technique faces additional challenges, with protein stability and physiological circulation being two of the largest.²

Non-Fouling Materials

One solution to these short circulation and immunological response challenges was suggested in the 1970s by Frank Davis of Rutgers University in his seminal work on the use of poly(ethylene glycol) (PEG) attachment to solve immunological problems in immunogenic proteins.⁶ His work led to what is commonly known today as PEGylation, whereby PEG is chemically conjugated to therapeutic proteins, medical implants, and devices in order to increase physiological circulation through increased hydrodynamic radius and prevent the foreign body response. The hydrophilicity of PEG allows it to surround itself with water which acts as a stealth agent preventing the human body's immunogenic response system from identifying it during delivery of the therapeutic protein. Furthermore, the increased hydrodynamic size has the added advantage of increasing drug half-life by slowing down the loss of the protein through renal clearance.

PEGylation, the most widely used non-fouling agent, is not without its own drawbacks. Bioactivity is reduced in PEGylated proteins and immunogenicity is becoming a larger problem with a significant and growing portion of the population possessing anti-PEG antibodies.⁷ An

attractive alternative to PEG is zwitterionic non-fouling materials such as poly(carboxybetaine) (pCB). Unlike PEG, which utilizes hydrogen bonding to hydrate its surface, pCB utilizes ionic solvation to interact more strongly with the surrounding water molecules.⁸ This significantly improves its non-fouling character especially in complex media like human blood.⁹

Zwitterionic Fusion Proteins

Zwitterionic peptides could be an attractive alternative to both pCB and PEG for several reasons. Two advantages are that these protective peptides are biodegradable and can be fused into a protein via genetic engineering in addition to chemical conjugation. Chains of alternate positively Lysine (K) and negatively Glutamate (E) charged amino acids in stoichiometrically equal amounts have been shown to result in an ultra-low fouling peptide.^{2,10}

Genetic fusion of proteins, the splicing of two genes together into a single piece of DNA capable of expressing one protein which shares characteristics of both starting proteins, has several advantages over chemical conjugation. The most significant advantage is size. The degree of protection from non-specific interactions in complex media is highly dependent on the proportional size of the stealth tag to the protein, be it PEG, pCB, or zwitterionic peptides. Peptide synthesis produces short peptides ranging generally from di-peptides to peptides of approximately 120 amino acid residues, which is significantly smaller than what is required for stealth function. However, as many proteins produced by cell-lines are larger than what is required, it follows that the genetic fusion will be able to produce a sufficiently large stealth tag.

Members of the Jiang Lab have experimented with this using an organophosphate hydrolyze (OPH), an organic bio-scavenger, as a protein fused with different lengths of EK peptides. Protein yield is challenging. A one-liter expression of OPH yields 50 - 100mg of purified protein, where as a one liter expression of OPH-EK yields can be as low as ~100 μ g. It

is hypothesized that the high amount of free E and K required for EK translation placed a metabolic burden on the host cell.

To address the metabolic burden concern, four different zwitterionic amino acid chains, all 10kDa in size, were screened for their impact on expression yield. They are (a) Glutamate (E) and Lysine (K) or EK, (b) EKG (Glycine), (c) EKGS (Serine), and (d) EKGSN (Asparagine)). Furthermore, we examined their impact on secondary structure and activity of EGFP to select against zwitterionic peptides that may negatively impact the adjoining fusion protein. The design of these chains further built on the work of White et al., who screened of over a 1000 proteins to determine from nature which are most common, and thus efficient at protecting proteins from non-specific binding.¹¹ The first adjustment from the original EK chain was to add in Glycine (G) and then Serine (S), and Asparagine (N). These three additional amino acids were chosen for their non-fouling characteristics as identified by White et. al.¹¹ Each of these were added stoichiometrically in order to preserve the zwitterionic nature of the chain. The four different fusion proteins examined in this study are as follows: EGFP-EK, EGFP-EKG, EGFP-EKGS, and EGFP-EKGSN. The EGFP was used as a control.

METHODS & MATERIALS

Materials

See Appendix for detailed list of Chemicals, Proteins and General Supplies.

Preparation for DNA Sequencing

After receiving the genes they were transformed into *E. coli* DH10B competent cells then spread on LB ampicillin plates for 16 hours at 37 degrees Celsius. Colonies were cultured for 16 hours at 37 degrees Celsius in 5mL Lysogeny Broth with 1x ampicillin (100ug/mL).

Subsequently, 5mL cultures were mini-prepped according to the zymocam mini-prep procedure. The samples were then sent to Genewiz for sequencing.

Hydrophobic Interaction Chromatography (HIC) Column Screening

The following five different 1 mL HIC columns were tested on the BioRad Fast Protein Liquid Chromatography: Butyl Fast Flow, Butyl High Performance, Butyl S Fast Flow, Octyl Fast Flow, and Phenyl High Performance. The fractions with EGFP in them from each HIC column were then run through SDS Page gel electrophoresis to determine qualitatively which column performed best.

EGFP Fusion Protein Test Expression Protocol

Streak LB ampicillin plates with cell stock and incubate at 37C for 16 hours. Pick one colony into 5mL LB/amp, incubate on the shaker at 37C for 16 hours. Add 1mL of LB/amp culture to 30mL Terrific Broth with 1x ampicillin. Incubate at 37C on shaker until the Optical Density (OD) at 600nm reaches 0.5 – 1.0, at which point the sample is induced with 30μL Isopropyl β-D-1-thiogalactopyranoside (IPTG) and then incubated at 30C on shaker until harvest. Four hours after induction OD600 readings are done again and the samples are normalized to OD by adding 400μL of PBS per OD to the pellet from 1 mL of each culture. The

resultant OD normalized solutions were then assessed for their fluorescence by exciting them at 395nm and reading emission at 509nm in the Cytation plate reader.

EGFP and EGFP Fusion Protein Expression Procedure

Streak LB/amp plate with cell stock and incubate for 16 hours at 37C. Pick a colony and culture 5mL TB/amp for 6-8 hours on shaker in 37C room. Add 5mL culture + 1L TB/amp in Erlenmeyer flask in the 30C on shaker. Add 1mM IPTG to induce expression and incubate for 24 hours in the 30C on shaker. Harvest by pelleting cultured cells at 15000 rpm for 10 minutes and discarding supernatant. Resuspend in 5mL of PBS per 1g of wet cell pellet. Using liquid nitrogen freeze the solution in the falcon tubes. Then thaw in water bath with slow mixing for approximately 30min or until completely thawed. Repeat for a total of 3 freeze thaws. Split suspended cells into four aliquots. Sonicate for five seconds on and 15 seconds off for 20 minutes or until clarified. Spin down clarified lysate at 15000 rpm for 10min. Keep supernatant and discard cell debris. Precipitate the DNA by pouring supernatant out into four new falcon tubes and add 1mL of the 0.1mM protamine sulfate in PBS solution for every 10mL of lysate. Let sit for approximately 20 minutes on ice. Spin down 15000 rpm for 10 minutes and keep the supernatant. Slowly add 2M ammonium sulfate to sample at 4C. Spin down at 15000 rpm for 10min and discard the pellet.

EGFP and EGFP Fusion Protein Purification Procedure

Hydrophobic interaction chromatography was used in conjunction with the previously identified phenyl HP Column and a decreasing ammonium sulfate gradient from 2M to 0. In the case of GFP-EKG elution was done with water instead of PBS. Each HIC run results in 2 x 1mL fractions which are then combined and concentrated to give a 1mL sample. This 1mL sample was then run on the ENrich 650 size exclusion column (using PBS as the buffer). This results in

2 x 1mL samples which are then combined and run through CaptoQ anion exchange with an increasing gradient of PBS and sodium chloride from 0 – 1M which gives a final product of several milliliters. The resultant protein was then run through SDS-polyacrylamide gel electrophoresis (PAGE) to determine purity and BCA to determine total protein in solution.

SDS-PAGE

This was used to determine presence of desired protein and relative purity in given fractions during the purification procedures as well as during several steps of the expression protocols. 30 μ L of each test sample was placed in a labeled micro centrifuge tube (1.5mL). 98 μ L of 6 x SDS running buffer and 2 μ L β -mercaptoethanol in one micro centrifuge tube. 6 μ L of the resultant β -mercaptoethanol solution was then added to each sample to be tested. Each test sample was placed in a heat block at 95C for 10min. In the first well 5 μ L of the ladder SeeBluePlus2 and 35 μ L of each test sample were loaded in wells of NuPAGE Bolt 4-12% PAGE gel. 200 V was applied to the PAGE gel in MOPS running buffer for approximately 30min. Once complete the gel was removed from the plastic shell and rinsed in millipore water for 10min on a mixing table, then gel fixing buffer for 10min, then colloidal blue for approximately 16 hours, then de-stained with water for approximately one hour. Images of the gels were captured with the UVP BioDoc-It Imaging System.

Bicinchoninic Acid Assay (BCA)

In order to determine approximate quantity of protein present this assay was used with OPH, EGFP, INF and their fusion proteins. A standard curve was created with BSA by adding 0 μ g, 2 μ g, 4 μ g, 6 μ g, 8 μ g, and 10 μ g to wells of a 96 well plate. Each condition was done in triplicate. Next several different concentrations of the target protein are added with the goal being that at least one of the unknown concentrations falls within the linear range of the control.

Usually the unknown is concentrated down to approximately 1mL and 2 μ L, 4 μ L, 6 μ L, 8 μ L of that are tested. BCA reagents A and B are mixed 50:1 (A:B) and 200 μ L are added to each well with mixing. Then the plate is placed in the 37C warm room for 30 minutes. The plate is subsequently read at 562nm in the Cytation5 imaging reader by BioTek.

Circular Dichroism (CD) Structural Analysis

In order to compare secondary structure of fusion proteins to their base protein the Jasco 720 Spectropolarimeter was used. Multiple test iterations were conducted in order to determine the appropriate buffer, protein concentration, and buffer concentration that minimized background signal. These initial tests showed that the ideal protein concentration was 5 μ M in 10mM Potassium Phosphate buffer at pH 7.4. Following the Jasco published procedures provided in the Appendix both the EGFP and EGPF fusion proteins were run through the basic secondary structure program. The data were then smoothed and analyzed using the BeStSel free online CD analysis algorithm.¹²

RESULTS AND DISCUSSION

Genetic Engineering

The genes were purchased from Genescript. They delivered genes for the Enhanced Green Florescent Protein (EGFP) as well as EGFP fusion proteins which met the following design criteria. Each amino acid chain was 10kDa, each different chain whether EK, EKG, EKGS, or EKGSN was a perfectly repeating sequence such that, the EK chain followed this pattern (EKEKEKEKEK...) this ensured that there would not be any significant localized charge thus preserving the zwitterionic nature of the peptide. The amino acid chains were fused to the N-terminus of the EGFP. The exact protein sequences for all five proteins can be found in Appendix A. After receiving the genes, miniprep samples were sent out for DNA sequencing Genewiz (Sanger Sequencing) to verify the sequence.

Developing a Purification Protocol

Expression protocol being widely understood for EGFP once the DNA sequences had been independently verified a 1L expression of the base EGFP protein was conducted according to the procedure previously laid out. Using this EGFP lysate a screen was conducted on five different hydrophobic interaction chromatography columns to determine which was the best choice. The five columns examined were as follows: Butyl Fast Flow, Butyl High Performance, Butyl S Fast Flow, Octyl Fast Flow, and Phenyl High Performance. The fractions with EGFP in them from each HIC column were then run through SDS page (**Figure 1**) to determine qualitatively which column performed best.

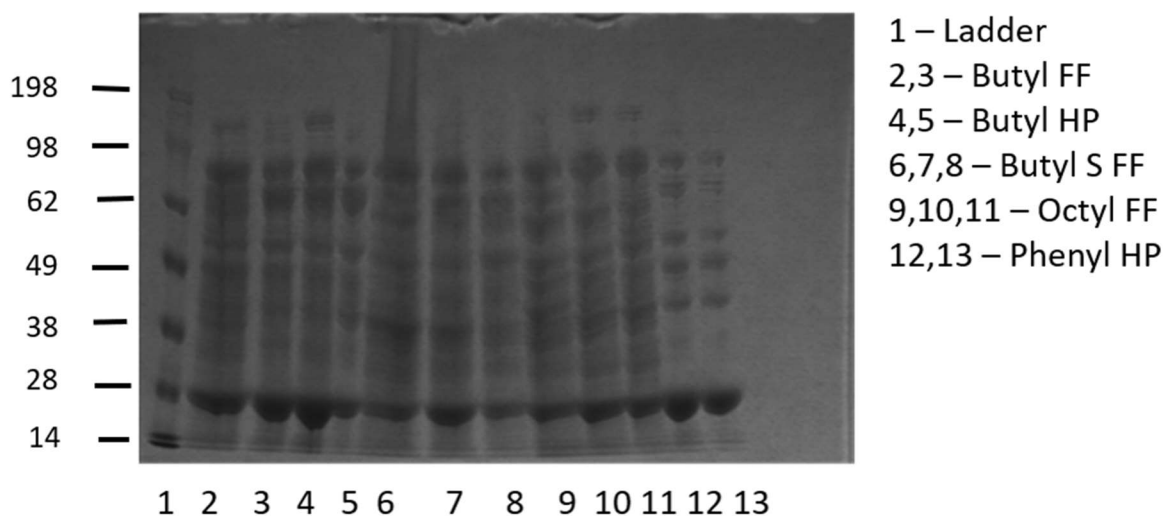


Figure 1. SDS Page gel from HIC column Screen

The large band located at 28kDa in each column is the EGFP all the other bands above and below are impurities. The gel clearly shows that the Phenyl HP column produced the protein with the fewest contaminants. The next step in the continued search for a purification protocol was to run the Phenyl HP column's purified EGFP through size exclusion chromatography SEC to remove more of the impurities. **Figure 2** below shows the results of an SDS page gel run on the three resultant fractions containing EGFP.

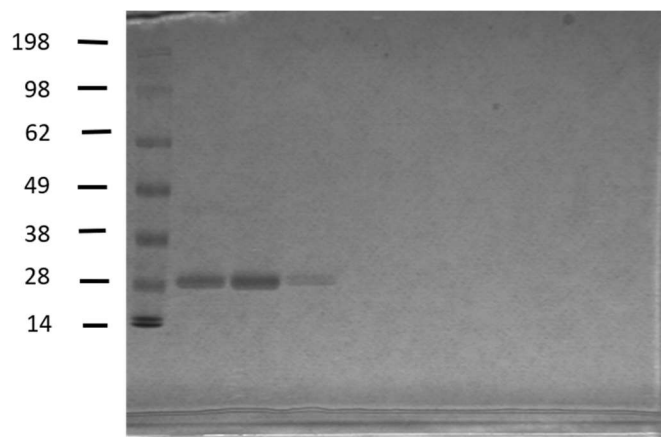


Figure 2. SDS Page gel of SEC fractions containing EGFP

Analysis of Figures 1 and 2 clearly show that SEC removed the majority of remaining impurities in the sample. The sample was still not pure enough to conduct structural analysis so

an Anion Exchange column was used as a polishing step. Gel electrophoresis was conducted again on the Anion Exchange column's resultant EGFP as seen in **Figure 3** below.

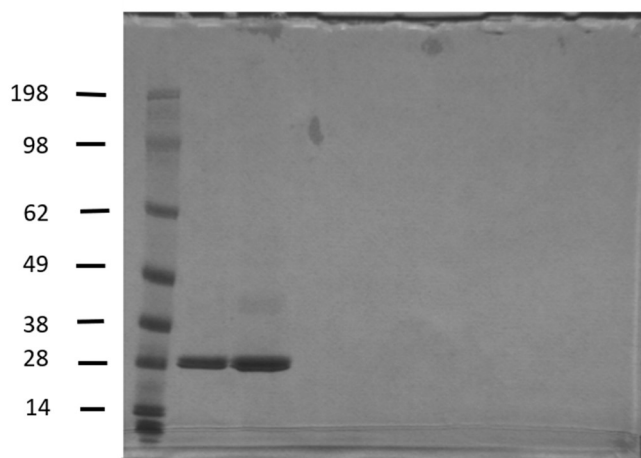


Figure 3. SDS Page gel of Anion Exchange fractions containing EGFP

This clearly shows that the results are very pure. The order of the steps was altered through several other iterations to determine a better scheme, however, none of the variations improved on the efficiency or results of this scheme. Interestingly, the addition of the super hydrophilic zwitterionic tails delayed the elution from the HIC column. The EGFP-EKG stuck to the column so strongly that the only way to remove it was by rinsing the column with water.

Test Expressions

Purification protocol developed, the next step was to begin test expressions of all fusion proteins. The first round of testing showed that there were issues with following the traditional expression protocol potentially due to EK toxicity as EK expression enhanced *E. coli* cell death. (unpublished data). Literature research showed that altering the starter culture to a solid state LB plate instead of a test tube liquid media was an effective method of combating toxic expressers.¹³⁻¹⁵ Following the protocol, a test expression was done on both the plate method and the traditional tube method of expression for EGFP, all fusion protein variants and the empty

vector (pUC19) as a negative control. The OD normalized SDS page gel results are shown below in **Figure 4**.

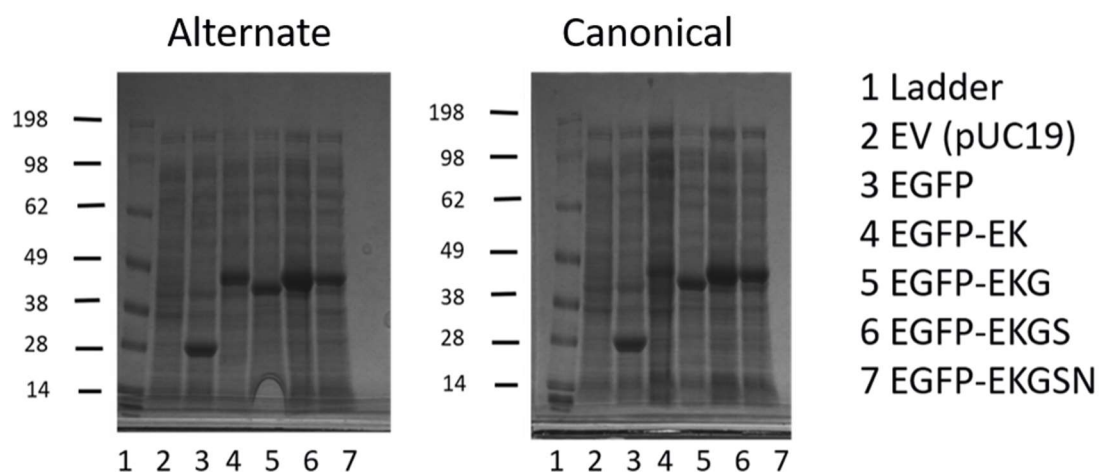


Figure 4. OD normalized SDS Page results for all Test Expressions

Interestingly, the fusion proteins all appeared to be slightly higher than the expected 38kDa. This lines up with the results of OPH-EK SDS Page gel electrophoresis experiments conducted in the lab. It is hypothesized that the zwitterionic tail has reduced binding with SDS due to its non-fouling properties, which will cause a lower charge per mass ratio on the protein resulting in a higher apparent molecular weight. The toxic expresser protocol also appears to have fewer impurities, this is especially evident in the EGFP-EK sample. But it was still unclear as to which protocol would produce higher yield, for that it was necessary to conduct a fluorescence assay with each sample normalized to EGFP. The results are shown in **Figure 5**.

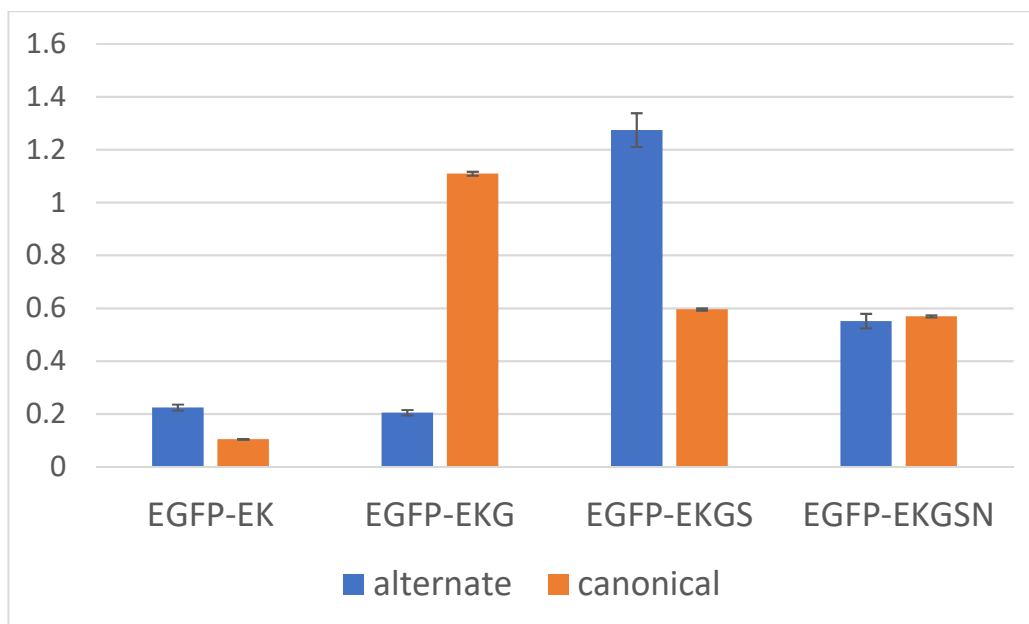


Figure 5. Test Expression yield results of GFP Fusion proteins and controls.

These data showed that with the exception of EGFP-EKG, which had higher fluorescence for the canonical protocol, the toxic expresser plate protocol outperformed the traditional tube culture method for these fusion proteins. This toxic expression method has since been found to be more successful with another fusion protein the lab has been studying, interferon-EKGSN (40kDa tail length).

Expression Yield Comparison

The fluorescence data were a good indication that the yield issues experienced with the EK fusion protein might have been resolved with this alternate expression protocol, but also that the addition of the other amino acids seems to have a positive impact on expression yield. However, further analysis was necessary to verify these results. One liter expressions following the protocol for expression and purification as laid out in the methods section were executed with the EGFP and all four EGFP fusion proteins. The resulting purified proteins were then run through SDS Page Gel Electrophoresis to verify purity and the results are shown below in

Figure 6.

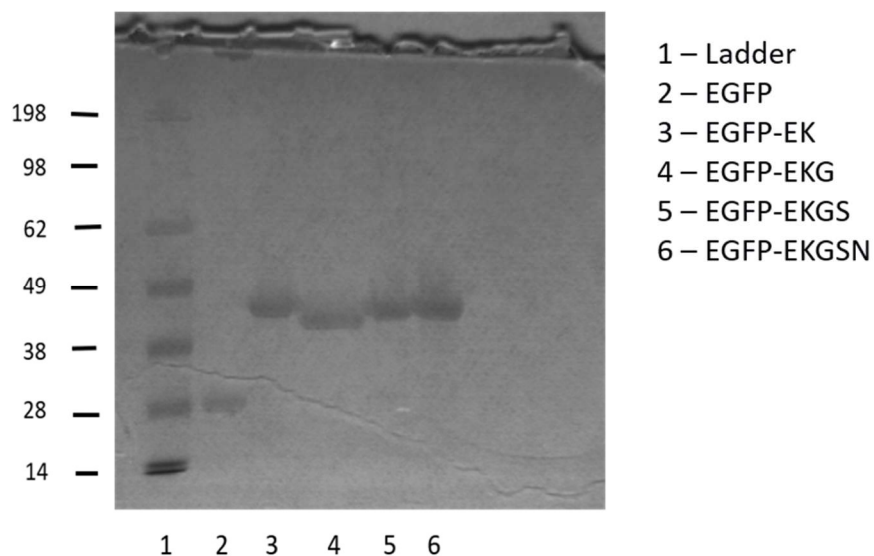


Figure 6. SDS Page Gel of Purified EGFP and all Fusion Proteins

The EGFP clearly runs on the gel at 28kDa, however the fusion proteins all seem to run slightly higher than their expected 38kDa. This behavior has been observed in both OPH-EK and Interferon-EKGSN as well as in other lab groups who study these zwitterionic fusion proteins, which is thought to be due to the reduced interaction between the zwitterionic tail and the SDS causing this altered motility through the gel. Subsequently, BCA was conducted on the purified proteins to determine yield. The results are detailed below in **Figure 7**.

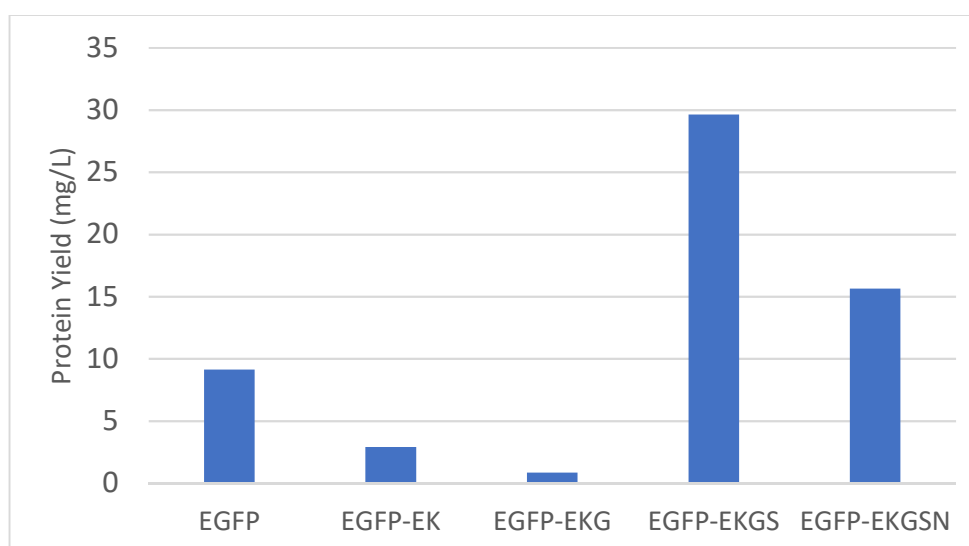


Figure 7. Yield Results for EGFP and all Fusion Proteins

The values for EGFP-EK and EGFP-EKG are clearly lower than for all other fusion proteins as well as being lower than the EGFP non-fusion protein. This was expected from the OPH-EK research conducted in the lab previously, however the addition of G to the sequence did not appear to increase yield at all. The reasons for this are still unclear and could indicate that both EK and EKG somehow are more toxic expressers than EKGS or EKGSN. Both EGFP-EKGS and EGFP-EKGSN show significantly higher yield than the EGFP non fusion protein. These results are very promising, but the question still remains whether the amino acid tail have a negative effect on the structure of the protein and more importantly the function of the protein.

Structural Analysis

To understand the impacts of the fusion tail on the structure of the protein, circular dichroism (CD) analysis was conducted. Utilizing the methods as laid out in the methods section above, the EGFP and all four fusion proteins were run through CD. Several trial runs were conducted to determine buffer type and concentration as well as concentration of the sample to achieve optimum results with minimal background noise. Based on these trial runs the following conditions were utilized: protein concentration of 5 μ M in 10mM potassium phosphate buffer at pH 7.4. The histograms for all five proteins are located in the Appendix. A side by side comparison is located below in **Figure 8**.

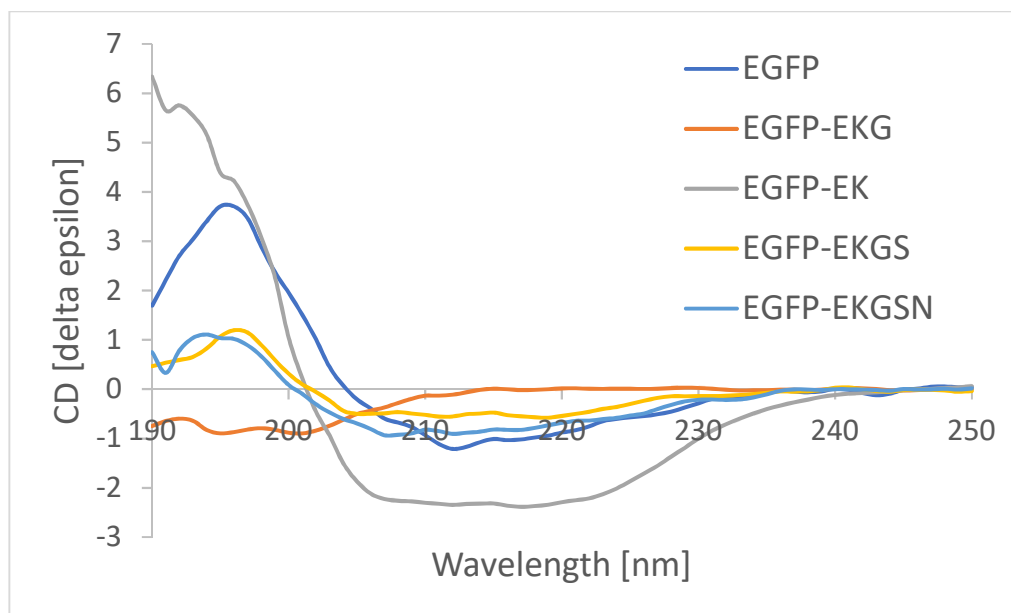


Figure 8. Circular Dichroism Spectroscopy

The general shape of the curves appears to be similar for all but the EKG fusion protein which shows distinct differences from the EGFP and other fusion proteins. The other observable difference is the magnitude of the maximums and minimums between the EGFP and the EGFP-EK fusion protein which exhibits an order of magnitude increase in both the maximum and minimum values. The BeStSel free online CD analysis algorithm was used to further analyze the data.¹² The results of this analysis are located below in **Figure 9**. The best fit plots and data table can be found in the Appendix.

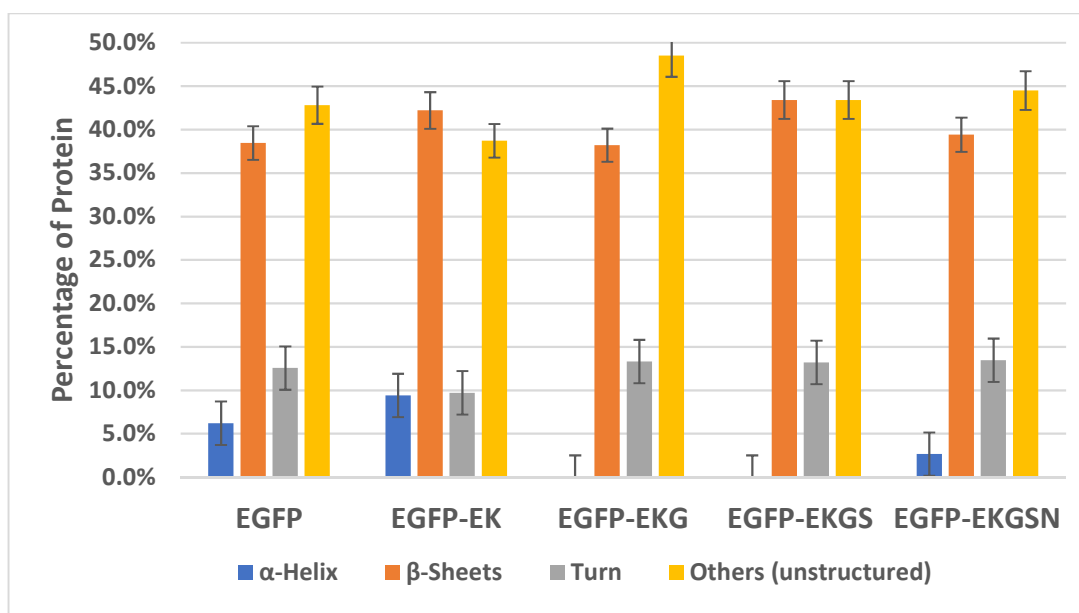


Figure 9. Circular Dichroism Analysis of EGFP and Fusion Proteins

From this analysis the EK and EKGSN fusion proteins appear to be the closest in structure to the EGFP. All of the fusion proteins exhibit similar secondary structures to the EGFP with most of the differences falling within the standard deviation. The EKGS and EKG both have an absence of any α -Helix structure, however the EGFP only showed 5% of the total structure as α -Helix. The only real outlier is the EKG fusion protein which demonstrated a 6% increase in unstructured configuration. Due to the small scale of the variances the data is inconclusive as to whether or not the fusion tail will negatively impact protein function for any of the fusion candidates. With the data still not definitive as to which amino acid tail to use, it was necessary to examine the activity of the fusion proteins compared to the unfused EGFP.

Activity Assay

Building on the structural analysis, potential impacts on protein function from the 10kDa tails were studied by a simple fluorescence assay. Following the protocol detailed in the methods section all samples were diluted to the same 1.5 μ M concentration in PBS. Then the sample was read in the Cytation plate imager with an absorbance wavelength of 485nm and an

emission wavelength of 530nm as per the company's recommended settings for EGFP. The fluorescence was then normalized to that of the EGFP and the experiment was repeated three times. All samples were purified within two days of the fluorescence assay and the Bicinchoninic Acid Assay (BCA) was performed the same day as the fluorescence assay to ensure protein sample freshness and accuracy of concentration numbers. BCA results and concentration calculations can be found in the Appendix. Additionally, another set of protein samples were placed in the 4°C fridge for two months and then a BCA, dilution to 1.4 μM in PBS, and fluorescence assay in triplicate was performed to examine the potential protective impacts of the tail on protein shelf life. The results are displayed below in **Figure 10**.

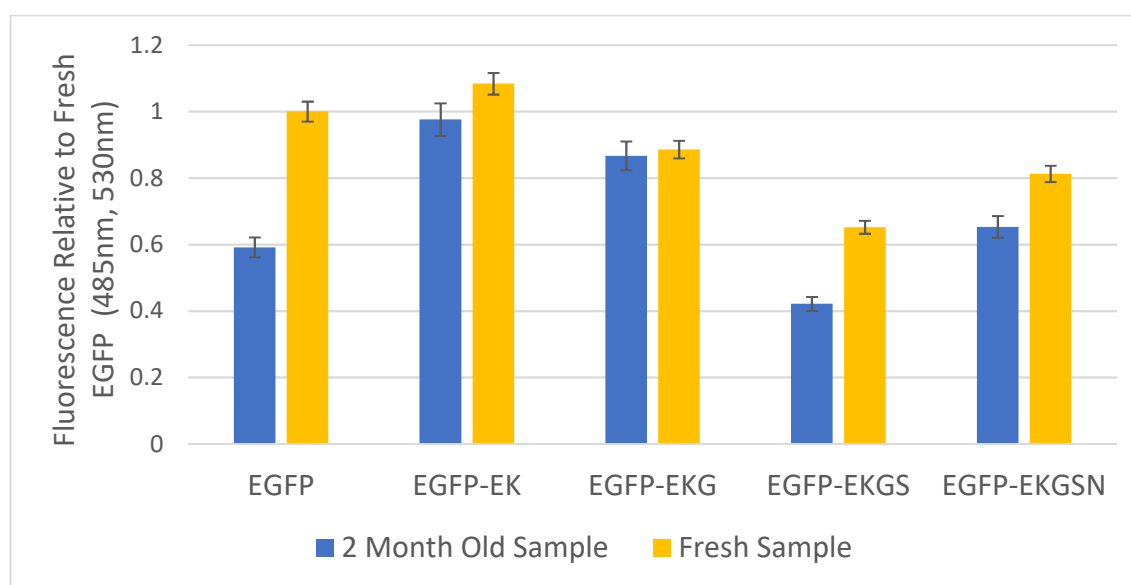


Figure 10. Fluorescence of Fusion Proteins at 1.4 μM in PBS

Intriguingly, EK and EKG tails seem to have the least impact on protein activity closely followed by EKGSN and lastly EKGS. Both EK and EKG fusion proteins fell within the standard deviation range of the EGFP indicating minimal impact to protein function. This data supports the initial design principles behind a simple EK tail. The EKGS performed the worst fluorescing at only 65% of the EGFP fluorescence. This could indicate that EKGS will

negatively impact performance of the target protein. At 82 ± 5.0 % relative fluorescence EKGSN appears to have minimal impact on protein function.

The 41 ± 4.5 % reduction in fluorescence of EGFP after two months at 4°C is to be expected for protein stored for that length of time. All fusion proteins did experience some decrease in fluorescence between the fresh and old samples. The percentage decrease, however was significantly less than that of the non-fusion protein EGFP. Percentage relative fluorescence decreases ranged from 23 ± 6.4 % for EGFP-EKGS to 2.2 ± 7.2 % for EGFP-EKG with EGFP-EKGSN falling at 16 ± 3.7 %. These data indicate that the zwitterionic amino acid tails could confer a protective effect on the protein which could be useful in pharmaceutical applications. Thermal stability testing should be conducted in the future to examine the effect of the tail on that aspect of the fusion protein.

Future Efforts

The next step is to take the knowledge gained from this study and apply it to a medically relevant protein. This protein would need to be small relative to the tail and, for broadest impact, something commonly utilized in medicine today that could benefit from increased circulation time and a substitute for PEGylation. The zwitterionic tail needs to be larger for broader impact both on the protein of interest as well as being more applicable to a wider range of protective and therapeutic proteins. Two possible candidates that the Jiang lab is currently researching are: granulocyte colony-stimulating factor (GCSF) known as Filgrastim which stimulates bone marrow to produce white blood cells (approximate size 18.8 kDa)¹⁶; and interferon alfa-2a, the PEGylated version is also known as Pegasys which is used to treat hepatitis C and B (approximate size 40kDa pegylated and 19kDa unpegylated).^{17,18} These candidates fused to a 40kDa EKGSN tail are currently undergoing analysis and preparation for future animal studies.

This EKGSN tail could also be fused to organophosphate hydrolyze (OPH)(36kDa) the bio scavenger initially fused to EK (30kDa) which showed the expression yield challenges which spawned much of the research in this thesis. By fusing either two 40kDa EKGSN tails or potentially one 80kDa EKGSN tail the same zwitterionic non-fouling affects that the initial EK tail were designed to achieve could be accomplished with the added benefit of having no negative impact on yield.

CONCLUSIONS

The primary purpose of this study was to screen four potential candidates for a bio-degradable zwitterionic fusion tail that overcomes the expression and yield issues identified by the initial OPH-EK fusion protein study. The four amino acid chains examined (EK, EKG, EKGS, and EKGSN) were fused to EGFP as a vehicle to study expression, purification, structure, and activity. EKGS and EKGSN both showed the most promising expression yield results with both outperforming the non-fusion EGFP yield. Structural analysis showed that all fusion proteins were fairly similar to the non-fusion EGFP with the exception of EKG. Finally, protein function/activity was examined through a fluorescence assay, which showed that EKGSN had significantly higher relative fluorescence than EKGS. In conclusion, EKGSN is the best candidate based on EGFP. The future study may focus on (a) revisiting the sequence using other proteins, (b) increasing the length of the identified peptide and (c) creating fusion proteins for application-relevant protective and therapeutic proteins to increase circulation time and prevent the foreign body response. The ultimate goal is to replace PEGylation.

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APPENDICES

Appendix A

Protein Sequences

EGFP

MSKGEELFTGVVPILVELDGDVNGHKFSVSGEGEGDATYGK
 LTLKFICTTGKLPVPWPTLVTTLTYGVCFSRYPDHMKQHD
 FFKSAMPEGYVQERTIFFKDDGNYKTRAEVKFEGDTLVNRI
 ELKGIDFKEDGNILGHKLEYNYNSHNVYIMADKQKNGIKVNF
 KIRHNIEDGSVQLADHYQQNTPIGDGPVLLPDNHYLSTQSAL
 SKDPNEKRDHMLLEFVTAAGITHGMDELYK

EGFP-EK

MEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKE
 KEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKEKMSKGE
 ELFTGVVPILVELDGDVNGHKFSVSGEGEGDATYGKLTTLKFI
 CTTGKLPVPWPTLVTTLTYGVCFSRYPDHMKQHDFFKS
 AMPEGYVQERTIFFKDDGNYKTRAEVKFEGDTLVNRIELKGI
 DFKEDGNILGHKLEYNYNSHNVYIMADKQKNGIKVNFKIRHN
 IEDGSVQLADHYQQNTPIGDGPVLLPDNHYLSTQSALS
 SKDPNEKRDHMLLEFVTAAGITHGMDELYK

EGFP-EKG

MEKGEKGEKGEKGEKGEKGEKGEKGEKGEKGEKGEKGEKGE
 KGEKGEKGEKGEKGEKGEKGEKGEKGEKGEKGEKGEKGEK
 EKGEKGEKGEKGEKGM SKGEELFTGVVPILVELDGDVNGHK
 FSVSGEGEGDATYGKLTTLKFICTTGKLPVPWPTLVTTLTYG
 VCFSRYPDHMKQHDFFKSAMPEGYVQERTIFFKDDGNYKT
 RAEVKFEGDTLVNRIELKGIDFKEDGNILGHKLEYNYNSHNV
 YIMADKQKNGIKVNFKIRHNIEDGSVQLADHYQQNTPIGDGP
 VLLPDNHYLSTQSALS
 SKDPNEKRDHMLLEFVTAAGITH
 GMDELYK

EGFP-EKGS

MEKGSEKGSSEKGSSEKGSSEKGSSEKGSSEKGSSEKGSSEK
 GSEKGSSEKGSSEKGSSEKGSSEKGSSEKGSSEKGSSEKGS
 SEKGSSEKGSSEKGSSEKGSSEKGSSEKGSSEKGSSEKGSSE
 KGSSEKGSSEKGSSEKGSSEKGSSEKGSSEKGSSEKGSSEKGS

SVSGEGEGDATYGKLTTLKFICTTGKLPVPWPTLVTTLTYGV
QCFSRYPDHMKQHDFFKSAMPEGYVQERTIFFKDDGNYKT
RAEVKFEGDTLVNRIELKGIDFKEDGNILGHKLEYNYN SHNV
YIMADKQKNGIKVNFKIRHNIEDG SVQLADHYQQNTPIGDGP
VLLPDNHYLSTQSALS KDPNEKRDHMLLEFVTAAGITH
GMDELYK

EGFP-EKGSN

MEKGSNEKGSNEKGSNEKGSNEKGSNEKGSNEKGS
NEKGSNEKGSNEKGSNEKGSNEKGSNEKGSNEKGS
NEKGSNEKGSNEKGSNEKGSNEKGSNEKGSNMSKGE
ELFTGVVPILVELDGDVNGHKFSVSGEGEGDATYGKLTTLKFI
CTTGKLPVPWPTLVTTLTYGVQCFSRYPDHMKQHDFFKS
AMPEGYVQERTIFFKDDGNYKT RAEVKFEGDTLVNRIELKGI
DFKEDGNILGHKLEYNYN SHNVYIMADKQKNGIKVNFKIRHN
IEDG SVQLADHYQQNTPIGDGPVLLPDNHYLSTQSALS KDP
NEKRDHMLLEFVTAAGITHGMDELYK

Appendix B

Methods and Materials Expanded

Chemicals

Bacto Agar was purchased from Becton, Dickinson and Company (Sparks, Maryland)

Bacto Tryptone – Pancreatic Digest of Casein – was purchased from Becton, Dickinson and Company (Sparks, Maryland)

Bacto Yeast Extract – Extract of autolyzed Yeast Cells – was purchased from Becton, Dickinson and Company (Sparks, Maryland)

Potassium Phosphate Dibasic Anhydrous (CAS 7758-11-4) was purchased from Fisher Chemical (Fair Lawn, New Jersey)

Potassium Phosphate Monobasic (CAS 7778-77-0) was purchased from Fisher Chemical (Fair Lawn, New Jersey)

2-Mercaptoethanol (CAS 60-24-2) was purchased from Sigma Aldrich (Milwaukee, Wisconsin)

Sodium Chloride (CAS 7647-14-5) was purchased from Fisher Scientific

Tween 20 (CAS 9005-64-5) was purchased from Fisher Scientific

UltraPure Agrose was purchased from Invitrogen (Carlsbad, California)

Hydrochloric Acid 1N (CAS 7647-01-0) was purchased from Fisher Chemical (Fair Lawn, New Jersey)

Sodium Hydroxide 1N (CAS 1310-73-2) was purchased from Fisher Chemical (Fair Lawn, New Jersey)

Phosphate Buffered Saline 10x Solution was purchased from Fisher BioReagents (Fair Lawn, New Jersey)

Pierce BCA Protein Assay Reagent A and Reagent B were purchased from Thermo Scientific (Rockford, Illinois)

Ethidium Bromide 10mg/ml (CAS 1239-45-8) was purchased from BioRad

Protamine Sulfate, Salmon Milt purchased from EMD Milipore Corp (Darmstadt, Germany)

Proteins

Bovine Serum Albumin (CAS 9048-46-8) lyophilized powder ($\geq 96\%$) was purchased from Sigma Aldrich (Milwaukee, Wisconsin)

Organophosphate Hydrolyze (OPH) and fusion proteins (wild type) expressed from a cell stock created by Erik Liu a member of the Jiang Group.

Green Fluorescent Protein (GFP) and fusion proteins expressed from DNA purchased from GenScript

Interferon and fusion proteins expressed from DNA purchased from GenScript

General supplies

ZR Plasmid Miniprep Classic Kit was purchased from Zymo Research

Zymoclean Gel DNA Recovery Kit was purchased from Zymo Research

100bp DNA ladder was purchased from GoldBio

SeeBlue Plus2 Prestained Protein Gel Electrophoresis standard was purchased from Invitrogen (Carlsbad, California)

SmartSpec 3000 Spectrophotometer from BioRad

Sterile Syringe Filters w/ 0.2 μ m Cellulose Acetate Membrane were purchased from VWR International.

MJ Research Peltier Thermal Cycler (PTC_200) was borrowed from the Lindstrom Group
Sonicator was purchased from Fisher Scientific

UV Transilluminator UVP was purchased from BioDoc-It Imaging Systems

Centrifuge 5804 R (15 amp version) was purchased from Eppendorf

Cytation5 Imaging Reader was purchased from BioTek

Scale was purchased from Mettler Toledo

Isotemp hot plate was purchased from Fisher Scientific

Spectropolarimeter Jasco J-720 was rented from the University of Washington for Protein

Structural analysis.

Genetic Engineering to Create OPH-EKG fusion proteins

1. Polymerase Chain Reaction (PCR) was performed on the OPH DNA that the Jiang lab had to add the appropriate primers which would enable the digestion and eventual ligation to the EKG, EKGG, and EKGGG DNA which had already been cloned into a PET_20 plasmid. Utilizing Erik Liu's OPH Plasmid (EL-5-82) 1 μ L plus 1 μ L of each primer (OPH_FWD3 and OPH_REV_NO STOP), 40 μ L of milipore water, 5 μ L of PFU ultra buffer, 1 μ L of dNTPs (nucleotides), and 1 μ L of PFU ultra (polymerase). This was all mixed in a 100 μ L micro centrifuge tube and placed in the Peltier Thermal Cycler (PTC_200) by MJ Research. On the following cycle 10 x {95C for 2min, 61C for 20 sec, and 72C for 90 sec} then 20 x {95C for 2min, 61C for 20 sec, and 72C for 90 sec} then 72C for 3 min.
2. Gel electrophoresis to determine if the PCR worked and to separate desired parts. (See below for detailed procedure).
3. DNA gel extraction to recover the DNA and separate desired segments. (See below for detailed procedure).
4. Digestion. The results from the PCR were then digested along with the EKG-PET_20, EKGG-PET20, and EKGGG-PET_20 plasmids. Using HindIII HF and NdeI as the

cutting enzymes and from NEB double digest finder it was determined that cutsmart buffer should be used. 10 μ L of each DNA were used OPH, EKG, EKGG, and EKGGG, to each was added 2.5 μ L of HindIII HF, 2.5 μ L of NdeI, 5 μ L of Cutsmart buffer, and 30 μ L of milipore water. This was allowed to sit for 4-6 hours in the 37C warm room.

5. Gel electrophoresis (to separate insert from vectors).
6. DNA gel extraction protocol (same as before).
7. Ligation. Next the OPH Gene was inserted into the EKG-PET_20 vector (also EKGG, and EKGGG). Using approximately 15ng of insert (OPH gene) to 50ng vector (EKG-PET_20). Quantity of each determined by OD readings. Both quantities of insert and vector were added to 2 μ L of ligase buffer and 1 μ L of ligase and then volume totaled with milipore water to 20 μ L. This then sat at room temperature for 30min.
8. Transformation. Then 5 μ L of each ligation product was added to DH10B competent cells sitting on ice for 15min.
9. Plate Colonies. Next the transformed cells were plated on Luria Broth (LB) plates and placed in the 37C over night.
10. Pick Colonies and conduct overnight culture. Isolated colonies were then selected and placed in 5mL LB and 5 μ L of 1000x ampicillin. This was then placed on a slow shaker over night in the 37C warm room.
11. Miniprep. Utilized the ZR Plasmid Miniprep Classic quick protocol (Catalog Nos. D4015, D4016 & D4054) Ver 1.0.0 from Zymo Research.
12. DNA Sequencing. Miniprep samples were then sent out for sequencing to Genewiz.

Gel electrophoresis

Utilized to determine presence of DNA constructs during cloning steps.

1. Utilizing UltraPure Agarose a gel was cast at either 2.0%(for 100bp-2kb) or 4.0% (for 10bp-400bp) with 30mL .5x TAE. The solution was microwaved until all the agarose was dissolved and then 2 μ L of 10mg/ml Ethidium Bromide from BioRad(Cat# 161-0433) was added. Solution was gently mixed and then poured into the mold and allowed to set for approximately 30min.
2. Next approximately 50 μ L of each sample was mixed with 10 μ L of 6 x dye.
3. Depending on the size of the target DNA either the 100bp ladder or the 1kb ladder was used in the first well. Then 30 μ L of each sample + dye solution was added to the other wells. Reservoirs were filled with the buffer and the cover was placed on the electrophoresis machine.
4. The electrophoresis was allowed to proceed for approximately 30min and then the gel was imaged under UV light on with the assistance of the UVP BioDoc-It Imaging System.
5. Desired bands were then cut out via razor blades and the Zymoclean Gel DNA Recovery protocol was used to recover the DNA.

Gel DNA recovery

Utilized the Zymoclean Gel DNA Recovery Kit and protocol (Catalog Nos. D4001, D4002, D4007, & D4008) Ver 1.2.1 from Zymo Research.

OPH Expression and purification Procedure

1. Streak plate with cell stock. (PET20-VRN-AMP BL21) *E coli*. BL21 is the expression strain and DH10B is the Gene Strain.
2. Pick a colony and culture 5mL TB and 5uL amp for 6-8 hours. (Make 1L TB during the day so that it is cool after autoclave inoculating by evening) TB recipe: 12g Bacto Tryptone, 24g yeast, 4mL glycerol (pull up 8mL due to extreme viscous nature and

dispense all of it into the container), and approximately 900mL Millipore H₂O then autoclave. In a separate flask add 2.31g Potassium phosphate monobasic and 12.54g potassium phosphate dibasic in approximately 100mL Millipore H₂O and autoclave.

Combine the two flasks when you are ready to use it.

3. 1mL culture + 1L TB in Erlenmeyer flask overnight in the 30C on shaker.
4. Add inducing agent 1mM CoCl₂ [mw 237g/mol so ~ 0.237g dry weight in the 1L solution dissolve first in 1ml water then add to flask] and IPTG (located in Eric's stock container in the -20C) (stock solution is 1M so take 1 mL) 24 hours in the 30C on shaker.
5. Harvest and resuspend spin down and discard supernatant. To do this, pour resulting solution from Step 4 into 4x50ml falcon tubes 10min at 15000 rpm. For the resuspension add HEPS CoCl₂ solution (located at bench) 25mL to each falcon tube resuspend by vortex'ing or shaking or pipetting.
6. Freeze thaw. Approximately 1hour and 30min in -80C then thaw in water bath with slow mixing until thawed. Repeat for a total of 3 freeze-thaw cycles.
7. Sonicate. Wipe down probe with ethanol and place all four falcon tube of lysate in an ice bucket packed with ice. Program the sonicator to run 5 sec on and 15 sec off for 20min. insert the probe into one of the tubes approximately 1 inch off the bottom of the tube and ensure that the tube is firmly packed into the ice with the probe not touching the sides of the tube. Run until all of the lysate has been clarified (if any of the liquid is still opaque then redo the sonication). At beginning of sonication combine 20mL HEPS CoCl₂ and 0.4g Protamine sulfate in a 50ml falcon tube and place on mixing table to dissolve for approximately an hour or more (don't inhale and remember it takes a long time to dissolve).

8. Spin down clarified lysate at 15000 rpm for 10min. keep supernatant and discard cell debris. Next precipitate the DNA by pouring supernatant out into 4 new falcon tubes and add 5mL of the protamine sulfate HEPS CoCl₂ solution to each. Let sit for approximately 20min on ice. Spin down again at 15000 rpm for 10min keep supernatant again (new falcon tubes).
9. Ammonium sulfate cut. At 60% saturation at 4C. Use EnCor calculator to determine approximate amount of dry ammonium sulfate to add to each of the falcon tubes from Step 8 (approximately 9-10g). place in cold room on mixer for 30min. Spin down at 15000 rpm for 10min and discard supernatant place in cold room/fridge (4C) until following day.
10. Equilibrate SEC in HEPS CoCl₂ buffer overnight.
11. Resuspend pellets with 7ml HEPS CoCl₂ buffer in first falcon tube pipette to resuspend then pour entire thing into second falcon tube and pipette to resuspend repeating until all of the pellets are resuspended in one falcon tube. Then spin down again keeping the supernatant and filter through a syringe filter. Size Exclusion Column (All day). Two to three runs of a proportional fraction of the entire volume through the SEC about 3-4mL. Pulling out desired fractions based off peaking on graph. Then set up a cleaning protocol to run overnight on the column. Take the desired fractions (approximately 10mL from each run totaling 20mL) and concentrate them through the thingamajiggers. Do NOT run the concentrators over 4500rpm. Use the swing bucket rotors to ensure the force is directly down the membrane. Place concentrated product (ideally below 5mL) back in the fridge 4C overnight.
12. Equilibrate column first. Anion Exchange Column (2-3 hours)

Enzyme-Linked Immunosorbent Assay (ELISA)

In order to detect the presence of interferon and fusion protein (interferon-EKGSN) a sandwich ELISA was utilized.

1. Coating with sample protein. 35 μ L of lysate (Empty Vector EV) was mixed with 665 μ L 50mM Sodium Bicarbonate then 50 μ L of this solution (100ng per well) was added to the first two columns of the ELISA plate.
2. This was repeated for the purified interferon with the same 100ng per well as the EV.
3. And again for the unknown Interferon-EKGSN.
4. Then the plate was placed in the 37C warm room for 2 hours.
5. Plate was dumped and rinsed three times with a 0.03% Tween in PBS solution (200 μ L per well). Then the plate was dumped until dry.
6. Blocking. 200 μ L of the block 3% BSA in PBS was added to each well and then the plate was placed in 4C overnight.
7. Plate was dumped and rinsed three times with a 0.03% Tween in PBS solution (200 μ L per well). Then the plate was dumped until dry.
8. Primary antibody. Next the primary interferon antibody was added diluted 1:3000 by adding 4.3 μ L of antiINF(biosource) in 1.3mL of the 3% BSA in PBS solution. 150 μ L of this solution was then added to the top row wells. Next 100 μ L of the 3% BSA in PBS solution was added to each of the wells in the bottom five rows. Then a serial dilution was conducted by pulling 50 μ L out of the top row and mixing three times in the next row. This was repeated until all 6 rows held exactly 100 μ L of solution. Then the plate was placed back in the 37C for 2 hours.
9. Plate was dumped and rinsed three times with a 0.03% Tween in PBS solution (200 μ L per well). Then the plate was dumped until dry.

10. Secondary antibody. Next the secondary (Goat anti Rabbit IgG (H+L) Peroxidase Conjugated 1mg/ml Thermo Scientific Product # 31466 lot# OD187360) was added by mixing 3 μ L of IgG with 7mL of the 3% BSA in PBS solution and gently mixing. Then 100 μ L of this solution was added every well in the odd numbered columns. The plate was placed back in the 37C for 90 minutes.
11. Plate was dumped and rinsed three times with a 0.03% Tween in PBS solution (200 μ L per well). Then the plate was dumped until dry.
12. Development. 50 μ L of 1-step ultra TMB blotting solution (Thermo Scientific Product # Lot# 37574) was added rapidly to every well and the plate was placed in a closed drawer to limit light exposure. A stopwatch was used and the plate was checked every 90 seconds. As soon as the empty vector began its colorimetric change 50 μ L of 2M sulfuric acid was added to every well to stop the reaction.
13. The plate was read at 450nm in a Cytation5 imaging reader by BioTek.

EGFP and EGFP Fusion Protein Time Course (Toxicity) Analysis

This was done to examine the potential toxicity of each of the fusion proteins.

1. Streak LB ampicillin plates with cell stock from each of the following: EGFP, EGFP-EK, and EGPF-EKGSN; place in 37C room overnight.
2. Pick three colonies of each and place each individual colony in a separate 5mL LB and 5 μ L ampicillin(1000x) overnight on shaker in 37C room.
3. Take 1mL of previous night's culture and add to 30mL TB plus 30 μ L ampicillin (1000x) place back in 37C room on shaker checking Optical Density (OD) at 600nm every 30min. This was done in triplicate with 3 samples designated for induction and three to not be inducted. This resulted in 6 total samples per protein.

4. Once the OD minus TB background OD reached 0.5 – 1.0 the three induction samples were induced with 30 μ L Isopropyl β -D-1-thiogalactopyranoside (IPTG) then placed on shaker in 30C room.
5. OD readings continued until a rough plateau had been reached.

Terrific Broth Recipe

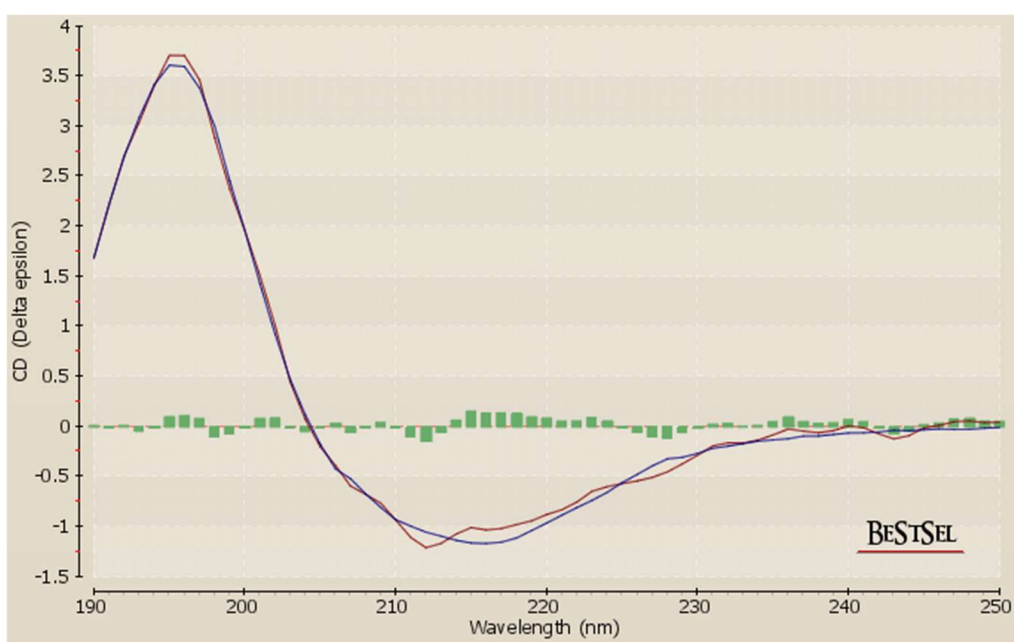
12g Bacto Tryptone, 24g yeast, 4mL glycerol (pull up 8mL due to extreme viscous nature and dispense all of it into the container), and approximately 900mL Millipore H₂O then autoclave. In a separate flask add 2.31g Potassium phosphate monobasic and 12.54g potassium phosphate dibasic in approximately 100mL Millipore H₂O and autoclave. Combine the two flasks when you are ready to use it.

Appendix C – CD Analysis for EGFP and EGFP Fusion Proteins from BeStSel

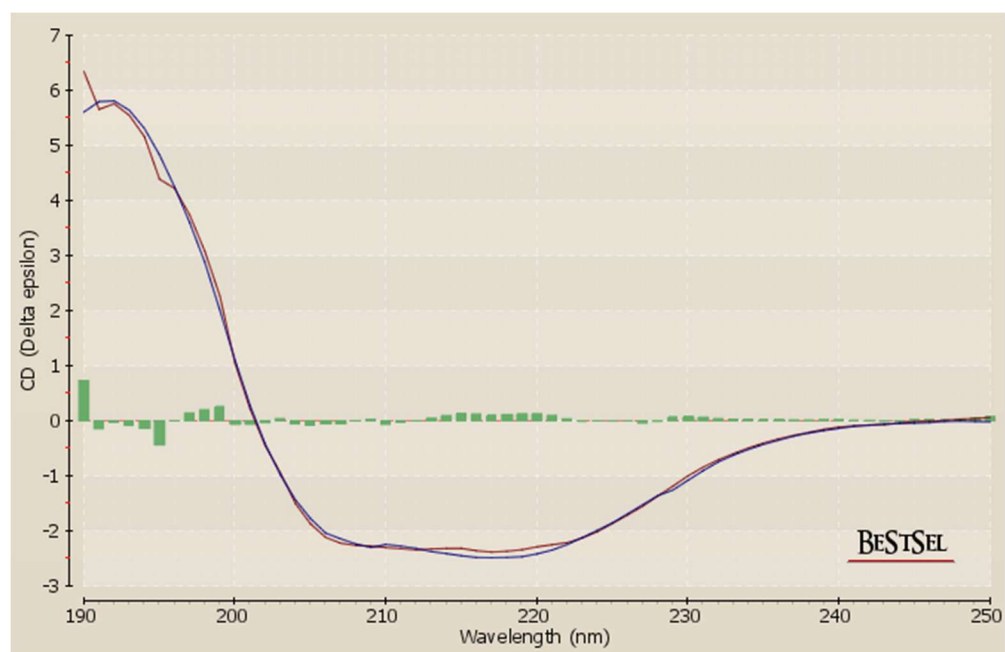
Table 1. BestSel Secondary Structure Analysis

Secondary Structure	GFP	GFP_EK	GFP_EKG	GFP_EKGS	GFP_EKGSN
α -Helix	6.2%	9.4%	0.0%	0.0%	2.7%
β Sheets	38.5%	42.2%	38.2%	43.4%	39.4%
Turn	12.6%	9.7%	13.3%	13.2%	13.5%
Others (unstructured)	42.8%	38.7%	48.5%	43.4%	44.5%

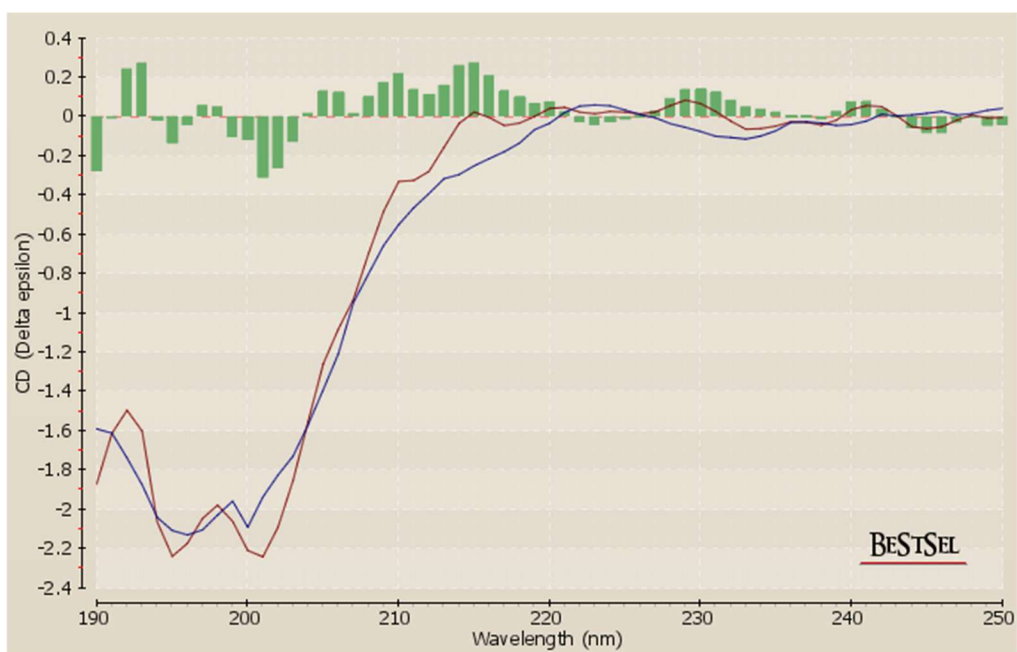
EGFP Secondary Structure Analysis (BeStSel)



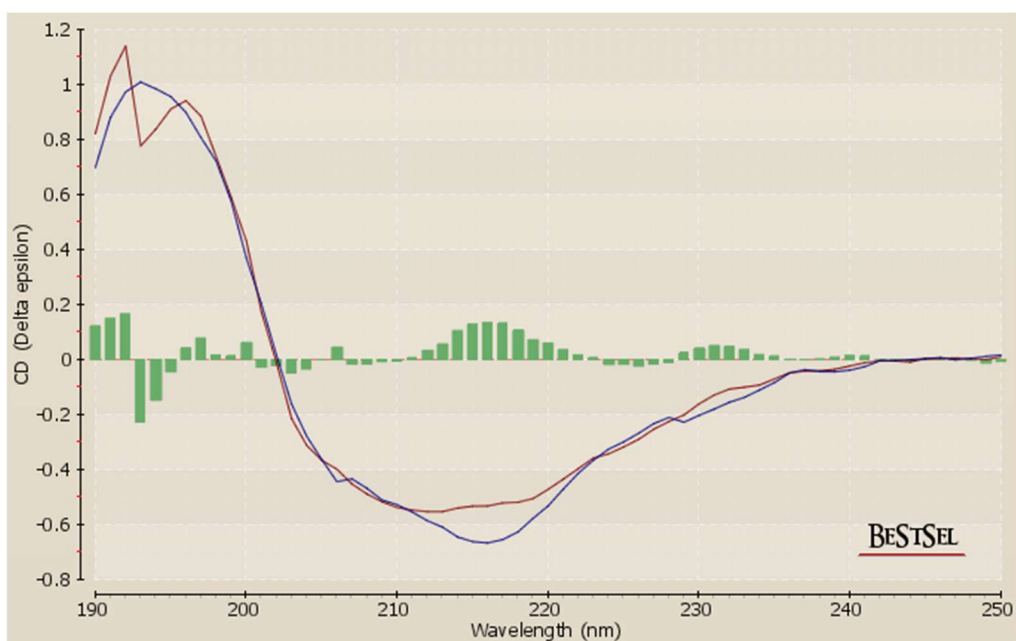
EGFP-EK Secondary Structure Analysis (BeStSel)



EGFP-EKG Secondary Structure Analysis (BeStSel)



EGFP-EKGS Secondary Structure Analysis (BeStSel)



EGFP-EKGSN Secondary Structure Analysis (BeStSel)

