THE INCORPORATION OF BORONOPHENYLALANINE INTO PHAGE DISPLAY LIBRARIES FOR IDENTIFYING INHIBITORS OF CD44

An Undergraduate Research Scholars Thesis

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ABSTRACT

The Incorporation of Boronophenylalanine into Phage Display Libraries for Identifying Inhibitors of CD44

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Many cancers, including breast and pancreatic, are known to display similar cancer stem cell markers, and the CD44 receptor is a common marker. The receptor binds specific biomolecules that lead to increased cell proliferation and survival. Boronophenylalanine, a noncanonical amino acid, is of interest because of boronic acid's novel and robust chemical properties, and its functional group boronic acid's ability to participated in reversible chemistry allows for recycling of protein targets and avoidance of immunogenic responses *in vivo*. Phage display will be the experimental tool for studying ligand capabilities of boronophenylalanine-containing peptides with CD44. Boronophenylalanine incorporation has been successfully tested using a sfGFP model, and two of the three plasmids required for phage production have been transformed into a top 10 cell line using microbiology techniques. Current efforts are towards transforming said cell line with the final plasmid and producing phages. After at least 3 rounds of selection and amplification, we hope produce a boronophenylalanine-incorporated heptapeptide library, and through sequencing of the phages, we hope to gain insight about this noncanonical amino acid's integration into peptides in developing reversible covalent inhibitors of CD44.

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I thank the graduate students of Dr. Liu's research group for being there for me whenever Trae was not available. Their contribution to the project was much appreciated.

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NOMENCLATURE

aaRS	aminoacyl-tRNA synthetase
Amp^r	ampicillin resistance
BoF	boronophenylalanine
BoFRS	boronophenylalanine-tRNA synthetase
CSC	cancer stem cell
Cmp^r	chloramphenicol resistance
Kan^r	kanamycin resistance
ncAA	noncanonical Amino Acid
OD	optical density
PylRS	pyrrolysine-tRNA synthetase
$tRNA^{BoF}$	boronophenylalanine specific tRNA
$tRNA^{Pyl}$	pyrrolysine specific tRNA

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CHAPTER I

INTRODUCTION

Cancer is a heterogenous disease that results from many genetic abnormalities in cells that disrupts cell homeostasis. The abnormalities associated with the majority of cancer types involve mutations that promote gain-of-function in oncogenes and loss-of-function in tumor suppressor genes; these mutations cause alterations in cell physiology resulting in acquired capabilities that fall into six categories: self-sufficiency in growth signals, insensitivity to anti-growth signals, tissue invasion and metastasis, sustained angiogenesis, and limitless replicative potential[1]. In developing better treatments and therapies for different cancers, it is important to know which cells are contributing to the progression of the disease, and the CSC model offers further insight on cancer development and proliferation. According to the CSC model, cancerous tissue may be organized in a similar fashion as normal tissues; cancer stem cells differentiate into diverse lineages with the bulk of progeny not contributing directly to the promotion of the disease[2]. Tumorigenic cancer cells often display CSC markers which allow researchers to identify which cells are actually contributing to the progression of the disease[3].

Of the six acquired capabilities, self-sufficiency in growth signals involves overexpression of receptors associated with cell growth and proliferation, and studies have shown that the glycoprotein transmembrane receptor CD44 is the most common CSC surface marker making it a focal point of cancer research[1,4]. Overexpression of this receptor leads to unregulated cell growth due to increased ligand-induced cell proliferation and growth which promotes cancer development. Hyaluronic acid and other grow-promoting biomolecules bind to

the receptor inducing cell proliferation, and many therapeutic strategies are aimed at preventing such binding[5].

Phage display can be a powerful tool for the production of peptide libraries that can be used for rapid screening of potential ligands[6]. The phage's DNA can be modified so that it codes for variation of peptides/proteins that will be displayed on the surface. This modification allows for a protein or peptide to be fused with a phage coat protein on its surface[7]. The displaying ability of the phages allow for the direct study of the structure-activity relationship of peptides/proteins with diverse biological molecules related to disease (Fig. 1). Herein, we will take a novel approach to this method by aiming to display an ncAA containing heptapeptide fused to the pIII surface protein of the phages for developing reversible covalent inhibitors of CD44.

One of the most common approaches for incorporation of ncAAs is the exploitation of amber and ochre nonsense codons. This is achieved through the use of an orthogonal aaRS and its cognate amber suppressing tRNA that are simultaneously expressed, and PylRS and $tRNA^{Pyl}$ have been confirmed orthogonal in all domains of life[8]. The use of a mutant archaic PylRS-$tRNA^{Pyl}$ pair to incorporate the BoF in a phage display system will be attempted. Boronic acid's novel chemical properties have allowed for its use in the synthesis of sugar ligands with selective recognition, and has been successfully incorporated in *E.* coli proteins[9]. Boronic acid's chemical properties have been exploited in many studies, but the most relevant property to this project is its ability to form reversible covalent bonds (Fig**.** 2). Boronic ester bond formation can be reversed which grants researchers the ability recycle proteins of interest and avoid immunological responses in living systems, both of which are important in drug discovery research[10]. The incorporation of ncAAs into a phage display system in an effort to develop

biological regulators of cancerous cells is a relatively new approach for examining the structure-activity relationship of biological systems. The experimental design is expected to help us shine light on this new research front. Through this project, we aimed at 3 objectives: testing the incorporation of BoF in a model protein, genetic incorporation of BoF in phage displayed peptides through amber suppression, and identifying potential reversible covalent inhibitors of CD44 through phage display.

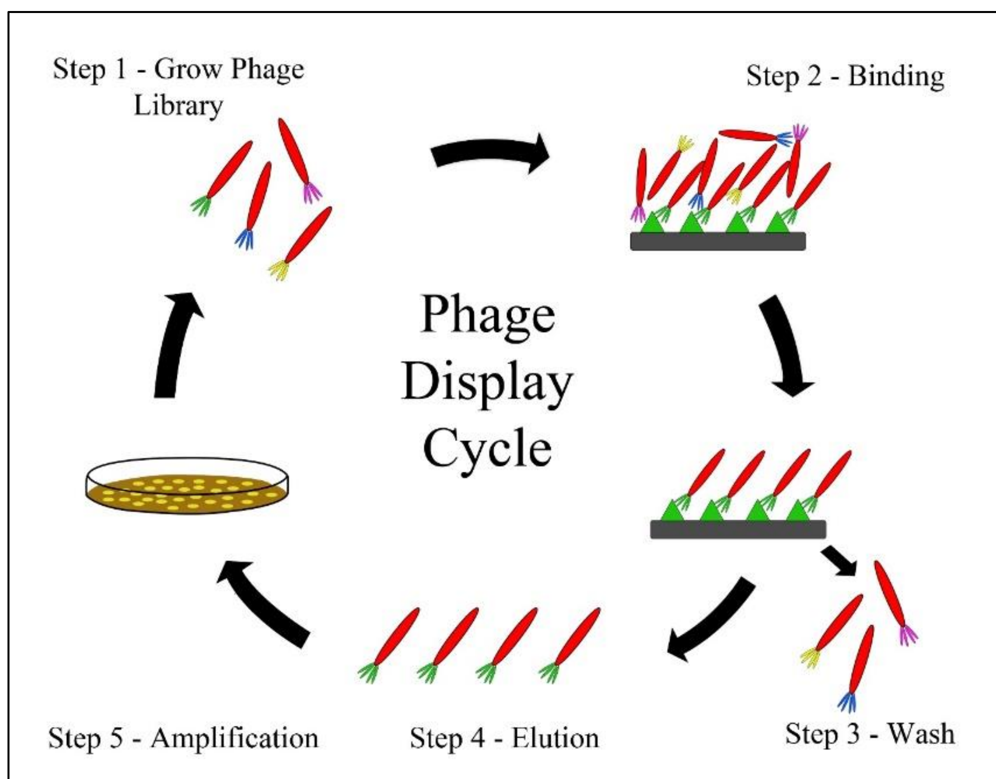

Figure 1. The Phage Display Cycle. 1) Phages expressing the pIII surface protein fused to the peptide library to be evolved are produced. 2) The phage library is exposed to an immobilized target in which some phages bind stronger than others to the target. 3) Low affinity binders are washed away. 4) High affinity binders are eluded and isolated. 5) The high affinity binders are used to infect bacteria and are amplified. Random mutations may occur in the peptide library gene after this amplification. These mutations introduce more variation in the peptide library allowing for more rounds of selection to be conducted for identifying high affinity binders to the target.

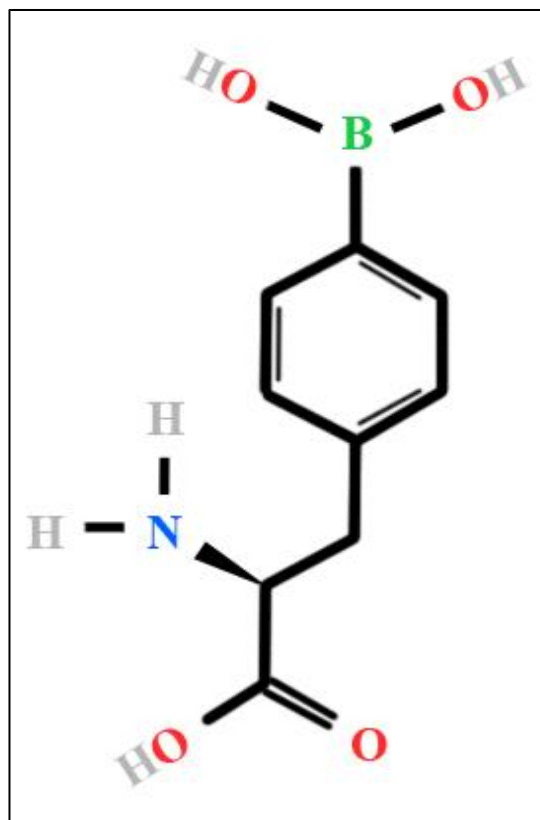

Figure 2. The structure of Boronophenylalanine. The functional group of this noncanonical amino acid is boronic acid and is directly responsible for its novel chemical properties.

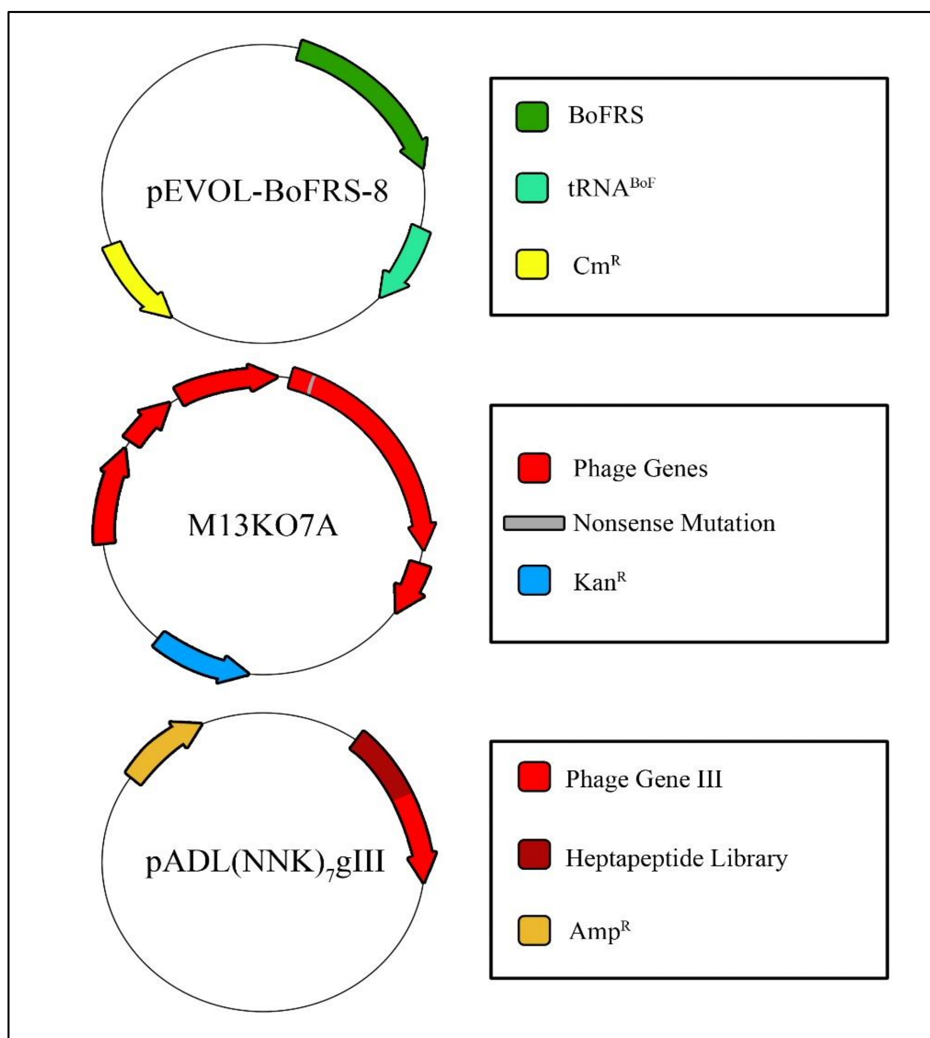

Figure 3. Essential Plasmids. Phage production required the transformation of 3 plasmids in *E. coli*. The pEVOL-BoFRS-8 plasmid contained genes encoding BoFRS and tRNA^{BoF} which are required for TAG codon site-specific BoF incorporation as well as a chloramphenicol resistance gene. The M13KO7A plasmid contained genes encoding for general phage proteins including a nonfunctional gene III which had a nonsense mutation. The plasmid also contained a kanamycin resistance gene. The pADL(NNK)$_7$gIII plasmid contained the gene encoding the pIII surface protein fused to the gene encoding the highly diverse and assessible heptapeptide. The codon sequence of the heptapeptide contained the amber codon, TAG, at a random position. The plasmid also contained a gene for ampicillin resistance.

CHAPTER II

METHODS

Transformation of Top 10 sfGFP-D134TAG cells with pEVOL-BoFRS-8

Before the production of phages, the pEVOL-BoFRS-8 plasmid's integrity needed to be tested in order to examine the BoF incorporation. The plasmid contained the genes encoding BoFRS and $tRNA^{BoF}$ which are mutants of PylRS and $tRNA^{Pyl}$ that are responsible for BoF incorporation in peptides with TAG-codon specificity. The plasmid also has a Cmp^{r} gene (Fig**.** 3) The sfGFP-D134TAG plasmid contained a TAG-nonsense mutation in the sfGFP gene and an Amp^{r} gene (Fig. 4). Top 10 sfGFP-D134TAG cells were inoculated to 5mL of LB broth media supplemented with ampicillin (100 µg/mL) and incubated overnight at 37 °C.

The next day the cells were made chemically competent. A 1mL sample of the overnight broth culture was inoculated to 94 mL of fresh LB broth supplemented with ampicillin (100 µg/mL). The culture was incubated at 37 °C until optimal OD_{600} (~0.5) was reached. After reaching this OD, 4 aliquots were made and placed on ice for ~5 minutes. The aliquots were centrifuged at 5000g for 10 minutes at 4°C before being placed back on ice. The cells were resuspended in 25 mL of cold 0.1 M $CaCl_2$ in each of the four samples and were left on ice for 20 minutes. The samples were centrifuged again using the aforementioned settings. Finally, the 4 chemically competent cells samples were each resuspended in 200 µL of cold 0.1M $CaCl_2$. One of the chemically competent cell aliquots was used for the transformation with pEVOL-BoFRS-8, and the other three were stored.

The 200 µL competent cell sample was exposed to 66.46 ng of pEVOL-BoFRS-8 and was left at room temperature for 30 minutes. The mixture was then placed in a 42 °C water bath

for 2 minutes and placed on ice for 1 minute. The sample solution was transferred to 2 mL of 2YT broth and was incubated for 30 minutes at 37 °C. The recovery broth was plated on LB agar supplemented with chloramphenicol (34 mg/mL) and ampicillin (100 mg/mL), and the plate was incubated at 37 °C overnight.

In order to quantify BoF incorporation by pEVOL-BoFRS-8, sfGFP expression was induced. A colony from the LB agar plate was inoculated to 5 mL of 2YT broth supplemented with chloramphenicol (34 µg/mL) and ampicillin (100 µg/mL) and incubated overnight at 37°C. A fresh 8 mL 2YT broth culture supplemented with ampicillin (100 µg/mL) and chloramphenicol (34 µg/mL) was prepared with 500µL of the overnight culture. The broth culture incubated for ~1 hour to a final OD_{600} of 0.43 before 16 mg of arabinose were added (2% by volume). The mixture was separated into 2 separate 4 mL culture tubes so that each contained about 2% arabinose. One tube was labeled the control and the other the experimental. In the experimental culture, 1.25 M BoF was added, and both cultures were incubated overnight at 37 °C. Both cultures were pelleted for 10 minutes at 4000 rpm before being resuspended in 900 µL of resuspension buffer, 100 µL of 11.5 mg/mL lysozyme solution, and 1 µL of 100 mM PMSF. The solutions were transferred into 2 different Eppendorf tubes and pelleted for 2 minutes at 14000 rpm. The supernatant of each of the 2 tubes were transferred to fresh Eppendorf tubes. Due to the presence of visible cellular debris in each of the solutions, they were subjected to 0.45 µm paper-syringe filtration. The fluorescence was quantified using relative fluorescent units (RFU), and a graph comparing the experimental and control fluorescence outputs was produced. Samples from both the experimental and control supernatants were used for SDS-PAGE and mass spectrometry.

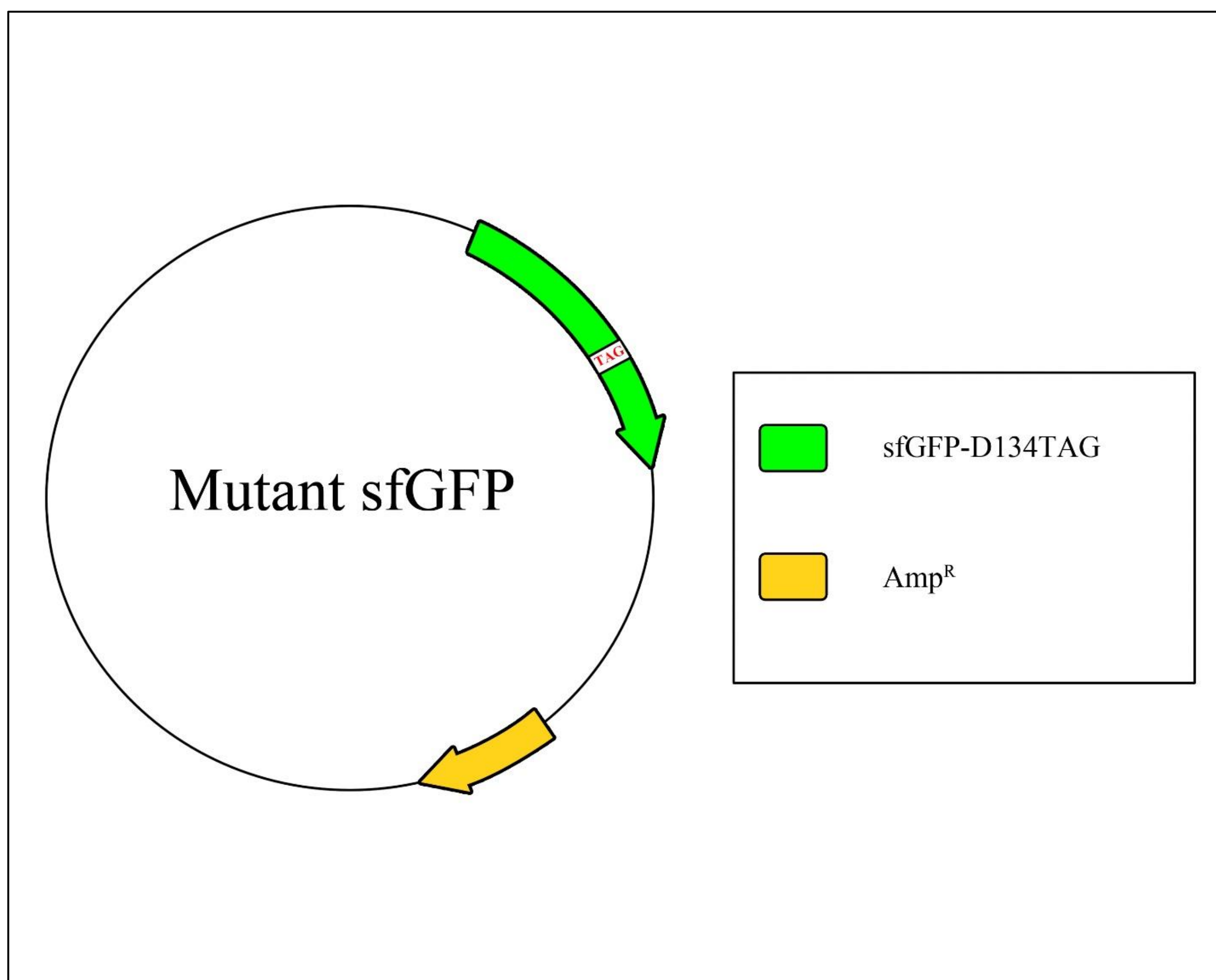

Figure 4. sfGFP-D134TAG Plasmid Map. The plasmid contained a mutant sfGFP gene and a gene for ampicillin resistance. The mutant sfGFP codon sequence contained the stop codon TAG at position 134, and the BoFRS/tRNA^{BoF} genes of the pEVOL-BoFRS-8 plasmid facilitated incorporation of BoF at said position.

Genetic Incorporation of BoF into Phage-Displayed Peptides

The production of ncAA-displaying phages required the transformation of top 10 cells with 3 distinct plasmids[11]: 1) pEVOL-BoFRS which contained genes encoding BoFRS, $tRNA^{BoF}$, and Cmp^r, 2) M13KO7A which contained general phage genes, a TAA mutated gene III, and a Kan^r gene 3) $pADL(NNK)_7gIII$ which contained the genes for the heptapeptide library and Amp^r (Fig. 3).

Top 10 chemical competent cells were exposed to 69.84 ng of the pEVOL-BoFRS-8 plasmid and were left at room temperature for 30 minutes. The cells were then heat-shocked for 30 seconds at 42 °C before being returned to the ice for 5 minutes. The cells were inoculated to 100 µL of SOC media and incubated for 1 hr at 37 °C. Following the recovery period, the cells were platted on LB agar supplemented with chloramphenicol (34 µg/mL). The plate was incubated at 37 °C overnight. The following day, one colony from the plate was inoculated to 5 mL of 2YT media supplemented with chloramphenicol (34 µg/mL) and incubated overnight at 37 °C in preparation for the M13KO7A helper phage plasmid transformation.

Cells from the overnight culture were made chemically competent and transformed. A 50 µL sample of the pEVOL-BoFRS-8 overnight transformant culture was inoculated to 5 mL of 2YT supplemented with chloramphenicol (34 µg/mL) and incubated at 37 °C until an optimal OD_{600} was reached (~0.46). The culture was placed on ice for 20 minutes with swirling for even cooling. After the cooling period, cells were pelleted for at 5000g for 10 minutes at 4 °C before being resuspended in 10 mL of cold 0.1M $MgCl_2$. The cells were then pelleted at 5000g for 10 min at 4 °C before being resuspended in 20 mL of cold 0.1 M $CaCl_2$. The cell solution was left on ice for 10 minutes and was then pelleted at 5000g for 10 min at 4 °C. Cells were resuspended

in 5 mL of cold 15% glycerol, 85 mM $CaCl_2$ and pelleted at 5000g for 10 min at 4 °C. Cells were resuspended in 200 µL of cold 15% glycerol, 85 mM $CaCl_2$ and aliquoted into 100 µL samples in two Eppendorf tubes with one being used for the transformation with the M13KO7A plasmid.

The 100 µL aliquot of chemical competent pEVOL-BoFRS transformant cells were exposed to 50.0 ng of M13KO7A and were left on ice for 30 minutes. The cells were then heat-shocked for 30 seconds at 42 °C before being returned to the ice for 5 minutes. After the wait, the cells were inoculated to 100 µL of SOC media and incubated for 1 hr at 37 °C. Following the recovery period, the cells were platted on LB agar infused chloramphenicol (34 µg/mL) and kanamycin (25 µg/mL). The plate was incubated overnight at 37 °C. A colony from the plate was inoculated to 5 mL of 2YT media supplemented with chloramphenicol (34 µg/mL) and kanamycin (25 µg/mL) and incubated overnight at 37 °C in preparation for the pADL$(NNK)_7$gIII plasmid transformation.

Cells containing BoFRS and M13K07A were made electrocompetent in preparation for the peptide library transformation. Two 5 mL overnight cultures were prepared in 2YT supplemented with chloramphenicol (34 µg/mL) and kanamycin (25 µg/mL) and incubated at 37 °C. The following day, the both cultures were inoculated to 1 L of no salt LB broth and were rapid-shake incubated at 37 °C until optimal OD_{600} was reached (0.42). The 1 L broth culture was then chilled on ice for 10 minutes with continuous swirling before being aliquoted into 3 centrifuge bottles in a cold room. The aliquots were then pelleted at 4000 rpm for 5 minutes at 0 °C. The cells of each aliquot were resuspended in 50 mL of 10% glycerol. The resuspension volume was brought to about 150 mL before being split into two centrifuge bottles. The two resuspensions were pelleted at 4000 rpm for 5 minutes at 0 °C, and each were then resuspended in 100 mL of 10% glycerol. After this 2nd resuspension, the solutions were combined into one

centrifuge bottle and pelleted at 4000 rpm for 5 min at 0 °C. The cells were then resuspended into 100 mL of 10% glycerol before being pelleted at 4000 rpm for 5 min at 0 °C. Following this final glycerol wash, the cells were resuspended 3 mL before being aliquoted in 30-120 mL samples and flash frozen. The aliquots were stored in -80 °C in preparation for the electroporation transformation. The final transformation with pADL(NNK)$_7$gIII for phage production and subsequent selection/amplification were not conducted.

CHAPTER III

RESULTS

Quantifying Incorporation of pEVOL-BoFRS in sfGFP-D134TAG -Top 10 Cells

After the induction of sfGFP expression, the experimental and control cultures were placed under UV light, and the relative fluorescence of each were quantified using computer software and was expressed in relative fluorescent units (Fig. 5). The control culture showed no green light fluorescence under UV light, and the experimental culture fluoresced strongly. The graph produced from the computer program showed that the experimental culture had a relative fluorescence of ~6000 RFU, and the control culture had a relative fluorescence of ~400 RFU. In order to further prove that boronophenylalanine was incorporated in sfGFP, SDS PAGE was conducted (Fig. 6), and mass spectrometry was performed on samples for both cultures (Fig. 7).

The experimental mass spectrometry data displayed a strong peak at 21-23 min, but this peak is absent in the control data. When sfGFP forms the chromophore responsible for fluorescence, it loses a water so that was taken into consideration in calculating the theoretical mass of the protein. The theorical mass of sfGFP was 27920.224 Da, and the calculation was as followed: 27732.23 Da (estimated mass without BoF incorporation) + 206.01 Da (estimated mass of BoF) – 18.016 (mass of water that was loss) = 27920.20 Da (theoretical mass of sfGFP with BoF incorporation). The deconvoluted experimental mass spectrometry data indicated that the mass of the sfGFP produced was 27884.9 Da which 35.324 Da less than the calculated theoretical mass. The most plausible explanation for this difference is that BoF residue participated in condensation reactions with two neighboring lysine residues which resulted in the loss of 2 more water molecules.

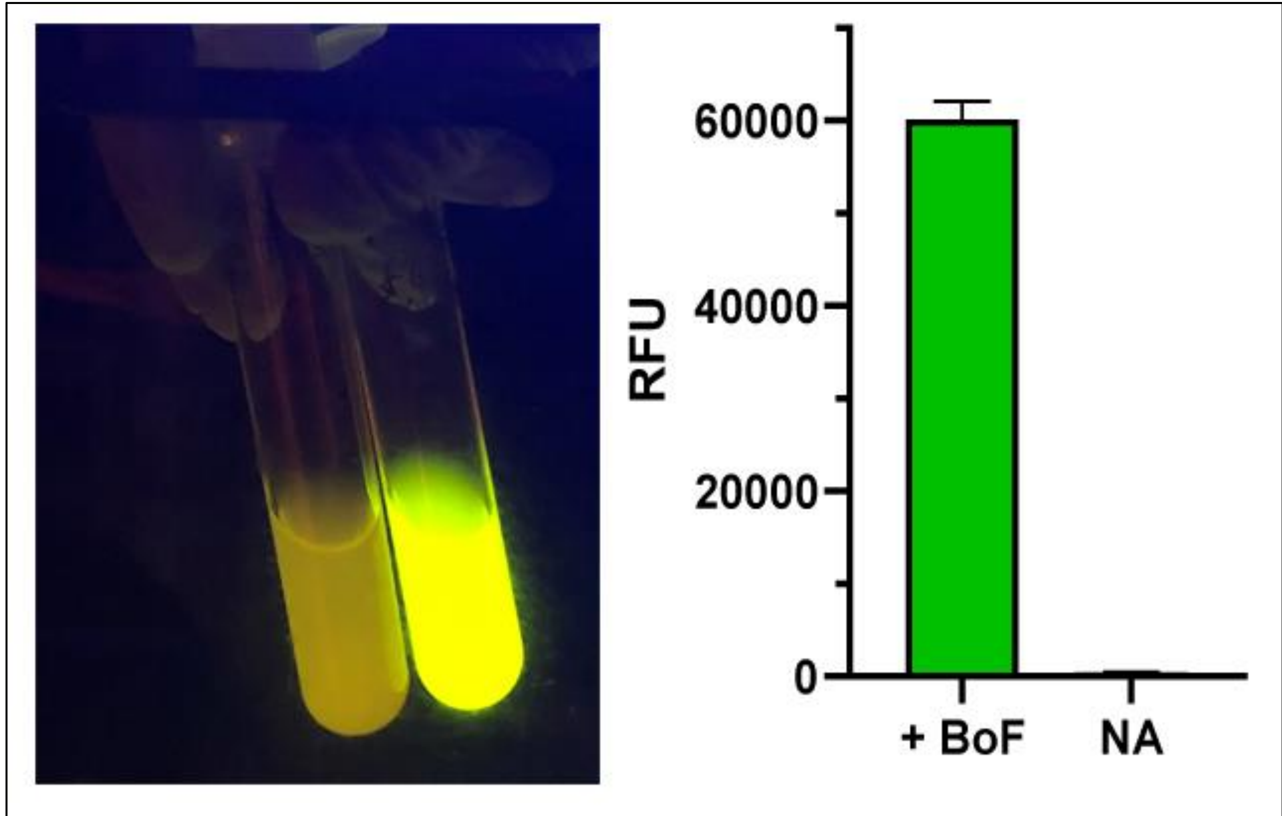

Figure 5. sfGFP-D134TAG Fluorescence Data. Relative fluorescence units (RFU) were generated by computer. The relative fluorescence of experimental and control cultures were ~6000 and 400 RFU respectively. The experimental culture was incubated in the presence of BoF, and the control culture was not.

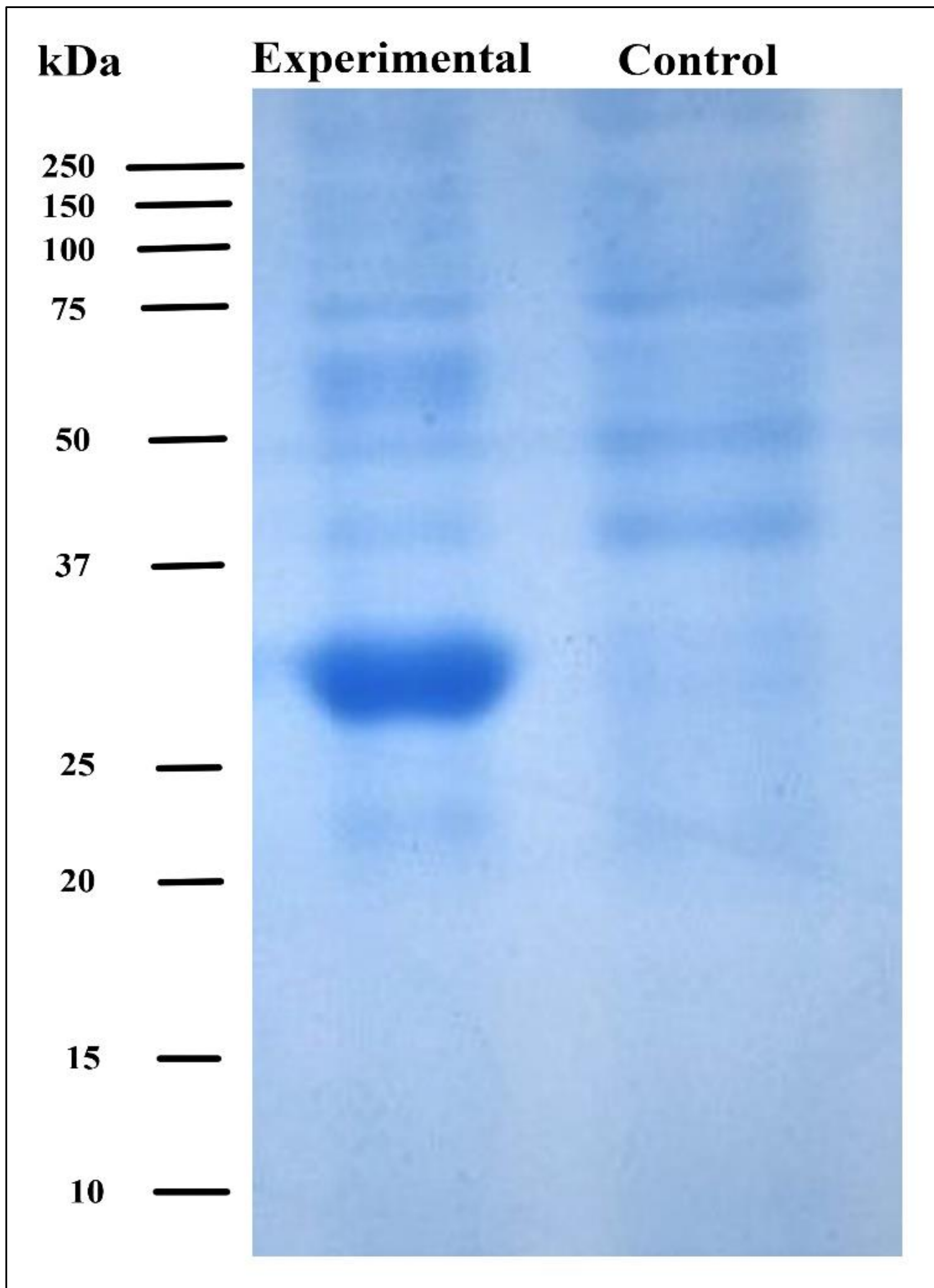

Figure 6. SDS-PHAGE. At ~28kDa, a band appears in the experimental lane while that band is absent in the control lane

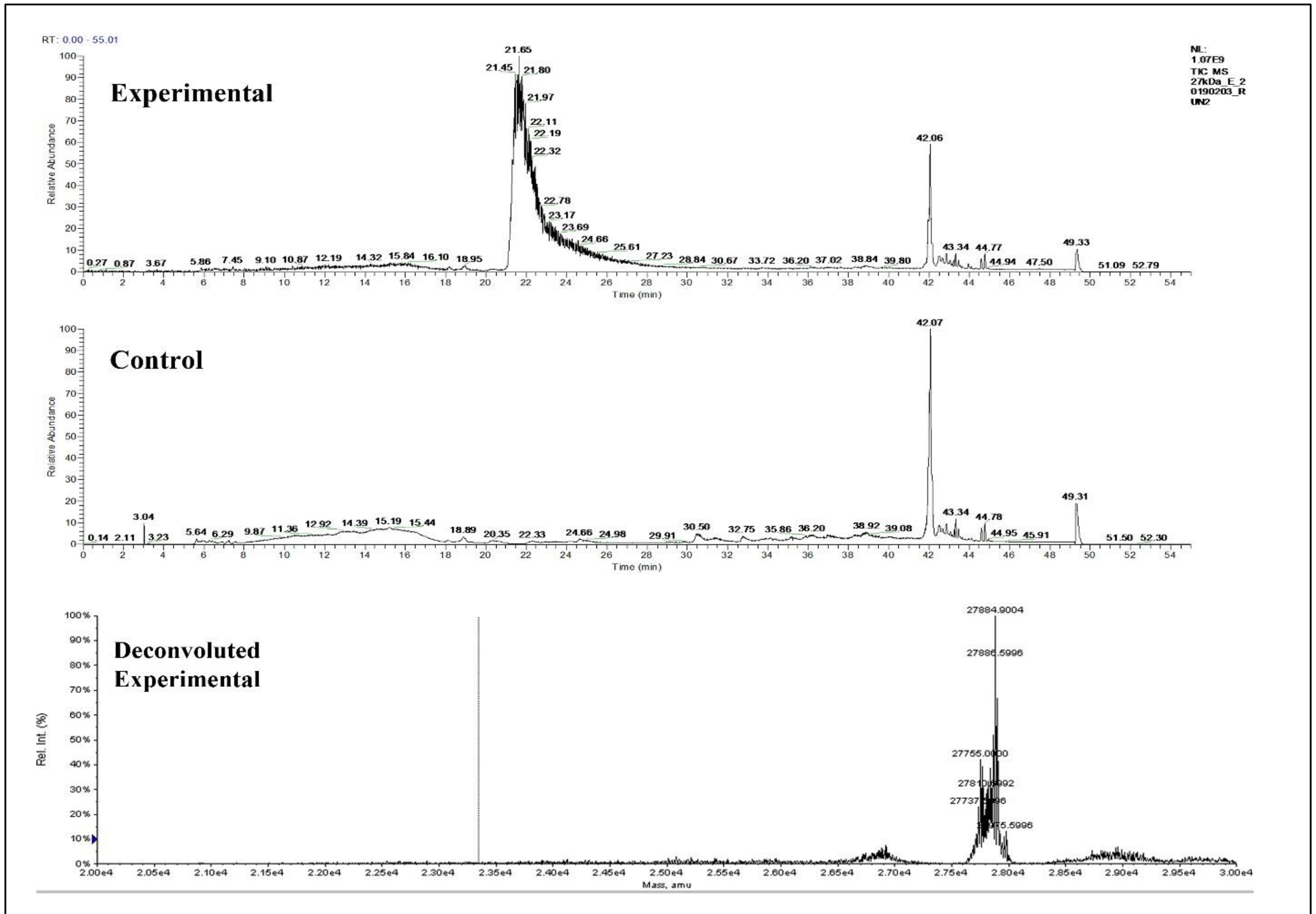

Figure 7. Mass Spectrometry of sfGFP-D134TAG with BoF Incorporation. The absence of a strong peak between 21-23min in the control data when compared to the experimental data indicates that the sfGFP was absent in the control sample. The deconvoluted experimental data suggests that the estimated mass of the sfGFP was ~27.9 kDa.

Phage Display Progress

Both the pEVOL-BoFRS and M13KO7A plasmids have been successfully transformed into a top 10 cell line, evident through growth on a kanamycin and chloramphenicol supplemented LB agar plate. (Fig. 8). Unfortunately the third and final plasmid needed for phage production was not able to be transformed into the existing cell line, resulting in no phages for selections against the CD44. We hope to have BoF-incorporated heptapeptide phage library by the end of summer 2020 if the circumstances surrounding COVID19 allow for it.

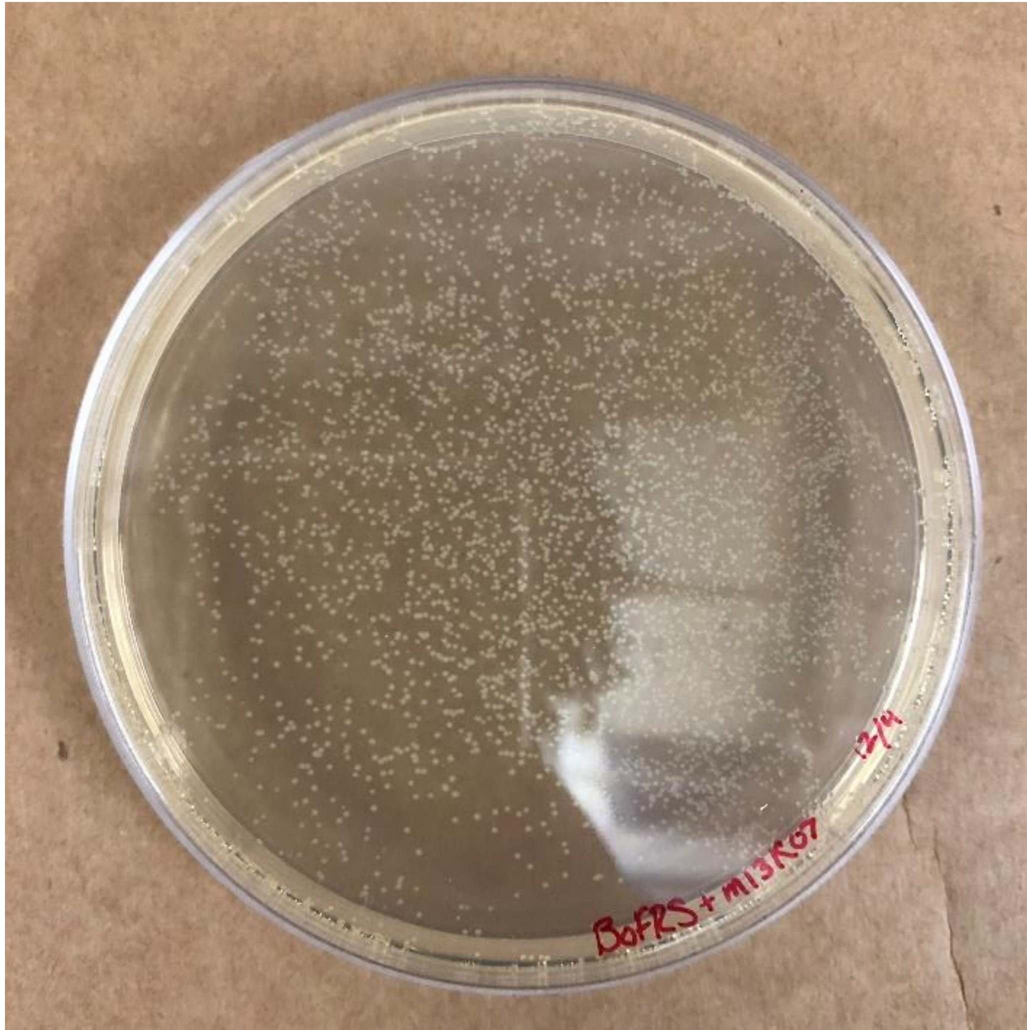

Figure 8. pEVOL-BoFRS-8/M13KO7A Transformants on LB agar plate. Two of the three plasmids required for phage production have been successful transformed into top 10 cells. The cells grew on LB agar supplemented with 34 μg/mL chloramphenicol and 25 μg/mL kanamycin.

CHAPTER IV

CONCLUSION

sfGFP Model for Testing Incorporation

The results from using the sfGFP-D134TAG model suggest that the pEVOL-BoFRS can facilitate the incorporation of BoF utilizing amber suppression with BoFRS/ $tRNA^{BoF}$. When BoF was present, sfGFP-D134TAG/pEVOL-BoFRS-8 transformant cultures fluoresced under UV light 15 times greater than in the absence of the ncAA. The experimental and control wells of the SDS-PAGE gel were different in that the band between 25-31 kDa, indicating the expression of sfGFP, was only present in the experimental well, and the mass spectrometry data indicated that BoF was incorporated in sfGFP assuming that the residue participated in condensation reactions with two neighboring lysine residues.

Future Research

Due to the unforeseen events surrounding the COVID-19 virus in spring 2020, phages have not been produced. The last plasmid, $pADL(NNK)_7gIII$, needs to be incorporated in the existing pEVOL-BoFRS-8/M13KO7A transformant cell line in order to produce phages. Once $pADL(NNK)_7gIII$ is transformed in the existing cell line through electroporation, phage expression will be induced. The phages will have a functionally assessible randomized heptapeptide containing BoF fused to the pIII protein. We hope that the high variation (4.48×10^8) and the incorporation of BoF in the randomized peptide will allow for rapid identification of reversible covalent inhibitors of CD44 through a phage display assay.

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