Purification of Phage-Displayed HSA-Specific Peptide for Biosensor Production

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Abstract

The goal of this project is to produce a carbon nanotube transistor-biosensor that can detect blood, which can be used by the police or military. This biosensor would be able to detect human serum albumin (HSA) and send a distress signal for help. HSA is the most abundant component of blood plasma, making it an ideal target for detection. The specific goal of this study is to purify an HSA-specific heptapeptide, BR-1, from a M13 filamentous phage that was obtained through phage display. The specificity of BR-1 for HSA was confirmed by performing an Enzyme-linked Immunosorbent Assay (ELISA), however, several peptide ELISAs were performed beforehand in order to find the optimal conditions for the ELISA (biotin amounts on peptide for streptavidin-biotin system and wash and blocking buffers). The BR-1 peptide was previously sequenced in this lab, and this sequence was utilized for this experiment. Primers were designed for a polymerase chain reaction process that incorporated the peptide sequence into the expression vector pMal-c5x. This DNA was then subsequently purified, ligated, and sequenced again to confirm the presence of the peptide sequence in the vector. The majority of the peptide sequence was incorporated into the vector; however, this process will need to be repeated in order to incorporate the complete sequence into the vector. After the peptide sequence is incorporated into the pMal-c5x expression vector, this vector will then be transformed into *E. coli*, and will be induced to express this peptide fused to a maltose-binding protein (MBP). The peptide will subsequently be purified from the MBP using an amylose resin column. After this process is complete, the specificity of this purified peptide can be tested by performing another ELISA. Future efforts will be to use the peptide that our lab purified and incorporate it into a biosensor that can detect HSA.

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Introduction

Background of Biosensors

The age of information has brought great advancements in technology that have benefitted society in many ways. Some of the areas that has been touched by these advancements are biosensing and bioelectronics. This technology can be used to detect a wide variety of molecules using basic sensor technology, along with biological methods. This technology can be used to improve food safety, environmental monitoring, or implemented into clothing or watches, which can be used for medical monitoring (Cherenack et al. 2012; Yang et al. 2015). Despite all the recent advancements that have been made in biosensor technology, there is still no type of commercial use of biosensors that can detect blood for armed forces or the police. Recent literature has shown that the technology is available to create such a biosensor, but it has not been performed yet for this purpose or on a large scale. The aim of this project will be to create a biosensor that can detect an element of blood. This will be done by finding a peptide that has a high affinity for a component of blood through phage display, such as human serum albumin, and functionalizing it to a single wall carbon nanotube system.

Biosensors and Carbon Nanotubes

A classic biosensor can be separated into two main components: the biological sensitive element and the transducer. The biological element may include proteins, peptides, or microorganisms (Yang et al. 2015). This part of the sensor utilizes common interactions that are found in nature such as between a peptide-protein or an antigen-antibody (Hu et al. 2007; Cortes et al. 2013). The transducer is the part of the sensor that converts the concentration of the molecules that are being sensed to a signal that can be detected (Yang et al. 2015). For example, a signal can be a change in current or

impedance when a molecule is bound to the biological sensing element (Cortes et al. 2013; Manzetti et al. 2015; Yang et al. 2015). A type of transducer that has become more common due to its chemical and electrical properties is a carbon nanotube (CNT) transducer (Chen & Huang 2009). CNTs can be described as sheets of graphite which are rolled into tubes and possess a high length to diameter ratio, strong Vander Waal forces, and conductive and semi-conductive properties. The most important structures that these nanotubes form are single wall carbon nanotubes (SWNTs) and multiwall carbon nanotubes (MWNTs). The SWNT is a cylinder-shaped structure with only one layer, while the MWNT is a cylinder-shaped structure with a collection of concentric SWNTs. MWNTs have several appearances such as coaxial cylindrically curved, coaxial polygonised, or scrolls (Belin & Epron 2005). For biosensors, SWCNTs have an advantage over the MWCNT in that it is easier to transfer a chemical signal after binding of the analyte, since it only has one layer (Manzetti et al. 2015).

Biofunctionalization of CNTs

Biofunctionalization of the CNT is done in order to create the biologically sensitive element of the biosensor. This can be accomplished through several different methods, such as physical adsorption or covalent modification. Physical adsorption takes advantage of the strong intermolecular forces of the CNT, by having the protein or enzyme adhere to the CNT via strong Vander Waal forces. This method involves no chemical methods and has little effect on protein or enzymatic activity. This method is not always used, since these interactions rely on Vander Waal forces which can be affected by factors such as temperature, pH, or ionic strength, and may cause the protein or peptide to dissociate from the electrode. Covalent modification, on the other hand, is attaching the biological molecule to the CNT using a covalent bond. This is sometimes

preferred over physical adsorption, because the covalent modification process is more robust and produces a more stable product than physically adsorbed biological elements, therefore they give the sensor a longer lifetime (Yang et al. 2015).

The covalent modification process can be done in several ways. Hu and coworkers did an experiment, where they created a biosensor by using MWCNTs by biofunctionalizing them with biotin and rabbit IgG. The biotin could then be detected using avidin (which has a high affinity for biotin) conjugated to a fluorescent tag, and IgG could also be detected by using an anti-IgG antibody conjugated to a fluorescent tag. They oxidized the CNTs by mixing them with an acidic mixture of sulfuric acid and nitric acid. They then filtered it, washed it, and dried the fibers in a vacuum. Then they were suspended in DMF and sonicated. After sonication, MWCNTs were mixed with a 1-ethyl-3-(3-dimethyl aminopropyl) carbodiimide/dimethylformamide (EDAC/DMF) solution and then a N- hydroxy succinimide (NHS)/DMF solution. This was then followed by sonication and washing steps. (Hu et al. 2007). These are important steps for functionalization, because EDAC (or EDC) reacts with the carboxylated group of the CNT to form an unstable amine-reactive intermediate. In order to stabilize this compound, NHS is added to convert this intermediate to a semi-stable amine-reactive NHS ester. After the EDAC/NHS and washing/sonication steps, the protein or peptide of interest can be added to the CNT compound to form an amide bond between the CNT and the protein (Gao & Kyratzis 2008). Therefore, steps allow experimenters to covalently bind the ligand of interest to the carbon nanotube (Hu et al. 2007).

CNTs are used for biosensors, because they can be biofunctionalized and act as an electrical transducer. The CNT may possess a metallic or semi-conductive configuration

depending on diameter and chirality. These nanotubes can be rolled at three different angles: arm chair, zig-zag, or chiral, and it is the difference in this angle that determines the mechanical, optical, and electrical properties of the CNT (Belin & Epron 2005). These properties, along with the chemical properties, make using CNTs ideal for a biosensor, since they can conduct an electrical signal to produce a sensor (Cortes et al. 2013).

Data Acquisition

The last element of the biosensor is the conversion of this physical data to a digital form. A system that allows digitalized data to be read from a biosensor is known as a Data Acquisition (DAQ) computer system (Kos et al. 2012). For CNT biosensors, a DAQ system can be used to read the impedance of the CNT before and after exposure to the target molecule. The impedance of the CNT should change if the composition of the groups attached to the CNTs change, which should be expected if the biologically sensitive element binds to its target molecule. In this process, the DAQ system circuit can be created by having the DAQ system hooked up to the CNT using alligator clips. The DAQ system will then send the desired voltage through the CNT, process this information, and give the measured impedance. This process was used by Cortes, Olszewki and Fagan in the detection of human serum albumin (HSA) using a CNT biosensor. This experiment successfully created a biosensor where the DAQ system detected a specific impedance signature for HSA compared to other fluids such as water, coffee, or urine (Cortes et al. 2013).

Phage Display

As mentioned previously, the goal of the experiment performed by Cortes and colleagues was to create a CNT biosensor to detect HSA. For their biosensor, they used a

peptide that had a high affinity for HSA, which was found through the process of phage display (Cortes et al. 2013). Phage display is a method to analyze protein-protein interactions by using a bacteriophage to express a certain protein or peptide on its surface to interact with a target (Willats 2002). Therefore, this is a process that can find a high affinity protein for a biosensor.

Bacteriophages

As reviewed by Clark and March, bacteriophage are viruses that infect bacteria, such as *E. coli*. When infecting bacteria, bacteriophage (phage) are able to use the bacterium to replicate the phage itself. There are three different types of phage, that can be used for processes that study protein-protein interactions. They are the M13 filamentous phage, lambda, and the T7 phage. Although the other phage can be used, the filamentous phage is used quite often due to several advantages. One main advantage of using M13 filamentous phage is that their size is not limited and can increase in length to accommodate the DNA that are within them. Therefore, if they are used as cloning vehicles and several genes are inserted into their genome, this will be easily accomplished since that assembly process will just produce a longer phage (Willats 2002)(Clark & March 2006).

Filamentous Phage

Filamentous phage are approximately 1 micron in length and less than 10 nm in diameter. This type of phage structure consists of a protein coat that surrounds the genetic material of the virus, which is single stranded DNA (Sidhu 2001). Eleven phage genes code for proteins that are important for DNA replication, assembly, structure, and ssDNA binding. There are several genes that encode for capsid proteins which are important for the structure of the phage and for the infection process. Protein pVIII is a major capsid

protein that covers majority of the phage. Approximately 2700 molecules of pVIII covers the average filamentous phage, and each protein consists of 50 amino acids. The proteins are arranged in an overlapping, roof-shingle manner with the amino-terminal portion of the protein on the outside of the particle and the carboxy terminal pointed inwards. There are also several minor capsid proteins that are present on the ends of the phage and there are approximately 5 molecules of each. These are pVII protein and protein pIX at one end, and protein pIII and protein pVI at the other end. (Barbas III et al. 2001). While pVIII is the most abundant capsid protein, pIII is the largest (pIII is composed of 406 amino acids, while pVIII is composed of 50 amino acids. pIII, an important protein for infection of *E. coli*, has three domains that are separated by glycine-rich areas. The second N-terminal domain (N2), binds to the F-pilus of the *E. coli*, which is important for host cell recognition and initiating the infection process. The other N terminal domain (N1), initiates translocation of viral DNA into *E. coli* by interacting with some of the bacterial periplasmic proteins. The third domain is the C-terminal domain and it is important for the structural integrity of the phage particle and is necessary for the phage infection process (Barbas III et al. 2001; Sidhu 2001; Rakonjac et al. 2011).

Filamentous phage can be used for the process of phage display in order to study protein-protein interactions This can be done because there is a direct physical link between the genotype, the genes in the vector, and the phenotype, the physical proteins that are expressed on the phage coat which the genotypes code for. When a nucleotide sequence is inserted and fused to the gIII or the gVIII genes, it will cause a peptide or protein to be fused to the N-terminus of pIII or pVIII on the phage. This can aid in finding a peptide, protein, or antibody that has a strong affinity for the ligand of their

choice, and then be able to isolate it, sequence it, and reproduce it, and this process is termed as "phage display (Clark & March 2006; Willats 2002)."

Types of Phage Display Expression

Phage display can be performed in several ways that differ in the expression of the amount of proteins on the phage. There is phage expression, where fusion of a gene for a protein/peptide and a gene for a coat protein are fused. This produces phage particles where all of the coat proteins express the fusion protein (only the genes that had been fused). This approach can be advantageous to use, since there is a higher number of expressed fusion proteins, but the functionality of the phage coat protein may be affected. Therefore, to overcome this problem, other types of expression have been discovered. Hybrid phage expression is phage-based where gene fusion is an additional element of the phage genome (the plasmid has both g1 and g1/x fusion). This will express some of the wildtype coat proteins and some of the coat proteins that possess the protein or peptide displayed (Sidhu 2001). Lastly, instead of the genes being on the same phagemid, there can be two phages that infect the bacteria, one is the phage that possesses the gene fusions and one the contributes other phenotypes that the phage will need. The helper phage in this case has a defective origin of replication, the whole helper phage will not be produced, but wild type protein coat genes can be expressed. This creates a hybrid phage, whose proteins were formed from two plasmids instead of one (Willats 2002; Hamzeh-Mivehroud et al. 2013).

Uses of Peptide Phage Display

One of the main advantages of peptide phage display, is the ability to generate a peptide with affinity for the target from a random phage peptide library and this method requires no special design. Peptide libraries offer highly discriminatory peptides that are

highly specific and can even discriminate between different conformations of a protein, which is ideal for creating biosensors. Phage display is also a cheap method, since commercial phage display libraries are available and easy to use (Pavan & Berti 2012). In an experiment that was performed by Ahmed and colleagues, the researchers used peptide phage display in order to find a peptide that had a high affinity for their ligands, which were cytokines. This process helped them produce these peptides, and the affinity of the peptide for the ligand was then confirmed by performing M13 Enyzme-linked Immunosorbant Assay (ELISA)s, direct ELISAs, and a dot plot assay (Ahmed et al. 2014)

Phage Display Process

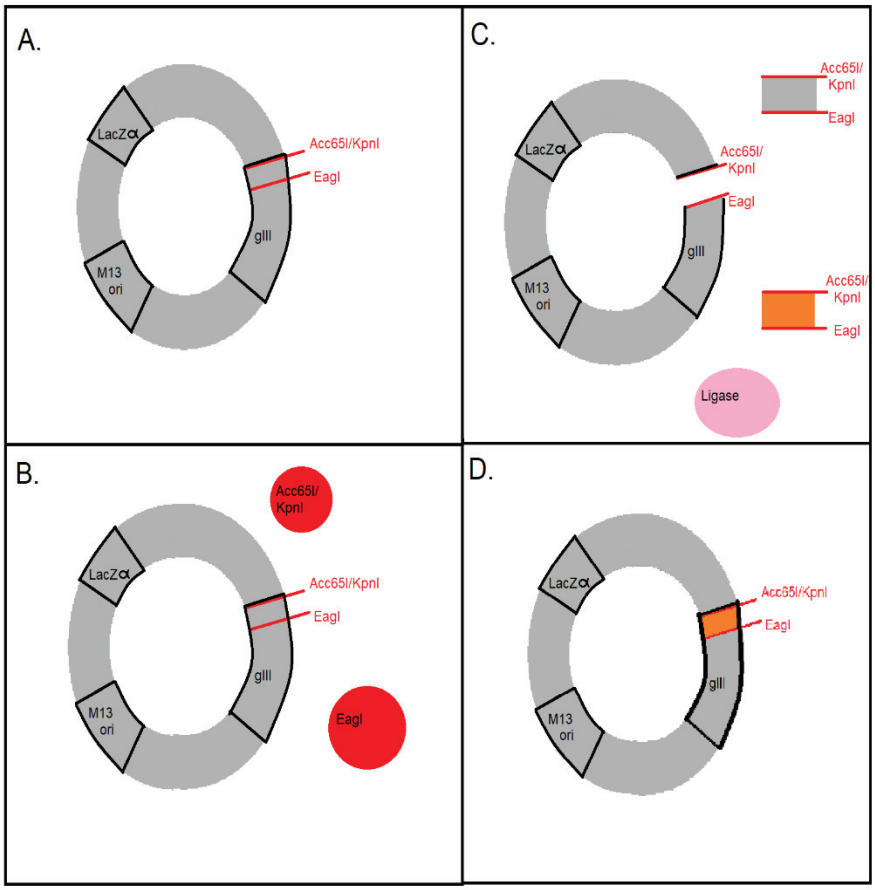
Phage display starts with the incorporation of a nucleotide sequence that encodes a certain protein/peptide to the phage or phagemid genome. The nucleotide sequence is fused to the gene of the phage coat particle, which causes the protein/peptide to be expressed on the surface of the phage. This protein is attached to the phage coat protein. This process allows the development of libraries of variant nucleotide sequences with large diversity to display variant proteins on the surface of the phage. Once the library is constructed, they can be screened for their ability to bind to a specific ligand (Willats 2002; Sidhu 2001; Noren & Noren 2001).

This whole process of vector manipulation can be easily accomplished, since the sequence of phage genomes such as M13KE are known and are used for creating phage display libraries. Phage vectors, like M13KE, possess restriction sites that have been placed at the 5’ end of gene III for display of peptide sequences (New England BioLabs Inc. A 2017). This allows experimenters to use compatible restriction enzymes to cut and

insert genes into the phagemid. These peptide sequences will be incorporated into the vector and will become N-terminal pIII fusions (New England Biolabs Inc. F 2002). The libraries constructed in the M13KE vector possess five copies of the protein that are displayed on all copies of the pIII coat protein. Restriction sites, Accl65I/Kpnl and EagI sites, have been incorporated into the M13KE vector flanking the pIII leader peptidase sequence cleavage site. In order to use these restriction sites to cut this specific region, the Acc651/Kpnl site was deleted in the original position on the vector (Figure 1). With all of these conditions present, the protein/peptide library can be constructed by cloning a synthetic oligonucleotide into the vector that possesses the same restriction sites as the M13KE vector (Noren & Noren 2001).

The peptide or proteins that are displayed on pIII must be short, because if they are longer than 30-50 amino acids, the length of the amino acid chain will be too large and cause complications for pIII infectivity function. Therefore, this is an important item to consider when choosing a peptide library for the phage display process (New England BioLabs Inc. A 2017).

Once a phage library with fusion proteins has been created, the next step is to isolate the phages that are desired from the rest of the library. This is done by a biopanning process, in which the phage with displayed proteins/ peptides that have a high affinity for the target will be selected. Successive rounds of the biopanning process are performed to get high affinity binders. The first step in biopanning is immobilization of the target. This can be achieved by simply putting the target into a MaxiSorp^{TM} plate, and allowing the immobilization process to occur via passive adsorption (Willats 2002). The next step of the process is to block the wells, so that the phage particles and the



<u>Figure 1</u>. M13KE as a Vector for a Peptide Library. M13KE vector can be manipulated to create peptide libraries for phage display. A) This panel shows the M13KE vector, with the restriction sites on gIII. B) Shows the introduction of the restriction enzymes *Acc65* I/*Kpn* I and *Eag* I, which will cut the corresponding restriction sites on the vector. C) This panel shows the introduction of a nucleotide sequence with the same restriction sites on the ends that can be ligated into the spot where the original nucleotide sequence was located. D) The new nucleotide sequence has been ligated into the vector, and now this phage will display a different fusion protein/peptide on pIII than the original sequence.

proteins that they are displaying do not bind nonspecifically to the wells. One item that should be taken into consideration when blocking the well, is if the blocking agent has similar properties to the target. That is why there are a variety of blocking agents that can be used, such as serum albumin, ovalbumin, casein, or milk powder (Dennis et al. 2002;)Lim et al. 2018);Rahbarnia et al. 2016). After the plates have been blocked, the phage library can be introduced to the well. Following binding to the target, the plate is washed to remove the non-bound phage (Lim et al. 2018; Willats 2002; Hamzeh-Mivehroud et al. 2013). The specific phage with the proteins/peptides that are bound to the target is then eluted. This is done by disrupting the interaction between the target and the ligand on the phage, which can be performed by changing the pH, changing the salt concentration, or using a detergent (Hamzeh-Mivehroud et al. 2013). Phage display selects for and produces peptides with high affinity for the ligand of the experimenter's choice (Pavan & Berti 2012; New England BioLabs Inc. A 2017).

The infection process is important for amplifying the phage displaying the desired peptide. Researchers can then either continue the biopanning process or purify the peptide. The filamentous phage infects the bacteria in a non-lytic manner, which means that it will not kill the bacteria in the process (Sidhu 2001). In the infection process, the N2 domain of the phage pIII coat protein binds to the F pilus of the bacterium (Figure 2 B). The pilus retracts and brings the pIII protein into the periplasmic space of the bacterium, where it will interact with the bacterium's inner membrane proteins. These cytoplasmic proteins are known as Tol proteins, and there are three of them: Tol A, Tol Q, and Tol R (Rakonjac et al. 2011). Tol Q is a membrane protein that weaves through the inner membrane three times and most of its residues are located in the cytoplasm. Tol

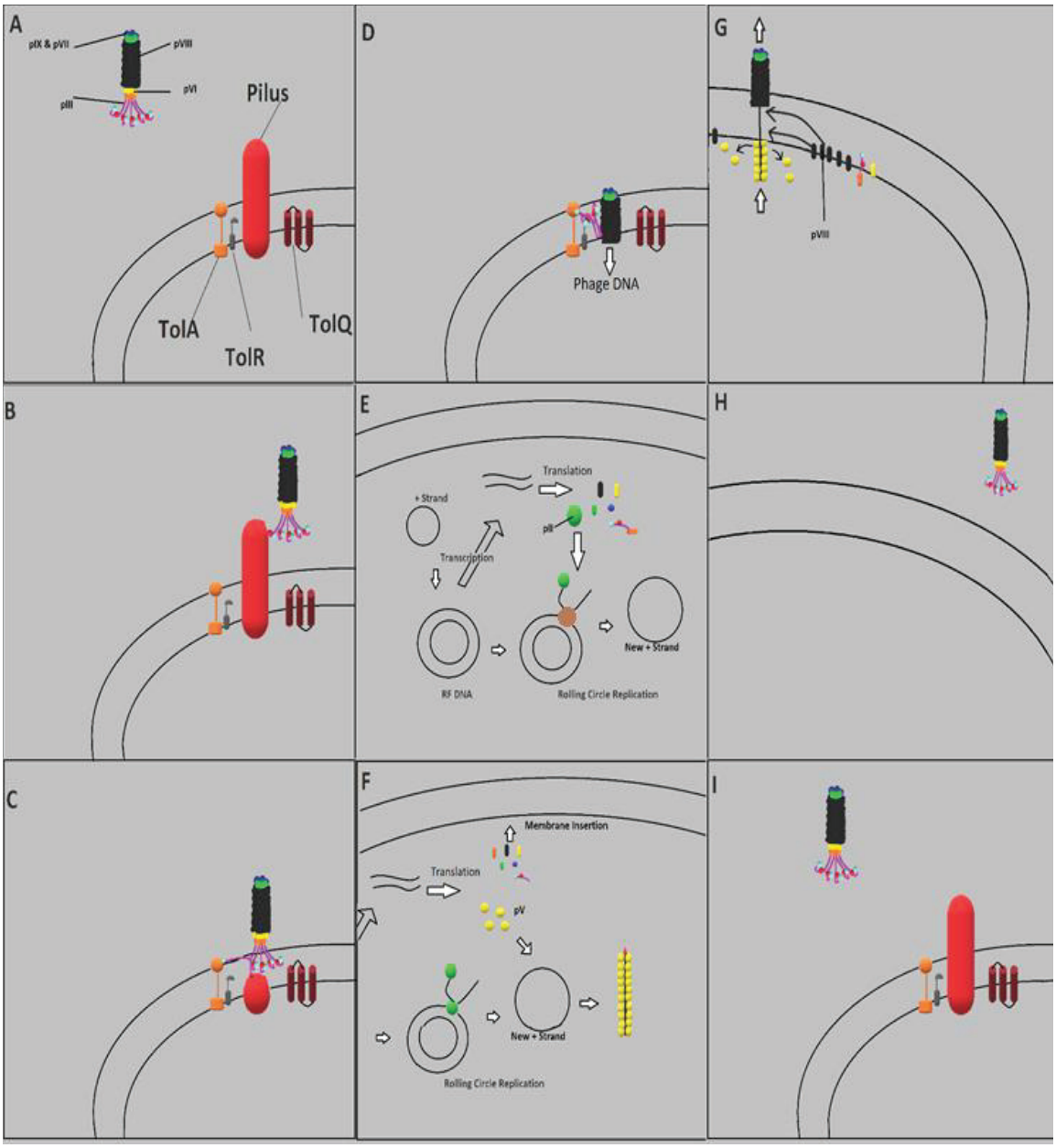


Figure 2. The Infection Replication, and Assembly Process of the F filamentous phage. A) The phage in close proximity to the *E. coli*. B) The N2 domain of pIII binds to the pilus of the *E. coli*. C) The phage is brought down to the periplasmic space by the retracting pilus and is now able to interact with the Tol proteins. D) The phage DNA enters the cell. E) This depicts the positive strand of phage DNA which can then take part in protein synthesis or DNA replication via rolling circle replication. F) The pV that is

being created converts the new + strands into a pV-DNA intermediate, in order to prevent new RF DNA from being created. G) The next step is where the phage is exiting the cell and is shedding off the pV, and the membrane inserted proteins assemble onto the new phage. H) and I) These steps show the new phage molecule outside of the *E. coli*, and now ready to infect a new cell.

R is anchored to the inner membrane by only one transmembrane region and the bulk of its residues are in the periplasmic space. Tol A is also anchored to the inner membrane by one domain and is mostly located in the periplasmic space, but, unlike Tol R, Tol A spans through the entire periplasmic space and has another domain anchored to the outer membrane of the bacterial cell. The three domains that Tol A possess' are D1 which is a transmembrane region of the inner membrane, D2 which spans the periplasm, and D3 which interacts with the outer membrane (Barbas III et al. 2001). These proteins are all shown and labeled in Figure 2A.

Typically, the N2 domain of the pIII coat protein is associated with N1 in a horseshoe shape, however, when N2 binds to the F pilus, this event displaces N1 and makes it free to interact with Tol A-D3 (Figure 2C). This is due to a conformational change by cis-trans isomerization of one of the prolines (pro $_{213}$) in the N2 domain (Rakonjac et al. 2011);Marvin et al. 2014). The subsequent steps of the infection process still remain to be elucidated by scientists. However, this whole process is known to aid in bringing the phage ssDNA into the cell (Barbas III et al. 2001).

Once the ssDNA is brought into the cell (Figure 2D), the replication and protein synthesis process can begin. This starts when the positive ssDNA strand of the phage enters the cytoplasm of the bacterium. Bacterial enzymes, host DNA polymerase, create a complementary negative strand of DNA, which adds a negative strand to the positive strand to make double stranded DNA (dsDNA). A bacterial gyrase enzyme then induces the formation of negative supercoiling. The product of this DNA replication process is supercoiled dsDNA. The negative strand of this dsDNA will be used for transcription, so that all the phage proteins can be produced. Some of the phage proteins that are

produced are going to help with replication, pII, pV, and pX, while the other eight proteins produced are going to be targeted to the inner and outer membranes (Rakonjac et al. 2011). Next, the pII protein that is created nicks the positive strand of DNA at a specific intergenic region, which causes the newly formed 3' hydroxyl end of the DNA to be able to act as a primer for the synthesis of DNA. Host DNA polymerase (brown circle in Figure 2E) then comes in and the rolling circle mode of replication begins, and a new positive strand of DNA is synthesized. pII then displaces and circularizes the positive DNA strand after one round of replication. This new positive ssDNA product can then be converted to dsDNA that is covalently closed and supercoiled by bacterial enzymes. This is a way in which a pool of progeny of the dsDNA replicative form (RF) molecules can be produced within the bacterium (Figure 2E). Another phage protein that has a role in replication is pX, but it is not yet known how it contributes to the process (Barbas III et al. 2001; Rakonjac et al. 2011).

RF DNA synthesis continues until a certain amount of the protein, pV, has been produced. Once a certain concentration of pV is reached in the bacterium, dimers of pV will start to interact and bind to the ssDNA that is produced and prevent the conversion to double stranded RF DNA. They will bind to all points of the DNA molecule, except for a hairpin loop at the end, which is used as a packaging signal for assembly (Rakonjac et al. 2011). This DNA-pV complex that is made during this process becomes the substrate for the upcoming phage assembly reaction (Barbas III et al. 2001). This process is shown in Figure 2F.

The other phage proteins that are created from the negative strand of DNA, are inserted into the inner or outer membrane, as they are being made during the DNA

replication and protein synthesis process (Figure 2F). Some of these proteins, such as pVIII and pIII, possess signal peptides that aid in bringing the proteins to the membrane, and then are removed. Other proteins, such as pVI, pVII, and pIX, do not possess signal peptides and their mechanism of translocating to the inner or outer membrane still needs to be elucidated (Barbas III et al. 2001; Rakonjac et al. 2011).

The last portion of the infection and replication process is phage assembly. The assembly process typically takes place in sites where the inner and outer bacterial membrane are in close contact with each other, which is where the phage coat proteins have been sent. Phage proteins pVII and pIX work together with the first set of pVIII molecules and interact with the DNA packaging signal on the end of the DNA-pV complex. Although the order of these events is still unclear, it is known that the packaging signal side of the DNA-pV complex exits the bacterium first and that pIX and pVII will be attached to this end of the phage particle. After initiation, the next step is elongation. The elongation phase is essentially a set of reactions in which the phage proteins are assembled onto the phage and it is extruded from the bacterium (Rakonjac et al. 2011). As the DNA-pV complex moves across the membranes, pV is removed and then replaced with pVIII proteins (Figure 2G). When the DNA is completely coated with pVIII, pVI and pIII are added to the end of the phage particle (Sidhu 2001). This is the last event before the phage exits the bacterium (Barbas III et al. 2001)

The infection, replication, and assembly process are the crux of the amplification process that takes place during biopanning. This can be done by a phage titering process that utilized a liquid culture of *E. coli* and plating the phage and *E. coli* mixture on agar plates. The liquid culture that can be used is *E. coli* ER2738 strain (Lim et al. 2018). This

strain of *E. coli* that is used does not have a complete *lacZ* gene and would prevent the *E. coli* from producing β-galactosidase by itself. However, the phagemid possesses the *laZα* portion of the gene. Therefore, by using the α-complementing strain with the other incomplete gene, β-galactosidase will be produced when the phage infects the *E. coli* (New England BioLabs Inc. A 2017). The phage and *E. coli* are then plated with 5-Bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal) and isopropyl-β-D-thiogalactoside (IPTG). IPTG is an inducer for the *lac* operon, therefore when it is added to this mixture, it will cause β-galactosidase production. X-gal is a modified galactose sugar that is colorless, but when β-galactosidase metabolizes it, the resulting product is a blue color. Therefore, after an incubation period, blue plaques will form on the bacterial lawn, and these plaques will consist of the phage and *E. coli*. These will be the phage with an affinity peptide attached to it and can then be re-entered into the biopanning cycle to select the phage with a higher affinity or the phage can be used for sequencing to look at the composition of the peptide (New England BioLabs Inc. A 2017; Noren & Noren 2001).

The next step in this process is extracting the fused peptide from the bacteriophage. This can be done by using the common recombinant DNA technology that is available today. The first step that can be done is to purify the phage DNA and then amplify it using polymerase chain reaction (PCR). After large quantities of the DNA have been collected and converted into dsDNA via the amplification process, the DNA will be digested with the same restriction enzymes that were used previously with this M13KE vector, for sites Accl65I/Kpnl and EagI (Xu et al. 2014). Then the fragment of DNA can be isolated by using 8% nondenaturing polyacrylamide gel (PAGE), using low molecular

weight DNA ladder (New England BioLabs Inc. B 1988a). A vector, known as a pMAL pIII vector, can receive the gene fragment from the M13KE vector. This is done by digesting the pMAL pIII vector with restriction enzymes that are also for *Acc65* I/*Kpn* I and *Eag* I, introducing the isolated gene fragment to this vector, and lastly using a ligase to successfully insert the gene fragment into the vector (New England BioLabs Inc. C 2018.; New England BioLabs Inc. B 1988b)

In the pMAL pIII vector, the target gene is ligated downstream of the *malE* gene on the vector, which encodes for maltose binding protein (MBP). The insertion of this gene causes a fusion of the gene fragment and of the *malE* gene, which will cause the expression of a fusion protein. The insertion of this gene will also interact with the *lacZ* gene on the DNA, which causes inactivation of the *lacZα*. This is useful since an α-complementing method can be performed by transforming this vector into a bacterium and use α-complementation, IPTG, and X-gal in order to isolate the MBP fusion protein (Zwick et al. 2013). After obtaining plaques, the peptide of interest can then be isolated through two purification techniques. The first one is using amylose affinity chromatography to purify the MBP fusion protein. This can be performed, because MBP has a high affinity for amylose, therefore can be easily separated by using column chromatography. The fusion protein can then be eluted with maltose, and following this step, the peptide can be separated from MBP using a protease. The pMal pIII vector contains a sequence that codes for the recognition site of a specific protease, so that a protease cleavage site will connect the MBP and the target peptide. This allows researchers to separate the two using a protease specific for this cleavage site (New

England BioLabs Inc. B 1988a; González-Techera, A. Umpiérrez-Failache et al. 2015; New England BioLabs Inc. C 2018).

Once the peptide of interest has been obtained, it can then be sequenced to observe the sequence homology that the peptide may have for its ligand. There are certain binding partners that are termed as natural binding partners, which are normally found in nature. Targets with the same sequence homology of the natural binding partner are desired and are sought after in phage display. Natural binding partners that have developed this relationship are said to be coevolved. Directed evolution of peptides, on the other hand, would be slightly different, since this would involve inducing mutations of proteins/peptides, in hopes of causing a mutation that will increase the affinity (Willats 2002). This type of site directed mutagenesis process is a common practice that has been used after the phage display process has been performed to help obtain a peptide with an even higher affinity for the ligand or to gain a better understanding of which amino acids are important for binding (Azzazy & Highsmith 2002; Willats 2002).

Recent Uses of the Combination of Phage Display and Biosensors

Recently, technology such as phage display and biosensors have been utilized together in order to produce a peptide, which would be used for the biological sensitive element of the biosensor, and then use that peptide to detect a certain compound. One recent experiment used this technology for the detection of the cholera toxin. Lim and workers wanted to be able to detect part of the cholera toxin molecule by using a biosensor, in order to find if this approach to detecting the molecule could be a facile alternative to the current method of hybridoma production, which is time-consuming and laborious. This toxin is produced by *Vibrio cholerae* and is a causative agent for diarrhea.

These researchers used a peptide phage library to first find a peptide against subunit-B of the toxin. After the biopanning process, they obtained several peptides, and then tested for the one with the highest affinity for subunit-B of the cholera toxin by performing ELISAs. Then they were able to use localized surface plasmon resonance and surface-enhanced Raman spectroscopy to detect if the peptide was binding to the B-subunit of the toxin (Lim et al. 2018). Another recent experiment that has utilized these types of technologies was performed by Kim and workers. This group wanted to make a biosensor that was capable of detecting trinitrotoluene (TNT), since many current methods require rather expensive technology. By using peptide phage display, they were able to obtain a tripeptide receptor that had a high affinity for TNT. This receptor was then bound to a layer of polydiacetylene (PDA), which was functionalized on a SWCNT field effect transistor (FET). This enabled them to be able to successfully detect TNT using this real time biosensor with great sensitivity (to the 1 fM)(Kim et al. 2011).

Combining phage display and biosensor technology has the potential to help many people in a variety of ways. There are potential medical and safety uses for this technology, such as detecting a pathogenic molecule or detecting blood of a person that works in a hazardous field (Lim et al. 2018; Cortes et al. 2013). That is why it is important for these fields of technology to be continuously studied and researched today and in the future.

Human Serum Albumin

HSA is an important globular protein that is produced in the liver and is the most abundant protein in plasma. It is large enough to not be filtered by the kidney, which is of great importance to pharmacological experiments, because if the drug can bind to HSA it

will be present in the circulation for a longer period of time (Fasano et al. 2005). As reviewed by Curry, serum albumin is an important molecule for the maintenance of osmotic blood pressure. It is also an excellent transporter that can carry molecules and proteins such as fatty acids, endogenous compounds, hormones, bilirubin, and some renal toxins (Curry 2009).

The modular structure of HSA, which allows it to be an extraordinary ligand binder, makes it useful in a variety of experimental designs. It is a protein composed of 585 amino acids that has three main homologous domains that form an alpha helical "heart shape," and these domains further divide into two subdomains, A and B. The protein is held together in its structure by highly linked disulfides (Fasano et al. 2005, Minomo et al. 2011, Lin et al. 2017). The binding of molecules usually occurs on two sites known as Sudlow sites, and there also a number of fatty acid binding sites across the molecule (Figure 3) (Lin et al. 2017, Minomo et al. 2011).

There are many qualities of HSA that have made it an important molecule to detect and find a high affinity protein/peptide against. Some groups want to find a high affinity binding peptide to HSA for the pharmokinetic advantages of a binding a small drug to HSA. In one experiment, Dennis and coworkers wanted to find a peptide that would bind to HSA, so that it would not be filtered easily by the kidney. This could lead to applications that can significantly extend a pharmaceutical's half-life in the human body. Peptide phage display libraries expressing random peptide sequences expressed as pVIII fusion proteins were used for this experiment. These phage libraries were screened against rat, rabbit, and human albumin molecules. The affinity of some of these clones were confirmed using M13 phage ELISAs, peptide competition assays, and surface

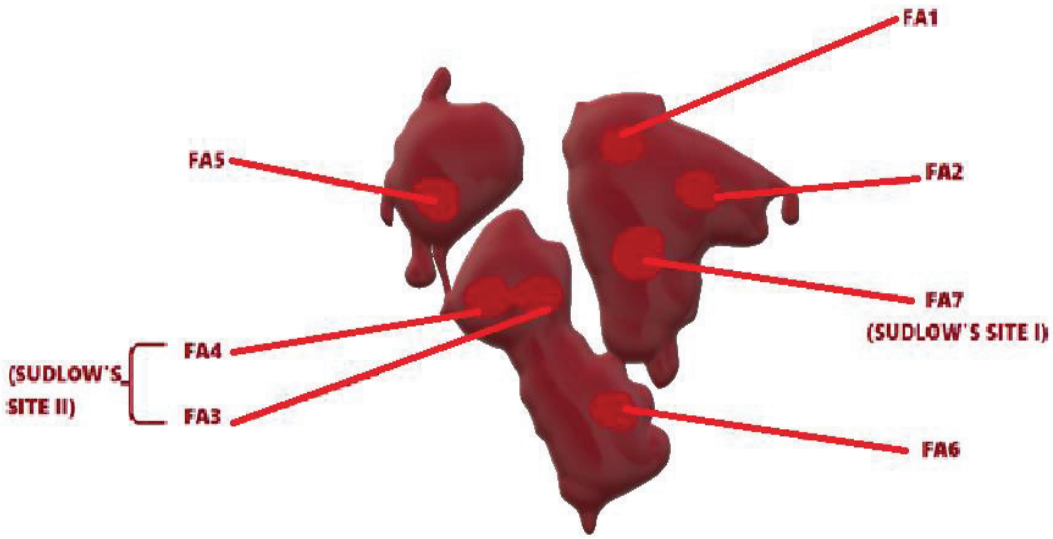


Figure 3. Structure of HSA and Binding Sites. HSA possesses many sites for ligand binding such as the fatty acid binding sites (FA#) and Sudlow's sites.

plasmon resonance to measure the affinities of the peptides. M13 ELISA is an experiment where the peptide that is expressed on the phage coat is tested for its affinity to a ligand and the peptide competition assays are performed in a similar manner. The M13 ELISA involved adding the phage clones obtained from phage display to Maxisorp^{TM} plates that were coated with a certain albumin and blocked. The wells were washed with PBS, 0.05% Tween 20 to wash off unbound phage. Horse-radish peroxidase (HRP) coupled to anti-M13 antibody was then added to the wells, and the colorimetric substrate ABTS/H_2O_2 substrate was then added to the wells. The color change of the wells could then be measured using a spectrophotometer. Surface Plasmon Resonance (SPR) was performed by having the albumin molecules bound to a biosensor chip, and then having light focused on the chip, reflected, and detected by a photodiode array. The angle of the light was then recorded. Then a solution-phase of the ligand was then injected on the chip. The angle of the light changed due to the electrons that absorb the light in the chip resonating differently due to the ligand being bound to the target molecule. This difference in angles then showed the experimenters if the peptide was bound or not (Dennis et al. 2002, Margulies et al. 2015). These procedures allowed the experimenters to assess the affinity of certain peptides and to select the best ones to continue experimenting with. The researchers then used SA21, one of the high affinity peptides obtained from phage display, which was stable in citrated rabbit serum *in vitro* for over a 24 hour period at 37 ºC, to see if it would show improved pharmokinetics. Controls such as an unrelated peptide and an engineered variant that binds to albumin were tested with SA21, by injecting the samples into New Zealand white rabbits via intravenous bolus doses. Blood samples were then collected at serial time points from one

minute prior to dosing to 21 days postdosing. The results of this experiment showed that the peptide obtained through phage display showed a significantly higher half-life compared to the controls. The peptide showed a longer half-life of 2.3 hours compared to the unrelated control peptide with a half-life of 7.6 minutes and the engineered variant with a half-life of 30 minutes (Dennis et al. 2002).

In another experiment, researchers created a biosensor that was able to detect HSA by creating a smart electronic yarn. In this experiment, they dipped a cotton yarn in a SWNT, poly (sodium 4-styrene sulfonate)-water solution, and an anti-HSA antibody solution. The yarn was then frozen and dried using a vacuum, which was done to prevent the antibodies from denaturing. This biosensor was able to successfully detect HSA concentrations as low as 119 nM, which was significant, since HSA concentrations in blood range from 446-746 μM. Unfortunately, although this experiment provided great results, there were a couple drawbacks to this biosensor, such as the limited lifetime of the antibodies and the sensor only being single use (Shim et al. 2008). The limited lifetime of the antibody is also problematic, because this would make the sensors difficult to manufacture and use for commercial purposes.

Another experiment involved Cortes and coworkers trying to create a biosensor that could detect HSA, since HSA is the most abundant protein in plasma, which would make it the best candidate to try to detect. The peptide that was used for this experiment was GK-1 and it was found previously by different experimenters, and its specific binding affinity for HSA was tested (Pingali, Aruna 1996, Olszewski 2013). This was investigated using an ELISA. The wells were made of polyvinyl chloride which were then coated with the target (HSA), and nonfat dry milk block was used for avoiding

undesired peptide-wall interactions. A biotinylated form of the peptide was then added to the wells, and after the binding period, removal of the unbound B-GK-1 was performed by washing. Following this step, HRP-streptavidin was added, and subsequently unbound HRP-streptavidin was washed. TMB colorimetric substrate was then added to the wells and quantification of the color change was performed using a spectrophotometer. A competitive ELISA was also performed in order to test the affinity of the peptide for HSA versus other analytes, such as apple juice, coffee, and NaCl. This competitive ELISA was performed by using similar methods that were done in the previous peptide ELISA. Then by using a condensation process involving a carbodiimide agent, the GK-1 and B-GK-1 were covalently attached to carboxylated MWNT. CNT-GK-1 was then exposed to biotinylated HSA in order to monitor and confirm the attachment in the biologically functionalized nanocomplex. Using mass balance analysis, B-GK-1 attached to the CNT was then quantified. Then the CNT-GK-1 complex was incorporated into a 74% polyester and 26% glace finished cotton thread, which was done by dipping the thread into 10 mg/ml of a bio-complex solution which was done assisted with an ultrasonication step, and then allowed to dry overnight. After they were dry, the threads were attached to a DAQ instrument, exposed to blood, and the resistance was measured. The ELISA results showed that GK-1 had a high affinity for HSA compared to the other targets. The DAQ results revealed that there was an increase in measured electrical resistance for the CNT-GK-1 and HSA, compared to the plain COOH-MWNT when in contact with apple juice, which means that the MWCNT-GK-1 complex exhibits a certain degree of selectivity towards human blood. Lastly, DAQ results also showed that when the MWCNT-GK-1 was exposed to different analytes, that blood induced an increase in

resistance, as opposed to the others that caused a decrease in resistance. This strong interaction is what causes the increase, instead of the decrease in resistance. Therefore, this group was able to successfully create a biosensor with a peptide that was derived from phage display that could detect HSA (Cortes et al. 2013).

Aims and Hypothesis

The goal of this project is to contribute to the collaborative works that have been completed and accomplished by several graduate students of YSU beforehand. In order to continue work on the biological element of the biosensor, the specificity of the peptide for HSA must be confirmed. Another task that was necessary was to find an economically feasible method of producing the peptide, rather than going to a company to produce it (this would be important for commercial production of this biosensor). Therefore, for this project, ELISAs were performed to show the specificity of BR1 for HSA and the peptide was purified using two different peptide incorporation methods. One peptide purification process involved using PCR to incorporate the sequence into an expression vector, and the other involved cloning purchased DNA, with the peptide sequence, into an expression vector. Both of these methods then utilized similar affinity chromatography methods to express and purify the peptide. Therefore, the first hypothesis for this project was that BR1 will be shown to have a high affinity and specificity for HSA, making its use for biosensor production a viable option. The second hypothesis was that the peptide will be expressed and purified using methods that would make producing the peptide cheaper than purchasing it.

Materials

Items that were purchased from Sigma-Aldrich (St. Louis, MO) included: Tryptone, yeast extract, glycerol, potassium phosphate monobasic and dibasic, tween 20, TBE Buffer (Tris-borate-EDTA), Ethylenediaminetetraacetic acid (EDTA), Ammonium hydroxide, formic acid, acetonitrile, 3,3',5,5'-Tetramethylbenzidine (TMB), agarose, sulfuric acid, sodium phosphate monobasic and dibasic, streptavidin-horse radish peroxidase, albumin from human serum, bovine serum albumin, sodium chloride, and boric acid. The Wizard® *Plus* SV Minipreps DNA Purification System was purchased from Promega Corporation (Madison, WI). GenomeLab Dye Terminator Cycle Sequencing with Quick Start Kit was purchased from Beckman Coulter® (Brea, CA). Several sets of primers were designed using Genome Compiler Corporation and produced by Integrated DNA Technologies for PCR, sequencing, and DNA incorporation (Coralville IA). The Q5® High-Fidelity 2X Master Mix, *Bam*H I restriction enzyme, T4 Polynucleotide Kinase, T4 DNA ligase, Cutsmart buffer, *Bam*H I, Quick-load 1 Kb Ladder, M13KE phage, and the pMAL™ vector kit was purchased from New England Biolabs (Beverly, MA). The N-morpholinothene sulfonic acid (MES) BupH MES buffered saline, EZ-Link®Hydrazide-PEG4-Biotin, 4′-hydroxyazobenzene-2-carboxylic acid, avidin, Pierce spin column Avidin Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), anti-M13 monoclonal antibody conjugated with biotin, ampicillin, casein block, and trifluoroacetic acid (TFA) were purchased from Thermo Scientific (Rochester, NY). The BR-1 peptide was purchased from Genscript Peptide (Nanjing, China). The anti-M13 monoclonal antibody conjugated to Horse Radish Peroxidase (HRP) and 0.05 M sodium carbonate was purchased from GE-Healthcare (Chicago, IL). The 6X Loading Buffer was purchased from Amresco (Solon, OH). DNA was stained using Gel Green from

EmbiTec. The QIAquick Gel Extraction Kit was purchased from Qiagen (Hilden, Germany). The agar and tris were purchased from Fisher Scientific (Hampton, NH), and the non-sterile 96 well plates were purchased from Corning Incorporated (Corning, NY).

Methods

Peptide ELISA

Peptide ELISAs were performed in order to see if the BR-1 peptide had a high specificity for HSA. There were several different peptide ELISAs performed, differing in certain steps, in order to optimize the ELISA process and troubleshoot any issues. The positive HSA (Pos. HSA) and the streptavidin blank (SA blank) wells on the polyvinyl chloride plate were coated with 100 µl of 10 µg/ml of HSA diluted in 0.05 M Na_2CO_3 (pH 9.6) buffer. The positive control phage wells were coated with 100 µl of 2x 10^{11} pfu/ml phage (90 µl) in 10X Na_2CO_3 (10 µl) .The No HSA wells and the antibody blank (Ab blank) wells had 100 µl of blocking buffer (1% Yeast Extract in PBST) coated in each of these wells. The plate was then incubated in a humidified chamber overnight at 4°C. A circle of water was put surrounding all of the test wells in order to prevent evaporation. The next day, the wells were aspirated and washed once with wash buffer (PBST), and then the plate was incubated for 30 minutes at 37°C. The wells were aspirated and washed three times with wash buffer. The Pos. HSA and No HSA wells were coated with 10 µg/ml of biotinylated peptide diluted with wash buffer (PBST), and the Ab blank, SA blank, and the positive phage control were coated with wash buffer. The plate was then incubated for 2 hours at room temperature. The wells were washed six times with wash buffer. Following this wash step, 100 µl of the diluted (1:5000 in sample buffer) HRP/anti-M13 monoclonal antibody peroxidase conjugate was to the positive control and Ab blank wells. The Sample buffer used for each peptide ELISA was either 1 X PBS + 10% Casein or 1% Yeast Extract in 1x PBS. A 1:10,000 dilution of HRP-Streptavidin in sample buffer to the Pos. HSA, No HSA, and SA blank wells. This was then left to be incubated overnight at 4°C in a humified chamber. The next day, the wells

were washed six times with wash buffer, and then 100 µl of TMB substrate solution was added. When a color change was observed, 50 µl of the stop solution, 2N H_2SO_4 was added, and then the absorbance was read at 450 nm.

As mentioned earlier, several more ELISAs followed this one differing in certain steps. The ELISA above was performed twice, followed by an ELISA utilizing a different source of HSA. After these ELISAs were performed, ELISAs using a different sample of biotinylated BR-1 (BBR1) were used and using different blocks (casein) were performed. Another peptide ELISA was performed at a later time using all stocks of BBR1 and the conditions listed in the previous paragraph. After this ELISA was performed, another ELISA utilizing a peptide with high amounts of biotin on it with the blocks and wash buffers that deviated from the original protocol was performed.

ELISA Testing the Streptavidin

This ELISA was performed in order to see if the streptavidin used for the peptide ELISA was working properly. The Ab blank wells and SA blank wells were coated with 100 µl of 10 µg/ml of protein (HSA) in 1X 0.05 M Na_2CO_3 (pH 9.6) buffer. The phage positive control wells were coated with 100 µl of $2x10^{11}$ pfu/ml phage (90 µl) in 10X Na_2CO_3 (10 µl). After this step the SAv positive control well was coated with a 1:10,000 dilution of HRP-Streptavidin in 1X Na_2CO_3. The anti-M13 positive control well was coated with diluted anti-M13 biotinylated antibody (1:5000) in 1X Na_2CO_3. After the appropriate wells were filled with the corresponding reagents, the plate was incubated overnight at 4°C in a humidified chamber. A circle of water was put surrounding all of the test wells in order to prevent evaporation. The next day, the wells were aspirated and washed once with 1X PBS. Each well was then blocked with 200 µl of 1% BSA, and then

incubated at 37°C for 30 min. The wells were then washed six times with 1X PBS, and then 100 µl of diluted HRP/anti-M13 monoclonal antibody peroxidase conjugate in sample buffer was added to the phage positive control and the Ab blank wells. The a 1:10,000 dilution of HRP-Streptavidin in sample buffer was added to the positive control anti-M13 biotinylated antibody well and SA well. 200 µl of 1X PBS was then added to the SA positive well. The plate was then incubated overnight. The next day, the wells were washed six times with wash buffer, and then 100 µl of TMB substrate solution was added. When a color change was observed, 50 µl of the stop solution, 2N H_2SO_4 was added, and then the absorbance was read at 450 nm.

Biotinylation of Peptide

Biotinylation of the peptide was performed in order to do ELISAs to see if the peptide could bind to HSA with a high affinity. A solution of 500 mM 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) in reaction buffer (10 mg EDC in 100 µL N-morpholinothene sulfonic acid, MES) was prepared, and then a 50 mM biotin hydrazide solution and a 1 ml solution of 0.5/ml or peptide was added to a glass test tube. This solution was then mixed and rocked, and then 3 µl of the EDC solution was added to the reaction, mixed and then rocked overnight. Next, the sample was diluted by doing a 1:1 dilution of the sample in 2.5% TFA and then added to a Pierce Graphite Spin Column. This was then put into a 1.5 ml screw cap microfuge tube. The column was then centrifuged at 2,000 x g for 1 minute to remove storage buffer, and then 100 µl of 1 M NH_4OH was added and then centrifuged at 2,000 x g for 1 minute. After this step was over, the flow-through was discarded. Next 100 µl of 1% TFA was added to the column, centrifuged at 2,000 x g for 1 minute, the flow-through was discarded, and then this step was repeated. Next, the sample was diluted by a 1:1 dilution with 2.5% TFA and 500 µl

of the sample, which was added to the spin column. The column was placed in a new collection tube, and the 10 minutes was given for binding with periodic vortex mixing in order to keep the graphite suspended as much as possible. A centrifugation step followed where the column was centrifuged at 1,000 x g for 3 minutes and then the flow-through was discarded. The column was then placed into a new collection tube and then washed with 200 µl of 1.0% TFA, and then this was centrifuged at 2,000 x g for 1 minute. The flow-though was then discarded and then this step was repeated. The column was then placed into a new collection tube, but this time 100 µl of 0.1% formic acid in 50% acetonitrile was added and centrifuged at 2,000 x g for 1 minute. The eluate that was obtained was transferred into a microcentrifuge tube. This whole step was repeated three more times using the same collection tube each time to obtain 400 µl of eluate. The sample was then surrounded by foil to contain and conduct heat, and then dried in a vacuum evaporator. The sample was then suspended in 1 ml of MES after evaporation (Thermo 2013).

HABA/Avidin Assay

For this assay, BR1 was diluted, since the 0.5 mg/ml concentration is too high to use for this assay, therefore the peptide was diluted 1:10 in MES. After all of the reagents were prepared for this experiment, the spectrophotometer was blanked with 1 ml of diH_2O. Next 900 µl of the mixed HABA/Avidin solution was measured at 500 nm and recorded as A_{500} HABA/avidin. MES (100 µl) was added to the well containing the HABA/Avidin mix. The absorbance was then read for this new mixture and the value was recorded as A_{500} HABA/avidin/biotin once it remained constant for 15 seconds. The cuvette used was rinsed 5 times with water and dried with a kimwipe. 900 µl of HABA/Avidin solution was then added to the cuvette and measured at 500nm, and this

measurement was recorded as A_{500} HABA/avidin. A 100 µl volume of the biotinylated peptide was added to the HABA/avidin mixture and mixed. The absorbance of this new mixture was the measured and recorded as A_{500} HABA/avidin/biotin once the value remained constant for 15 seconds. Calculations were then performed using Beer Lambert's law to find mmol of biotin in original sample/mmol of molecules in original sample. Formulas for calculations are shown in Figure 4 (Thermo 2012).

After the concentration of mmol of biotin per mmol of molecules of BR1 was found, another peptide ELISA was performed using all of the sample of peptide and the same method listed previously.

Incorporation of a Peptide Sequence into pMal-c5x via PCR

This process was performed in order to transfer the sequence of the peptide into an expression vector, and transform the vector into an *E. coli* strain, which could then produce the peptide. The sequence of the BR1 peptide (Rees, 2016) was incorporated into a primer for the pMal-c5x vector. These primers were designed using Genome Compiler Corporation's website and sent to Integrated DNA Technologies (IDT) to be manufactured (Table 1). Once the primers were received, they were used to perform PCR of the vector, resulting in the insertion of the peptide into the pMal-c5x vector (Figure 5).

All of the reaction components were assembled on ice before the reaction was quickly transferred to the thermocycler. For 25 µl reaction, 12.5 µl of 2 X Q5 master mix was mixed with 1.25 µl of 10 uM of BR1_1 and BR1_2 primers, 1 µl of either a 1:10, 1:100, or 1:1000 dilution of the pMal-c5x vector, and 9 µl of Nuclease-Free Water filled the tube until the final volume was 25 µl. The tube was then mixed by flicking the tube, and then centrifuged to move the contents to the bottom of the tube. A thermocycler was

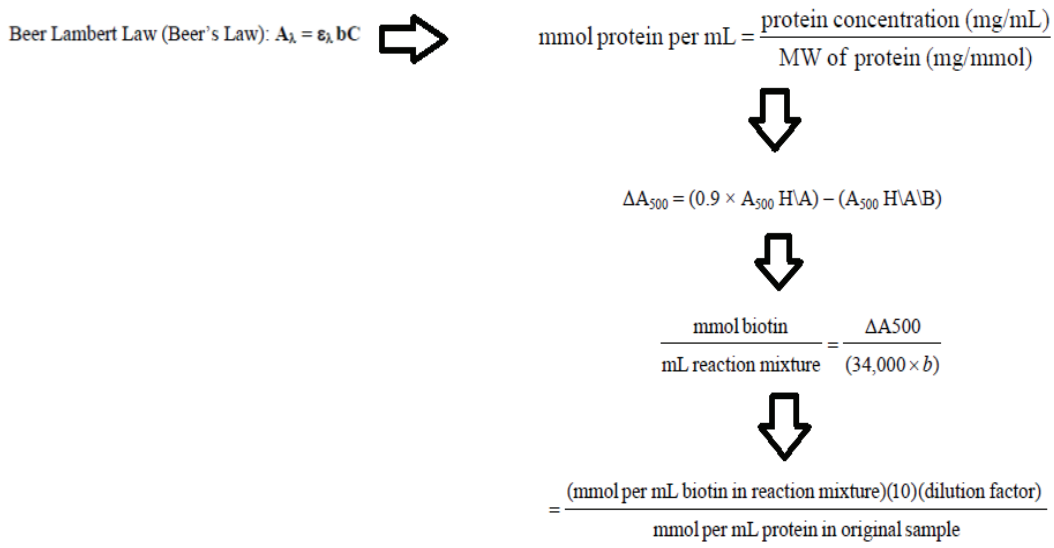


Figure 4. Calculations for the Biotin Incorporation Assay. These are the calculations and derivations that were used for the calculations in this experiment. In the equations, A_{500} represents absorbance at 500 nm, H represents HABA, A represents Avidin, and B represents biotin.

Table 1. Primers Designed for the PCR Process.

Name	Sequence	Scale	Purification
BR1_1/Alex 1	GCGAATCATCATCAGGCGTCTTAACAAATAAAACGAAAGGCTCAGTC	25 nmol	Standard Desalting
BR1_2/Alex 2	CCTTCCCTCGATCCCGAG	25 nmol	Standard Desalting

[a]BR1_1 and BR1_2 are the primers that will be used to incorporate the BR1 sequence into the pMal-c5x vector. The BR1_1 primer possesses the peptide sequence.

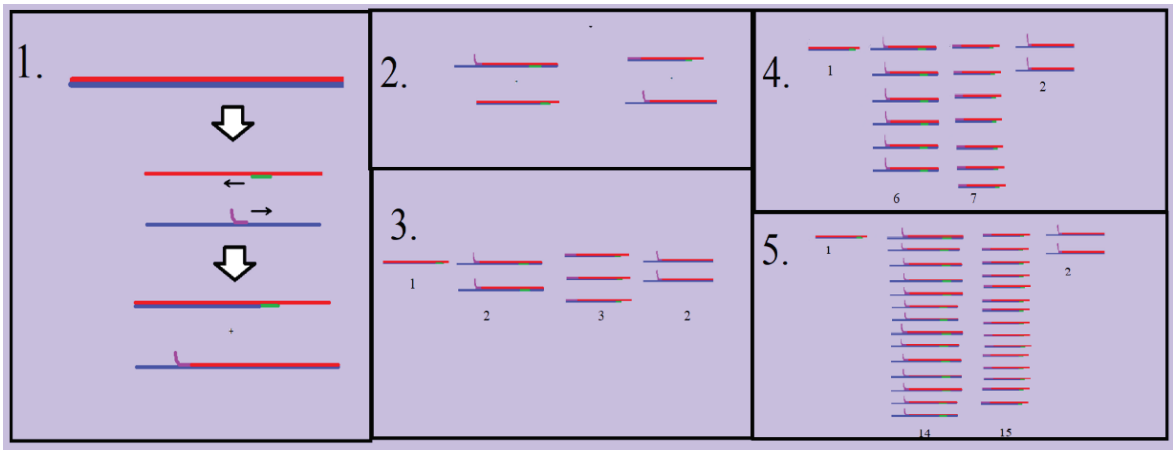


Figure 5. Cycles of the PCR Reaction. This figure displays the PCR products produced in the first several cycles, along with the number of different types of products produced. In the first round of the PCR reaction, the primers bind to the DNA and use DNA polymerase for extension. This allows the incorporation of the peptide sequence in one of the newly produced strands. The peptide sequence is not compatible with the vector DNA, therefore there would be a "lip" attached to the primer sequences (purple primer). In the second cycle, four different PCR products are produced, two have the BR1 sequence incorporated into the strands of DNA, one having a complementary strand to the BR1 sequence. In the third round of the PCR process, ratios of the PCR products from the previous cycle are now different. There are a total of seven strands of DNA with the peptide sequence incorporated with them, and three of these strands now have complementary strands to the BR1 sequence. In cycles four and five, the major PCR products begin to emerge, where there are now 15 strands of DNA with the peptide sequence incorporated into them with a complementary strand that also have the peptide sequence. There are also 14 strands with the peptide sequence incorporated into one of the strands, which will produce 14 strands of the peptide sequence in both strands in the next cycle.

then used for the following conditions; 98°C for 30 seconds, 98°C for 10 seconds, 63°C for 15 seconds, 72°C for 1 minute, and then these temperatures and times were cycled 34 more times. The reaction mixture was held at 72°C for 2 minutes, and following this step it was held at 10°C until it was stored at -20°C (New England BioLabs Inc. D 2015).

Gel Electrophoresis

Gel electrophoresis was used to confirm the presence of the PCR product. Agarose (0.5 g) and 50 ml of 1 X tris/borate/EDTA (TBE) (VWR/Amresco) electrophoresis buffer was added to a 200 ml flask. This mixture was microwaved for 1 minute, swirled, and then microwaved for another 10 seconds, until the mixture was clear. It was cooled for 10 minutes at room temperature. We added 5 µl of Gel Green. A gel comb was placed in the cast on the side next to the negative electrode and the gel was then poured so that about half the height of the comb teeth was submerged. After the gel solidified, the comb was removed, and TBE buffer was poured into both reservoirs and over the surface of the agarose. The TBE buffer covered the gel to a depth of 2-3mm. Loading buffer and tracking dye were then added to the DNA samples and then the samples were loaded into the wells. In the gel, 10 µl of the Quick Load ladder, 2 µl of loading dye and 3 µl of the PCR product was then added to the other wells. After the gel ran for 30 minutes at 100 mV, the gel was removed and viewed in an UV light chamber.

Gel Extraction and DNA Purification

The fragment of DNA was excised from gel using a razor and placed in a pre-weighed microcentrifuge tube. The tube was weighed to find the mass of the gel bands (0.18 g). Following this step, three volumes of QG buffer were added to the tube (540 µl). The tube was then incubated for 10 minutes at 50°C and vortexed every 2-3 min to help dissolve the gel. Sodium acetate (10 µl of 3 M) was added to the tube and the

contents were transferred to the QIAquick spin column and centrifuged 1 minute at 10,000 x g. The flow-through was discarded. The centrifugation step was repeated for the volume that could not fit in the spin column the first time. PE buffer (750 µl) was added to the spin column and centrifuged again for 1 min at 10,000 x g. After this step, the flow-through was discarded and the column was placed in a new tube. EB buffer (50 µl) was then added and then incubated for 4 minutes at room temperature. The tube was then centrifuged at 1 minute at 10,000 x g, the spin column was discarded, and a gel was run on this sample to confirm that DNA content was not lost (Qiagen 2001) .

Ligation

DNA that was previously purified was used in this reaction to ligate the ends of the plasmid. This was done by mixing 5 µl of DNA, 3 µl of nuclease free water, and 1 µl of polynucleotide kinase. This process was performed in order to add a phosphate group to the 5' end of the DNA so that it can ligate. This mixture incubated in a microcentrifuge tube for 30 minutes. T4 DNA Ligase buffer (2 µl) was added to this mixture and incubated overnight at 16ºC (New England Biolabs Inc. E 2015).

Transformation

E. coli cells were used for the transformation process. In a microcentrifuge tube, 2 µl of DNA and 100 µl of the *E. coli* cells were incubated for 30 minutes on ice. The cells were then heat-shocked by placing them into a hot water bath for 50 seconds at 42ºC. The cells were then placed back on ice. LB broth (900 µl) was then added to the tube, and then placed in a shaker for an hour. The bacteria were then plated and placed in an incubator.

The next day, four colonies of bacteria were transferred into a tube with 5 µl of ampicillin and 5 ml of LB broth. The tubes were then placed in a rotating roller drum

inside an incubator at a temperature of 37ºC. The next day, the samples were centrifuged for 7 minutes at 6738 x g. The supernatant was then poured off and excess supernatant was removed. The samples were then frozen at -20ºC.

Plasmid Prep

The pellet obtained from transformed *E. coli* was obtained and then resuspended in 250 µl of cell resuspension solution in a sterile 1.5 ml microcentrifuge tube. The tube was vortexed to completely resuspend the pellet. Cell lysis solution (250 µl) was added mixed 4 times by inverting the tube. The tube was then incubated 1-5 min until the cell suspension cleared. Alkaline protease solution (10 µl) was added and mixed 4 times by inversion of the tube. The mixture was then incubated at room temperature for 5 minutes. Neutralization solution (350 µl) was added and was immediately mixed by inverting the tube 4 times. The lysate was then centrifuged at 14,000 x g for 10 minutes at room temperature.

Approximately 850 µl of the clear lysate was transferred to a prepared spin column and centrifuged at 14,000 x g for 1 minute at room temperature. The flow-through was then discarded, the spin column was reinserted into the collection tube, and 750 µl of column wash solution (diluted with 95% ethanol) was then added to the spin column. The tube was then centrifuged at 14,000 x g for 1 minute, the flow-through was then discarded again, and after reinserting the collection tube, the wash procedure was then repeated, except using 250 µl of column wash solution. The tube was then centrifuged at 14,000 x g for 2 minutes at room temperature. The spin column was transferred to a new, sterile 1.5 ml microcentrifuge tube, and 100 µl of nuclease-free water was added. This was then centrifuged for 1 minute at room temperature at 14, 000

x g. After eluting the DNA in this step, the sample was then of DNA stored at -20°C (Promega Corporation 2010).

Digestion

Digestion of the DNA was performed using restriction enzyme *Bam*H I, which should no longer be able to cut the plasmid construct. The purified vector (10 µl) was added to 1 µl of *Bam*H I, 2 µl of cutsmart buffer, and 7 µl of nuclease free water. This was also performed with the original pMal-c5x as a control, and for this reaction there was 2 µl of the vector, 1 µl of *Bam*H I, 2 µl of the cutsmart buffer, and 5 µl of nuclease free water. These reactions incubated for an hour at 37^{o}C. After this incubation period, these samples were run on an agarose gel.

Sequencing

Sequencing was performed to confirm that the peptide sequence was in the vector. For this reaction to take place, special primers for the new vector were recommended in the pMal-c5x manual and ordered from Integrated DNA Technologies (IDT). These primers are forward and reverse primers near the malE gene and the multiple cloning site (Table 2).

The DNA samples (10 fmol) obtained after the plasmid prep were transferred to microcentrifuge tubes and water (dH_2O) was added to the sample, bringing the total volume to 10 µl. Primers (2 µl at 1.6 µl) were added to the DNA sample bringing the total volume to 12 µl. DTCS Quick Start Master Mix (8 µl) containing DNA polymerase, dNTPs, reaction buffer components, and dyed labeled terminators was, added bringing the total volume of the DNA sample to 20 µl.

Table 2. Primers for the Sanger Sequencing Reaction.

Forward Primer	5′d(GGTCGTCAGACTGTCGATGAAGCC)3′
Reverse Primer	5′d(TGTCCTACTCAGGAGAGCGTTCAC)3′

The reaction components were then thoroughly mixed and centrifuged. The contents were placed in a thermal cycler set for 30 cycles of 96°C for 20 seconds, 50 °C for 20 seconds, 60 °C for 4 minutes, and followed by holding at 4 °C. The Stop Solution/Glycogen mixture (5 µl), containing 2 µl of 3 M Sodium Acetate (pH 5.2), 2 µl of 100 mM Na2-EDTA (pH 8.0) and 1 µl of 20 mg/ml of glycogen, was added to the sample. The sequencing reaction then went into a sterile microcentrifuge tube and was mixed. Cold (from -20 °C freezer) 95% (v/v) ethanol (60 µl) was added, mixed, and the contents were then centrifuged at 14,000 rpm x g at 4 °C for 15 minutes.

After centrifugation, the supernatant was removed, and the pellet was rinsed twice with 200 µl of 70% (v/v) ethanol from a -20°C freezer. For each rinse the sample was centrifuged at 14,000 rpm x g at 4°C for 2 minutes. The supernatant was completely removed with a micropipette. The sample was then vacuum dried for 10 minutes, and then resuspended in 40 µl the Sample Loading Solution. Ed Budde, member of the Youngstown State University Biology Department, transferred the samples into the sample plate, and loaded it into the sequencer (Beckman Coulter 2004).

Results

ELISAs

In order to confirm that peptide BR1 has a high specificity for HSA, several ELISAs were performed. This was necessary to accomplish before peptide purification procedures began. These peptide ELISAs were performed using purchased peptide (BR1) that had been biotinylated by previous lab members or were newly biotinylated (Rees 2016). Figure 6 shows two of the eight peptide ELISAs that were performed. These initial peptide ELISAs did not yield the desired results, however these ELISAs did assist in troubleshooting the difficulties that this lab was having with the procedure.

Figure 6 shows two ELISAs that were performed to test the specificity of BR-1 to HSA. The positive control wells were performed in one well of each ELISA, while the blank and test wells were performed in triplicate. In Figure 6A, biotinylated BR1 "AH" was tested for its specificity for HSA. The block used was 1% Yeast Extract in PBS-0.05% Tween 20, the wash buffer was 1 X PBS-0.05% Tween 20, and the sample buffer was 0.1% Tween 20 in PBS. Unfortunately, there was no significant binding in the Positive HSA (Pos. HSA) well. The average absorbance of the Pos. HSA well was 0.059 +-0.006 at 450 nm, compared to the No HSA well which was 0.054 +-0.011 at 450 nm. These values became negative when the background noise detected in the SAv blank wells was subtracted from these values (Pos. HSA = -0.002 +-0.006 at 450 nm and No HSA = -0.008 +- 0.011) at 450 nm. The p-value obtained from this ELISA for the No HSA and Pos. HSA well was 0.46, which showed that the difference between the two was negligible. In Figure 6B, the graph shows a similar ELISA, except that casein block was substituted for yeast extract block, PBS wash buffer, and biotinylated BR1 "FK" was used. This ELISA also yielded results that showed no binding of the peptide to HSA. The

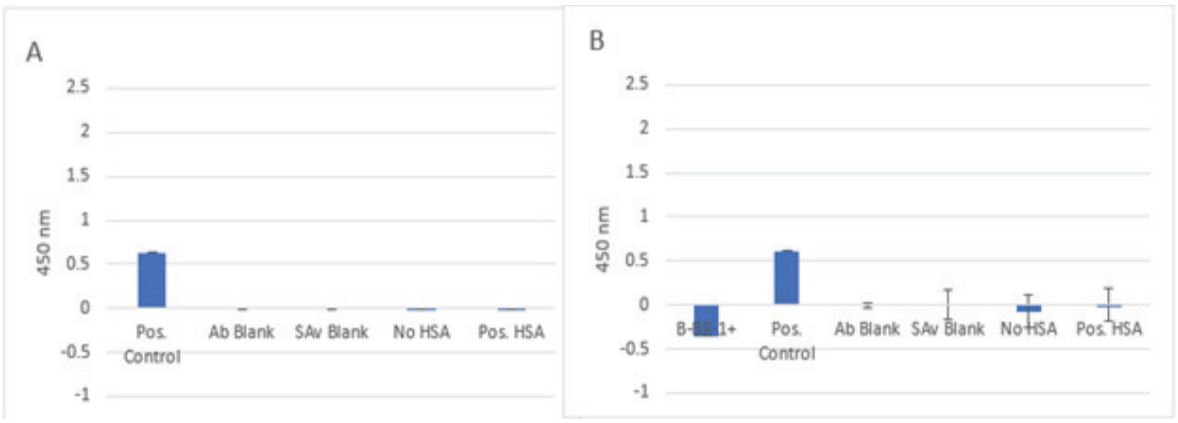
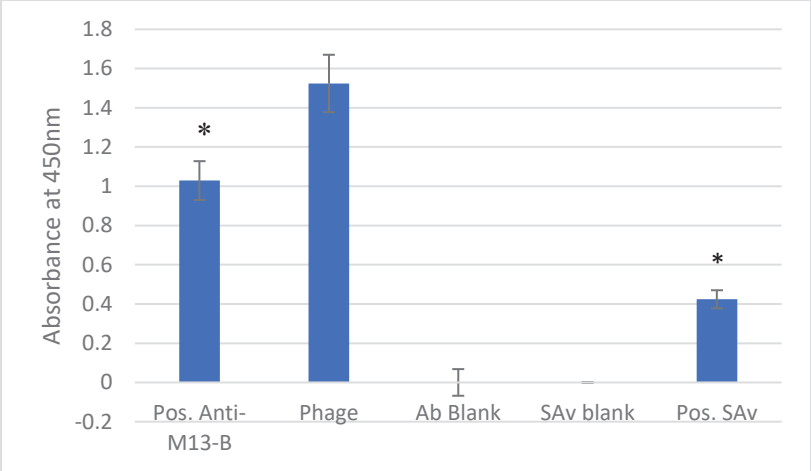


Figure 6. Peptide ELISAs against HSA. ELISAs were performed to test the specificity of BR-1 for HSA. The ELISA performed in A used the AH peptide and yeast extract in block and wash buffer, while the ELISA performed in B used the FK peptide with casein in the blocks and wash buffer. The positive control was coated with $2x10^{11}$ pfu/100ul of M13 phage diluted in 10X Na_2CO_3 to facilitate binding to the plate. The SAv blank and Pos. HSA were coated with HSA (diluted in 0.05 M Na_2CO_3). The B-BR1 (in 6B) well was coated with the biotinylated peptide diluted in 10X Na_2CO_3. The other wells received blocking buffer (1% Yeast Extract in PBST in 6A or 1% casein in TBS in 6B). After the incubation period the wells were washed (1X PBS + 0.1% Tween 20 in 6A or 1X PBS in 6B) and blocked, and then the Pos. HSA and No HSA wells received biotinylated peptide (1X PBS + 0.1% Tween 20 in 6A or 1X PBS in 6B), while the others received wash buffer. The Pos. control and Ab blank wells were then incubated with an anti-M13 antibody conjugated to HRP diluted in sample buffer (1X PBS + (1:10 dilution of Yeast Extract) + 0.05% Tween-20 in 6A or 1 X PBS + 10% Casein in 6B), while the other wells were incubated with streptavidin-HRP diluted in sample buffer. After incubation, the wells were then washed, and then received the substrate TMB to cause a color change which was stopped with 2N H_2SO_4 and measured at 450 nm. Absorbance from the blank

wells were subtracted from all of the wells. The Positive control and the B-BR1 well were performed in singlets, while the rest were performed in triplicate.

average absorbance for the Pos. HSA well was 0.206 +- 0.216 at 450 nm, while the average of the No HSA well was 0.493 +- 0.204 at 450 nm. These values became negative when the background noise detected in the SAv blank wells was subtracted from these values (Pos. HSA = -0.003+-0.216 at 450 nm and No HSA = -0.072+-0.204 at 450 nm). The p-value for this second ELISA was 0.67. The positive control for both of these ELISAs was M13 phage bound to the plate using sodium carbonate buffer and an anti-M13 phage antibody conjugated to a peroxidase enzyme. The positive controls in both figures (6A and 6B) showed absorbance peaks at around 0.6 at 450 nm. Also shown in ELISA 6B is another positive control, the FK biotinylated peptide bound to the plate. Unfortunately, the result for this control was around 0.065 at 450 nm, which then became -0.36 when the background noise was subtracted.

Even though there were difficulties with the ELISAs performed previously, there are many factors that can contribute to an ELISA not working. Therefore, another ELISA was performed to understand which factors might be causing the negative results (Figure 7). All wells in this test were performed in duplicate. In this ELISA, the positive control was M13 phage and an anti-M13 phage antibody conjugated to a peroxidase enzyme. The phage well absorbance was recorded at 1.524+-0.146 at 450 nm. One variable that was tested was the streptavidin conjugated to horseradish-peroxidase. In order to determine if the streptavidin component was functional, biotinylated anti-M13 phage antibody (Pos. Anti-M13-B) was bound to the plate, the streptavidin conjugated to horseradish-peroxidase was allowed to bind to that antibody, and following this step was the addition of TMB substrate. This yielded a positive result (1.029 +- 0.100) that was significantly



<u>Figure 7.</u> ELISA to Test the Streptavidin for Biotin Activity. This ELISA was performed in order to see if both components of the streptavidin HRP were working. The Pos. Anti-M13- B well was coated with an anti-M13 antibody that was conjugated to a biotin molecule (diluted in 1X Na_2CO_3), The phage well was coated with M13 phage. The M13 and the SAv blank wells were performed as described in Figure 6A. The Pos. SAv well was coated with streptavidin-HRP diluted in 1X Na_2CO_3, and the AB blank well was coated with blocking buffer (1% BSA). After an incubation step, all wells were washed and blocked. Anti-M13 antibody conjugated to HRP was added to the phage and Ab blank wells. Streptavidin-HRP was added to the Pos. Anti-M13-B and SAv blank. All antibodies were diluted in sample buffer (PBS + 0.1% BSA). The Pos. SAv well received only wash buffer. After incubation, the wells were washed, and TMB substrate solution added to cause a color change, which was stopped with H_2SO_4 and measured at 450 nm. Absorbance from the blank wells were subtracted from all of the wells. All wells in this ELISA were performed in duplicate. Asterisks over the positive wells indicate there was significantly higher absorbance in those test wells than the blank wells, and this was confirmed with a T test ($p<0.05$).

higher than our SAv blank control (p-value=0.0046). The SAv blank was also exposed the streptavidin conjugated to horseradish-peroxidase, but with no biotin in the well the streptavidin did not bind. The horseradish-peroxidase component of the streptavidin conjugated to horseradish-peroxidase was also tested by having it bound directly to the plate and later exposing that to TMB. This also yielded a positive result (0.424+-0.046 at 450 nm) that was significantly higher than the negative control (SAv blank) with a p-value of 0.0058. The phage, Pos. Anti-M13-B, and Pos. SAv wells all had the background absorbances detected in the blank wells subtracted from them. This ELISA showed that the streptavidin conjugated to horseradish-peroxidase was functional, the positive and negative controls work, and that these controls worked in the presence of these blocks and wash buffers. Therefore, the issue must be with the peptide or the biotin on the peptide.

Biotinylation Incorporation Assay

For this experiment three biotinylated BR1 samples the lab possessed were compared to the recently biotinylated peptide. The peptides tested in this assay were EP, FK, BR, and AH. A biotinylation incorporation assay was completed in order to find the value of mmol biotin/mmol BR1 (Table 3). The results of this experiment showed that the highest value of mmol of biotin/mmol of BR1 was for the BR peptide which had 0.083184 mmol biotin/ mmol BR1. Therefore, this stock of peptide was the one that would be used for future ELISAs. The results of this assay also revealed that the biotinylation experiment that was performed in this work was not successful and yielded a negative number. Therefore, another assay should be performed again using a higher concentration of the peptide to find a more accurate amount of biotin for AH and EP.

Table 3 Biotinylation Incorporation Assay Results for Four Different Preparations of Biotinylated BR-1.

Sample	[b]FK	[c]EP	[d]BR	[e]AH
[a]mmol biotin/mmol BR-1	0.004415	-0.06246	0.083184	-6.6E-06

[a]Biotinylation Incorporation Assay was used in order to determine the mmol biotin/mmol BR-1. The four samples of B-BR-1 were AH, EP, BR, and FK. Briefly, using a spectrophotometer, samples of a HABA/avidin solution, MES and HABA/Avidin solution, HABA/avidin and biotin solution, and HABA/avidin and biotinylated peptide were measured at 500 nm. This was performed for each peptide, and then the Beer Lambert Law was used to find the amount of biotin per peptide.

[b]FK peptide was biotinylated by Floyd Kenney (Kenney, 2018), and was used in Figure 6B.

[c]EP peptide was biotinylated by Errek Pham and Alex Huber during this project.

[d]BR was the peptide biotinylated by Bill Rees (Rees, 2016).

[e]AH peptide was prepared by Alex Huber during this project and was used in Figure 6A.

Peptide ELISA Following Biotinylation Process

The peptide ELISA shown in Figure 8 yielded excellent results, which allowed the project to move forward into the peptide purification process. For this ELISA, the normal controls were used, along with testing of all of the stocks of biotinylated peptide that the lab possessed. The results showed that in several cases (FK, BR, AH 10x), BR-1 had a high specificity for HSA. In these conditions (yeast extract block and sample buffer with 10% yeast extract in PBS, as previously used in Figure 6A), the FK HSA showed a significant amount of binding at a concentration of 10 µg/ml with an average absorbance of 0.224+-0.010 at 450 nm compared to the FK no HSA with an average absorbance at 0.062+-0.004 at 450 nm (p-value of 0.002). The peptide AH also was shown to have a high specificity for HSA at these conditions with an average absorbance of 1.176 +-0.108 at 450 nm compared to the AH no HSA well with an average absorbance of 0.600 +- 0.073 at 450 nm (p-value of 0.024), however, the concentration was 80 µg/ml, which was ten times higher than the previous concentrations used with this peptide. The peptide that showed a very significant specificity for HSA was the 10 µg/ml of BR peptide, which showed an average absorbance of 0.703+-0.005 at 450 nm compared to the BR no HSA wells which had an average absorbance of 0.207+-0.004 at 450 nm (p-value of 7.5 x $10^{-5)}$. Unfortunately, the EP peptide showed no significant binding to HSA with an average absorbance of 0.012 +-0.004 at 450 compared to the EP no HSA well with an average absorbance of 0.005+-0.003 at 450 nm. For further testing of the BR-1 peptide, the BR biotinylated sample was the peptide that was used.

The poor results in Figure 6B could be attributed to the use of casein block. Casein is an effective block that is commercially available. Therefore, the ELISA using casein block was repeated using with the BR biotinylated peptide. PBS with 0.1 % casein

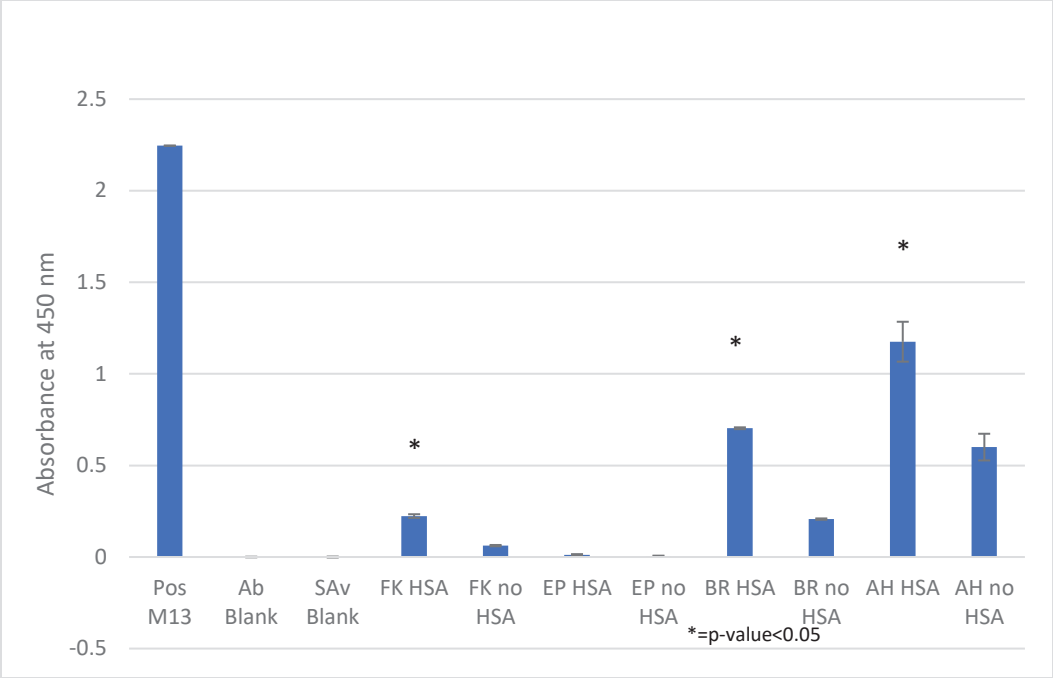


Figure 8. Peptide ELISA Using All Stocks of Peptide. This ELISA was performed after the biotinylation incorporation and HABA/Avidin assay. The method was identical to that performed in figure 6A. All of the stocks of the BR-1 peptide (FK , EP, BR, and AH) were tested. The FK peptide had 0.004 mmol biotin/mmol BR-1, the EP has a low amount of biotin and shows a low absorbance when it is used at 1X, the BR peptide has 0.08 mmol biotin/mmol BR-1, and AH was used at 10X, compared to 1X in Figure 6A. The positive control was performed in singlet, the blanks were performed in triplicate, and the rest of the wells were performed in duplicate. Asterisks over the positive HSA wells indicate there was significantly higher absorbance in those test wells than the no HSA wells, and this was confirmed with a T test ($p<0.05$).

was also used as a sample buffer, instead of the original solutions that included Tween 20 and yeast block. The results for this ELISA are shown in Figure 9. Along with using the normal Pos. M13 control, the Pos. biotinylated BR1 control was also used for this test. This test unfortunately did not show any significant binding for the BR peptide to the Pos. HSA with an average absorbance of 0.004+-0.005 at 450 nm when compared to the no HSA well with an average absorbance of 0.005+-0.002. The positive B-BR1 well also showed a fairly low absorbance, especially when subtracted from the background noise that was detected from the blank wells, with an absorbance -0.02 at 450 nm. This data indicates that the casein block and sample buffer may have been inhibiting the binding of peptide in these ELISAs.

PCR, Gel Extraction and Purification, Digestion, and Sequencing

In this project the use of PCR was attempted in order to incorporate the peptide sequence into the pMal-c5x expression vector. This was performed by using two primers for the pMal-c5x vector, except one of the primers had the sequence encoding BR-1 incorporated in it. In Figure 10A, it can be seen that the initial PCR process did produce 3 bands on the gel (1:10, 1:100. 1:1000 dilutions of the vector used in the reaction), which showed the process did occur with the unique primers that were designed for this experiment. Figure 10B showed that after the gel extraction and purification method, DNA was not lost. The 5.6 kb bands were excised and purified from the gel, because of the presence of other contaminating fragments. The purified DNA was treated with polynucleotide kinase in order to add 5' phosphate groups to the ends so that ligation and transformation could follow. In Figure 10C, after four transformants were selected and the DNA was purified by performing a plasmid prep, a *Bam*H I digestion was performed on the DNA, since the new vector construct would lack this restriction site. The results

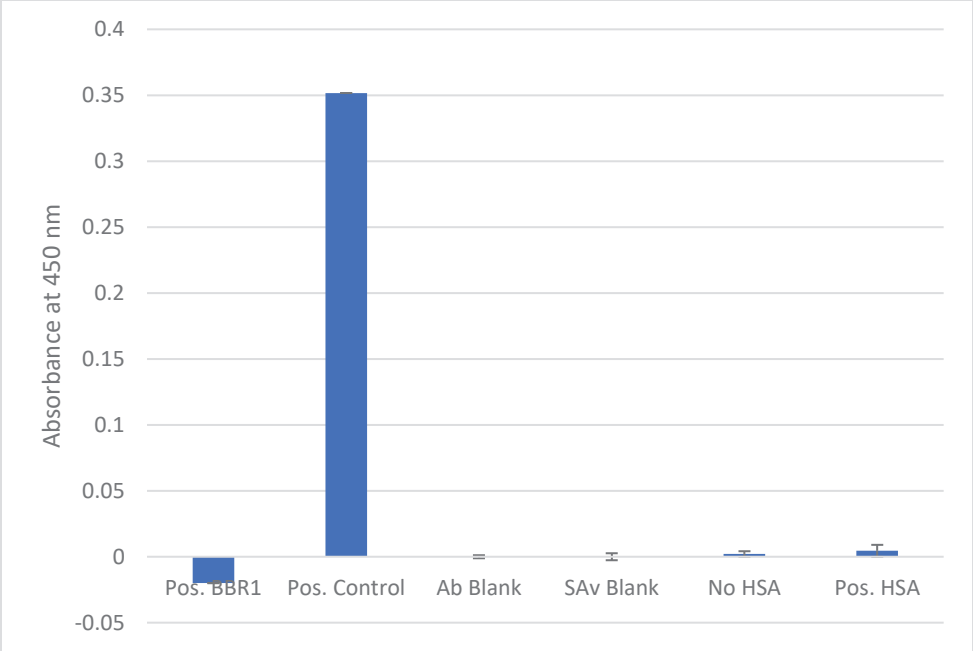


Figure 9. Peptide ELISA using BR Biotinylated Peptide and Casein Block and Sample buffers. This ELISA was to test if using casein in the blocks and wash buffer had any effect on the ELISA. The protocol is identical to that used in Figure 6B, with one exception: the BR peptide was used instead of the FK peptide. The Positive control and the B-BR1 well were performed in singlets, while the rest were performed in triplicate.

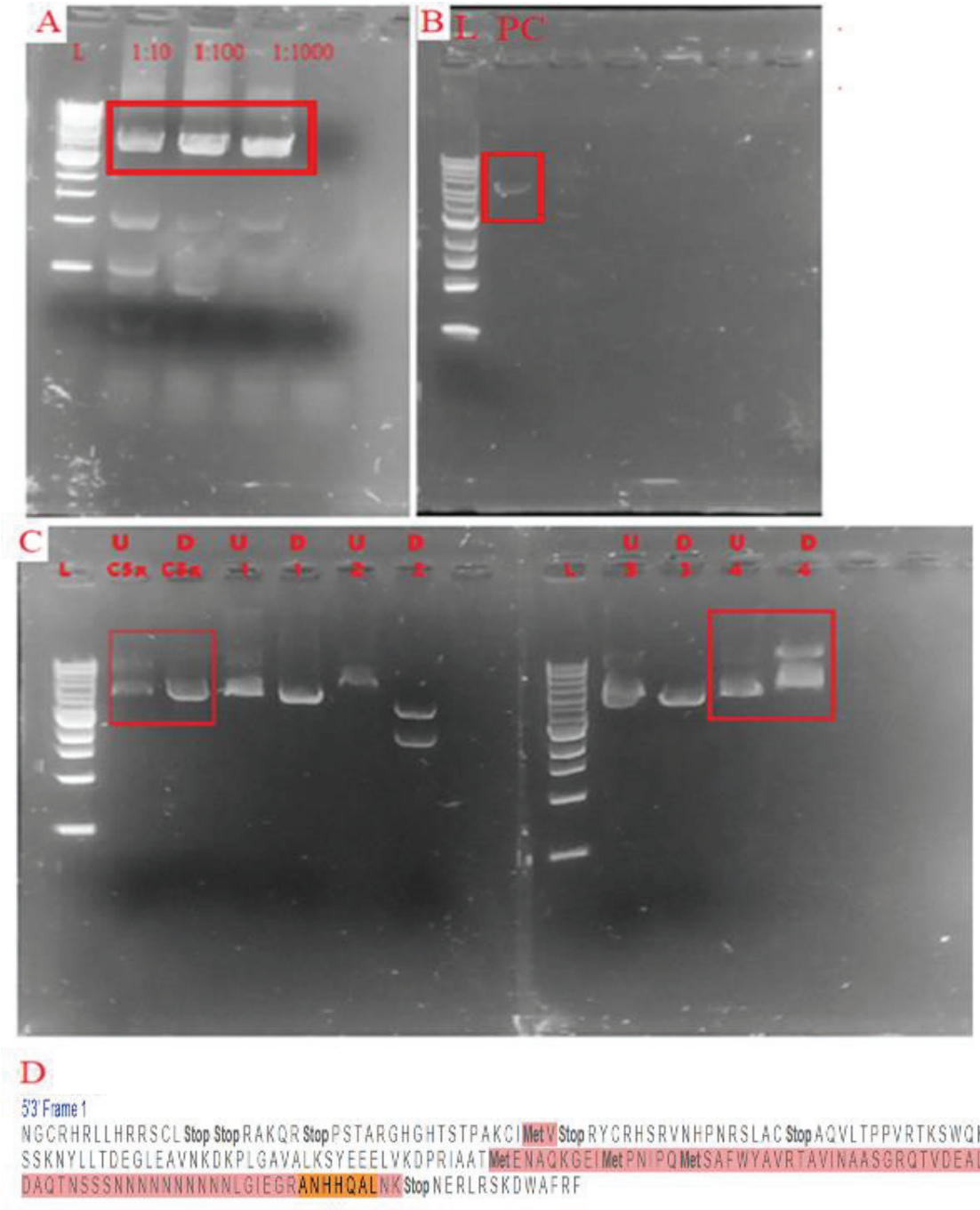


Figure 10. PCR, Gel Extraction and Purification, Digestion, and Sequencing Results. In Figure 10A, the agarose gel performed after the initial PCR process is shown. In this PCR process, the primers were designed for the pMal-c5x vector, however, one of the primers had the sequence for BR-1 attached to it. Forward primer, BR1_1, possessed

the peptide sequence, while BR1_2 was a normal reverse primer. The 25 ul reaction mixture contained Q5 master mix, the primers, either a 1:10, 1:100, or 1:1000 dilution of the pMal-c5x vector, and 9 ul of Nuclease-Free Water (Gel of DNA content from this process shown in 10A). Figure 10B shows an agarose gel of purified DNA which was obtained by excising the vector band from the gel and using purification kits to purify the sample of plasmid construct (PC) from all three lanes. Figure 10C shows an agarose gel with samples of the plasmid construct digested with *Bam*H I. This was performed by transforming the DNA and then performing a plasmid prep. A *Bam*H I digestion was performed, since this restriction site should no longer be in the plasmid construct. This was performed with the pMal-c5x (c5x on gel), and samples 1-4 (S1-S4 on gel). These samples were digested with *Bam*H I (D) and run on a gel next to the undigested (U) samples. Figure 10D. A sequencing reaction was performed using primers designed for this plasmid construct. Following the reaction clean-up step, Sanger Sequencing was performed.

show that out of the four DNA samples obtained, one sample showed promising results. This was sample 4 (labeled “4” on the gel), which showed similar migration patterns to the undigested pMal-c5x (labeled “c5x” on the gel) when digested with *Bam*H I. The other samples (1-3), all showed a degree of digestion. Also, in Figures 10A-C, the bands were visible around 5600 bp, which was promising, since that was approximately the size of the pMal-c5x vector. In Figure 10, the results of the Sanger sequencing showed that the full peptide sequence was almost successfully incorporated in the expression vector. There were errors located in the codon for the last amino acid, serine, which caused a codon for a leucine to be incorporated into the vector. The peptide sequence was ANHHQAL, instead of ANHHQAS (the peptide sequence for BR1).

Discussion

A biosensor that can detect blood, more specifically HSA, can be utilized for police and military purposes in case someone is shot on the field and is unresponsive. In this situation, the biosensor would be able to detect HSA and send a signal to a dispatcher, who can then send for help. The BR-1 peptide is a heptapeptide composed the amino acids ANHHQAS that was obtained through phage display. The bait protein used in the phage display process was HSA. This protein was selected because it is the most abundant plasma protein in blood, and a peptide capable of binding to HSA with a high specificity can be potentially used to create a biosensor that detects HSA.

The hypothesis of this project was to show that BR-1 has a high specificity for HSA and can be produced in high concentration to use in preparing a biosensor. An important goal was to purify the BR-1 peptide using the pMAl-c5x expression system. The objectives, in particular, were to first show that BR-1 has a high specificity for HSA via peptide ELISA. This task was successfully accomplished, although several ELISAs were performed in order to optimize the experiment. The other objective was to use PCR to incorporate the peptide sequence into the pMal-c5x expression vector, and transform it into bacteria, and use the amylose resin column to purify the peptide. In this work, majority of the peptide sequence was successfully incorporated into the vector. However, this task will need to be attempted again as the vector produced a nucleotide sequence that produced one incorrect amino acid in the peptide sequence.

The specificity of BR-1 for HSA was confirmed using a peptide ELISA (Figure 8), however, there were several ELISAs performed that showed negative results. This was partially due to the low amounts of biotin on the peptide's surface for the particular

samples that were used. For example, AH was the peptide that was utilized for the majority of the peptide ELISAs and this was shown to have a low amount of biotin on its surface in the biotin incorporation assay (Table 3). In Table 3 it can be seen that the AH peptide possessed -6.6E-06 mmol of biotin/mmol of BR-1, which shows that the biotin was undetectable. In Figure 8, a ten-fold higher concentration of the peptide bound specifically to HSA and was detected by streptavidin-peroxidase. If a higher concentration of this AH peptide was used for this biotinylation incorporation assay, this would most likely given a positive result for the test.

To prevent problems with obtaining a biotinylated peptide in future studies, two items should be considered. The first item that this lab might consider would be to adjust the vacuum evaporation set up. The normal equipment for vacuum evaporation could not be used due to the high acidity of the sample. The vacuum evaporation set up that was utilized for this project was a sidearm filter flask with the sidearm attached to a hose which was connected to an aspirator. The flask was sealed at the top with a rubber stopper, and this set up was heated on a hot plate. The sample was inside foil-wrapped Eppendorf tube, which was held upright by a 15 ml conical tube (Figure 11). This was the first time that this lab had used this specific set up for biotinylation, therefore more testing of this set up should be performed in order to find an optimal method for biotinylation of peptides. It is also possible that sample was lost during this process or that the biotinylated peptide function was affected by the heating process.

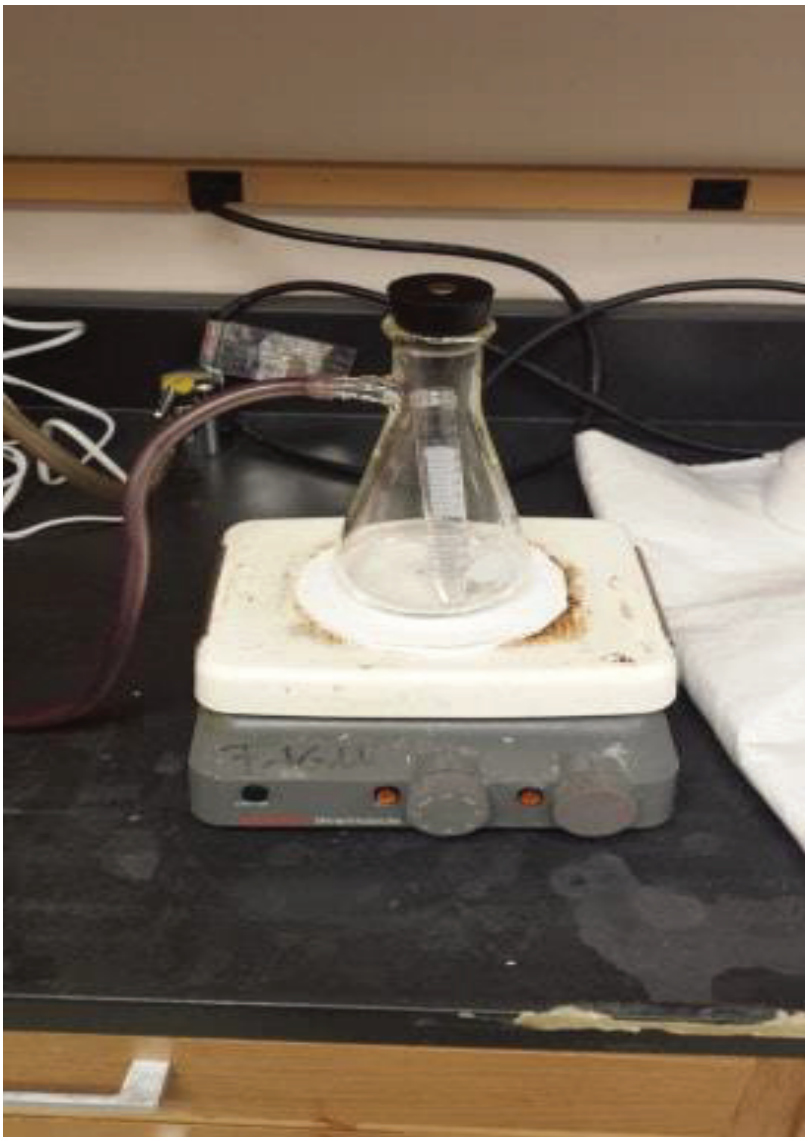


Figure 11. Vacuum Evaporation Set up. This is an image of the vacuum evaporation set up that was utilized for the biotinylation of BR-1 in this experiment.

For future immunoassays performed the lab should consider purchasing biotinylated peptide. In Ahmed et al, phage display was to find a peptide that could bind to human interleukin-4 receptor (IL-4R) and interleukin-13 (IL-13). The purpose of this experiment was to simply explore the potential uses of phage display in order to find high affinity binders with a high specificity for a ligand, instead of using a more expensive and labor intensive hybridoma alternative. In this paper they used a 12-mer random peptide library from New England Biolabs Inc, and performed peptide phage display against purified IL-4R-alpha and IL-13. Once the peptide sequence was obtained, the research group obtained biotinylated versions of the peptides, where the biotin molecule was specifically added to the N-terminus, from the company AUSPEP in Melbourne, Australia. This group was then able to perform direct ELISA in which they were able to first have the target protein, either IL-4R or IL-13 bound to the plate and incubated overnight. The next day they washed out the IL-2R solution was then discarded and the wells were then blocked with a BSA and NaN_3 blocking solution. After a 1-2 hour incubation step, the blocking buffer was discarded and washed with 0.3% TBST. Following this step the biotinylated peptide was then added to the wells and allowed time to incubate for 1-2 hours at room temperature. Lastly, the streptavidin-HRP was added to the wells, and then an OPD solution was used in order to measure the color change. This process yielded excellent results that showed with increased concentrations of biotinylated peptide, there was an increase in absorbance (Ahmed et al. 2014).

Another factor that may have also contributed to these peptide ELISAs not working may have been changes from the optimal protocol. Changes were made to the method in order to explore and troubleshoot the issues with the ELISAs, and this took

place before the amounts of biotin per peptide had been tested. Figures 8 and 9 peptide ELISAs were performed that utilized the biotinylated BR peptide. However, only one of the ELISAs worked. One of the changes that may have made a great impact in the results was using the casein block. Casein is a milk product that may contain biotin, which could disrupt the biotin-avidin assay. This may have caused high background noise in the blank and no HSA wells, which may have caused false negative results in the ELISA (Thermo 2017). Table 4 displays a chart that possesses the optimal ELISA protocol versus the modified protocol that should be avoided for further testing with the biotin-streptavidin system.

Typically for ELISAs, one of the more common blocks that are used is bovine serum albumin (BSA). However, BSA could not be used for this experiment since the ligand was HSA. Although there are a significant number of differences between BSA and HSA, BSA still shares many conserved sequences and domains as HSA, possessing approximately 76% sequence homology when compared to HSA. Therefore BSA would not have been an appropriate block (Bujacz 2012; Huang & Kim 2004; Chruszcz et al. 2013).

Another practice that is commonly performed after phage display to test the peptide specificity is an M13 Phage ELISA. These ELISAs utilize phage that are displaying the peptide or protein of interest after the biopanning process. These typically have the ligand bound to the plate, followed by exposure of the phage displaying the peptide, and then an anti-M13 antibody conjugated to HRP is utilized for detection. As mentioned previously, Ahmed et al used phage display to find a peptide that could bind to human interleukin-4 receptor (IL-4R) and interleukin-13 (IL-13). During this experiment,

Table 4 Comparison of Methods between Optimal ELISA procedure and Modified ELISA Procedure

Reagent/Concentration	Optimal Procedure	Modified Procedure
HSA	Sigma-Aldrich *	Sigma-Aldrich
Wash Buffer	1 X PBS-0.05% Tween 20 *	1X PBS
B-BR-1	BR peptide *	BR peptide*
Blocking Buffer	1% Yeast Extract in PBS-0.05% Tween 20 *	Casein(1% (w/v) casein) in TBS block from Thermo-Scientific
Sample Buffer	0.1% Tween 20 and PBS *	1 X PBS + 10% Casein

*= Solution or dilution made the day of experiment instead of using older solutions

the researchers also performed an M13 binding ELISA. However, instead of using purified peptide, the peptide was still displayed on the phage. In this way, they were able to avoid using a biotin-streptavidin system, and instead the researchers used an anti-M13 antibody conjugated to HRP. This process was performed with three different phage clones selected from the phage display process and 5 different phage titer concentrations. The results showed that with higher phage clone titers, there was a higher absorbance. It also showed that in the highest titer, there was a phage clone," N7," which showed a significantly higher absorbance than the other two clones(Ahmed et al. 2014).

This method was also previously utilized in this lab in which phage displaying the BR-1 peptide were used for the ELISA. The only significant difference between this ELISA and the typical ELISAs performed in this lab, was the use of phage displaying BR-1 instead of biotinylated BR-1 and the utilization of an anti-M13 antibody conjugated to HRP instead of a biotin-streptavidin system. The p-value obtained from this experiment was less than 0.05, showing that BR-1 had a high specificity for HSA while still displayed on phage pIII (Rees 2016).

Once a specific molecule is found through phage display, there are several ways to obtain the purified peptide. In an experiment that was performed by Dennis et al, peptides binding to HSA were sought for pharmokinetic purposes. HSA is a molecule that is large enough to not be filtered by the kidneys, therefore being able to bind small drugs to albumin molecules can potentially increase their half-life in the body. This group used a specially designed peptide phage library in order to find phage clones expressing a peptide that binds to HSA with a high specificity. After obtaining the sequence of the

peptide, the group used Fmoc (N-(9-fluorenyl) methoxycarbonyl)-based solid phase synthesis in order to obtain the peptide. This stepwise process involves the addition of one amino acid at a time, and each amino acid that is used has a Fmoc protecting group. After the amino acid and protecting group have been added, the protecting group is removed by adding piperidine, and then is removed from the reaction through a washing step. This allows the amine group of the amino acid to react with a new amino acid that is introduced (by creating an amide bond with the carboxyl end of the introduced fmoc-protected amino acid). The process is then repeated until the desired peptide is constructed. Once the last amino acid is added and the last fmoc group has been removed, the reaction can be treated with TFA to remove the peptide from the resin (Pingali, Aruna 1996; Schatzlein et al. 2001). Once Dennis and colleagues used Fmoc-based solid phase synthesis, they were able to obtain a biotinylated peptide that they were able to use for immunoassays such as a competitive ELISA. This ELISA allowed the group to show that competing ligands of albumins did not affect the binding of the peptide to rat or rabbit albumin (Dennis et al. 2002)

Another method of obtaining peptides found through phage display is by finding the sequence of the peptide and sending them to a company to be manufactured. This was seen in the experiment mentioned earlier performed by Ahmed et al, where they purchased their purified peptide along with their biotinylated versions from a company in Australia after obtaining the sequence of the peptide (Ahmed et al. 2014). Similar to this group, previous members in this lab purchased BR-1 from Genscript USA Inc., Piscataway, NJ for immunoassays, biotinylation procedures, and CNT coating procedures. Although there is a certain convenience to purchasing a purified peptide from

a company, the overall goal of this project is to produce a biosensor that could eventually become produced on a large scale for commercial use. Therefore, it would be advantageous for manufacturers producing this biosensor to be able to make large quantities of the peptide, rather than constantly purchasing the peptide from a company. That is why this lab group sought a cheaper way to obtain large amounts of the peptide. A significant method that has been commonly utilized by researchers using phage display is transforming an expression vector that possess the peptide or protein sequence into E. *coli*, inducing expression of the peptide, and purifying the peptide via affinity chromatography.

Xu et al utilized this type of peptide purification procedure when finding a peptide that could bind to anti-ochratoxin antibody. Ochratoxins are mycotoxins that are capable of contaminating a wide variety of crops, therefore strong and accurate detection methods are necessary for public safety. Chemical synthetic method may yield heterogeneous products which could affect the accuracy of the immunoassays. Finding an ochratoxin conjugate that can be produced using a method other than chemical synthesis was important to this group, since competitive immunoassays are one of the primary tests that are used for mycotoxin detection. This group produced a peptide specific for ochratoxin using phage display techniques and was able to use a NEB pMal-pIII shuttle vector to produce the peptide in larger quantities. This vector has compatible restriction sites to the phage library vector and is specifically designed for expression of peptides that are displayed on pIII. The use of this vector creates a mannose binding protein (MBP) fused to the peptide or protein of interest. The insertion of this gene will also interact with the *lacZ* gene on the DNA, which causes inactivation of the *lacZα*. This is useful, since an α-

complementing method can be performed by transforming this vector into a bacterium and use α-complementation, IPTG, and X-gal in order to isolate the MBP fusion protein. After DNA purification, the group was able to amplify the sequence of the peptide using PCR and specific primers that come with the phage display library kit. The DNA was then purified using a kit, and then digested with restriction enzymes. After it was purified by 8% polyacrylamide gel electrophoresis, the segment of DNA was ligated into the digested pMal-pIII vector (that had been digested with the same restriction enzymes). This plasmid construct was then transformed into *E. coli*. The culture was then induced with IPTG and then incubated. Periplasmic fusion proteins were then extracted using osmotic shock, and the peptide fused to the MBP was purified using amylose affinity chromatography. This allowed the group to successfully perform competitive qualitative and quantitative immunoassays that were able to detect ochratoxin in contaminated samples of crops with high sensitivity.

Although the pMal-pIII vector would have been an ideal expression vector to use for the project in this research, it has unfortunately been discontinued by NEB. A similar expression also sold by NEB is the pMal-c5x. This is an expression vector that works the same way as the pMal-pIII vector, except it was not specifically designed for transferring the genes from the M13KE vector. This vector was used in a paper written by Zhou et al. This group studied Bluetongue disease, which is caused by the RNA zoonotic virus known as the Bluetongue virus (BTV). This virus is can be distributed by midges and can infect sheep, cattle, camelids, and carnivores. This group wanted to find a peptide that can bind to the outer capsid protein VP2, which could potentially be used for sera screening. They utilized the L2 gene which encodes for the VP2 protein and divided it

into three segments, and expressed it with a MBP-tag, along with a His-tag. Briefly, three overlapping VP2 gene segments were synthesized and cloned into the pMal-c5x using restriction enzymes. *E. coli* strains were grown in LB medium and protein expression was then induced by IPTG. Cells were then centrifuged, pelleted, and suspended in PBS. The cells were then sonicated in an ice water bath. The MBP fusion proteins were then purified using the NEB MBP kit. This involved using amylose resin affinity chromatography. The sonicated bacterial supernatant was diluted with column buffer and loaded onto the column containing amylose resin. MBP binds to amylose, but has higher affinity for maltose. The column was then washed with column buffer and the MBP fusion proteins were eluted with maltose. These fusion proteins (segments of the L2 gene expressed as a fusion protein with the MBP) were then used for creating monoclonal antibody-secreting hybridomas against certain segments of the BTV VP2 protein. They were then able to test these hybridomas using immunoassays and confirm that they possessed monoclonal antibodies that could be used for serological diagnosis of BTV-4.

Tharuka et al. also performed studies that utilized the pMal-c5x vector. In their experiments, the researchers characterized IL-10 from big belly seahorse. Using the pMal-C5x vector allowed the researchers to be able to see how this cytokine affected a murine macrophage cell line by producing recombinant IL10 and observing how it affected the cell cultures. They were able to find that this recombinant protein increased STAT3 and SOCS3 expression when the cytokine was cultured with these cells (Tharuka et al. 2019).

In this study, one of the goals was to utilize the pMal-c5x vector in order to purify BR-1 peptide. While a new plasmid construct was produced, unfortunately, the desired

plasmid construct was altered by two bases. This caused the last codon of the peptide sequence to change from a serine to a lysine. This protocol almost worked and may be repeated in the future, since it can be a viable method for obtaining this plasmid construct. Future work may involve having primers purified by IDT to ensure that there are no errors introduced into the synthesized primers. As for current and future research, methods similar to Tharuka et al and Zhou et al are being performed, in which primers that encode the BR-1 sequence with compatible ends to the pMal-C5x restriction sites (*Xmn* I and *Hin*d III) have been ordered. These primers have been annealed and ligated into a digested pMal-C5x. If sequencing confirms that the peptide sequence is present, then the peptide purification process using the amylose resin affinity chromatography can be used.

The pMal-c5x is an expression vector that has been utilized successfully by many experiments and there are many benefits to using this expression system. Primarily, if large amounts of peptide or protein need to be produced, this system can be a cost-effective alternative to purchasing the peptide from a company. Once the plasmid construct is produced and the bacteria possess the transformed vector, a renewable source of the peptide has been created. The bacteria can be pelleted and then frozen for future use. Therefore, the limiting factor of this pMal-c5x kit would be the amylose resin. The amylose resin comes with this kit in a 15 ml bottle and this resin can be reused (New England BioLabs Inc. B 1988b). One ml of the amylose resin can bind to 6-8 mg of fusion protein. Therefore 15 ml should produce at least 100 mg of purified fusion peptide. This resin can also be regenerated by washing the column with three volumes of water, then 3 volumes 0.1% SDS, followed by 1 column volume of water, and lastly three

column volumes of buffer. Since this resin can be reused three to five times, this can potentially yield up to 500 mg of purified fusion peptide.

The chart in figure 12 shows the cost comparisons of purchasing the purified peptide versus producing it. Purchasing a low quantity of the peptide gives approximately 10-14 mg, costing $158 (this was the amount and cost from purchasing BR-1 previously). Producing the peptide with the amylose resin column will cost approximately $681 for the kit and primers. The amylose resin column can potentially produce approximately 500 mg of fusion peptide which is approximately 20 mg of purified peptide. Therefore, if only a small amount of peptide is needed for an experiment, it would be cheaper to purchase it. However, in order to allow mass production of a biosensor, much more peptide would be required. Therefore, in order to produce more peptide, more amylose resin must be purchased. In order to produce 100 mg of the peptide, the pMal kit, primers and extra amylose resin would cost approximately $1525, as opposed to ordering at least 100 mg which would cost approximately $1580. Therefore, saving money has already occurred here. If 200 mg of the peptide is needed, the savings is even more evident at $2580 to produce the peptide versus $3160 to purchase it. Therefore, since it is cheaper to produce the peptide for this purpose, efforts to incorporate this peptide sequence into this expression vector will be continued.

In conclusion, BR-1 was shown to bind to HSA with a high specificity by using a peptide ELISA. Due to the initial peptide ELISAs showing negative results, the amount of biotin per mg BR-1 peptide was investigated. The BR peptide was shown to possess the most biotin and the second most was the FK peptide. After confirming the specificity of BR-1 for HSA, the focus transitioned to the peptide purification portion of the project.

Method	Purchased	Cost	Total Initial Cost	Amount	Total Cost of 100mg	Total Cost of 200 mg
Purchase	BR-1	$158	$158	10-14 mg	$1,580	$3,160
Production	pMal kit	$645	$681	20 mg	$1,525	$2,580
	Primers	$18				
	Sequencing Primers	$18				
	Amylose Resin	$844 for 100mg $1055 for 200 mg				

Figure 12. Cost Analysis of Purchasing Versus Producing the Peptide. This chart serves to compare the cost of buying BR-1 versus purchasing it. If a low amount of the peptide is required, then it is cheaper to purchase the peptide. However, when 100 mg or 200 mg of the peptide is needed, it is cheaper to produce the peptide, since the cost to produce 20 mg of the peptide is reduced from $681 to $211 (the cost of one order of amylose resin).

The BR-1 peptide sequence was attempted to be incorporated into the pMal vector, so that it could be later purified using an amylose resin column. Unfortunately, due to errors in the PCR process, the last amino acid of BR-1 was altered to a leucine residue, instead of a serine. Other efforts have begun in an attempt to incorporate the peptide sequence into the vector by cloning a segment of purchased DNA (Primers that encode BR-1 annealed together with compatible ends for the pMal vector). If sequencing shows that the peptide sequence is in the pMal vector, then affinity chromatography can take place in order to purify BR-1. This will allow mass amounts of peptide to be able to be produced in a cost-effective manner. This will be important for the future goals of this project, which are to eventually produce a wearable biosensor, that can detect blood. This biosensor can be worn by all military and police. This technology can help protect the military and police, and potentially save lives.

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