Identifying Peptides that Bind to Human Serum Albumin Using Phage Display for the Development of Sensors that Detect Injury in Military Personnel.

by

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ABSTRACT

Phage display is a molecular technique that allows for the placement of proteins, peptides, or antibody fragments on the surface of a phage (a virus that infects bacteria). These studies used the Ph.D.-7 M13 phage library (New England BioLabs), which has random 7 amino acid peptides bound to the PIII capsid protein. Solid surface panning was used to select for phage displaying peptides that bind to the most abundant protein in blood, human serum albumin (HSA). An M13 ELISA was performed by binding HSA to poly-vinyl chloride (PVC) plate wells and allowing phage clones containing peptides specific for HSA to bind. Phage were cloned by picking plaques. Clone HSA 6 demonstrated significant binding specificity to HSA in an ELISA (P value ≤ 0.05) when compared to No HSA (non-specific binding) and sequencing of phage DNA demonstrated an amino acid sequence favorable to specific binding to HSA. The peptide was synthesized (BR-1), biotinylated and tested for specificity to HSA. A peptide ELISA for binding to HSA was performed using various blocking buffers: 1% yeast extract in PBST, 5% non-fat dry milk in PBS and 5% normal calf serum in PBS with Triton X-100. Peptide BR-1 bound specifically to HSA (P value ≤ 0.01) when compared to No HSA wells. All blocking buffers were suitable choices for preventing non-specific binding. An albumin ELISA was performed to determine if peptide BR-1 was specific for HSA, when compared to egg albumin, chicken albumin, rabbit albumin and cow albumin. The albumin ELISA exhibited BR-1 binding specificity to HSA (P value ≤ 0.01), when compared to BSA and OVA. These studies show that phage display is an efficient method for isolating peptides that bind with high affinity to HSA.

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Chapter 1: Introduction

Phage display allows for the selection of peptides, proteins, or antibody fragments with specific desirable binding characteristics against numerous biological targets via an *in vitro* selection process termed biopanning. This is accomplished by inserting the nucleotide sequence of the peptide to be displayed, to a gene in the genome that encodes a coat protein. This fusion allows for the display of the peptide on the surface of the desired phage coat protein, while the inserted nucleotide sequence remains within the virion (reviewed in Willats 2002). Our biological target of interest is human serum albumin (HSA), the most abundant protein in the plasma. Our library of bacteriophage, virus that infects bacteria, all express random peptides on their protein coats. Screening of the library for the specific peptides that bind to HSA, if successful, will allow us to select for bacteriophage expressing these specific peptides. Once we have a peptide specific for HSA, we can couple the peptides to nanofibers and develop a patch that soldiers can wear on major wound sites of the body. This will allow the detection of HSA (once blood has been spilled on the patch containing peptides), ultimately leading to the signaling of emergency medical personnel to assist wounded soldiers.

The bacteriophage used in this experiment come from the filamentous phage family, specifically M13, which is one of three total strains that could potentially be used. The filamentous phage include: M13, fl and fd. Ph.D. Phage Display Libraries is the library of choice, which is modified for pentavalent (5) display of peptides on the minor coat protein pIII. This library is the most suitable library for selection of higher affinity ligands because pIII libraries offer the advantage of reduced valency of peptides, when compared to pVIII libraries. The reduced valency of pIII libraries ensures that there will

be less binding sites for the peptide to bind to, which in turn allows for the selection of higher affinity binding of peptides. This library consists of 10^9 independent clones, which makes it possible to encode the majority of the possible HSA specific peptide sequences, allowing this library to be a very efficient tool for our project (Ph.D. Phage Display Libraries Instruction Manual). We propose that phage display using the diverse Ph.D. library of bacteriophage will allow for the selection of specific peptide ligands that will be able to detect HSA.

1.1 Phage Display

Phage display technology has made major contributions in immunology, cell biology, drug discovery and pharmacology in recent years. This is due to its simplicity, reduced costs and quick set up times. Phage display technology allows for the display of foreign polypeptides, antibody fragments, and proteins on the surface of bacteriophage. Expression of these various fragments is accomplished by incorporating the nucleotide sequence encoding the protein, peptide, or antibody fragment into a gene within the gene encoding a phage coat protein (Reviewed in Willats, 2002). The major vehicle that allows this technology to be possible is the bacteriophage. Bacteriophage are a group of viruses that infect bacteria and are the major tool for displaying peptides, proteins or antibody fragments to be bound to a specific target. One advantage of this approach is the amount of expressed foreign proteins, however the functionality of the coat protein can become compromised by the fusion of the phage. This could ultimately affect phage viability, because there is no wild type version of the coat protein being retained. Using hybrid phage technology can help avoid this problem.

1.1.A Hybrid Phage Technology

Hybrid phage can be produced having both wild type and coat proteins fused to foreign peptides. Hybrid phage can be created using a phagemid-based system where phagemids carry sequences encoding fusion proteins, and a helper phage is needed to provide the genes needed for single stranded DNA replication and the inclusion of phagemid DNA into bacteriophage Particles. Helper phage and phagemids are then co-infected together into host bacteria (Sidhu, 2001). However, there are disadvantages to using hybrid phage systems. Smaller numbers of proteins are displayed because of competition between fusion coat proteins and wild type coat proteins for inclusion in the newly synthesized phage (Reviewed in Willats, 2002). These disadvantages can be turned into advantages by using the resulting low valency phage to select for high avidity binding during the panning process (Rondot *et al*, 2001).

1.2 Bacteriophage Family

The Ff phage family, M13, fd and fl, are ideal cloning vehicles for assembly and display of longer phage particles. This family of phage has similar characteristics and undergo protein export through non-lytic propagation mechanisms. M13 bacteriophage are the most widely used phage for the display of oligopeptides. George P. Smith was the founder of this fundamental technology and introduced this method to the world in 1985 (Smith, 1985). Filamentous M13 bacteriophage use the Sec or SRP pathway to transport fusion proteins to the periplasm as an unfolded state. The sec pathway uses a series of steps to move proteins out of the cell.

Within the inner membrane, the coat proteins are imbedded with their C termini in the cytoplasm and their N termini in the periplasm. The remaining phage proteins form

a pore in which viral ssDNA is exported and subsequently surrounded by coat proteins into an assembling phage particle. After P6 and P3 coat proteins at the end of the phage particle are added, the process is terminated and the assembled phage particle is released into the extracellular environment (Sidhu, 2001).

There are some disadvantages when using Ff phage, since Ff phage undergo non-lytic propagation mechanisms, where all of the particles of the phage coat must be exported through the bacterial inner membrane before assembly of the mature phage particle. Only proteins that are stable enough to endure such propagation mechanisms are able to be displayed (Danner and Belasco, 2001). Lambda and T7 lytic phage can be used to avoid this limitation. Lytic phage undergo different propagation mechanisms in which capsid assembly occurs exclusively in the cytoplasm, which avoids the limitations of protein export. Another advantage to using lambda phage is their ability to display larger proteins at high densities (Zucconi *et al*, 2001). Despite some of these limitations, the Ff bacteriophage allows for a highly flexible platform for display and is a very robust virus. The Ff bacteriophage, such as M13, allow for display on any of its five coat proteins (Sidhu, 2001; Rodi and Makowski, 1999). P3 of the M13 bacteriophage allows for display of a small number of larger proteins where p8 allows for large numbers of smaller proteins (Reviewed in Willats, 2002).

1.3 M13 Phage Biology

1.3.A Structure of Ff bacteriophage

The Ff bacteriophage genome is a single stranded DNA molecule composed of 6400 nucleotides encased in a semi-flexible protein cylinder. One end of the bacteriophage contains 5 molecules of gene VII protein (PVII) and gene IX protein (pIX)

and the other end of the phage contains 5 molecules of gene III and gene VI proteins (pIII and pVI). The packaging signal (PS) of the DNA is found at the end of the phage where coat proteins pVII and pIX are located. This is the first part of the phage to be assembled (Reviewed in Webster, 2001). The other end of the phage is composed of 5 molecules each of pIII and pVI. Coat protein pIII is made up of three domains designated N1, N2 and CT. N1 is required for translocation of the DNA into the cytoplasm and helps aid in the insertion of coat proteins into the membrane during infection. N2, the second domain, functions in helping bind to the F pilus (Deng *et al*, 1999). On the host bacteria N1 and N2 interact with each other to form a knob-like structure at the pIII site of the phage. N1 and N2 are essential for the infectivity of the phage and if one of these domains is removed the phage is rendered noninfectious (Gray *et al*, 1981; Armstrong *et al*, 1981). CT, the third domain of pIII, is essential for phage particle stability (Crissman and Smith 1984; Kremser and Rasched 1994).

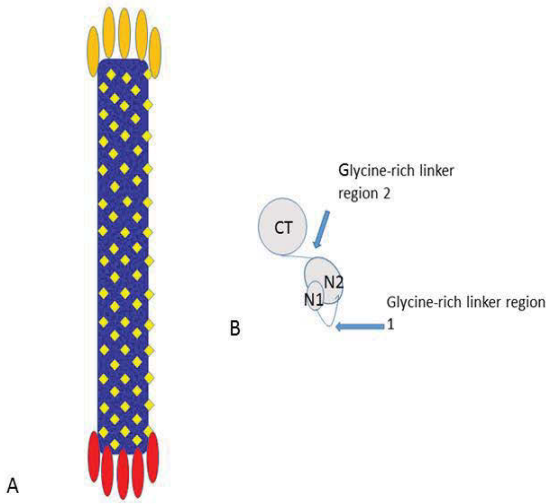

Fig.1.**A**: **Filamentous Bacteriophage Structure.** Filamentous phage is a rod-like virus consisting of circular ssDNA genome. Filamentous phage consists of several thousand copies of major coat proteins. PVIII is the major coat protein in between two minor coat proteins, PIX on one end and Plll on the other end. **B**: Representation of minor coat protein PIII. Two N-terminal domains, consisting of N2 and N1. Their major role is phage infectivity. They aid in binding of the F-pilus and TolA membrane protein of the bacterial host during conjugation. The C-terminal domain (CT) is essential for virion assembly at the inner bacterial membrane. It aids in capping together PIV and the capsid end (reviewed in Bratkovic, 2009).

1.3.B Infection Process

The F pilus, a protein tube composed of pilin subunits, is essential in the infectious process of the bacteriophage. Infection is a multistep process involving the F conjugative pilus and the bacterial cytoplasmic membrane proteins TolQ, R and A (Webster, 2001). The F pilus is required for the transfer of DNA, termed conjugation, and is initiated by the tip of the F pilus interacting with the bacterial outer membrane. The F pilus retracts resulting in the donor and recipient cells drawing together to assist the processes required for replication and the transfer of a single strand of the F plasmid DNA. Tol bacterial proteins Q, R and A are necessary for maintaining the integrity of the bacterial outer membrane. These bacterial Tol proteins are essential for translocation of the phage coat proteins into the cytoplasmic membrane and for translocation of the phage DNA into the cytoplasm during the infection process (Russel *et al*, 1988; Click and Webster 1998). Infection begins when the F pilus binds to the N2 domain of the phage pIII protein, though the exact binding site on the N2 domain is not known (Deng *et al*, 1999). The pilus is withdrawn after the phage has bound to the F pilus, resulting in the transport of the pIII end of the phage particle to the periplasm (Reviewed in Webster, 2001). Once binding of domain N2 to the F pilus is initiated, domains N1 and N2 are released which allows N1 to interact with domain 3 of protein TolA (TolA-D3). TolA-D3 is considered the coreceptor and the tip of the F pilus is the receptor for phage attachment. In order for interaction of the F pilus with TolA-D3 to occur, the N2 domain of pIII must displace the N1 domain. The F pilus' most important function is to bring the phage into a position where TolA-D3 can interact successfully with pIII-N1 domain. It

can also be concluded that bacterial proteins TolQ, R and A are absolutely essential for DNA entrance into the cytoplasm and coat protein into the membrane (Russel *et al*, 1988; Click and Webster 1997).

1.3.C Assembly Process

The phage assembly process occurs at both inner and outer membranes, and requires five capsid proteins, three assembly proteins, ATP, a proton motive force and thioredoxin, a bacterial protein (Reviewed by Webster, 2001, Feng *et al*, 1997). The inner and outer membranes, which are in close contact with each other, are termed the bacterial adhesion zones and this is the exact location where assembly takes place (Lopez and Webster 1983). The process of phage assembly has been divided into three process'; initiation, elongation, and termination.

1.3.C.1 Initiation

Initiation is the starting point of replication or translocation and involves proteins pVII and pIX, as well as pVIII molecules to interact with the DNA packaging signal (PS). The reason for this mechanisms results from the fact that this is the first part of the phage to appear from the bacterium host (Lopez and Webster 1983). PS located on single-stranded circular DNA allows it to be encapsulated into the phage and thus is essential for efficient phage assembly (Reviewed by Webster, 2001).

1.3.C.2 Elongation

Proceeding initiation, the phage will become elongated, resulting from a set of reactions that replace pV dimers with PVIII as the DNA is expressed through the membrane. ATP hydrolysis and thioredoxin aid in the interaction of the positively charged carboxy-terminal portions of pVIII molecules and DNA while the

transmembrane components interact with one another to form the capsid tube (Reviewed in Webster, 2001). As the process of elongation continues, the phage particles are transported through the pIV exit pore. The construction of the phage is complete and ends once the end of the DNA is reached.

1.3.C.3 Termination

The CT domain of pIII is required for the release and termination processes of phage assembly. The CT domain is composed of two subdomains which are both involved in termination of assembly (Rakonjac *et al*, 1999). Termination, a two-step process, first involves pVI and pIII interactions with pVIII molecules that are assembled around the end of the DNA to form a complex known as the pretermination complex. After the pre-termination complex is formed, structural change occurs in the CT region, allowing pIII to dissociate from the membrane. The CT region of pIII is anchored in the membrane at the end of the phage particle. Consequently, this CT region must flip around to interact with the phage to be the first part of pIII to exit. (Reviewed in Webster, 2001)

1.4 Display of proteins and peptides on coat proteins

The M13 phage is composed of five coat proteins; pIII, pVI, pVII, pVIII and pIX, that express multiple binding sites for proteins and peptides, all of which have been used for displaying foreign peptides, proteins, and antibody fragments (Sidhu, 2001; Rodi and Makowski, 1999). Most commonly, the M13 phage displays proteins and peptides that are fused to either pVIII or pIII coat proteins (Reviewed in Webster, 2001). Display on pVIII is limited to peptides containing 6 to 8 amino acids of length.

One reason for size constraints on pVIII may be that the rate of successful targeting and translocation of chimeric pVIII into the membrane is affected by

interference of the inserts. Large numbers of proteins smaller in size can be displayed on pVIII, while larger proteins can be displayed on coat protein pIII (Reviewed in Willats; 2002). PIII is the most commonly used capsid protein for display of peptides and proteins. There are multiple disadvantages and advantages to go along with displaying molecules on the pIII surface. PIII molecules that contain large inserts package into the phage significantly well, however, the phage can only display five peptides. Insertion of peptides and proteins take place between the signal sequence and the beginning of the first domain (N1) of pIII. Insertion places the foreign protein at the end of the packaged particles which creates less steric hindrance when it passes through the exit pore pIV (Reviewed in Webster, 2001). Large inserts create problems for the bacteriophage. These large inserts lower phage infectivity and could possibly make the phage non-infective, which limits the ability to select particular proteins to be displayed (Parmley and smith, 1988). Two N-terminal domains (N1 and N2) and one C-terminal domain (CT), make up the PIII coat protein. The ability of the bacteriophage to carry out the infection process requires N2 and N1 N-terminal domains. They aid in the binding of PIII to the TolA membrane protein and to the F pilus of the bacterial host. The C-terminal domain helps virion assembly on the inner bacterial membrane by capping the end of the capsid together with pVI (Willats, 2002). Foreign proteins may also be inserted between N-terminal domains N1 and N2, as well as in between CT and N2 domains of pIII. When inserting in between these domains, infectivity is retained but lowered.

1.5 Phage displaying peptide libraries

George P. Smith introduced the idea of using bacteriophage coat proteins to display random peptides. He showed that a peptide could be fused to the minor coat

protein pIII and bind to a ligand on a surface, the process called panning. By fusing random peptides to coat proteins, they can be screened by panning to identify peptides that bind to a ligand of choice (Reviewed in Scott, 2001). Since George Smiths discovery, phage displaying peptide libraries have expanded and can now be categorized into two different libraries; Synthetic and recombinant-displayed libraries. Synthetic peptide libraries are synthesized from monomeric components and can be made from natural or unnatural amino acids. Recombinant libraries are encoded by oligonucleotides that have been introduced into RNA, phage or phagemid genome or a receptor-encoding plasmid. This introduction allows each peptide in the library to be physically linked to a nucleic acid which can be amplified and decoded by DNA sequencing. There are advantages and disadvantages in both peptide libraries. Synthetic peptide libraries offer the advantage of having libraries that can include a wide variety of non-amino acid units which add chemical variations to the library and some synthetic libraries can be screened in as little as one day. Recombinant-displaying libraries offer the advantage of displaying longer peptides but have drawbacks of poor display and/or production of said peptides. Nonetheless, both libraries are suitable for targeting peptides against biological targets of choice.

1.6 Ph.D. Phage Display Library

The Ph.D. phage library we have selected (Ph.D.-7, New England Biolabs) uses the M13 phage vector to display five peptides composed of seven amino acids that are fused to the N-terminal end of the minor coat protein pIII. Coat protein II is found at one end of the M13 virion and is responsible for initiating the infection process by binding to the F-pilus of the bacterial cell. It is important that the peptides being displayed on PIII

are shorter than 50 amino acid residues, as phage infectivity can be affected by larger peptides. Coat protein pIII libraries offer reduced valency when compared to pVIII libraries. This allows the Ph.D. system to be more suitable for higher affinity ligand binding and the library contains 10^9 clones unique peptides. This library has been constructed to contain peptides that allow for binding sequences against multiple proteins which include: enzymes, cell-surface receptors, and monoclonal antibodies. There are several advantages with the Ph.D. library which include: its ability to produce multiple DNA sequences which encode the peptide to be displayed on the surface of the phage and its versatility which allows it to be used for applications in epitope mapping, anti-microbial/viral peptides, small molecule binders and enzyme substrates. More importantly, it has been used to discover bioactive peptides by using *in vivo* and *in vitro* biopanning approaches which include: peptide antagonists and cell targeting peptides.

1.7 Ecoli ER 2738

The host strain we will be using is *E.coli* ER2738 which is an F+ strain that has a rapid growth rate and allows for M13 production. Our M13 phage is a male-specific coliphage (infect bacteria that contain the F plasmid), which requires the use of *E.coli* which has been grown on selective media for the presence of the F-factor. This F-factor of ER2738 contains a mini-transposon, which is a segment of DNA that has a repeat of insertion sequence elements at each end. The mini-transposon within the F-factor includes genes for tetracycline resistance, so cells holding the F-factor can be chosen by plating and multiplying the bacterium in tetracycline-containing medium (PHD Phage Displaying Libraries).

1.8 Human Serum Albumin

Human Serum Albumin (HSA) is one of the most abundant proteins in human plasma and has astonishing ligand binding capabilities (Reviewed in Fasano *et al*, 2005). The unique structural organization of HSA allows for a vast number of ligand binding sites. Since HSA have these defined qualities, it remains an important protein for our research. If we can isolate bacteriophage with bound peptides that are specific for HSA and can readily detect HSA, we can couple HSA specific peptides to carbon nanotubes. Once the peptides are coupled to carbon nanotubes, the carbon nanotubes containing peptides will be developed into a patch that military personnel can wear in major vital organ areas. When the solider becomes wounded, blood will spill onto the developed patch, HSA specific peptides will detect the presence of HSA leading to a radio transmission that alerts emergency medical personnel.

1.9 Types of Carbon Nanotubes and their Applications

Binding of peptides to Carbon nanotubes (CNTs) was previously done in 1991 by physicist, Sumio Ijima in order to obtain a peptide that recognizes nanographite structures such as carbon nanotubes and carbon nanohorns (Seker and Demir, 2011). This and other work has had led to advances in technological and biomedical applications. Carbon nanotubes (CNTs) are composed of seamless cylinders of graphene sheets rolled up to construct a tube. These sheets possess properties of chemical, physical, and mechanical integrities that have rapidly gained interest in the past decade (Reviewed by Liu, 2009). There are two different types of nanotubes and their graphene layers characterize the structure of the two. Single-walled carbon nanotubes (SWNTs), the carbon nanotube of choice for coupling our peptides and multi-walled carbon nanotubes (MWNTs). SWNTs

have the advantage of being flexible enough to facilitate multiple binding sites, which in turn, improves binding affinity of nanotubes with targeted ligands. Carbon nanotubes (CNTs) have shown to be semiconductive depending on the arrangement of the walls of the tubes and the diameter and helicity (Odom *et al,* 1998). Carbon nanotubes need to be functionalized in some way in order for them to be useful in the biomedical field. Functionalization of CNTs has a profound effect on the electronic and electrochemical properties of CNTs when they are used for nanosensors and nanoelectronic devices (Sanz *et al*, 2012).

CNTs, engineered by the Chemical Engineering Department at Youngstown State University will be used in our study to couple to our peptides that have been selected for specific binding properties to HSA. The major application of our peptides and CNTs will be tailored to the use of soldiers in the battle field. Patches containing CNTs coupled to peptides with HSA binding specificities will be worn by soldiers and when activated by blood, will be able to alert emergency medical professionals of the location to send aid to the wounded soldier.

Chapter 2: Materials

Phage display peptide library kit (cat. # E8100S) was purchased from New England Biolabs (Beverly, MA; www.neb.com). SA-HRP (cat. # S-2438), Triton X-100 (cat. #T-6878), chicken serum albumin (CSA) (cat. # A3014), rabbit serum albumin (RbSA) (cat. #A0764), bovine serum albumin (BSA) (cat. #A-1009), albumin from chicken egg white (OVA) (cat. #A5503), yeast extract (cat. #Y4250), tetracycline hydrochloride (cat. #T3383), tetramethylbenzidine (TMB) (cat. #T0440), glycerol (cat. #G5516), dimethyl sulfoxide (DMSO) (cat. #D2650), sodium chloride (cat. #S271-1), sodium phosphate monobasic (cat. #S5011), sodium phosphate (cat. #S5136) were purchased from Sigma Chemical Company (St. Louis, MO). Casein in TBS blocking buffer (cat. #37532), polyacrylamide desalting columns (cat. #43426), EZ-Link® Hydrazide-PEG_4-Biotin (cat. #21360), N-morpholinothene sulfonic acid (MES) BupH MES buffered saline pack (cat. #28390), Pierce graphite spin column (cat. #88302), Avidin (cat. #21121), 4'-hydroxyazobenzene-2-carboxylic acid (HABA) (cat. #28010), 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), trifluoroacetic acid (TFA) were purchased from Thermo Scientific (Rochester, NY). 96 well Polyvinyl chloride plates (cat. #353912) was purchased from Corning (NY). 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) (cat. #0428) agarose for gel electrophoresis and EZ-vision 3 dye were purchased from Amresco (Solon, OH). Non-fat dry milk was purchased from Food Club. Calcium chloride dihydrate was purchased from Mallinckredt (cat. #AB008814). Wizard® *Plus* SV Minipreps DNA Purification System. GenomeLab Dye Terminator Cycle Sequencing with Quick Start Kit was purchased from Beckman Coulter® (Brea, CA).

Chapter 3: Methods

3.1 Bacterial growth and storage

E.coli host strain ER2738 (F' *proA+B+lacIq* Δ*(lacZ)M15 zzf::Tn10 (TetR)/fhuA2 glnV* Δ*(lac-proAB)* Δ*(hsdMS-mcrB)5* [rk-mk-McrBC-]) was streaked onto LB agar plates containing 10µg/ml of tetracycline to insure only ER2738 E. coli that express the F plasmid (which contains the tetracycline resistance gene) would grow and incubated overnight(ON) at 37ºC. The Plate was then wrapped with parafilm and stored at 4ºC in dark conditions for a maximum of 1 month (Chung *et al,* 1989).

3.*2 Determining ER2738 mid logrhythmic phase*

E.coli host strain ER2738 was incubated in 40ml of LB broth and 40µl of 20mg/ml tetracycline was added to the mixture. The medium containing the broth and tetracycline was incubated at 37ºC with shaking at 250 rpm.An overnight culture was added (800 µl) to a 300ml culture flask and incubated at 37ºC with shaking at 250 rpm for 6 hours. Mid log phase growth was determined by monitoring absorbance at 595 nm.

3.3 Phage Amplification

E.coli host strain ER2738 cells (from an LB tet plate) were inoculated into 20 ml of LB-tet media in a 20ml erlenmeyer and incubated ON at 37ºC shaking at 250 rpm. The following day, 40ml of Terrific broth with 40µl of 20mg/ml tetracycline in a 125 ml flask was inoculated with 800µl of the ON culture and incubated at 37ºC with shaking at 250 rpm until the cells reached early log phase ($OD_{595} = 0.01 - 0.05$). Once cells reached early log phase, the mixture was shaken slowly (100 rpm) at 37ºC for 10 minutes to allow sheared F pili to regenerate. Phage library particles ($4x10^9$ PhD 7 NEB library) were added to 20ml cells (approximately 10^{10}cells in 10 ml) and mixed by swirling.

Incubation at 37ºC with shaking at 250 rpm continued until the OD reached .4-.5 at 595nm. The bacteria cells were removed by centrifugation at 4,500 x *g* for 10 minutes and the supernatant was transferred to a fresh tube. Centrifugation was repeated and the top 32 ml of the supernatant was transferred to a new tube. A small portion (500 µl) of supernatant was saved and mixed with 500 µl of glycerol for storage at -20^{o}C. Eight ml of 2.5 M NaCl/20% PEG-8000 (w/v) was added to the remaining solution. The phage were precipitated by placing the sample on ice for 1 hour. Centrifugation at 12,000 x *g* at 4ºC for 15 minutes was then performed to pellet the phage. The supernatant was removed and the pellet was resuspended in 2 mL of TBS, transferred to an Eppendorf tube and spun briefly to remove any cell debris. The supernatant was then transferred to a fresh tube and 200 µL of 2.5 M NaCl/20% PEG-8000 was added and the tube was incubated on ice for 15-60 minutes. The mixture was spun at 12,000-14,000 rpm in a benchtop centrifuge for 10 minutes, the supernatant was discarded, spun briefly again and the remaining supernatant was removed by using a pipette. The pellet was resuspended in 200 µL TBS and 200 µL of sterile glycerol for storage at -20^{o}C and labeled Amp B (Maniatis *et al,* 1982).

3.4 Phage Titering

Phage titering experiments were performed to determine the number of pfu/ml (plaque forming units) in our amplified aliquots. A water bath was prewarmed to 45ºC while LB plates were incubated at 37ºC until ready for use. Top agar was melted using an autoclave and 3 mL of top agar was dispensed into sterile culture tubes, one per expected dilution. Culture tubes were maintained at 45ºC until needed. Xgal (40mg/ml) and IPTG (40mg/ml) were placed into each culture tube containing 3ml of top agar. Serial 100 fold

dilutions of the phage were performed. The phage dilution (20 µL) was transferred into 200 µL ON bacteria, vortexed and incubated for 1-5 minutes. The infected cells were transferred to the culture tubes containing top agar, Xgal and IPTG, vortexed, then poured onto LB-tet plates. The plates were cooled at room temperature, incubated overnight at 37ºC and then blue plaques were counted (Barbas *et al*, 2001).

3.5 ELISA

An ELISA was performed to determine if our library possessed phage that had peptides specific for human serum albumin (HSA). A Polyvinyl chloride plate was used and 100 µL of 10 µg/ml of HSA in 0.05 M Na_2CO_3(pH 9.6) buffer was coated onto the test and blank wells ON at 4ºC. Positive control wells were coated with 100 µL of $2x10^{11}$ pfu/ml phage in Na_2CO_3. To the No HSA wells, 200 µL of block buffer (1% Casein in TBS) was added. 230 µL of block (1% Casein in TBS) was added to the pre-adsorbtion wells and the plate was incubated at room temperature for 2 hours in a humidified chamber. The coating solution was aspirated and all wells except the No HSA well were washed once with wash buffer (PBS). Test, Blank, and Positive control wells were filled with 200 µL of blocking buffer (1% Casein-TBS). Phage ($2x10^{11}$ pfu/ml in 200 µL TBS) was added to pre-adsorption wells. The plate was incubated at 37ºC for 30 minutes. The blocking buffer was aspirated from the Test and No HSA wells which were then coated with 100 µL of pre-adsorbed phage. Sample buffer was added to the Blank and Positive Control wells (Phage only) and the plate was incubated at room temperature for 2 hours. The wells were washed 6 times with wash buffer (PBS) and 100 µL of 1:5000 diluted anti-M13 Monoclonal Peroxidase conjugate in PBS-0.1% Casein was added to all wells, then incubated for 2 hours at RT. The microplate was washed 6 times

with wash buffer and 100 µL of 3,3',5,5',-Tetramethylbenzidine substrate solution was added to all wells. The plate incubated at room temperature until a suitable blue color formed. Stop solution (50 µL 2N H_2SO_4) was added and absorbance was read at 450nm using a microplate reader (Dennis *et al*, 2002)

3.6 Amp B HSA Biopanning

A biopanning procedure was conducted to isolate phage that have high specificity and affinity for human serum albumin (HSA). A solution of 10 µg/ml of the target (HSA) in 0.05 M $NaHCO_3$, pH 9.6, was prepared and 100 µl of this solution was added to each of 6 wells of the 96 well microtiter plate. Block solution (230 µL casein only), was added to 2 pre-adsorption wells and the microtiter plate was stored at 4°C ON in a humidified container to coat wells with target. Following an ON incubation, all wells containing HSA were aspirated and filled with 230 µl of blocking buffer (1% Casein-TBS in 4 wells for the first and third panning and 1% OVA-TBS in 2 wells for the second panning). For the Pre-adsorption wells, a 100-fold representation of the library ($2x10^{11}$ pfu) was diluted with 100 µl of PBS + 0.1% casein. Blocking solution was aspirated from the two casein only pre-adsorption wells, followed by washing the wells 6 times with PBS. Phage were added to the pre-adsorption wells, rocked gently for 2 hours at room temperature and then transferred into the first panning wells (wells blocked with casein). For the first round of panning, the first two wells containing HSA and casein were washed 10 times with PBS. Phage from the pre-adsorption wells were transferred to the first panning wells (wells blocked with casein) and rocked gently at RT for 2 hours. The non-binding phage were aspirated. Phage that bound to the wells were eluted by adding 100 µl of elution buffer (500 mM KCl and 10 mM HCl, pH2) and gently rocked for 5 minutes at RT. The

eluate was transferred into a microcentrifuge tube and neutralized with 25 µl of 20 M Tris-HCl, pH 8. For P1 and P2 binding phage, a small amount (~ 1 µl) of the eluate (P1 or P2) was saved and stored in 100 µl of PBS in fridge to titer later. For P2 and P3, specific (binding) phage were transferred to the next panning wells and the procedure was repeated. The P3 eluate was saved and titered to obtain the pfu/ml. Plaques from the P3 plate were picked, DNA was extracted and sequenced to determine the amino acid composition of the peptides expressed by the phage. (Aaron K.Sato *et al*, 2002)

3.7 RF M13 Isolation

After P3 plaques were extracted from the tittered plates, an RF M13 Isolation procedure was performed to obtain DNA for sequencing. An ON culture of ER2738 in 2ml of LB was prepared and 500 µL of ON was used to inoculate 50ml of Terrific Broth. The mixture was incubated and shaken for 2-3 hours at 37°C. Phage (50 µl) was added to the ER2738 Terrific Broth culture and grown for 5-6 hours at 37°C while shaking. The supernatant was spun for 10 minutes at 10K x *g*. Supernatant was removed, stored in fridge and pellet was drained and frozen ON at -20°C.

3.8 Wizard® Plus SV Minipreps DNA Purification System

DNA purification was performed using a Wizard® *Plus* SV Minipreps DNA Purification System kit. A DNA pellet that was frozen ON was resuspended with 250 µl Cell Resuspension Solution (50mM Tris-HCL (pH 7.5), 10mM EDTA and 100µg/ml RNase A). Cell Lysis Solution (250 µl; 0.2M NaOH and 1% SDS) was added to each sample and the sample was inverted 4 times to mix. Alkaline Protease Solution (10µl) was added and the tube was inverted 4 times to mix. The solution was incubated for 5 minutes at room temperature. Neutralization Solution (350µl; 4.09M guanidine

hydrochloride, 0.759M potassium acetate, 2.12M glacial acetic acid) was added to the tube and the tube was inverted 4 times to mix. The solution was centrifuged at 14k x *g* for 10 minutes at room temperature. A spin column was inserted into a collection tube and the lysate was added to the column. The spin column was centrifuged at 14k x *g* for 1 minute at room temperature, solution flowthrough was discarded and the column was reinserted into a collection tube. Wash Solution (750µl; 162.8mM potassium acetate, 22.6mM Tris-HCl (pH 7.5), and 0.109mM EDTA (pH 8.0) 35ml of 95% ethanol) was added and the column was centrifuged at 14k x *g* for 1 minute. Flowthrough was discarded and the column was reinserted into a collection tube. The wash solution step was repeated with 250 µl of Wash Solution. The column was centrifuged at 14k x *g* for 2 minutes at room temperature and the spin column was transferred to a sterile microcentrifuge tube. Nuclease-Free Water (100µl) was added to the spin column and centrifuged at 14k x *g* for 1 minute at room temperature. The purified DNA was then stored at -20°C and used for DNA sequencing (Wizard® *Plus* SV Minipreps DNA Purification System quick protocol by Promega).

3.9 Gel Electrophoresis

Agarose (1%) in Tris-phosphate buffer (0.08M with 0.002M EDTA,TPE) was prepared and cooled to 50°C. The warm agarose solution was poured into a mold and the comb was clamped into the mold to form sample wells. The gel sat at room temperature for 30-45 minutes and the comb was removed. TPE was added to the electrophoresis tank to cover the gel to a depth of 1mm. The samples were mixed with loading buffer/tracking dye (EZ vision 3 dye) (Amresco) and loaded into the slots of the gel. The gel was run at

60 V for 1-2 hours and the DNA strands were visualized using UV-illumination (Molecular Cloning).

3.10 DNA sequencing

Ed Budde, Molecular Biology Instrumentation Specialist in the Molecular Biology Analytical Core Laboratory at Youngstown State University, OH, performed DNA sequencing using the GenomeLab™ Dye Terminator Cycle sequencing with the Quick Start kit. To prepare the DNA sequencing template, 260 ng of DNA was mixed with water to make a final volume of 10 µl. Primers, 96 gIII and 28 gIII (2µl of 1pmol/µl) were added, followed by 8.0µl of DTCS Quick Start Master Mix, bringing the total volume to 20µl. The contents were mixed thoroughly, then briefly centrifuged and added to a thermal cycling machine for 30 cycles at 96°C for 20 seconds, 50 °C for 20 seconds and 60°C for 4 mins. Following thermal cycling, 5µl of Stop solution (Sodium Acetate 2µl of 1.2 mM, pH 5.2, 2 µL of 40 mM $Na_2 - EDTA$, pH 8.0, and 1 µL of 4 mg/ml of glycogen) was added. The sequencing reaction was transferred to microfuge tubes and mixed thoroughly. Cold 95% ethanol/dH20 (60 µL) was added to the sequencing reaction and mixed thoroughly, followed by immediate centrifugation at 14,000 rpm at 4°C for 15 minutes. Following centrifugation, the supernatant was removed with a micropipette. The pellet that remained was rinsed 2 times with 200µL of 70% ethanol/dH20 with intermediate centrifugation at 14,000 rpm at 4°C for a minimum of 2 minutes. Following centrifugation, the supernatant was removed with a micropipette. The microfuge tube was vacuum dried for 10 minutes and the pellet was resuspended in 40 µL of Sample Loading Solution (GenomeLab™ Dye Terminator Cycle sequencing with Quick Start kit). The sequencer samples were separated into appropriate wells in the sample plate and the plate

was added to the sequencer (Beckman Coulter CEQ™ 2000XL DNA Analysis System). The information was sent back in an SCF format and analyzed in Gene Studio (Gene Studio.com)

3.11 Biotinylation of peptide BR-1

Immediately before use, 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. Biotin hydrazide (50 mM, 25 µL) solution and 1ml of peptide solution (0.5 mg/mL = 50 µL of 10 mg/ml + 950 µL H2O) was added to a glass test tube, mixed and rocked. The EDC solution prepared previously (100 mg/mL) 3 µL was added to the reaction, mixed and rocked overnight. Sample was diluted 1:1 in 2.5% TFA and added to a Pierce Graphite Spin Column and placed into a 1.5 mL screw cap microfuge tube. The column was centrifuged at 2,000 × *g* for 1 minute to remove storage buffer. 1M NH_4OH (100µL) was added and centrifuged at 2,000 × *g* for 1 minute, flow-through was discarded and this step was repeated. Acetonitrile (100µL) was added to the column and centrifuged at 2,000 × *g* for 1 minute, followed by discarding flow-through. 1% TFA (100µL) was added to column and centrifuged at 2000 × *g* for 1 minute. The flow-through was discarded and this step was repeated one more time. The sample was diluted 1:1 in 2.5% TFA and 500 µL of the sample was added to the spin column. The column was placed into a new collection tube and binding was allowed for 10 minutes with periodic vortex mixing to keep the graphite suspended as much as possible. The column was centrifuged at 1,000 × *g* for 3 minutes and the flow-through was discarded. The column was placed into a new collection tube and washed by adding 200µL of 1.0% TFA, followed by centrifugation at 2000 × *g* for 1 minute. The flow-through was

discarded and this step was repeated once. The column was placed into new collection tube, 100µL of 0.1% formic acid in 50% acetonitrile was added and centrifuged at 2000 × *g* for 1 minute. The eluate was transferred to a microfuge tube. This step was repeated three more times using the same collection tube for a total elution volume of 400µL (x 2 = 800 µL). The sample was surrounded by foil to maintain and conduct heat and dried in a vacuum evaporator (a plastic bell jar placed on a heating block at a low setting, with a vacuum attached, Fisher Maxima Dry 15 psi, 40 L/min, 65 torr.). The sample was suspended in 1 ml MES after evaporation.

3.12 Biotin Incorporation Assay

Biotinylated peptide and biotinylated HSA concentrations were measured performing a biotin incorporation assay. Using a spectrophotometer, 900 µl of the 4'-hydroxyazobenzene-2-carboxylic acid avidin (HABA/avidin) solution was added to a cuvette and mixed thoroughly on a shaker plate. The absorbance was measured at 500 nm and recorded as A_{500} HABA/avidin. MES (100 µl) was added to the well containing the HABA/avidin, mixed and the absorbance was measured again and the value was recorded as A_{500} HABA/avidin/biotin. For the sample measurements, 900 µl of the HABA/avidin solution was added to a cuvette and the absorbance was measured at 500 nm and recorded as A_{500} HABA/avidin. Biotinylated sample, 100 µl (Peptide, 0.5 mg/ml, was diluted by taking 10 µl sample in 90 µl MES) was added to the well containing the HABA/avidin and mixed thoroughly by pipetting and shaking gently. The absorbance was measured again and the value was recorded as A_{500} HABA/avidin/biotin. This method was repeated for the biotinylated HSA.

3.13 Peptide ELISA

An ELISA was performed to determine if our biotinylated peptide BR-1 is specific for human serum albumin (HSA) (Dennis, *et al*, 2002). A Polyvinyl chloride plate was used and 100 µL of 10 µg/ml of HSA in 0.05 M Na_2CO_3(pH 9.6) buffer was coated onto the test, streptavidin and antibody blank wells ON at 4ºC. Positive control wells were coated with 100 µL of $2x10^{11}$ pfu/ml phage in Na_2CO_3. To the No HSA wells, 200 µL of block buffer was added (Non-fat dry milk (NFDM) 5% in PBS) was added in one series, 1% Yeast Extract in PBST in another series of wells, and 5% Normal Calf Serum in 1X PBS + 0.1% Triton X-100 in another series of wells. The plate was incubated ON at 4^{o}C. The coating solution was aspirated and all wells except the No HSA well were washed once with wash buffer (PBST). Test, Blank, and Positive control wells were filled with 200 µL of blocking buffer and incubated for 1 Hr at RT. Blocking buffer was aspirated and the wells were washed 3 times with wash buffer. Following the washes, 100 µg/mL biotinylated peptide in PBST was added to the test and No HSA wells, PBST was added to the blank and positive control wells and the plate was incubated for 2 hours at RT. Wells were aspirated, washed 6 times with wash buffer and 100 µL diluted (1:5000 in sample buffer) HRP/anti-M13 monoclonal antibody was added to the positive control and Ab blank wells and a 1:10,000 dilution of HRP-Streptavidin (Sigma) in sample buffer was added to the test (+ HSA), no HSA, and SA blank wells and incubated at 4^{o}C ON. The microplate was washed 6 times with wash buffer and 100 µL of 3,3',5,5',-Tetramethylbenzidine substrate solution (Sigma) was added to all wells. The plate was incubated at room temperature until a suitable blue color formed. Stop solution (50 µL 2N H_2SO_4) was added and absorbance was read at 450nm

using a microplate reader (An ELISA using BR-1 against various albumins was performed using the same protocol but with 1% yeast extract in PBST as the blocking buffer.

Chapter 4: Results

Three rounds of biopanning against HSA with PhD 7 phage library were performed in order to select for clones specific for binding to human serum albumin (HSA). To further increase specificity toward HSA, a preadsorbtion step against casein blocking buffer was added to remove casein specific phage. Following preadsorption, the remaining phage were panned in wells coated with 10 µl HSA and blocked with 1% Casein in TBS for panning rounds 1 and 3, with and 1% OVA in TBS for round 2. Switching blocking buffers each round offers the removal of blocking buffer specific phage, resulting in increased phage specificity towards HSA. The phage were not amplified between panning rounds, as previous studies using amplification between rounds resulted in high levels of non-specfic phage (Sang *et al*, 2014). Biopanning was successful in enriching phage specific for HSA and yielded phage titers of $4.x10^4$ pfu/ml following panning round 3 prior to amplification of the panned phage.

Ten plaques were picked from the 3rd round of panning (P3), amplified and PEG purified. The titers for the purified and amplified clones HSA 2,3 and 6 were $4.1x10^{13}$, $3.85x10^{13}$, $5.0x10^{13}$ pfu/ml, respectively. In order to confirm that the phage clones bind specifically to HSA following amplification, an ELISA was performed and the results are shown in Figure 1. After the wells were seeded with HSA, blocking buffer (1% Casein in TBS) was added to the wells. After incubation, $2x10^{11}$ pfu/ml of phage were added and the plate was incubated at 37°C for 30 minutes. Binding of phage was detected by the addition of anti-M13 monoclonal peroxidase conjugate followed by the addition of TMB substrate. Absorbance was measured at 450nm wavelengths. Testing of HSA phage clones 2,3 and 6 yielded results that confirm specificity for HSA. HSA 6 (Figure 2.)

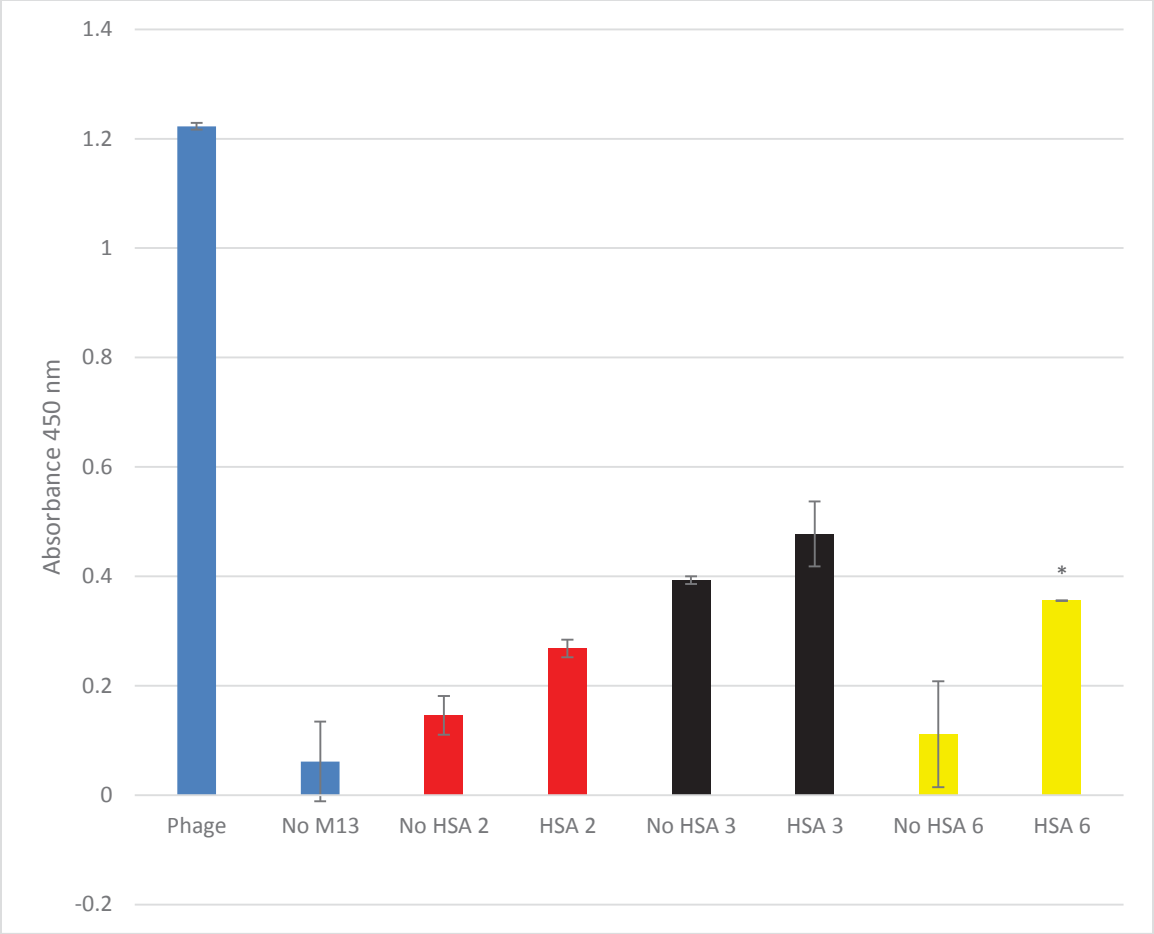

Fig.2. M13 ELISA of HSA Specific Phage Clones 2, 3 and 6. Following 3 rounds of panning and amplification, phage clones were picked and tested for binding to HSA, as compared to no HSA (non-specific binding), by ELISA. Positive control (phage) is amplified PhD7 library bound directly to the polyvinyl chloride plate with sodium carbonate buffer. The negative control (No M13) is HSA without phage library. After the wells were incubated with HSA, blocking buffer (1% Casein in TBS) was added to the wells. After incubation, $2x10^{11}$ pfu/ml of phage were added to the pre-adsorption wells and the plate was incubated at 37ºC for 30 minutes. Following incubation, the pre-adsorbed phage were moved to the No HSA and HSA wells, incubated for 2 hours at RT, and the wells were washed with washing buffer. Anti-M13 Monoclonal Peroxidase

conjugate was added to the wells and incubated. (TMB) 3,3',5,5',-Tetramethylbenzidine substrate solution was added as a color changing substrate and stopped with 2N H_2SO_4 and the absorbance measured using a microplate reader. All clones selected demonstrated specific binding to HSA. Absorbance from the No M13 Blank wells was subtracted from the absorbance of all other conditions prior to data calculation. HSA 6 absorbance = $0.356 \pm .059$ SD. The figure shows the data from one of two similar experiments. Each condition was performed in triplicate and the data is expressed as the mean +/- the standard deviation. The P value was obtained by performing a two tailed T test comparing HSA vs No HSA average absorbance values. (* = P value ≤ 0.05). This data is representative of the data seen in three separate experiments.

showed the best results based on binding specificity (HSA 6 absorbance = 0.417 ± .059 SD, $P \leq 0.05$).

DNA purification was performed using the *Wizard® Plus SV Minipreps DNA Purification System* by following the manufactures instructions. Gel electrophoresis was performed using 1% agarose in Tris-phosphate buffer (0.08M with 0.002M EDTA,TPE) to determine the molecular weight and integrity of the DNA (Figure 3). Using the GenomeLab™ Dye Terminator Cycle sequencing with Quick Start kit, DNA sequencing was performed using the 96 gIII and 28 gIII sequencing primers provided by the library manufacturer to determine the composition of peptides HSA 2,3 and 6 (Table 1).

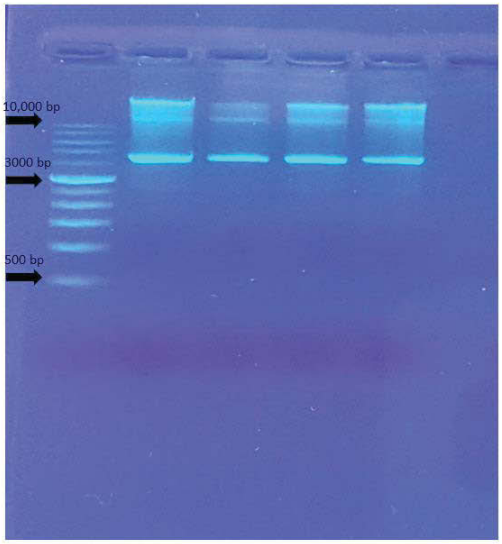

Fig.3. Gel Electrophoresis of HSA Clones 2,3,4 and 6. RF M13 phage DNA was extracted from clones 2,3,4 and 6 and purified to determine the DNA integrity and molecular weight. Lane 1 is the 10kb DNA ladder, lane 2 is phage clone 2, lane 3 is phage clone 3, lane 4 is phage clone 4, and lane 5 is phage clone 6.

Phage Clone	Peptide Sequence
2	NTPHTSA
3	FYSKQAI
6	ANHHQAS

Table 1. DNA Sequences of Phage Peptides from HSA 2,3 and 6. DNA sequencing was performed on three phage clones demonstrating specificity for HSA.

DNA purification and sequencing showed that HSA 6 peptide variant had no plastic binding amino acid residues (Trp, Tyr) (Adey *et al*, 1995). HSA also possessed hydrophobic residues to aid in peptide binding to HSA (Sato *et al,* 2002). Due to the sequence results and the specificity shown in the ELISA, phage clone 6 peptide was chosen for synthesis (BR-1 from NEO Scientific).

To detect peptide BR-1 binding in an ELISA, the peptide was biotinylated and a colorimetric biotin incorporation assay was performed. Briefly, MES was added to a HABA/avidin solution, mixed thoroughly and placed into a cuvette to measure the absorbance of the blank using a spectrophotometer at 500nm. Following the blank, BR-1 was diluted in MES, mixed thoroughly in HABA/avidin solution and the absorbance was measured at 500nm. To obtain the percent biotinylation, the following equation was used:

$$\%\ Biotinylated = \frac{\text{Abs of HABA avidin MES blank} - \text{Abs of HABA avidin biotinylated sample}}{\text{Amount of peptide added}} x\ 100$$

Biotinylation of peptide BR-1 yielded results of 110.46% biotinylation. We suspect the biotin percentage was above 100% because of free biotin remaining in the sample.

An ELISA was performed to determine if biotinylated peptide BR-1 was specific for HSA. Briefly, HSA was seeded in the wells of a polyvinyl chloride plate, the No HSA wells were blocked with three different blocking buffers: 5% NFDM in PBS, 1% yeast extract in PBST and 5% normal calf serum in PBS and 0.1% triton x-100 and incubated overnight at 4°C. Following incubation, the wells were washed and the HSA wells blocked using the aforementioned blocking buffers. Anti-M13 peroxidase antibody was used to detect the positive control (M13). Streptavidin-peroxidase was used to detect biotinylated peptide bound. TMB, 3,3',5,5',-Tetramethylbenzidine substrate solution was added to induce a color change and stopped using 2N H_2SO_4, and absorbance read at

450nm. Multiple blocking buffers were used in order to verify which blocking buffer was best suited for blocking non-specific binding. The results were significant in that BR-1 showed high specificity for HSA, when compared to the no HSA wells using all blocking buffers (Figure 4).

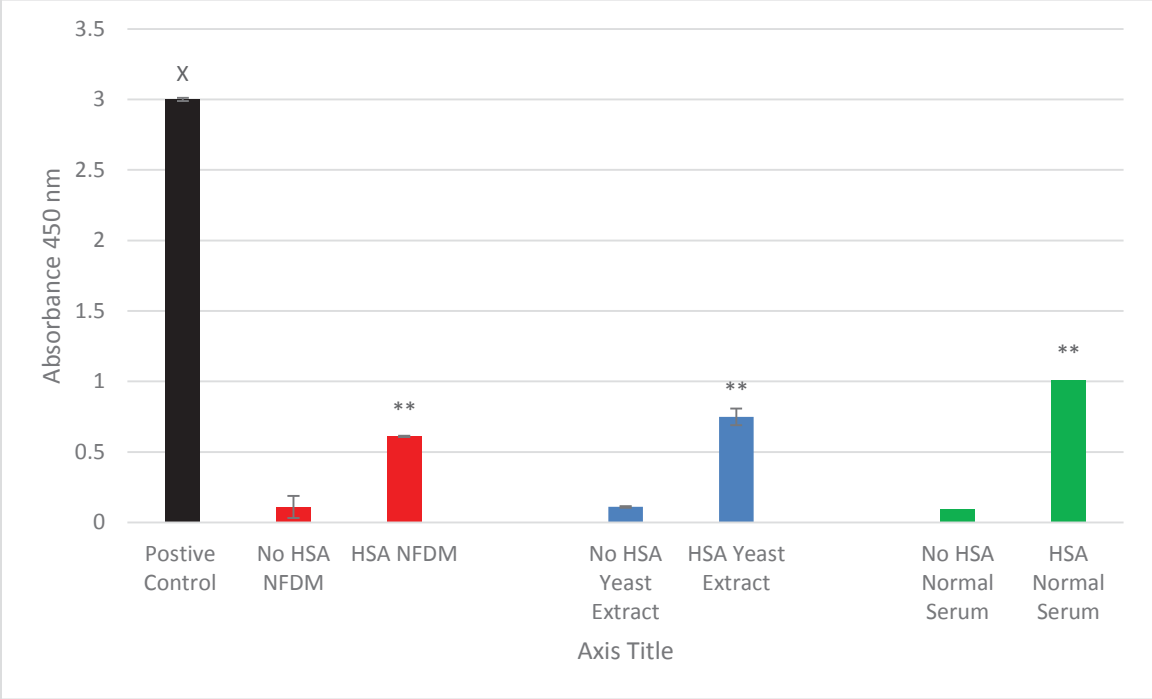

Fig.4. Peptide BR-1 ELISA Blocking Buffer Comparison. Peptide BR-1 was biotinylated and its specificity was demonstrated by performing a peptide ELISA. Briefly, HSA was seeded in the wells of a polyvinyl chloride plate, the No HSA wells were blocked with three different blocking buffers: 5% NFDM in PBS, 1% yeast extract in PBST and 5% normal calf serum in PBS and 0.1% triton x-100 and incubated overnight at 4°C. Following incubation, the wells were washed and the HSA wells blocked using the aforementioned blocking buffers. Anti-M13 peroxidase antibody was used to detect the positive control (M13). Streptavidin-peroxidase was used to detect biotinylated peptide bound. TMB, 3,3',5,5',-Tetramethylbenzidine substrate solution was added to induce a color change and stopped using 2N H_2SO_4, and absorbance read at 450nm. Absorbance from the No M13 Blank wells was subtracted from the absorbance of all other conditions prior to data calculation. Binding of biotinylated peptide BR-1 to

HSA is shown with 5% NFDM blocking buffer (absorbance = 0.609 ± 0.078 SD), 1% yeast extract + PBST blocking buffer (absorbance = 0.749 ± 0.059 SD), 5% normal calf serum blocking buffer (1.011 ± 0.059 SD). The figure shows the data from one of three similar experiments. Each condition was performed in triplicate and the data is expressed as the mean +/- the standard deviation. The P value was obtained by performing a two tailed T test comparing HSA vs No HSA average absorbance values of each blocking buffer used (** = P value ≤ 0.01). X= Absorbance greater than 3.

The results were a highly significant ($p \leq 0.01$) when binding to no HSA was compared to binding to HSA using each of the blocking buffers in at least one of three assays. However, results were significant in all three trials when using the 1% yeast extract in PBST blocking buffer, so subsequent ELISAs were performed using 1% yeast extract as the blocking buffer.

In order to determine whether peptide BR-1 was specific for HSA or also bound to various other albumins, a peptide ELISA was performed to examine binding of BR-1 to human serum albumin (HSA), chicken serum albumin (CSA), chicken egg white albumin (OVA), bovine serum albumin (BSA), and rabbit serum albumin (RbSA) (Figure 5).

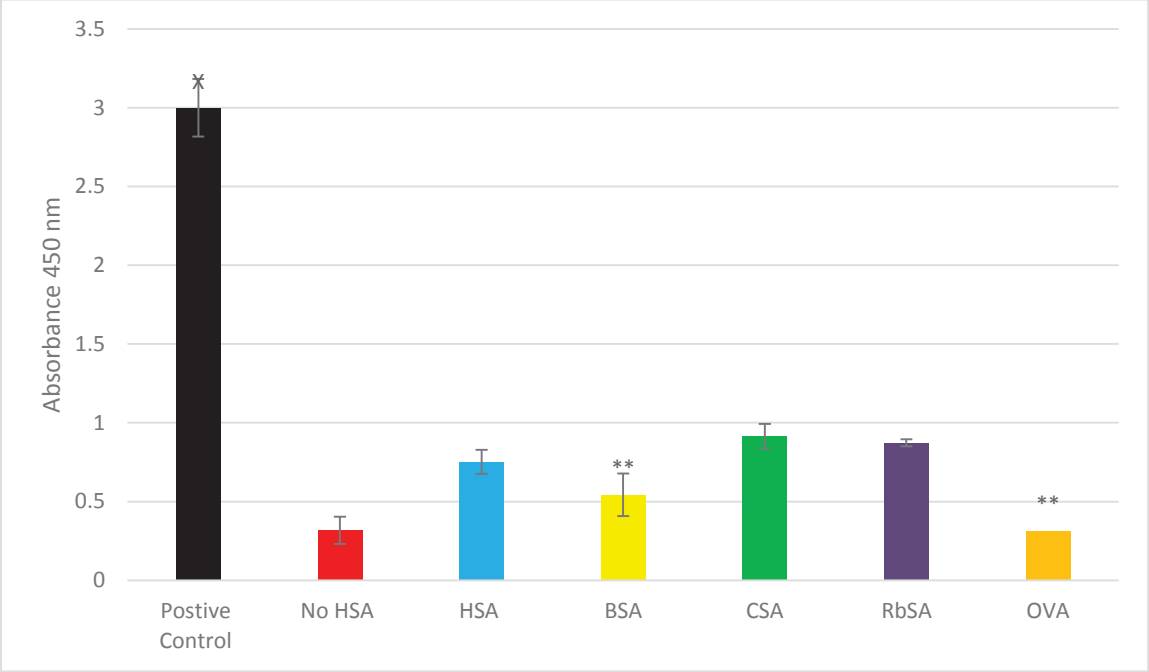

Fig.5. **BR-1 Peptide ELISA against Various Albumins.** Peptide BR-1 was biotinylated and its specificity was demonstrated by performing a peptide ELISA. HSA was placed in the wells of a polyvinyl chloride plate, the No HSA wells were blocked with three different blocking buffers: 5% NFDM in PBS, 1% yeast extract in PBST and 5% normal calf serum in PBS containing 0.1% triton x-100 and incubated overnight at 4°C. Following incubation, the wells were washed and the HSA wells blocked using the aforementioned blocking buffers. Biotinylated peptide BR-1 was added to all wells, except the positive control. Anti-M13 peroxidase antibody was used to detect the positive control (M13). Streptavidin-peroxidase was used to detect biotinylated peptide bound. TMB, 3,3',5,5',-Tetramethylbenzidine substrate solution was added to induce a color change, stopped using 2N H_2SO_4, and the absorbance was read at 450nm. Absorbance from the No M13 Blank wells was subtracted from the absorbance of all other conditions prior to data calculation. Binding of biotinylated peptide BR-1 to HSA is shown with 5%

NFDM blocking buffer (absorbance = 0.609 ± 0.078 SD), 1% yeast extract + PBST blocking buffer (absorbance = 0.749 ± 0.059 SD), 5% normal calf serum blocking buffer (1.011 ± 0.059 SD). The figure shows the data from one of three similar experiments. Each condition was performed in triplicate and the data is expressed as the mean +/- the standard deviation. The P value was obtained by performing a two tailed T test comparing HSA vs No HSA average absorbance values of each blocking buffer used (** = P value ≤ 0.01). X= Absorbance greater than 3.

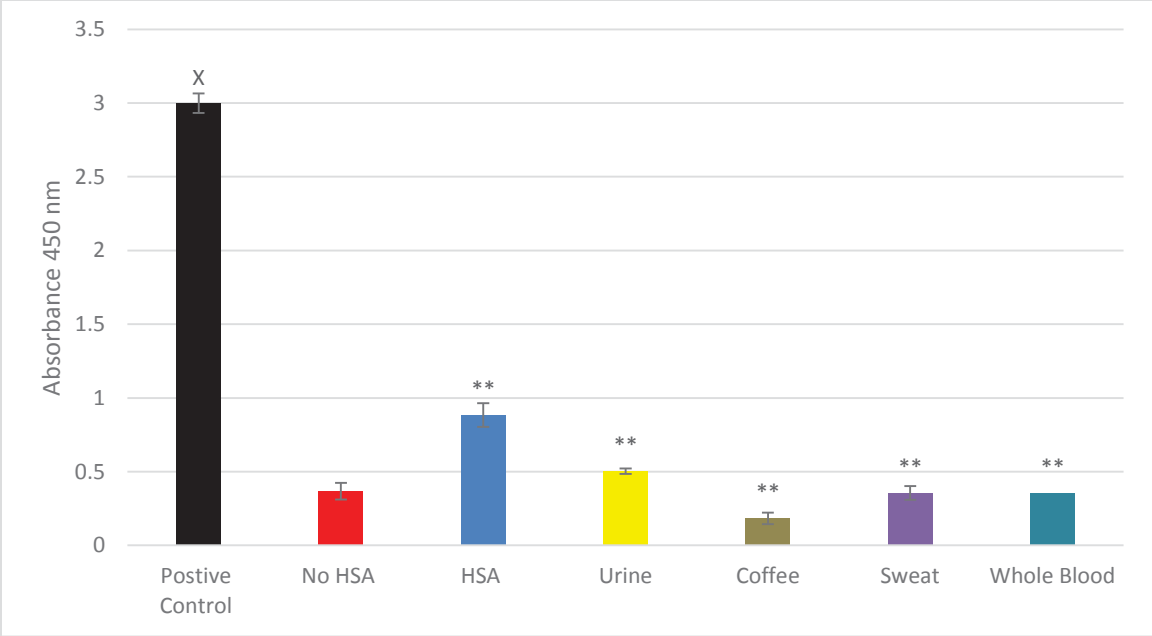

Fig.6. BR-1 Peptide ELISA against Various Solutions. An ELISA was performed to determine binding of peptide BR-1 to various solutions, as described in Figure, with the minor modification of the use of 1% yeast extract and PBST blocking buffer. Absorbance from the No M13 Blank wells was subtracted from the absorbance of all other conditions prior to data calculation. Absorbance for No HSA = 0.368 ± 0.067 SD, Urine = 0.504 ± 0.081 SD, Coffee = 0.183 ± 0.019 SD, Sweat = 0.355 ± 0.040 SD, Whole Blood = 0.355 ± 0.047 SD. The figure shows the data from one of two similar experiments. Each condition was performed in triplicate and the data is expressed as the mean +/- the standard deviation. The P value was obtained by performing a two tailed T test comparing HSA vs No HSA and HSA vs urine, coffee, sweat, and whole blood average absorbance values. (** = P value ≤ 0.01). X= Absorbance greater than 3.

Peptide BR-1 showed binding specificity towards HSA, when compared to all solutions with high significance (p value ≤ 0.01), as demonstrated using a two tailed T test.

Chapter 5: Discussion

Phage display is a technique that allows for the display and identification of proteins, peptides or antibody fragments against various biological targets (Zhou *et al*, 2015, Bhardwaj *et al*, 1994, Dennis *et al*, 2002, Shanmugam *et al*, 2011). In this study we have used phage display technologies for the identification of peptides that bind to human serum albumin (HSA) for the development of sensors that detect injury in military personnel. HSA is one of the most abundant proteins in human plasma and boasts astonishing ligand binding capabilities (Reviewed in Fasano *et al*, 2005). HSA is synthesized in the liver and is exported into the blood at concentrations of 7.0×10^{-4} M. It's high concentrations make it a perfect candidate as a target for peptide binding detection of blood. To achieve the goal of identifying peptides that bind to HSA, the PhD 7 library was used. The library contains bacteriophage that each express 5 identical peptides (seven amino acids in length) on the minor coat protein pIII. Each phage displays a unique randomly synthesized peptide and the library contains 10^9 unique peptides displayed on phage. We selected for phage that bind to HSA by performing solid-state panning of phage against purified HSA coated on poly-L lysine treated tissue culture plates. Our biopanning procedure was unique, in that amplification was not performed between panning rounds but was performed after all three rounds were completed. Sang and coworkers (Sang *et al,* 2014) discovered that, when selecting for HSA specific phage, intermediate amplification led to enrichment of non-specific binding phage. To avoid this limitation, our library was screened against HSA for three rounds with amplification only after the third panning round. Also noteworthy was the addition of a pre-adsorption step prior to panning. We exposed the library to wells containing only

casein block prior to starting the first panning step. This allowed us to actively remove blocking buffer specific phage. Tween-20 incorporated into washing buffers and blocking buffers is a common practice, but has been shown by Hakami and coworkers (Hakami *et al,* 2015) to promote non-specific binding to polystyrene plates. Hakami and coworkers investigated the use of detergents, polyethylene glycol and their effects on binding interactions of the bacteriophage to the target of interest. An ELISA using varying concentrations of the detergents; Tween-20, Triton-X 100, and Igepal CA-630 mixed with bacteriophage clones was performed and phage binding was examined. The authors determined that the use of detergents can result in nonspecific binding of phage particles to the surface of the wells. To circumvent this issue and to promote enrichment of phage towards HSA, we did not include Tween-20 in our wash buffer. We also employed blocking techniques that switch blocking buffers every round using Casein in TBS, OVA in TBS and Casein in TBS sequentially. With the addition of these techniques we were able to successfully enrich for phage containing peptides specific to HSA. Following panning we had $4.x10^4$ pfu/ml of phage prior to amplification.

When tittering the phage from the third round of panning, plaques were picked, amplified and PEG purified. An ELISA was performed on 10 picked phage clones to demonstrate binding specificity to HSA (Figure 1). Clones 2,3 and 6 demonstrated binding specificity towards HSA with phage clone 6 having the highest specificity with a p value of ≤ 0.05 (Fig.1) When comparing binding to wells containing no HSA. The others were excluded from the results because of poor binding specificity (data not shown). Phage clones 2,3 and 6 were chosen for RF M13 DNA extraction, DNA purification and sequencing to determine the amino acid composition of the peptides. The

peptide sequences are shown in Table 1. The amino acid composition of the peptides is important in the promotion of high affinity and specific binding to HSA. Sato and coworkers (Sato *et al*, 2002) used phage display to identify cyclic peptide ligands for the use of affinity purification of HSA. An ELISA was performed to investigate the dissociation constants of the varying peptide loops to HSA. To do this, Sato and coworkers created alanine mutant peptides and tested binding affinity to HSA in various pH levels and unexpectedly found that peptides containing alanine mutants bound HSA with higher affinity than all other peptides tested. Additionally, they demonstrated that alanine residues at key positions in the peptide may aid in peptide binding to HSA. Our phage clones all contain alanine residues, with clone HSA 6 containing two alanine residues and demonstrating the greatest binding specificity.

Adey and coworkers isolated plastic binding phage by performing three rounds of panning with one amplification step immediately following the first round of panning and compared the absorbance of plastic binding phage to specific phage library particles by performing an ELISA. The ELISA compared absorbance values of plastic binding phage to a normal library of phage when using the various washing solutions; Tween-20, milk BSA and PBS, and blocking solutions; none, BSA or milk. The authors found that plastic binding phage bound more specifically to the polystyrene microtiter plate when using non-ionic detergents; Tween-20, NP-40 and Triton X-100. Also, an acid elution procedure after one round of panning was performed to demonstrate phage recovery of plastic binding phage compared to specific phage library particles and the results demonstrated that the amino acid residues tyrosine and tryptophan promote plastic binding and should be avoided (Adey *et al*, 1995). Based off of these studies, we were

selective in choosing which peptide to synthesize and move on with future experiments. Phage clones HSA 2, 3 and 6 all contained 2 or more hydrophobic residues with clone HSA 3 containing one tyrosine plastic binding residue. Because of the tyrosine residue, HSA 3 was excluded from our selection. Clone HSA 6 contained 2 alanine residues, no plastic binding residues and performed successfully in the ELISA experiment. Phage clone 6 peptide was therefore chosen to be synthesized (manufactured by NEO scientific) and was named BR-1.

A peptide ELISA was performed to confirm the specificity of peptide BR-1 to HSA. In other studies using phage display (Tang *et al,* 2013, Zhou *et al,* 2015), BSA in PBS is used as the primary blocking buffer, however HSA is similar in morphology and functionality, so the traditional BSA blocking buffer was not an option. When investigating the use of this ELISA, three different blocking buffers were used (5% NFDM, 1 % yeast extract in PBST and 5% normal calf serum) to determine which buffer blocked non-specific binding the best (Fig. 3). Briefly, HSA was seeded in the wells of a polyvinyl chloride plate, wells were blocked with one of the previously mentioned blocking buffers, peptide Br-1 was incubated in wells containing HSA and the absorbance values were measured and compared. Previous studies have employed similar strategies to determine the variability in ELISA results when using multiple blocking buffers such as (Vogt *et al,* 1987). Vogt and coworkers tested 8 different blocking buffers including instant dry milk, similar to the 5% NFDM used in our experiment, and demonstrated that instant dry milk, among 2 others tested, was their preferred blocking buffer. Loizou and coworkers (Loizo *et al,* 1985) performed an ELISA to measure anti-cardiolipin antibodies using 10% fetal calf serum as the blocking buffer, similar to our

5% normal calf serum, and succeeded in eliminating non-specific binding. Yeast extract (1%) blocking buffer was also tested in these studies, as it was available in the lab and it contains proteins and amino acids which are important for blocking nonspecific binding. The results showed that the peptide was specific for HSA when using all blocking buffers, giving us highly significant p values (≤ 0.01) for all three. We decided to continue our future ELISA experiments using 1% yeast extract in PBST, because it was the most consistent throughout the experiments (three similar experiments with all conditions tested in triplicate).

An albumin ELISA was developed to determine if peptide BR-1 is specific for only HSA, or if it would also bind to other albumins. Rabbit (RbSA), bovine (BSA), chicken egg (OVA), chicken serum (CSA) albumins were tested and compared to human serum albumin. Sato and coworkers performed a similar experiment comparing dissociation constants of two peptides for HSA over other albumins (bovine, goat, pig, rabbit, rat, mouse, chicken egg, and rhesus). The peptides were isolated using phage display and biopanning procedures, as previously described, to develop an improved method of affinity purification of HSA. Their peptide DX-236 bound with high affinity toward all albumins while DX-321 showed a strong preference for HSA. Both peptides had no significant binding to OVA, similar to the data seen with our peptide BR-1(Sato *et al,* 2002). The results for BR-1 showed similar binding to HSA, CSA and RbSA, while showing preference towards HSA over OVA and BSA (P value ≤ 0.01).

A solutions ELISA was performed to determine the specificity of BR-1 towards HSA compared to other solutions soldiers might come in contact with on a day to day basis urine, sweat, coffee and whole blood were tested. This is similar to our previous study by

(Cortes *et al,* 2013) which examined HSA, apple juice, black coffee and NaCl solution (sweat substitute). This study described our previous work using phage display to identify peptides that bind to HSA. In our previous studies, the peptide used was synthesized from a peptide sequence identified by the use of amine-functionalized POROS® particles by N-fluorenylmethoxycarbonyl (FMOC) chemistry (Pingali *et al,* 1996). They successfully proved that peptide Gk-1 bound with high specificity towards HSA when compared to apple juice, coffee and NaCl solutions, showing a similar trend to our data. Peptide BR-1 bound to HSA with high significance ($p \leq 0.01$), when compared to all other solutions. However, BR-1 did not bind to whole blood, as would be expected. One reason for this could be the amount of proteins and cells in whole blood are causing blocking of the peptide binding to HSA. We anticipate better results in future studies using an electrical response to BR-1 peptide, as BR-1 had similar binding specificity to GK-1. Our previous studies also were able to successfully demonstrate that peptide GK-1 had a unique and specific electrical response to whole blood compared to other solutions. This final experiment will be a future aim of our study with peptide Br-1.

In conclusion, we were able to successfully isolate peptide BR-1 using phage display technology and biopanning procedures, and to demonstrate its specificity towards HSA. Our results suggest intermediate amplification between biopanning rounds isn't necessary for a successful biopanning experiment and should be considered as an alternative procedure to circumvent enrichment of nonspecific binding phage. Like HSA specific peptides tested by Adey and coworkers (Adey *et al,* 1995), our peptides have hydrophobic and alanine residues and do show increased binding specificity towards HSA when performing an ELISA to detect nonspecific binding to other solutions. When

comparing blocking buffers: 5% NFDM, 1% yeast extract in PBST and 5% normal calf serum, all are suitable choices for sufficient blocking of nonspecific binding, with the yeast extract blocking buffer demonstrating the most consistent results. Future experiments include the purification and expression of peptide BR-1 in a soluble form using a pMAL vector and a competitive ELISA will be performed to demonstrate at what concentration of HSA the peptide binding is inhibited. Solid state detection will also be performed to confirm peptide Br-1 blood detection when compared to other solutions. With successful solid state detection the use of BR-1 bound to carbon nanotubes will be investigated as a possible mechanism for producing biosensor to be worn on major vital organ areas for all military personnel.

Chapter 6: References

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