THE USE OF PHAGE DISPLAY TO IDENTIFY SPECIFIC PEPTIDE LIGANDS

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ABSTRACT

Phage display allows the expression of peptides on filamentous phage when the peptide of interest is fused to the gene for a virus coat protein. The goal of this project was to use phage display to produce peptide molecules that bind specifically to human serum albumin. An M13 library displaying random linear heptapeptides linked to coat protein pIII (Ph.D.-7^{TM}, New England BioLabs) was amplified by infecting *E. coli* host strain ER2738 to produce phage library stocks Amp LA ($2.1x10^{12}$ pfu/ml) and Amp LS ($1.0x10^{9}$ pfu/ml). The amplified phage were titered on LB plates containing X-gal, IPTG, and tetracycline to ensure expression of the F pilus. Three rounds of biopanning on HSA-coated polyvinyl chloride plates and amplification of the virus was performed (Amp P3A and Amp P3S). Individual plaques were picked and amplified to produce clones (HSA1-HSA8). We developed a phage concentration ELISA to test for the quantity of phage needed to detect binding in an ELISA using peroxidase-conjugated antibodies against M13. Phage were diluted in sodium carbonate to bind the protein to the plate, followed by block and anti-M13-PO. It was found that a concentration greater than 10^{6} pfu/ml was needed to give a positive M13 ELISA. We then tested the conditions needed to produce an ELISA that measures phage binding to a specific ligand (M13 ELISA). Three different plates were tested. Although MaxiSorp® plates are thought to give a good signal and more consistent data, we found that specific binding of Amp LA was best when using polyvinyl chloride plates. The influence of blocking buffer (ovalbumin and casein) was also tested. Casein block and sample buffer gave a good signal in the presence of HSA with the least non-specific binding. These studies demonstrate that phage display is an effective method for producing HSA-binding peptides.

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LIST OF SYMBOLS AND ABBREVIATIONS

Ab	antibody
Amp LA	Amplified phage library stock LA
Amp LS	Amplified phage library stock LS
Cat.	catalog
CDRs	complementarity determining regions
DHFR	dihydrofolate reductase
DMF	dimethylformamide
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dsDNA	double stranded deoxyribonucleic acid
dsFv	disulphide stabilized single chain variable fragments
dsRNA	double stranded ribonucleic acid
E. coli	*Escherichia coli*
ELISA	enzyme-linked Immunosorbent Assay
F pilus	fertility pilus
Fab	antigen binding antibody fragment
g	gravitational
H	heavy chain
HAT	hypoxanthine-aminopteri-thymidine
HGPRT	hypoxanthine-guanine phosphoribosyltransferase
HSA	human serum albumin

Ig	immunoglobulin
IPTG	Isopropyl β-D-1-thiogalactopyranoside
L	light chain
LB	Luria broth
mAb	monoclonal antibody
MWNTs	multi-walled nanotubes
NKT	Natural killer T cell
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PEG	poly (ethylene) glycol
pfu	plaque forming unit
pII	protein II
pIII	protein III
RF	replicative form
RNA	ribonucleic acid
rpm	revolution per minute
scFvs	single chain variable fragments
ssDNA	single stranded deoxyribonucleic acid
ssRNA	single stranded ribonucleic acid
SWNTs	single-walled nanotubes
TBS	tris buffered saline
T_C	T cytotoxic cell
Tet	Tetracycline

T_h	T helper cell
T_{reg}	T regulatory cell
V_H	variable domain, heavy chain
V_L	variable domain, light chain
X-gal	5-Bromo-4-chloro-3-indolyl β-D-galactopyranoside

CHAPTER I: INTRODUCTION

Phage display is a molecular technique used to select peptides with desired binding properties by studying the interaction of peptides or antibody fragments displayed on the surface of the phage with potential ligands (Cesareni, 1992). This technique was first discovered by George P. Smith in 1985, after he demonstrated the display of peptides on filamentous phage (Smith, 1985). A bacteriophage (phage) is a virus that infects bacteria. There are three types of bacteriophage; filamentous, lambda and T7 phage. The filamentous phage family includes three strains M13, f1 and fd. These strains are genetically and phenotypically similar. M13 filamentous phage is widely used for phage display (Haq *et al*., 2012). The utility of phage display in the selection peptides that will specifically bind to human serum albumin will be investigated in these studies.

In phage display, random peptide molecules are displayed on the surface of a bacteriophage by fusing a foreign protein gene to the phage coat protein gene. A collection of viruses, each having a unique peptide displayed, is called a phage library (Willats, 2002). Phage libraries are screened using affinity selection or bio-panning. This is a process which involves binding of the phage to an immobilized ligand, washing off unbound phage, elution of specifically bound phage and amplification of the ligand specific phage by growth in bacteria. The phage display cycle is performed three to four times to select a phage population that is highly enriched and with high binding specificity. The isolated individual clone's specificity can be analyzed by enzyme-linked immunosorbent assay (ELISA) and characterized by DNA sequencing (Hoogenboom *et al*., 1998). The unique DNA sequences are then analyzed using bioinformatics tools to select clones that can withstand different environmental conditions. The positive clones

can then be transferred to a protein expression vector and the ligand-specific proteins purified using affinity chromatography.

Prior to the development of phage display, manipulation of the natural immune response was the preferred method for the development of specific receptor (antibodies). The immune system produces specific antigen receptors that can be used to identify foreign molecules. Phage display has been developed to provide a less expensive and faster method to produce specific receptors for ligands.

1.1 PRODUCTION OF ANTIBODY BY THE IMMUNE SYSTEM

1.1.1 Adaptive (Acquired) Immunity

In the human body, adaptive immunity is the third line of defense against pathogens, following barrier, chemical, and innate defenses, such as inflammation and phagocytosis. It develops slowly, because a foreign particle, such as a virus, must bind to specific receptors on immune cells and activate the cells, a process requiring a few days to a week. The molecule that binds to the receptor is called an antigen. Due to the specificity of the antigen receptors, the adaptive immune response can identify and eliminate unique pathogens from among many pathogens. While the immune response is specific, it is also diverse, that is, there are millions of cells each having a unique receptor, each capable of creating billions of antibodies, secreted proteins that retain the specificity of the antigen receptor. Acquired immunity also has memory. This means that it is able to produce a heightened response against specific microorganisms when they are encountered a second time. It is also capable of discriminating between self and non-self antigens, allowing the prevention of potentially fatal autoimmune diseases (an immune

response to self molecules). The acquired immune response requires interactions between antigen presenting cells and lymphocytes. There are two different types of specific lymphocytes, B cells and T cells (Sue *et al*., 2012).

B lymphocytes mature in the bone marrow and are responsible for the humoral branch of the acquired immune system. When a naïve B cell (first response to antigen) encounters an antigen, it proliferates and differentiates into either memory B cells or effector cells called plasma cells. Memory B cells express membrane bound antibody molecules of the original antigenic specificity. They have a long life span, and are responsible for the heightened response that is seen in the second encounter with the same antigen. Plasma cells have a short lifetime, living only for about a week, when they secrete antibodies at a rate greater than two thousand molecules per second (Kuby, 1997).

The T lymphocytes mature in the thymus and they are differentiated from other lymphocytes by the presence of a T-cell receptor on the cell surface. They undergo differentiation during immune response and develop into effector T cells. There are several subsets of T cells, helper ($T_{h)}$, Cytotoxic (T_c), regulatory (Treg), each with different functions. T cells can stimulate the activities of leukocytes through cell-to-cell contact or cytokines activating cell mediated immunity. The interaction between the B and T cells is essential for antibody response to antigens (Sue *et al*., 2012).

1.2 ANTIBODY PRODUCTION

An antibody (Ab) or immunoglobulin (Ig) is a protein produced by plasma cells to identify and neutralize foreign organisms, antigen, such as viruses. Antibodies are Y-shaped and contain a paratope, region on an antibody that recognizes an epitope on an

antigen, allowing antigen antibody binding. Antibodies are composed of two identical heavy chains (H) and two identical light chains (L). The antibodies have been grouped into five different isotypes based on which heavy chain they have; gamma (γ)-IgG, alpha (α)-IgA, delta (δ)-IgD, mu (μ)-IgM and epislon (ε)-IgE (Buss *et al*., 2012). The constant region domains, found in the heavy chains, are responsible for effector function while the complementarity-determining regions (CDRs) found in the variable region of each heavy and light chain combine to form the antigen binding site (Buss *et al*., 2012). The structure of an antibody is depicted in Figure 1.

1.2.1 Monoclonal antibodies

Monoclonal antibodies (mAb) are antibodies that arise from a single clone of B lymphoid cells and bind to a single epitope. The two major methods of monoclonal antibody production are by the use of hybridoma technology and phage display technology. Monoclonal antibodies have allowed the development of diagnostic tools and treatments that are specific for one molecule (Edwards, 1981).

The advantages of monoclonal antibodies are: they can be used to mark, identify, test for and purify unknown antigens, they can be purified, radioactively labeled and used to locate molecules or cells within the body, and once cloned, they can be manufactured in large amounts at a low cost.

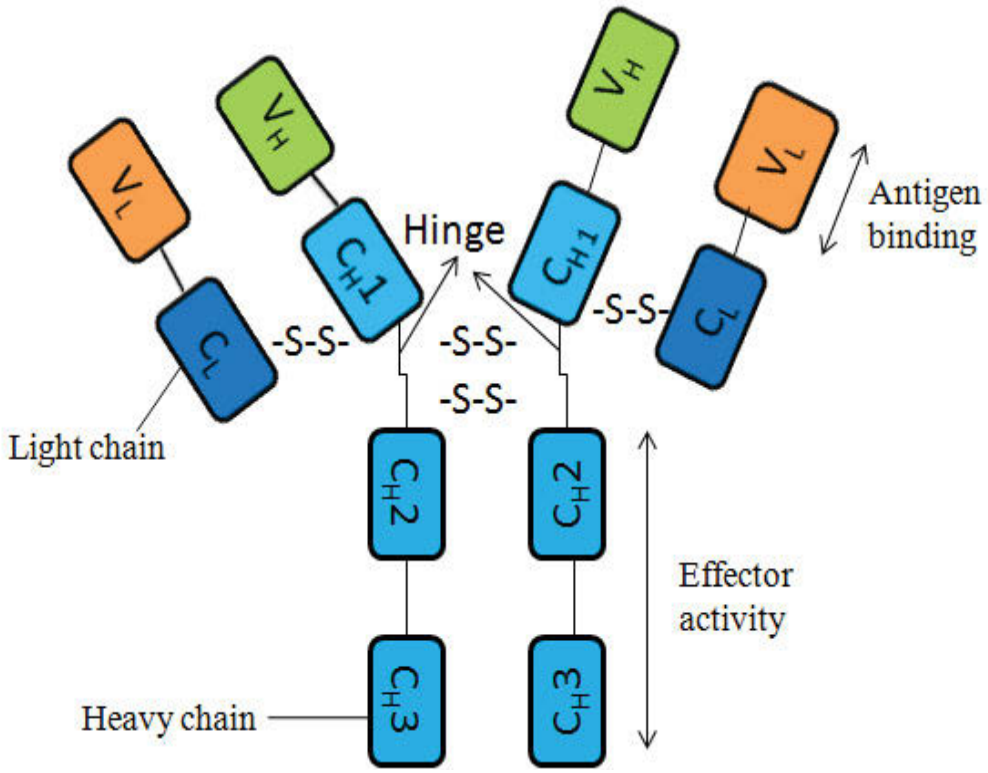

Figure 1: Structure of an antibody

The heavy (H) and light (L) chains contain a variable (V) region represented by green and tan segments, respectively. The blue and light blue segments represent the constant (C) regions of both the heavy and light chains. Some heavy chains contain the hinge region for flexibility. Antigen binds to an antibody at the antigen binding site (paratope) a region between the variable heavy chain (V_H) and variable light (V_L) chain. The effector function is mediated by the constant regions of the heavy chains.

The disadvantages of monoclonal antibodies are; they are expensive to produce and characterize, they are unable to differentiate groups of molecules if they share epitopes and they are large molecules requiring complex secondary, tertiary and quaternary structure

for function, making them less efficient in many practical procedures (Mason and Williams, 1980).

HYBRIDOMA TECHNOLOGY

1.3.1 Overview of Hybridoma Technology

Hybridoma technology was introduced in 1975, by George J. F. Kohler and Cesar Milstein, allowing the production of monoclonal antibodies that bind to a specific antigen (Köhler and Milstein, 1975). Hybridoma technology uses two types of cells, plasma cells and myeloma (cancerous plasma cells) cells. Plasma cells produce antibodies, while myeloma cells are capable of continuous proliferation (Ahmad *et al.,* 2012).

To produce monoclonal antibodies, a mouse is immunized with an antigen, and the spleen is removed. The spleen contains plasma cells producing antibodies against the antigen. These cells are then mixed with myeloma cells in the presence of polyethylene glycol (PEG), which acts as a fusion agent, to make hybrid cells. The hybrid cells will have both the properties of plasma cells and of the myeloma cells. The plasma cells have hypoxanthine-guanine phosphoribosyltransferase (HGPRT), an enzyme needed for the nucleic acid metabolism salvage pathway, which enables them to survive when incubated with aminopterine. However, B cells are not long lived cells. The myeloma cells are not able to grow in aminopterine, as they lack HGPRT, but they are able to grow for an

unlimited period of time in cell culture. Hybrid cells are selected from other unfused cells using hypoxanthine-aminopterin thymidine (HAT) medium, which allows only hybrid cells to grow (Pandey, 2010).

The HAT medium contains hypoxanthine, aminopterin and thymidine. The aminopterin inhibits dihydrofolate reductase (DHFR) enzyme which is necessary for nucleic acid metabolism by the *de novo* pathway. Therefore, the cell has to use the alternate salvage pathway, with the help of hypoxanthine and thymidine which provide the raw materials for DNA synthesis using this pathway. The unfused plasma cells undergo normal cell death as they are mortal cells. The HGPRT deficient cells, unfused myeloma, die in the presence of HAT medium as they cannot use the *salvage* pathway. The hybrid cells have HGPRT enzymes from the B cells; therefore they can survive in HAT medium. The hybrid cell can then be cloned to produce many daughter clones which eventually secrete monoclonal antibodies (Shanu *et al.,* 2011). This is depicted in Figure 2.

1.4 PHAGE DISPLAY

1.4.1 Overview of Phage Display Technology

Phage display technology is a laboratory technique used to study protein interactions using bacteriophage to produce the protein receptors. A link between the genotype and the phenotype of the phage is created by displaying proteins on the surface of the phage particle following fusion of the gene encoding the displayed protein to the phage coat protein gene (Smith, 1985).

This technique for the production and screening of proteins can be divided into three procedures: library creation, clone screening and selected clone analysis, a process called the phage display cycle (Hoogenboom *et al.*, 1998). Libraries of proteins are created by packaging a population of DNA for the displayed proteins fused to the coat protein gene of a phage or phagemid, a plasmid with phage origin of replication, (Clackson *et a*l., 1991). Clone screening and selection is done by a process called bio-panning or affinity selection. During this process, the phage library is exposed to a target and the phage with the appropriate specificity will bind to the immobilized target. The unbound phage are washed out by adjusting the detergent concentration and washing time (Petrenko and Smith, 2000).

The specific binders and non-specific binder are then eluted by either lowering or increasing pH to break non-covalent bond, using reducing reagents to disrupt the disulphide bond or by using enzyme cleavage for example trypsin cleavage. These three methods will disrupt the interaction between the phage and the target. The eluted phage is then amplified by infecting Gram negative bacteria, for example *Escherichia coli*. The amplified phage is less diverse and more enriched after three to four rounds of screening. The selected clones with the desired specificities are finally analyzed by enzyme-linked immunosorbent assay (ELISA) and sequencing (Willats, 2002).

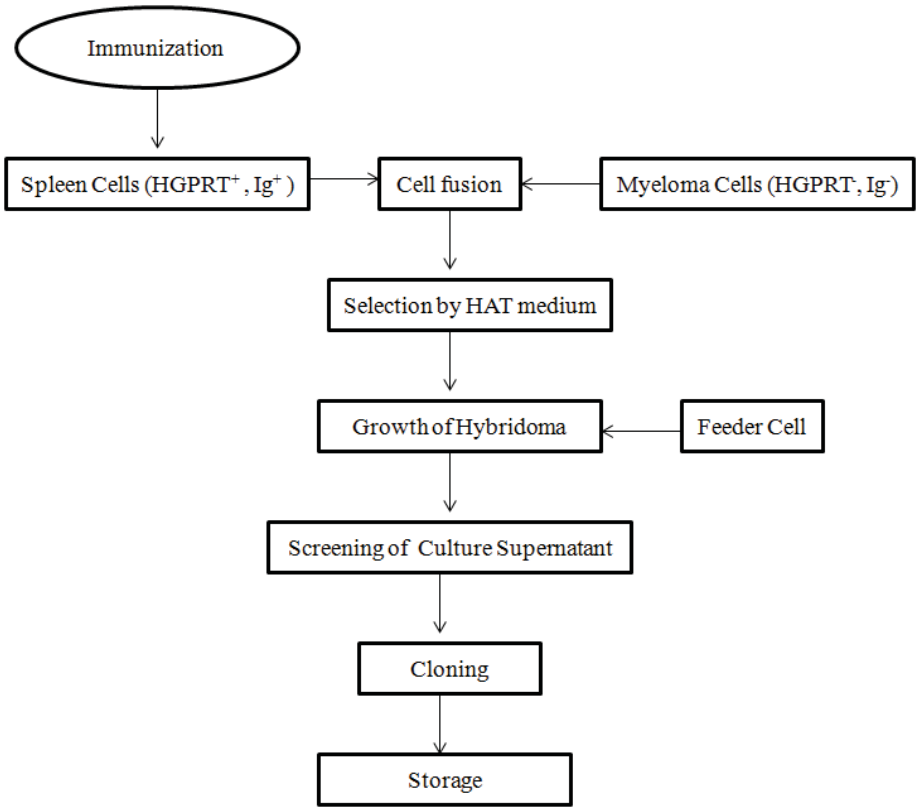

Figure 2: Hybridoma technology for the production of monoclonal antibodies

Immunization of mice is done three times before spleen cells extraction and preparation. The spleen cells collected have HGPRT and they secrete immunoglobulin (Ig). The spleen cells are then fused to myeloma cells (cancerous plasma cells) in the presence of polyethylene glycol (PEG). HAT medium is used to select unfused myeloma cells from hybrid cells. The unfused spleen cells die naturally, as they are short lived. The hybrid cells are then grown by feeding of the culture. The feeder cells help in the growth by providing growth factors. The culture supernatant is then screened by enzyme linked-immunosorbent assay (ELISA) to detect the antigen specific antibodies. The hybrid cells are cloned to assure that monoclonal antibodies are produced. The hybridoma cells can be stored in liquid nitrogen for several years with good recovery.

1.4.2 Hybridoma technology versus Phage Display Technology

Hybridoma technology was the first technique used to produce monoclonal antibodies. This technology has several limitations; hybridoma antibodies are mouse proteins, this limits their therapeutic use in human, as they will trigger human anti-mouse antibody reactions (Tjandra *et al*., 1990). This method is costly, time consuming and it is difficult to generate them against highly conserved and toxic antigens (Winter and Milstein, 1991). Hybridoma technology also has a low fusion efficiency, resulting in a limited number of monoclonal antibodies produced (Gorny, 2012).

Phage display technology is used to produce antibodies with properties that are difficult to obtain with hybridoma. The advantages of this *in vitro* technique include less turnaround time for antibody production and amplification of the antibody is greater, as it is performed in bacteria. The selection conditions can be controlled, for example by presentation of specific type of target, in contrast to animal immunization where there is little control over the produced antibodies. Phage display libraries contain diverse variants, as high as 10^{13} that can be screened against a wide range of biological and inorganic targets (Willats, 2012). This technique is cost effective, needs no special equipment, it is rapid to set up and the libraries can be purchased commercially (Whaley *et al*., 2000). However, the disadvantages of phage display include: it is a long, labor-intensive process and each bacteriophage displays only a few antibodies on its surface, yielding low numbers of monoclonal antibodies (Rondot *et al*., 2001).

1.4.3 Filamentous phage

Filamentous bacteriophage are viruses that belong to the genus Inovirus. These viruses have a circular DNA genome encased in a long protein capsid cylinder known as a coat and their genome may be dsDNA, ssDNA, dsRNA or ssRNA (Sidhu, 2001). Bacteriophage are viruses that infect Gram negative bacteria that contain a fertility factor that encodes conjugation pili. The phage binds to the pili and infects the bacterium and during infection they do not kill their host (Barbas III *et al.,* 2001).

There are three classes of filamentous phage; M13, f1 and fd, these viruses are all genetically and phenotypically closely related to each other (Rodi and Makowski, 1999). M13 is a phage that infects *E. coli.* It is the most studied filamentous phage and widely used in phage display technology. This is because of their small genome that makes them easy to manipulate. DNA can be inserted into non-essential regions resulting in the assembly of a longer phage particle. They can be produced in high amounts and are stable under harsh environments (Russel *et al.,* 2004).

1.4.4 M13 Bacteriophage Structure

The M13 phage is approximately 1μm in length and greater than 10 nm in diameter, containing a circular single-stranded DNA genome of 6407 nucleotides surrounded by coat protein. The protein coat is composed of two fusion protein pIII (minor coat protein) and pVIII (major coat protein). pVIII covers the length of the particle and contains approximately 2,700 proteins, pIII and pVI are at one end while

pVII and pIX are at the other end of the particle each containing approximately five proteins (Wickner *et al*., 1978).

All the five-coat proteins are responsible for host cell recognition and structural stability. pIII is the most complex protein other than its function in cell recognition and stability it also plays an important role in infection. It has two major domains, the N-terminal (N1 and N2) and the C-terminal domain. The N1 domain is responsible for viral DNA translocation into *E.coli*, the N2 domain enhances host cell recognition by binding to the F pilus present on the bacterium and the C-terminal domain is integrates pIII into the phage coat (Sidhu, 2001).

1.4.5 M13 LIFE CYCLE

The life cycle of M13 virus is divided into three phases; infection, replication, assembly and exportation. Infection phase is initiated by the binding of the N terminal of the phage pIII protein to the tip of the F pilus. F pilus is a protein tube on the surface of E. coli that allows the transfer of DNA into the bacteria a process called conjugation. During phage infection, TolQ, R and A proteins are necessary for maintaining the integrity of the bacteria outer membrane (Lazzaroni et al., 1999).

The pIII remains attached to the F pilus until the genome is transferred into the host cell cytoplasm while the pVIII strips off. The subsequent steps involved phage infection is unclear (Barbas III *et al*., 2001).

Viral genome replication phase starts once the viral single stranded DNA enters the cytoplasm. The viral single stranded DNA is acted upon by type II topoisimerase

enzyme, gyrase, to form a double stranded super-coiled covalently closed replicative form (RF) DNA molecule. This replicative form DNA is the template for phage DNA replication by rolling circle mechanism (Creighton, 1999). pII protein introduces a nick at a specific site in the double stranded replicative form DNA beginning the amplification of viral genome. The host DNA polymerase III copies the replicated strand resulting in more copies of the replicative DNA. pX is required for proper replication of phage DNA, it acts as pII inhibitor (Vos et al., 2007).

The assembly phase of the new viral particle is a membrane-associated event divided into three stages; initiation, elongation and termination. In the initiation stage, pVII, pVIII and pIX interact with packaging signal. The packaging signal allows for efficient assembly and ensures the single stranded DNA is encapsulated into the phage. During elongation stage, the amount of pV increases allowing it to bind to the newly synthesized single stranded DNA to prevent polymerase activity and block the conversion into replicative form DNA. At this stage pV proteins are stripped off and replaced by pVIII proteins. Finally the termination stage occurs when the end of the DNA is reached. pVI and pIII are added, pIII prevents elongation and releases the phage from the membrane (Barbas III *et al*., 2001).

Exportation phase releases the newly synthesized mature phage from the host's membrane through a process that is still unclear (Sidhu S., 2001).

1.5 PHAGE DISPLAY OF ANTIBODIES

Antibody phage display starts with the generation of random antibody libraries that can be isolated by affinity selection. There are two categories of antibody libraries

construction; single-pot and post-immunized libraries from mammalian V-gene encoding the antibody domains (Willats, 2002).

Post-immunized libraries are constructed using antibody gene sequences from animals that have been immunized with target of interest. The antibody sequence production process; affinity maturation and selection occurs *in-vivo* then the isolated V-genes are manipulated and packaged into phage vectors to produce expressed antibody fragments. One drawback of the method is that for each antigen a new library must be constructed (Hoogenboom *et al*., 1998).

Single-pot libraries are constructed using antibody gene sequences from unimmunized animals. There are two types of this library; naïve and synthetic libraries. Naïve libraries are constructed using V-genes that have undergone in-vivo rearrangement (Dennis et al., 2002). Synthetic libraries are made from un-arranged V-gene sequences based on complementarity binding region (CDR) sequence which is critical for binding by shaping the antigen combing site. These arranged antibody sequences are the manipulated then packaged into phage to express antibody fragments (Winter, 1998).

The different antibody formats that can be expressed on the surface of phage are: single chain variable fragments (scFvs), antigen binding antibody fragment (Fab) and disulphide stabilized single chain variable fragments (dsFv). These antibody fragments are currently used in affinity selection because full–length antibodies can be poorly expressed by phage display (Lee et al., 2007). The scFv fragments are smaller than Fab libraries making it more genetically stable. However, scFv's form dimers and trimers which can complicate selection and characterization. Fab lacks the tendency to dimerize,

which facilitates the performance of assays to screen the kinetics of binding (Holliger *et al.*, 1993).

1.6 PHAGE DISPLAY OF PEPTIDES

Peptide phage display is commonly designed using filamentous phage although lytic phage and phagemid vectors can also be used to generate random combinatorial libraries from which peptides can be isolated by bio-panning (Molek *et al*., 2011). There are two theories that describe the evolution of peptide phage display; convergent evolution and direct evolution (Willats, 2002).

Convergent evolution theory states that, peptides binding to a target can be isolated by affinity selection and the nucleotide sequence encoding the isolated peptide homology compared to the native sequence in the database. The limitation of this theory is that mimotopes, peptides without sequence resemblance but bind tight to the target as the natural binding partner, will be isolated (Kay *et al*., 2000).

Direct evolution theory for peptide isolation uses affinity selection to select for peptide that binds to the target (Greenwood *et al*., 1991). Then the nucleotide sequence encoding the selected peptides are altered by error prone PCR, DNA shuffling or phage population amplification in a host bacteria mutator strains. These altered sequences are used to create a second combinatorial library that will be screened by bio-panning to have peptides with altered and improved binding properties (Lowman and Wells, 1993).

1.7 COUPLED PEPTIDE TO NANOFIBER

Specific selected peptides by phage display are widely used in nanotechnology. In 1991 a Japanese physicist, Sumio Ijima, was the first to invent and bind carbon nanotubes to peptides while working at NEC Corporation in Japan (Seker and Demir, 2011). Carbon nanotubes are cylindrical; long hallow structure with walls made of graphene. There are two major types of carbon nanotubes; single-walled and multi-walled nanotubes. Single-walled nanotubes (SWNTs) have one layer of graphene cylinder while multi-walled nanotubes (MWNTs) have many layers (Sinha *et al*., 2006).

Some advantages of carbon nanotubes include; they are elastic, not sensitive to variations of temperature, extremely small in size and have high mechanical strength. The disadvantages of carbon nanotubes include the production of pure nanotubes is costly and there is lack of detailed understanding of synthesis mechanism of carbon nanotubes (Endo *et al*., 2008).

In this study, the carbon nanotubes will be synthesized in the Chemical Engineering Department, then coupled to the peptides selected from peptide display library and used to detect albumin in blood. This project will be applied as one of the occupation safety measures in the different fields. For example, in the battle field, the coupled peptide to nanotubes will be attached to uniforms of soldiers allowing for an emergency signal to be dispatched automatically. An ideal peptide should be specific towards a particular analyte, sensitive and it should be able to function well and maintain its integrity in a variety of environmental conditions.

1.8 SUMMARY

Recent advancement in technology and the wide use of nano-materials has led to the need of a faster, easier and safer ways of research and products discovery methods. Phage display is one of the modern techniques rapidly assimilated different fields. This technique is proving to be a gold standard for the identification and isolating protein ligands with the potential to become a great research tool and therapeutic antibodies (Hammers and Stanley, 2014).

In this study, we used phage titration to quantify the amount of phage that was in the phage stock library. The phage stock library was bought from the New England BioLabs®, USA and it had seven random amino acid peptides (heptapeptide). To select phage that bound to human serum albumin (HSA), affinity selection was done. A concentration ELISA was then developed to measure phage that bind to albumin. M13 ELISA was used to determine the specificity of the isolated clones and various ligands. ELISA was performed to test clones against HSA from multiple donors, this was to determine if the clones will recognize allelic variation of HSA. The quality of isolated DNA will be determined by performing electrophoresis and the number of unique clones was analyzed by sequencing and bioinformatics tools.

Further the positive clones will be transferred to a protein expression vector, pMAL-5. The pMAL-5 vector will express and purify by affinity chromatography, proteins produced from cloned gene which will be inserted downstream from the *malE* gene of *E. coli* that encodes maltose-binding protein (MBP) resulting MBP fusion protein expression. The purified proteins will then be coupled to carbon nanotubes to send a

signal once the peptide comes into contact with human albumin. This project once completed will be of great importance in occupational health, teaching and research.

CHAPTER II: MATERIALS

Phage display peptide library kit (cat. # E8100S) was purchased from New England BioLabs (Beverly, MA; www.neb.com). HRP/Anti-M13 monoclonal conjugate (product code: 27-9420-01) was purchased from GE Healthcare (UK). Bacto-Agar (cat. # 15-6783B) was purchased from Carolina Biological (Burlington, NC). Tryptone (cat. # T7293), yeast extract (cat. # Y4250), tetracycline hydrochloride (cat. # T3383), IPTG (cat. # I6758), human serum albumin (HSA) (cat. # A9511), glycerol (cat. # G5516), tetramethylbenzidine (TMB) (cat. # T0440), albumin from chicken egg white (OVA) (cat. # A7641), sodium chloride (cat. # S7653), sodium phosphate monobasic (cat. # 5011), dimethyl sulfoxide (DMSO) (cat. # D2650), dimethylformamide (DMF) (cat. # D8654), sodium carbonate (cat. # S2127) and sodium phosphate dibasic (cat. # 5136) were purchased from Sigma Chemical Company (St. Louis, MO). 96 well Polyvinyl chloride plates were purchased from Corning (NY). 96 well flat-bottomed MaxiSorp® plates, Blocker casein in TBS (product # 37532), aerosol barrier pipet tips were purchased from Thermo Fisher Scientific (Rochester, NY). X-gal (code: 0428) was purchased from Amresco (Solon, OH).

CHAPTER III: REAGENTS AND SOLUTIONS

10X Phosphate Buffered Saline (PBS)

Anhydrous sodium phosphate monobasic (2.22 g) and sodium phosphate dibasic (11.94 g) were weighed and mixed with 500ml Milli-Q water. The pH of the solution was adjusted to 7.3, then sodium chloride (87.66 g) was added and the final volume brought to 1 L with Milli-Q water. The solution was then filter sterilized and stored at room temperature.

Luria Bertani (LB) broth

Bacto-Tryptone (10 g), yeast extract (5 g) and NaCl (5 g) were weighed and dissolved in 1 L of Milli-Q water. The solution was sterilized by autoclaving then, stored at 4°C.

Top Agar

Bacto-Tryptone (10 g), yeast extract (5 g), NaCl (5 g) and Bacto-Agar (7 g) were weighed and dissolved in 1 L of Milli-Q water. The solution was sterilized by autoclaving and stored at 4°C. The solid was melted in microwave as needed.

Tetracycline Stock, 20mg/ml in 70% ethanol

Tetracycline (200mg) was weighed. A solution of Milli-Q water and ethanol (1:1) were premixed and added to tetracycline powder then the volume was brought upto 10mL with ethanol. The mixture was vortexed and stored at -20°C.

LB + Tet Plates

Bacto-Tryptone (10 g), yeast extract (5 g), NaCl (5 g) and Agar (15 g) were weighed. They were dissolved in 1L of Milli-Q water. Then autoclaved at 121°C for 15

min. Cooled the solution to 55°C, added tetracycline stock (1 ml) and poured into petri dishes. The plates were left to harden then inverted and stored at 4°C in the dark. Do not use plates if brown or black.

X-gal stock, 2%

X-gal (5-Bromo-4-chloro-3-indolyl-β-D-galactoside) (0.1 g) was dissolved into 5 ml of dimethyl sulfoxide (DMSO). Wrapped the aliquots with aluminum foil and stored at -20^{0}C. Do not filter.

Tris-Buffered Saline-Tween (TBS + 0.1% [v/v] Tween-20)

Tween-20 (1 ml) was dissolved in 1 L of TBS. The solution was autoclaved and store at room temperature.

20% (w/v) polyethylene glycol–8000, 2.5 M NaCl (PEG /NaCl)

NaCl (7.3 g) and PEG-8000 (10 g) was dissolved in 50 ml Milli-Q water. Filter sterilized and stored at room temperature.

Ovalbumin-Phosphate Buffered Saline (OVA-PBS), 1%

Chicken egg white albumin (0.1 g) was dissolved in PBS (10 ml), mixed, filter sterilized and stored at 4^{0}C.

Ovalbumin-Phosphate Buffered Saline, 1%/Tween-20, 0.05%

Chicken egg white albumin (0.05 g) was dissolved in 5 ml PBS and Tween-20 (2.5 µl) was added. Filter sterilized and stored at 4^{0}C.

Phosphate Buffered Saline/Tween 20, 0.05%

PBS (50ml) was measured and added to Milli-Q water (450 ml). Tween-20 (0.25 ml) was added swirl the bottle to mix and stored at room temperature.

2 N Sulphuric Acid

Milli-Q water (235.7 ml) was measured then carefully sulphuric acid (14.3 ml) was added. Always pour acid into water.

1 M IPTG

IPTG (2.38g) was added to Milli-Q water (10 ml), mixed, filter sterilized and stored at -20°C.

CHAPTER IV: METHODS

4.1 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 tetracycline (10 μg/ml) and incubated at 37^{o}C overnight. The plate was then wrapped with parafilm and stored at 4^{o}C in the dark for a maximum of 1 month (Chung *et al*., 1989).

4.2 Bacterial stock maintenance

LB broth (10 ml in a 250 ml Erlenmeyer flask) was inoculated with ER2738 from LB agar plates containing tetracycline (10 μg/ml) and incubated overnight at 37°C in a rotary shaker at 210 rpm. The culture was then transferred to a vial containing sterile glycerol and vortexed briefly to mix. The cells were stored at -20°C and in liquid nitrogen (Nagel and Kunz, 1972).

4.3 Phage amplification

E. coli ER2738 cells were inoculated in LB broth (20 ml) containing tetracycline (10 μg/ml) in a 50 ml conical tube and incubated at 37^{o}C overnight on a rotary shaker (210 rpm) with the tube tilted. LB broth (20 ml) in a 250 ml Erlenmeyer flask was inoculated with overnight culture (200 μl) and 1 μl of phage ($2.1x10^{11}$ pfu/ml) and incubated at 37^{o}C on a rotary shaker (210 rpm) for 4.5 hours. The cell culture (20 μl) was transferred to a centrifuge tube and the bacterial cells removed by centrifugation at 14,000 *x g* for 10 minutes at 4^{o}C. Without disturbing the cell pellet, the supernatant (16 ml) containing the virus was transferred into a new centrifuge tube and re-centrifuged at

14,000 *x g* for 10 min at 4°C. The supernatant was collected and the remaining bacteria were heat killed by incubating the tubes in a water bath 65°C for 15 minutes.

4.5 Phage titration

To determine the number of phage in a sample, 1 ul of an overnight culture of ER2738 in LB broth containing tetracycline (10 μg/ml) was inoculated into LB broth (10 ml) in a 250 ml Erlenmeyer flask and incubated at 37°C for 4 hours on a rotary shaker (210 rpm). While the cells were growing, top agar was melted in a microwave then 3 ml was dispensed into sterile culture tubes (one per expected phage dilution), the tubes were maintained at 45°C in a water bath. LB agar plates containing tetracycline (10 μg/ml) were pre-warmed for at least one hour at 37°C (Van Dorst et al., 2010). Ten-fold serial dilutions of phage were prepared in PBS. Dilutions were performed in duplicate. The ER2738 culture in LB broth (200 μl) was dispensed into microcentrifuge tubes then phage dilution (10 μl) was added. The mixture was vortexed immediately and incubated at room temperature for 5 minutes. X-gal (40 μl of 20% in DMSO), IPTG (40 μl of 20% in H_2O) and the ER2738 culture containing the phage (210μl) were added to the top agar. The tubes containing the mixture were vortexed immediately and poured onto the pre-warmed LB agar containing tetracycline (10μg/ml) plates. The plates were gently tilted to spread the mixture, cooled and incubated at 37°C overnight. The blue plaques were counted and converted to plaque forming units (pfu) per ml (Anderson *et al*., 2011).

4.6 Surface panning (Bio-Panning)

Surface panning was used to select for phage that bind to human serum albumin. A 96 well polyvinyl chloride plate was coated with 150 µl of the target (10 µg/ml solution of HSA in 0.05 M $NaHCO_3$, pH 9.6). The plate was then incubated overnight at 4^0C in a humidified container. The coating solution was removed and 200 µl of the blocking buffer (1% OVA-PBS or casein block) was added to the wells and incubated for 1 hour at 4^0C. The blocking buffer was removed and the wells were washed six times with TBST (TBS + 0.01% Tween 20). New England BioLabs phage library $2.1x10^{11}$ pfu/ml inTBST was pipetted onto the coated plate and rocked gently for 2 hours at room temperature (Thomas *et al.,* 2003). The unbound phage was removed by washing ten times with 200µl TBS-0.01% Tween 20 (wash buffer). The bound phage was then eluted with 100 µl elution buffer (500mM KCl, 10 mM HCl) with gentle rocking for 10 minutes at room temperature. The eluate was pipetted into a microfuge tube and neutralized with 250 µl of 2 M Tris-HCl, pH 8.0 (neutralization buffer). The eluted phage was titered to determine the number of phage after the first round of panning. The remaining eluate was amplified by adding it to an overnight culture of *E. coli* ER2738, grown in LB broth (20 ml) containing tetracycline (10 µg/ml) and then titered.

This phage display cycle of clone isolation and amplification was repeated two more times. In the second and third cycles, the eluted and amplified phage was used as the phage source. A wash buffer containing higher concentrations of Tween-20, TBST (TBS+0.05% Tween 20), was used in the last two rounds of panning. The eluate and amplified phage were titered to determine the number of phage after each round of

panning. After the third round of amplification the titered phage plates were wrapped with parafilm and stored at 4°C (Wu *et al*., 2011).

4.6 Plaque Amplification

Plaque amplification was performed to isolate individual clones. An overnight culture of *E. coli* ER2738 was diluted (1:100) in LB broth. The diluted culture (1ml) was dispensed into culture tubes, one for each clone to be characterized. A blue plaque from the titering plate was picked using a sterile glass Pasteur pipette and transferred to a tube containing the diluted culture. The tubes were incubated at 37°C with shaking at 210 rpm for 5 hours. The culture was transferred to microcentrifuge tubes and centrifuged at 14,000 *x g* for 30 seconds. The supernatant was transferred to a fresh tube and the centrifugation repeated. The upper 80% of the supernatant was transferred to a fresh tube using a pipette, diluted with sterile glycerol (1:1) and stored at –20°C.

4.7 Phage Concentration ELISA

A Phage concentration ELISA (Tables 1, 2 and Figure 3) was developed to measure the amount of phage needed to demonstrate binding to albumin. The wells of a 96 well polyvinyl chloride plate were coated with 100ul of blocking buffer (PBS-1% OVA) for the negative control or with 100µl of phage diluted in 0.05 M $NaHCO_3$ (1:10), pH 9.6. The plate was incubated overnight at 4^oC in a humidified chamber. The coating solution was removed and each well filled with 200µl of blocking buffer (PBS-1% OVA). The plate was incubated for 30 minutes at 37^oC. The blocking buffer was aspirated and the wells washed six times with 200µl wash buffer (PBS-0.05% Tween 20). Anti-M13

Peroxidase Conjugated antibody was diluted 1:5000 in OVA sample buffer (1% OVA-PBS + 0.05% Tween-20) and 100µl added to each well. The plate was incubated for 2 hours at room temperature and then washed six times with 200µl wash buffer (PBS-0.05% Tween 20). TMB substrate solution (100µl) was added to each well and incubated at room temperature for a maximum of 20 to 60 minutes, until a suitable blue color formed. Stop solution (2N H_2SO_4) (50µl), was added and the absorbance read at 450nm using an ELISA reader.

Table 1: Phage concentration ELISA plate map testing Amp LS

Order of event	Blank	Phage	Phage	Phage	Phage	Phage	Phage	Phage
1	PBS-1% OVA	$1.0x10^9$	$1.0x10^8$	$1.0x10^7$	$1.0x10^6$	$1.0x10^5$	$1.0x10^4$	$1.0x10^3$
2	PBS-1% OVA	PBS-1% OVA	PBS-1% OVA	PBS-1% OVA	PBS-1% OVA	PBS-1% OVA	PBS-1% OVA	PBS-1% OVA
3	Anti-M13/HRP	Anti-M13/HRP	Anti-M13/HRP	Anti-M13/HRP	Anti-M13/HRP	Anti-M13/HRP	Anti-M13/HRP	Anti-M13/HRP

Table 2: Phage concentration ELISA plate map testing Amp LA

Order of event	Blank	Phage	Phage	Phage	Phage	Phage
1	PBS-1% OVA	$1.0x10^7$	$1.0x10^6$	$1.0x10^5$	$1.0x10^4$	$1.0x10^3$
2	PBS-1% OVA	PBS-1% OVA	PBS-1% OVA	PBS-1% OVA	PBS-1% OVA	PBS-1% OVA
3	Anti-M13/HRP	Anti-M13/HRP	Anti-M13/HRP	Anti-M13/HRP	Anti-M13/HRP	Anti-M13/HRP

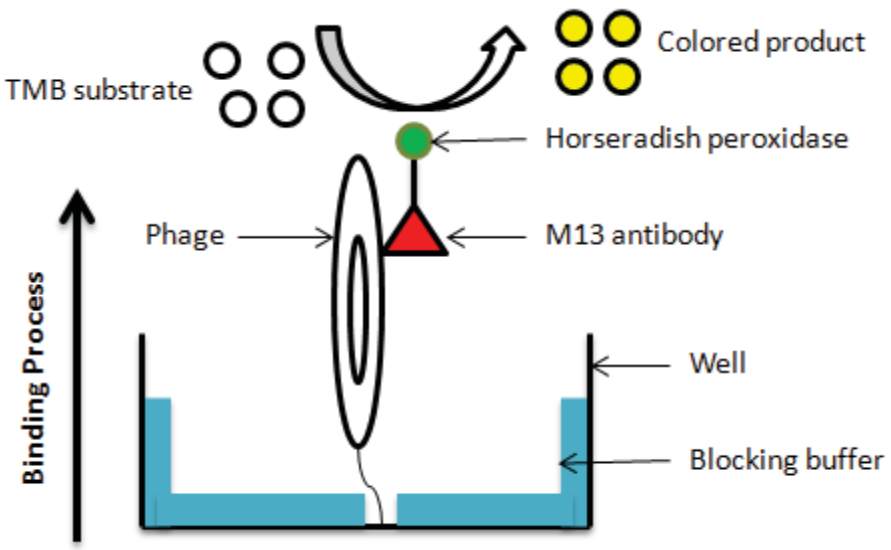

Figure 3: Phage concentration ELISA. Poly-vinyl chloride plate was coated with phage diluted in sodium carbonate (1:100) overnight. The wells was then blocked with PBS-1% OVA for 30 minutes at 37°C. The plate was washed 6 times with with 200µl wash buffer (PBS-0.05% Tween 20). Anti-M13 peroxidase conjugate antibody diluted in sample buffer (1:5000) was added to each well and the plate incubated at room temperature for 2 hours. 3.3'5.5'-tetramethylbenzidine (TMB) substrate (100µl) was added to each well and incubated at room temperature until a blue color formed. This reaction was stopped by adding 50µl stop solution (2N H_2SO_4) forming the yellow product and absorbance read at 450 nm.

4.8 M13 Phage ELISA

An M13 phage ELISA (Figure 4) was developed to determine the specificity of the isolated clones. A 96 well polyvinyl chloride, tissue culture, or MaxiSorp® plate was coated with the ligand, 10µg/ml HSA, in 0.05 M $NaHCO_3$ (pH 9.6) or PBS (1:100).

Positive control wells were coated with phage in 0.05 M $NaHCO_3$ pH 9.6 (100µl). The no HSA wells were coated with 0.05 M $NaHCO_3$ pH 9.6 (100µl) and incubated overnight at 4^0C in a humidified chamber. Triplicate wells were used for each clone (Kingsbury and Junghans, 1995).

The wells were washed once with 200µl phosphate buffered saline containing 0.05% Tween 20 (wash buffer). Block (200µl of 1% OVA in PBS or casein block) was added to the wells and the plate was incubated at 25^0C for 1 hour. The test and no HSA wells were coated with phage (100µl). The blank and positive control wells were coated with 100µl of Tris-Buffered saline-Tween (TBS + 0.1% Tween-20), and the plate was rocked for 2 hours at room temperature, allowing binding to occur between the HSA and phage. The unbound phage was washed off 6 times using wash buffer (phosphate buffered saline containing 0.05% Tween 20). Peroxidase conjugated anti-M13 monoclonal antibody (Dennis *et al*., 2002) diluted 1:5000 in OVA sample buffer (PBS+ 0.1% OVA+Tween 20) was added and the plate incubated overnight at 4^0C. The plate was washed 6 times with wash buffer, then 3.3'5.5'-tetramethylbenzidine (TMB) substrate (100µl) was added and the plate was incubated at room temperature until color developed. Immediately 50µl of stop solution (2N H_2SO_4) was added causing colometric change from blue to yellow and absorbance was read at 450nm using an ELISA reader (Wu et al., 2011).

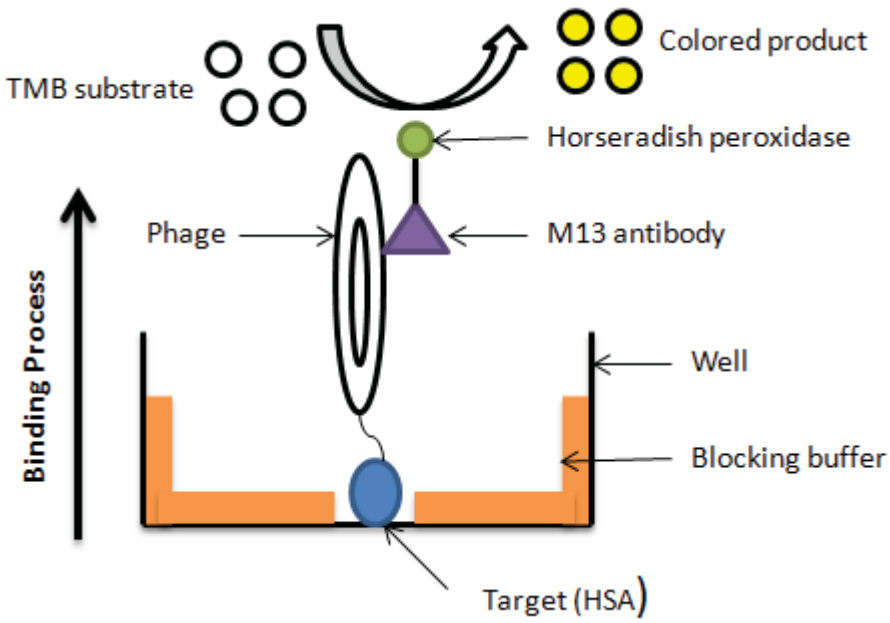

Figure 4: M13 ELISA. Poly-vinyl chloride, MaxiSorp® or tissue plate was coated with phage diluted in sodium carbonate or PBS (1:100) overnight. The wells was then blocked with PBS-1% OVA or casein for 30 minutes at 37°C. The plate was washed 6 times with with 200µl wash buffer (PBS-0.05% Tween 20). Anti-M13 Peroxidase Conjugated antibody diluted in sample buffer (1:5000) was added to each well and the plate incubated at room temperature for 2 hours. 3.3'5.5'-tetramethylbenzidine (TMB) substrate (100µl) was added to each well and incubated at room temperature until a blue color formed. This reaction was stopped by adding 50µl stop solution (2N H_2SO_4) giving the yellow product and absorbance read at 450 nm.

CHAPTER V: RESULTS

In order to isolate and identify peptide molecules that bind to human serum albumin (HSA), a phage display peptide library of random oligonucleotides encoding seven amino acid peptides (Ph.D.-7, New England Biolabs) was screened for binding to the protein. The M13 Phage used in this research project belongs to the family Inovirdae and to the genus Inovirus, This group of virus have a circular single-stranded DNA genome enclosed in a long capsid cylinder (Ackermann, 2009). M13 is a non-lytic phage that produces areas of diminished cell growth called plaques. The M13 phage vector used to prepare the library carries the lacZα gene, which encodes β-galactosidase (β-gal). Incubation of bacteria infected with the phage in the presence of IPTG will induce the production of β-gal and the β-gal will hydrolyze X-gal to create blue plaques (Rao *et al*., 2013). In this project, the *E. coli* host strain ER2738 was used. This bacterium is tetracycline resistant and has fertility pilus (F^+ pilus) that is used by the virus as a site of attachment during replication (Lin *et al*., 2011). The tetracycline resistance gene is found on the plasmid that encodes the pilus. Growth in the presence of tetracycline produces only bacterium that is expressing the pilus. The phage uses this pilus to attach to and enter the bacterium. During replication and extrusion from the bacterium, M13 virus have the advantage of being able to incorporate variable length circular DNA, as the capsid cylinder extends in length to accommodate longer DNA genomes.

The original phage library (L) ($2.1X10^{11}$ pfu/ml) was amplified to obtain a sufficient quantity of phage for this study. Amplification was performed by inoculating LB broth containing tetracycline (10 μg/ml) with an overnight culture of *E. coli* ER2738 and with the phage library. This mixture was incubated for 4.5 hours then centrifuged; the

supernatant was collected and the tube containing the supernatant incubated in a water bath 65^{o}C for 15 minutes to heat kill any bacteria in the sample (Belliveau et al., 1992). Two samples were obtained after amplification: Amp LA and Amp LS.

The original library stock (L), Amp LA and Amp LS were titered to determine the concentration of the virus. This was accomplished by counting the number of blue plaques on LB+Tet plates in the presence of *E. coli* ER2738, X-gal and IPTG (Figure 5). The amplified phage stock concentrations were $2.1x10^{12}$ pfu/ml (AmpLA) and $1.0x10^{10}$ pfu/ml (AmpLS). The concentration of Amp LA was higher, while the Amp LS was lower than that of the original phage stock (L) $2.1x10^{11}$ pfu/ml.

To screen the phage for high binding specificity, surface panning was performed (Dennis *et al.,* 2002). Phage library stocks Amp LA and Amp LS were affinity selected for binding to human serum albumin (Dennis *et al.*, 2002; Sato *et al.,* 2002). Phage that bound after the first round of panning were eluted and amplified. The amplified phage from the first round of panning was again allowed to bind to HSA, and the bound phage was eluted and amplified. This was repeated for a third round of panning and the eluted phage was amplified. After each round of panning the eluted and amplified phage were titered (Figures 6 and 7, Table 3 and 4). Phage that was eluted from the third round of panning were plated as described for titering and individual plaques were collected and amplified to produce clones for further analysis. The isolated clones were labeled HSA1 through HSA7. The concentrations of the isolated clones were determined (Table 5). HSA1 and HSA3 had the highest titers. These clones have been stored for future analysis.

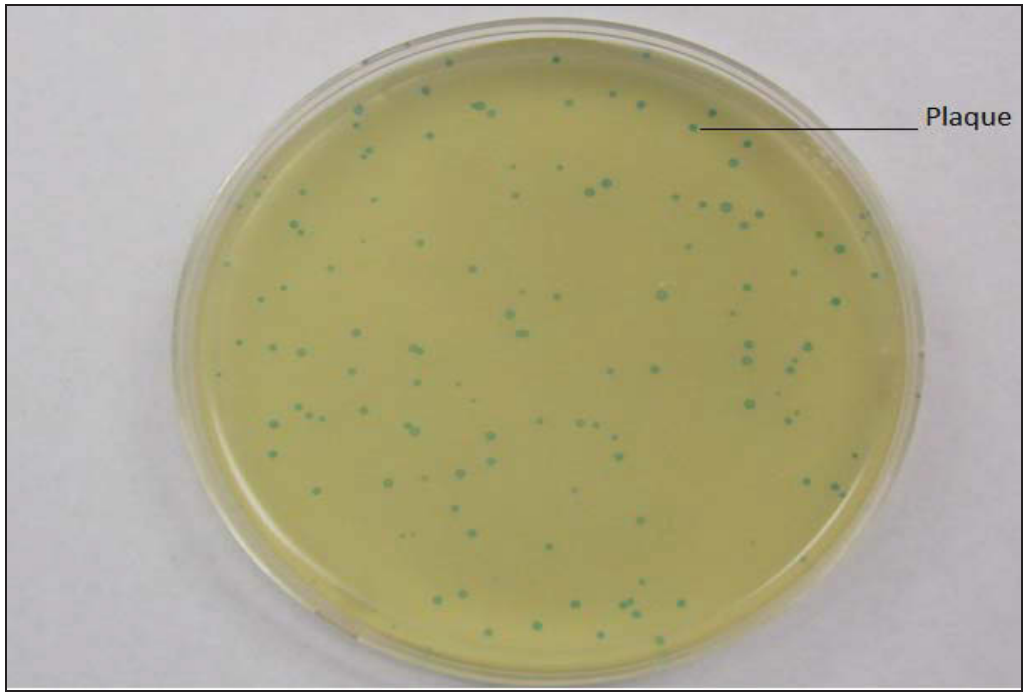

Figure 5: Phage titration plaques

An overnight culture of ER2738 in LB broth containing tetracycline (10 µg/ml) was inoculated into LB broth (10 ml) was incubated overnight at 37°C in a rotary shaker (210 rpm) for 4 hours. Top agar was melted and 3 ml dispensed into a sterile culture tubes maintained at 45^0C in a water bath. The LB+Tet plates were pre-warmed at 37^0C for 1 hour. Phage library stock (L) with an unknown concentration was diluted in sterile PBS (1:100). The diluted phage (10 µl) was added to LB-ER2738 culture (200 µl) and incubated at room temperature for 5 minutes. To the top agar the following were added; X-gal (40 µl), IPTG (40 µl) and LB-ER2738 culture containing the diluted phage (210 µl). The tubes were then vortexed and poured onto the pre-warmed LB+Tet plates. The plates were gently tilted to spread the mixture, cooled to harden and incubated at 37^oC overnight. The blue plaques were counted and converted to pfu/ml.

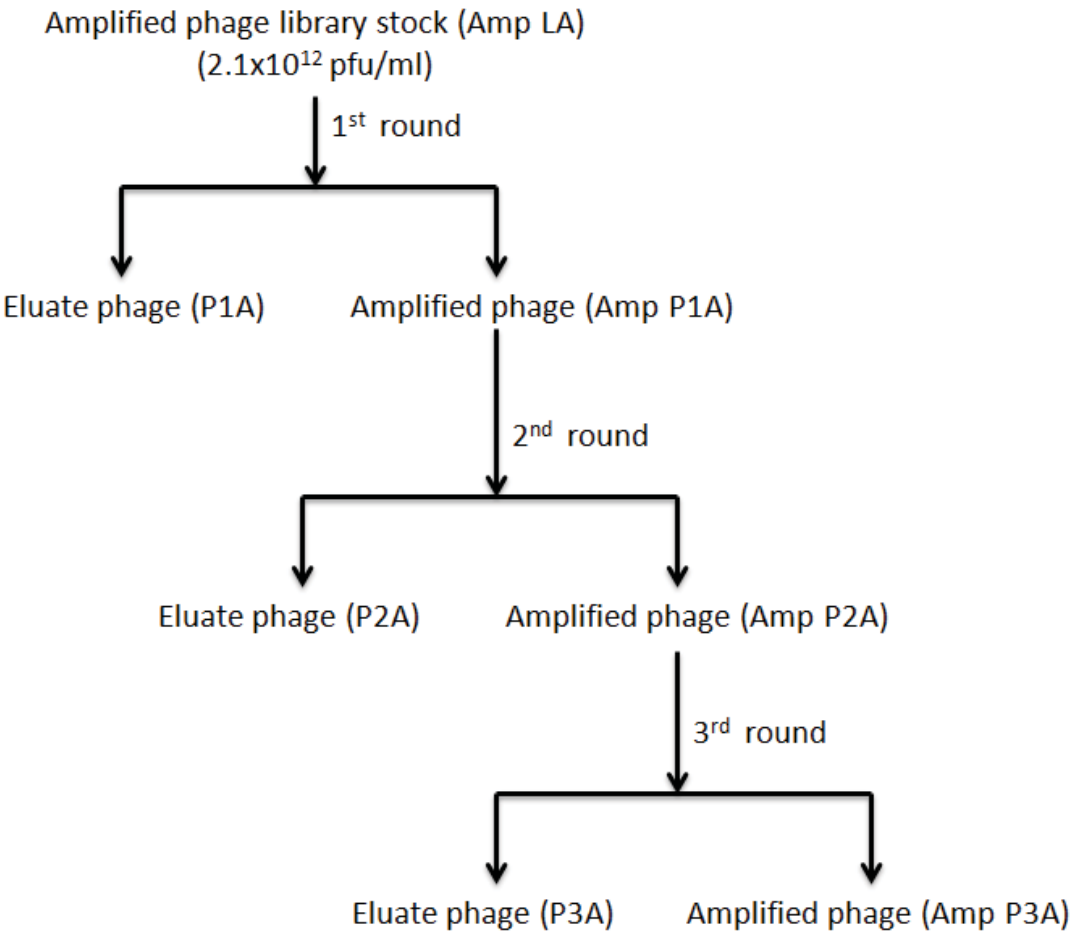

Figure 6: Surface panning of amplified phage library stock (Amp LA)

Human serum albumin (10 µg/ml) was diluted in sodium carbonate (1:100) and coated on polyvinyl chloride plate overnight with gentle agitation. Pour off the coating solution, filled with blocking buffer (PBS-1% OVA) and incubated for 1 hour at 4°C. The blocking solution was discarded and the plate washed six times with TBST (TBS + 0.1% Tween-20). Amplified phage library stock (Amp LA) with concentration $2.1x10^{12}$ pfu/ml was diluted in PBS (1:100) and added to the coated plate with gentle rocking for 1 hour at room temperature. Nonbinding phage was discarded and the plate washed ten times with TBST. Binding phage was eluted using elution buffer (500 mM KCl, 10 mM

HCl, pH 2) this was labeled eluate phage (P1A). A small amount of the eluate was titered as described in Figure 5. The rest of the eluate was amplified by adding it to ER2738 culture (20 ml) and incubating with vigorous shaking for 4.5 hours at 37°C. The culture was transferred to a centrifuge tube and centrifuged for 10 minutes at 12,000 *x g* at 4°C. The supernatant (16 ml) was transferred to a fresh tube. This amplified phage eluate (Amp P1A) was titered as described above. The wells were then coated with diluted HSA (10 μg/ml) in PBS (1:100) overnight. The second round of panning was done using amplified phage (Amp P1A) as the input phage and the concentration of the wash buffer was raised to TBS-0.5% Tween-20. The resulting second round eluate (P2A) was titered then amplified (Amp P2A) and titered. The plate was coated with HSA (10 μg/ml) for the third round of panning. The amplified phage (Amp P2A) was used as an input phage and the wash buffer was increased as used in the second round of panning. The eluted phage was titered (P3A) then amplified and titered (Amp P3A). The concentrations of eluted phage and amplified phage titers are shown in Table 3.

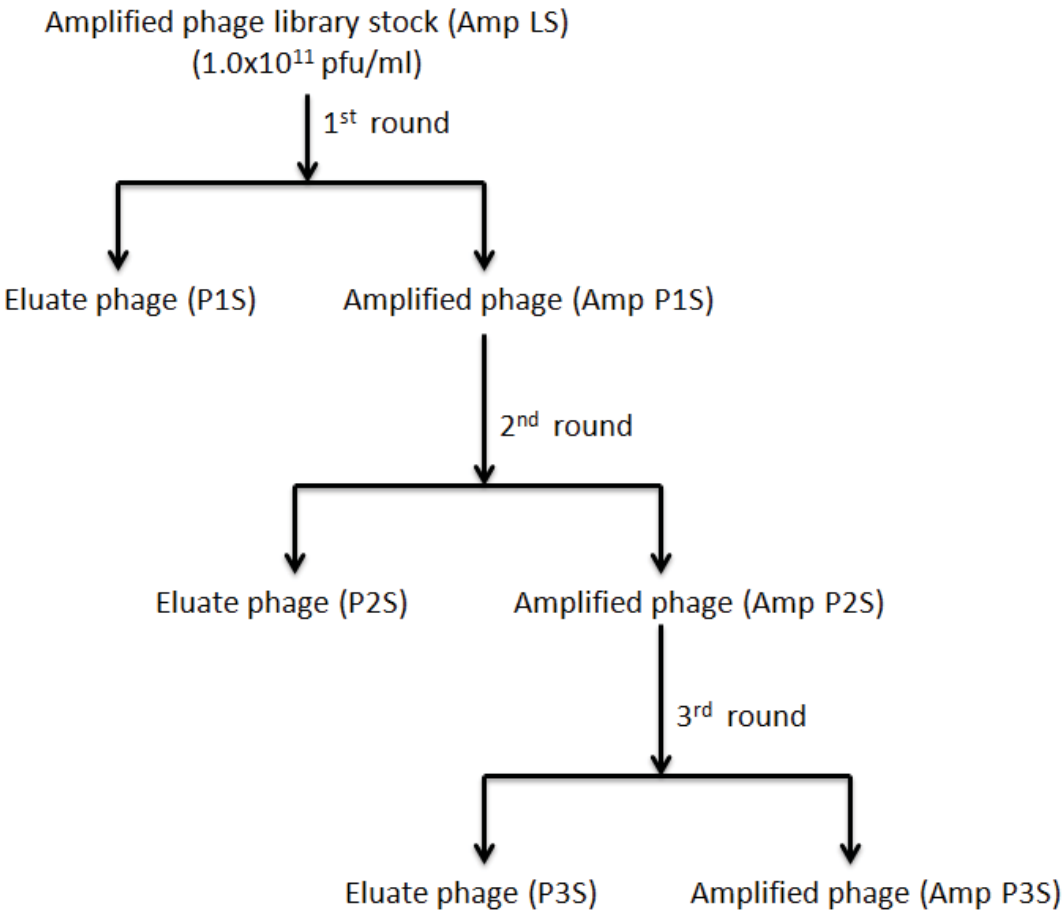

Figure 7: Surface panning of amplified phage library stock (Amp LS)

Human serum albumin specific amplified phage library stock (Amp LS) was isolated using biopanning as described in Figure 6. After coating the plate with HSA overnight, amplified phage library stock (Amp LS) with concentration (2.1x1011 pfu/ml) was diluted in PBS (1:100) and added to the plate. After the first round of panning the eluted phage (P1S) was titered then amplified (Amp P1S) and titered. The amplified phage (Amp P1S) was used as an input phage for the second round of panning. The eluted phage (P2S) was titered and then amplified (Amp P2S). This amplified phage (Amp P2S) was used as input phage for the third round of panning. The eluted phage (P3S) was titered then amplified (Amp P3). The concentration of wash buffer was

increased to TBS-0.5% Tween-20 for the second and third round of panning. The concentrations of eluted phage and amplified phage titers are shown in Table 4.

Table 3: Plaques concentration formed during Amp LA bio-panning process

Bio-panning rounds	Unamplified phage (pfu/ml)[a]	Amplified phage (pfu/ml)[b]
1st	$1.6x10^8$	$1.2x10^9$
2nd	$1.6x10^5$	$2.4x10^{10}$
3rd	$1.0x10^5$	$2.2x10^{11}$

[a] and [b]: The concentration of the phage from the 1st - 3rd round of panning were obtained by counting the blue plaques on LB/IPTG/X-gal plate and multiplying the counts by the dilution factor for that plate giving plaque forming units (pfu) per 0.01 ml.

Table 4: Plaques concentration formed during Amp LS bio-panning process

Bio-panning rounds	Unamplified phage (pfu/ml)[c]	Amplified phage (pfu/ml)[d]
1st	$1.8x10^6$	$1.9x10^{10}$
2nd	$1.1x10^6$	$1.4x10^{11}$
3rd	$2.1x10^8$	$3.1x10^{12}$

[c] and [d]: The concentration of the phage from the 1st - 3rd round of panning were obtained by counting the blue plaques on LB/IPTG/X-gal plate and multiplying the counts by the dilution factor for that plate giving plaque forming units (pfu) per 0.01 ml.

Table 5: Isolated plaques concentration

Plaques	Concentration (pfu/ml)
HSA1	$1.0x10^{10}$
HSA2	$3.1x10^{7}$
HSA3	$1.0x10^{10}$
HSA4	$2.0x10^{6}$
HSA5	$1.4x10^{8}$
HSA6	$1.1x10^{9}$
HSA7	$1.6x10^{8}$
HSA8	$2.1x10^{6}$

A method was developed (phage concentration ELISA) to test for the phage concentration needed to detect binding in an ELISA using peroxidase-conjugated antibodies against M13. The two amplified library stocks (Amp LA and Amp LS) with known concentrations were tested. For the amplified phage library stock (Amp LA) concentration ELISA (Figure 8), it was found that a concentration greater than 10^6 pfu/ml was needed to give a positive M13 ELISA. In a seconnd phage concentration ELISA using amplified phage library stock (Amp LS) (Figure 9), binding was also detected at a phage concentration greater than 10^6 pfu/ml. Figure 9 shows a linear increase in binding between the concentrations of 10^8 and the maximum concentration tested of 10^9 pfu/ml ($R^2 = 0.997$).

We next tested the conditions needed to produce an ELISA that measured phage binding to a specific ligand (M13 ELISA). The ELISA method was tested with the polyvinyl chloride plates that are normally used for protein ELISAs and also with MaxiSorp® plates, polystyrene plates thought to give improved signal and more consistent data (ThermoScientific product information) than polyvinyl chloride plates. In Figures 10 (polyvinyl chloride plates) and 11 (MaxiSorp® plates), amplified phage library (Amp LA) and amplified 3rd round of panning phage (Amp P3S) were bound to the plate directly with sodium carbonate buffer as postitive controls. Good binding of Amp LA was seen with the polyvinyl chloride plate under these conditions, but less binding was seen with the MaxiSorp® plates. The Amp P3S phage had low binding of protein to the wells in both plates, even though the phage concentration was similar to that seen in Amp LA. Specific binding of the library was shown by coupling HSA to the plate with sodium carbonate buffer and then testing for the binding of phage (L = original

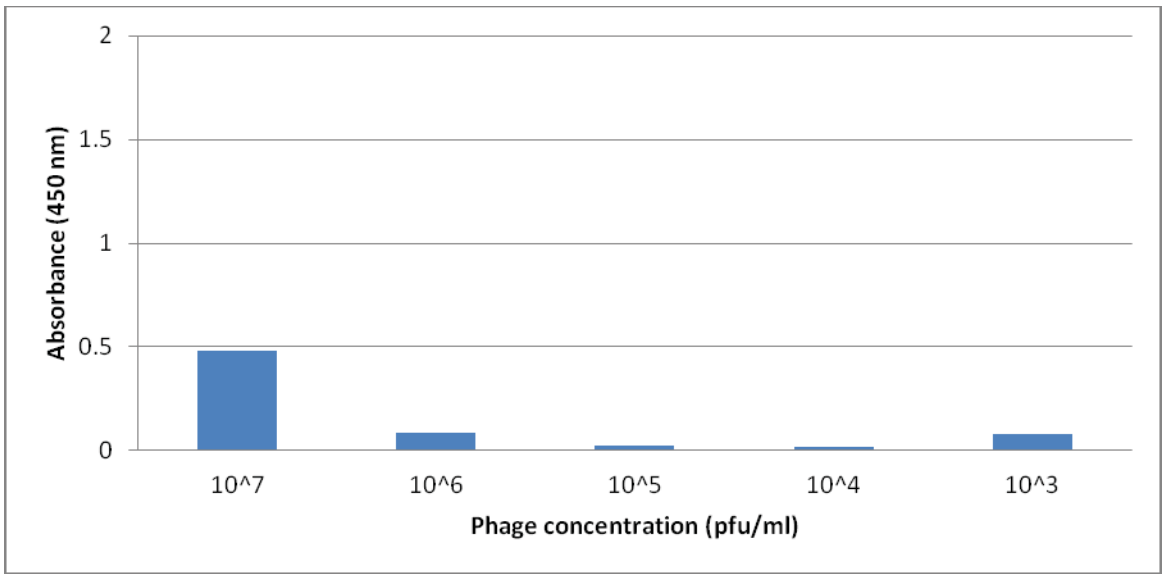

Figure 8: Amplified phage library stock (Amp LA) concentration ELISA

Poly-vinyl chloride plate was coated with various dilutions of amplified phage library stock (Amp LA) in sodium carbonate. The blank well was coated with blocking buffer (1% OVA-PBS). HRP/Anti-M13 monoclonal conjgate was added to all the wells. TMB substrate was added and the absorbance was read at O.D. 450 nm. All conditions were performed in triplicate. The blank absorbance was subtracted from the test absorbance.

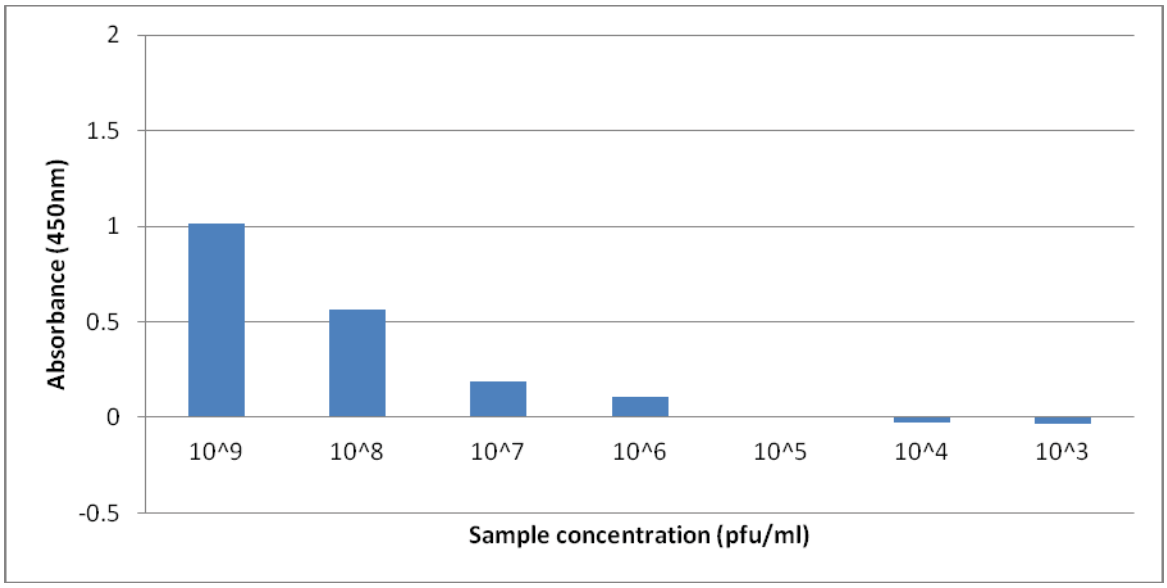

Figure 9: Amplified phage library stock (Amp LS) concentration ELISA

Various dilutions of amplified phage library stock (Amp LS) diluted in sodium carbonate was coated on a poly-vinyl chloride plate. An ELISA was performed as described in Figure 8. Absorbance was read at O.D. 450 nm.

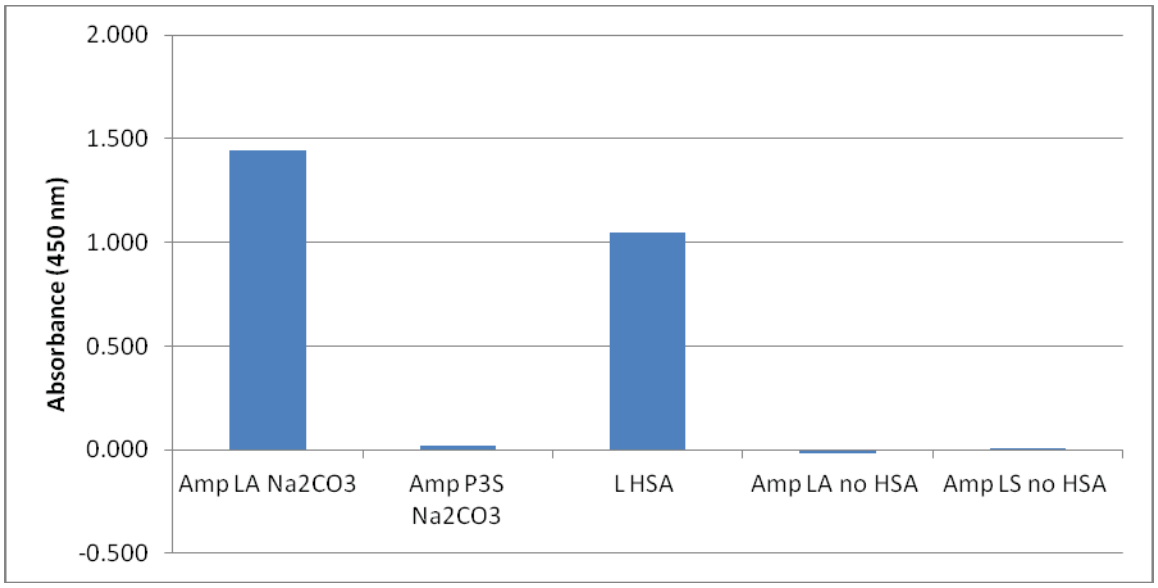

Figure 10: Amplified phage library stock (Amp LA) and amplified phage following the third round of panning for binding to HSA (Amp P3S) M13 ELISA performed on a polyvinyl chloride plate

Amplified phage library stock (Amp LA, $2.1x10^{12}$ pfu/ml) and amplified phage from the 3rd round of panning (Amp P3S, $3.1x10^{12}$ pfu/ml) were tested as positive controls by coating a 96 well polyvinyl chloride plate with phage (diluted 1:100) in the presence of sodium carbonate buffer (0.05 M $NaHCO_3$, pH 9.6). Binding of the original library (L, $2.1x10^{11}$) was tested in wells coated with HSA (10µg/ml HSA, in 0.05 M $NaHCO_3$, pH 9.6). All wells were incubated with blocking buffer (1% OVA in PBS). Phage to be tested for specific binding was added following the blocking step. The no HSA wells contained no ligand, only amplified phage (Amp LA or Amp LS, $1.0x10^{10}$). The blank wells were coated with human serum albumin and no phage was added. HRP/Anti-M13 monoclonal conjgate was added to all the wells. TMB was added and the

absorbance was read at O.D. 450 nm. All conditions were performed in triplicate. Blank absorbance was subtracted from all samples.

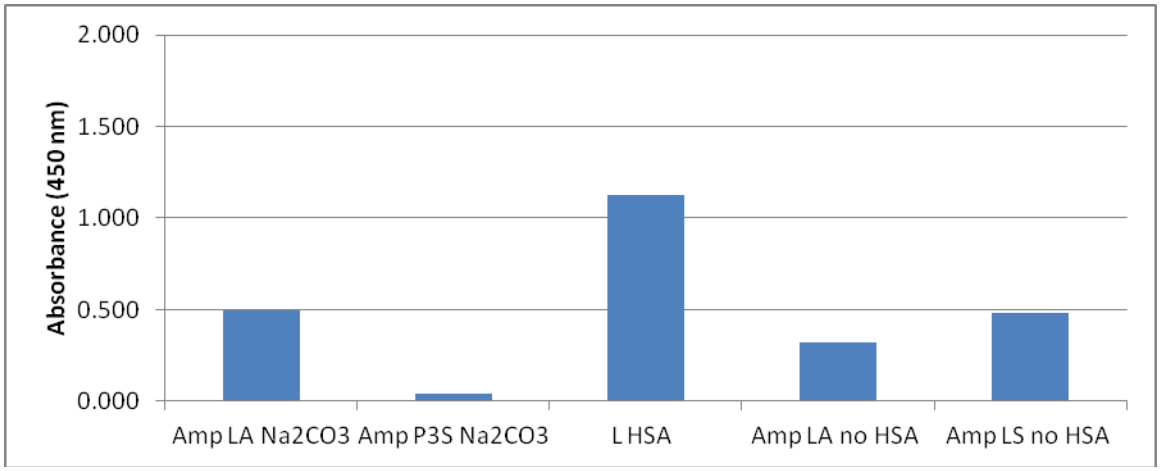

Figure 11: Amplified phage library stock (Amp LA) and Amplified 3rd panning phage (Amp P3S) M13 ELISA done on MaxiSorp® plate

Amplified phage library stock (Amp LA, $2.1x10^{12}$ pfu/ml) and amplified phage from the 3rd round of panning (Amp P3S, $3.1x10^{12}$ pfu/ml) were tested as positive controls by coating a 96 well MaxiSorp® plate with phage in the presence of sodium carbonate buffer. Binding of the original library to HSA was tested (L, $2.1x10^{11}$). The no HSA wells contained no ligand, only amplified phage (Amp LA or Amp LS, $1.0x10^{10}$). The ELISA was performed as described in Figure 10. Blank wells contained HSA and HRP/Anti-M13, but no phage. All conditions were performed in triplicate. Blank absorbance was subtracted from all samples.

library). However, when the amplified libraries (Amp LA and Amp LS) were incubated with wells that contained no HSA and were blocked with OVA, binding still was seen.

Binding of ligand to polyvinyl chloride plates is always performed in the presence of sodium carbonate. However, MaxiSorp® plates can be coated with ligand in the presence of PBS (Rao *et al*., 2012; Gaskin *et al*., 2000). In addition, negatively charged molecules like albumin might be better coated on positively charged plates, such as tissue culture plates which are coated with poly-L lysine. We determined their effect upon false positive results that could be seen with negative controls and on specific binding using an amplified library that had been panned against HSA once (Amp P1A). In Figures 12- 14, we tested polyvinyl chloride plates, tissue culture plates and MaxiSorp® plates using sodium carbonate for binding of ligands to the plate. In addition, in Figure 15, we tested MaxiSorp® plates using PBS for binding of ligand to the plates. All ELISAs were performed using OVA blocking buffer. The negative controls tested were the binding of an irrelevant peroxidase-labelled antibody (anti-mouse immunoglobulin) to HSA, and binding of phage to wells containing no HSA. Anti-mouse immunoglobulin did not prove to be a good negative control, possibly due to contaminating immunoglobulin in the HSA used. However, the least non-specific binding with anti-mouse immunoglobulin was seen when using the polyvinyl chloride plates. Specific binding of Amp P1A to HSA was not seen under any conditions.

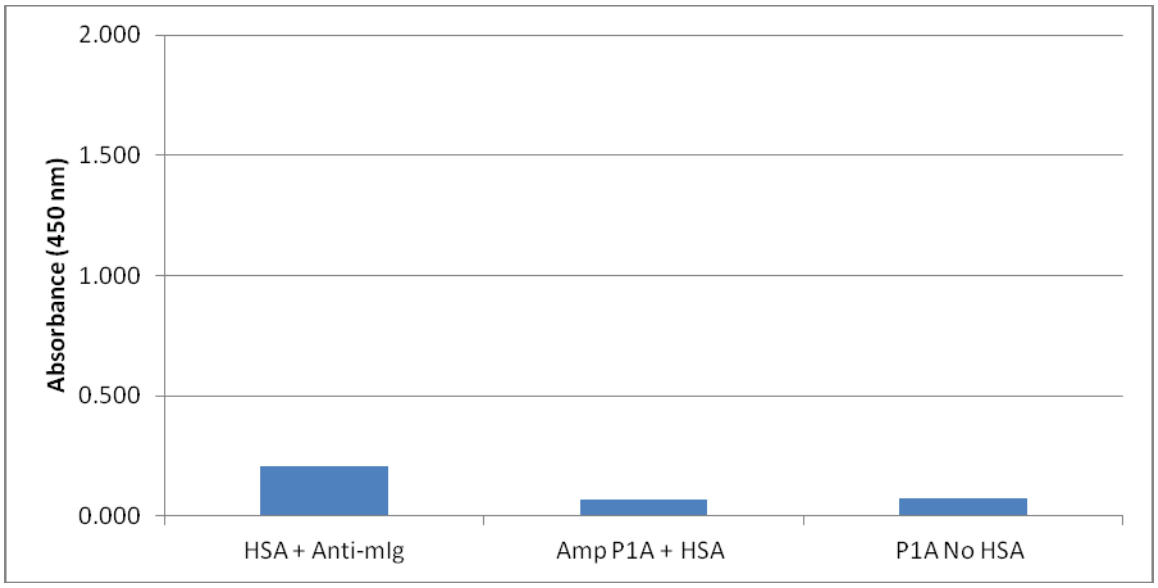

Figure 12: Amplified phage (Amp P1A) M13 ELISA of negative controls performed on a poly-vinyl chloride plate following one round of panning against human serum albumin

Negative controls for the amplified phage from the 1st round panning (Amp P1A) were tested using a 96 well poly-vinyl chloride plate and OVA (1% OVA in PBS) block. The amplified phage (Amp P1A) had a concentration of 1.2×10^9 pfu/ml. HSA coated wells were incubated with 10 ug/ml OVA in sodium carbonate buffer. Peroxidase-conjugated anti-mouse immunoglobulin was tested for use as a negative control. The no HSA well had no ligand, only amplified phage (Amp P1A). The blank well was coated with human serum albumin and no phage was added. HRP/Anti-M13 monoclonal conjgate was added to all the wells and the ELISA performed as described in Figure 10. All conditions were performed in triplicate. The blank absorbance was subtracted from the test absorbance.

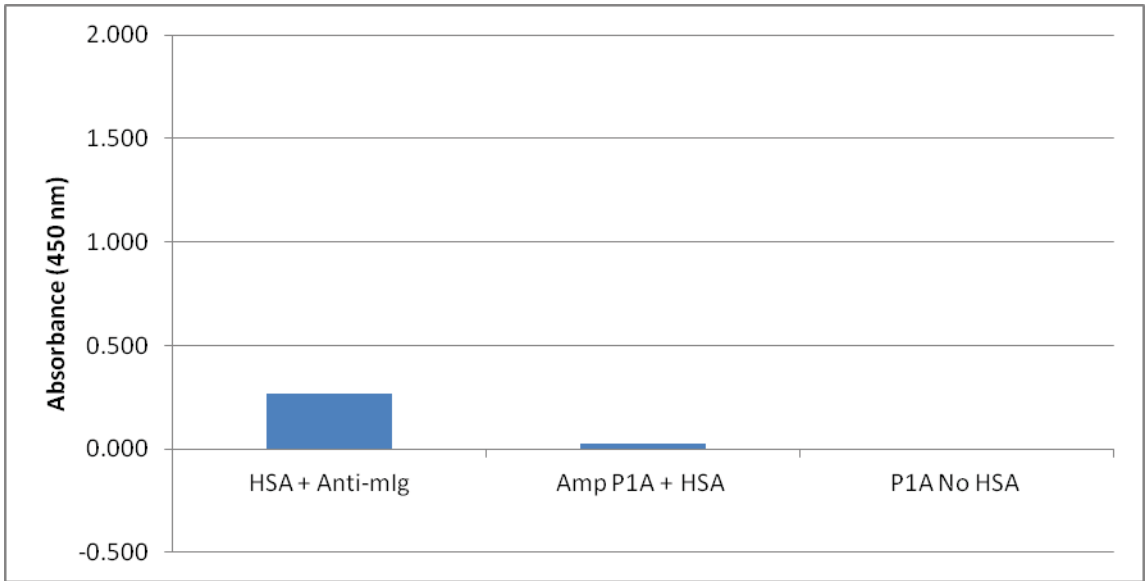

Figure 13: Amplified phage (Amp P1A) M13 ELISA of negative controls performed on a tissue culture plate

Negative controls for the amplified phage from the 1st round panning (Amp P1A) were tested using a 96 well tissue culture (poly-L lysine coated) plate and OVA (1% OVA in PBS) block. The amplified phage (Amp P1A) had a concentration of 1.2×10^9 pfu/ml. HSA coated wells were incubated with 10 ug/ml OVA in sodium carbonate buffer. Peroxidase-conjugated anti-mouse immunoglobulin was tested for use as a negative control. The no HSA well had no ligand, only amplified phage (Amp P1A). The blank well was coated with human serum albumin and no phage was added. HRP/Anti-M13 monoclonal conjgate was added to all the wells and the ELISA performed as described in Figure 10. All conditions were performed in triplicate. The blank absorbance was subtracted from the test absorbance.

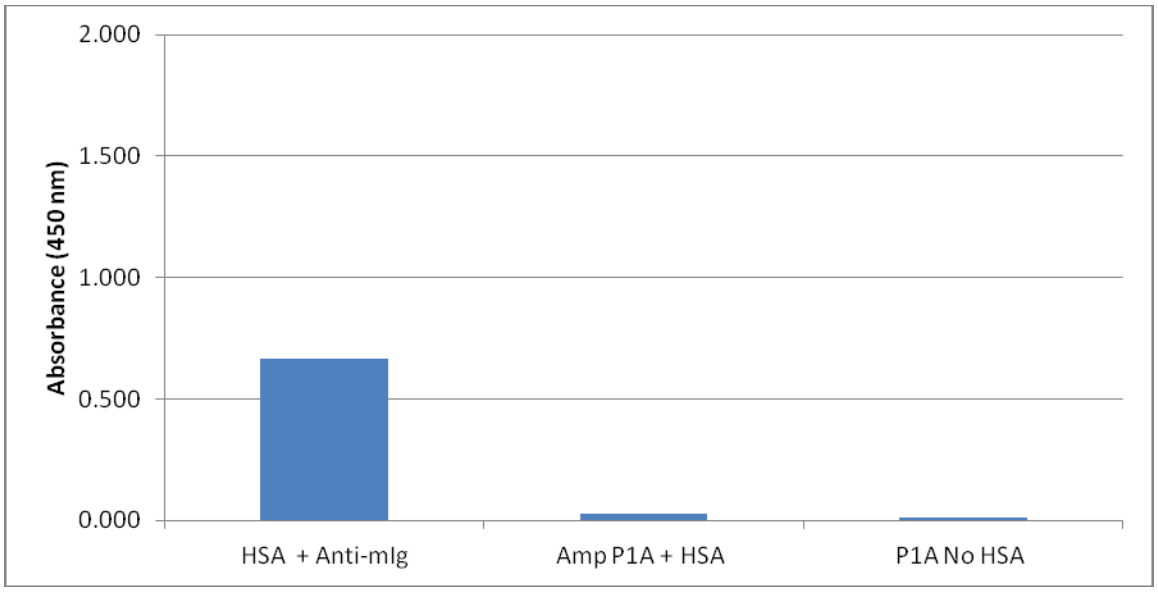

Figure 14: Amplified phage (Amp P1A) M13 ELISA of negative controls performed on a MaxiSorp® plate using sodium carbonate

Negative controls for the amplified phage from the 1st round panning (Amp P1A) were tested using a 96 well MaxiSorp® plate and OVA (1% OVA in PBS) block. The amplified phage (Amp P1A) had a concentration of $1.2x10^9$ pfu/ml. HSA coated wells were incubated with 10 ug/ml OVA in sodium carbonate buffer. Peroxidase-conjugated anti-mouse immunoglobulin was tested for use as a negative control. The no HSA well had no ligand, only amplified phage (Amp P1A). The blank well was coated with human serum albumin and no phage was added. HRP/Anti-M13 monoclonal conjgate was added to all the wells and the ELISA performed as described in Figure 10. All conditions were performed

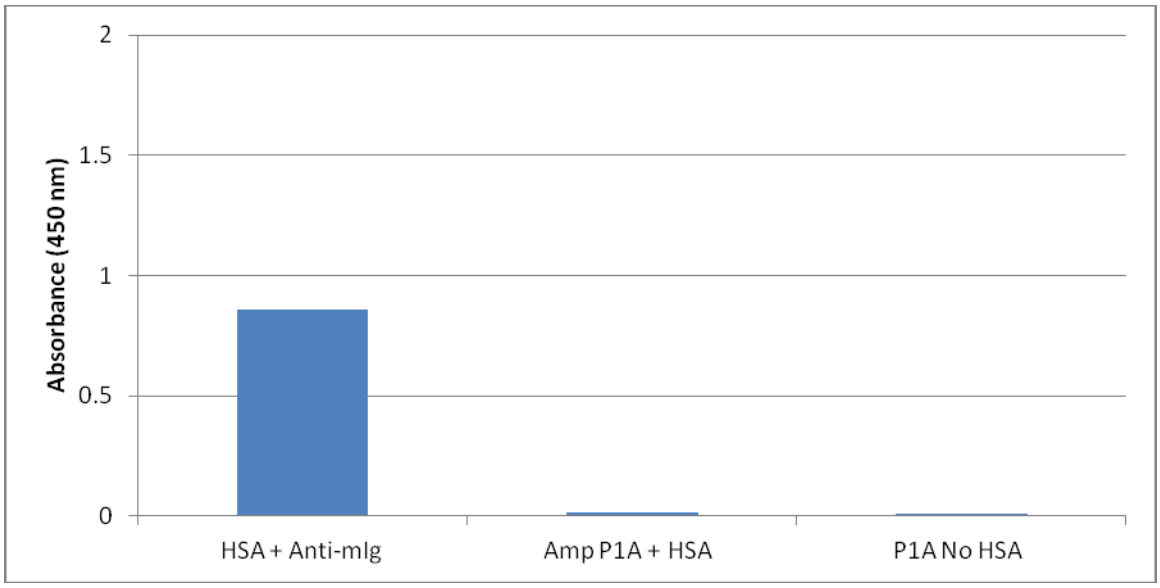

Figure 15: Amplified phage (Amp P1A) M13 ELISA of negative controls performed on a MaxiSorp® plate using phosphate buffered saline

Negative controls for the amplified phage from the 1st round panning (Amp P1A) were tested using a 96 well MaxiSorp® plate and OVA (1% OVA in PBS) block. The amplified phage (Amp P1A) had a concentration of $1.2x10^9$ pfu/ml. HSA coated wells were incubated with 10 ug/ml OVA in PBS. Peroxidase-conjugated anti-mouse immunoglobulin was tested for use as a negative control. The no HSA well had no ligand, only amplified phage (Amp P1A). The blank well was coated with human serum albumin and no phage was added. HRP/Anti-M13 monoclonal conjgate was added to all the wells and the ELISA performed as described in Figure 10. All conditions were performed in triplicate. The blank absorbance was subtracted from the test absorbance.

Using the polyvinyl chloride plates shown to be most effective in these tests, the amplified phage library that had been through three rounds of panning for binding to HSA (Amp P3A) was tested for binding specificity (Figure 16). Amp LS bound to the plate with sodium carbonate buffer was used as the positive control. HSA was bound to the wells with sodium carbonate buffer to test for phage binding. In this experiment, phage binding was not demonstrated in wells containing HSA or in the negative control wells without HSA.

The blocking and sample buffers used in an ELISA can also influence the appearance of false positive results and the sensitivity and specificity of the reactions. We compared the OVA block and OVA sample buffer (Figure 17) used in all previous assays to casein block and casein sample buffer (Figure 18) using the polyvinyl chloride plates, as they were shown to give maximum sensitivity and low false positives. As a positive control, the plates were coated with amplified phage library (Amp LS) diluted 1:100 in sodium carbonate. All wells were then coated with blocking buffer containing OVA or casein. The positive control was incubated with peroxidase conjugated anti-M13, as in previous experiments. Three negative controls were performed. In two controls, Amp LS was added to wells that were not coated with HSA, followed by either peroxidase conjugated anti-M13 (Amp LS, no HSA) or peroxidase conjugated anti-mouse immunoglobulin (Amp LS, no HSA, anti-mouse Ig). The third control contained no HSA or phage, only peroxidase conjugated anti-mouse immunoglobulin (anti-mIg, no phage, no HSA). Absorbance in the positive control wells was slightly higher in the ELISA using casein blck and sample buffer (an absorbance of 0.773 compared to 0.560). The

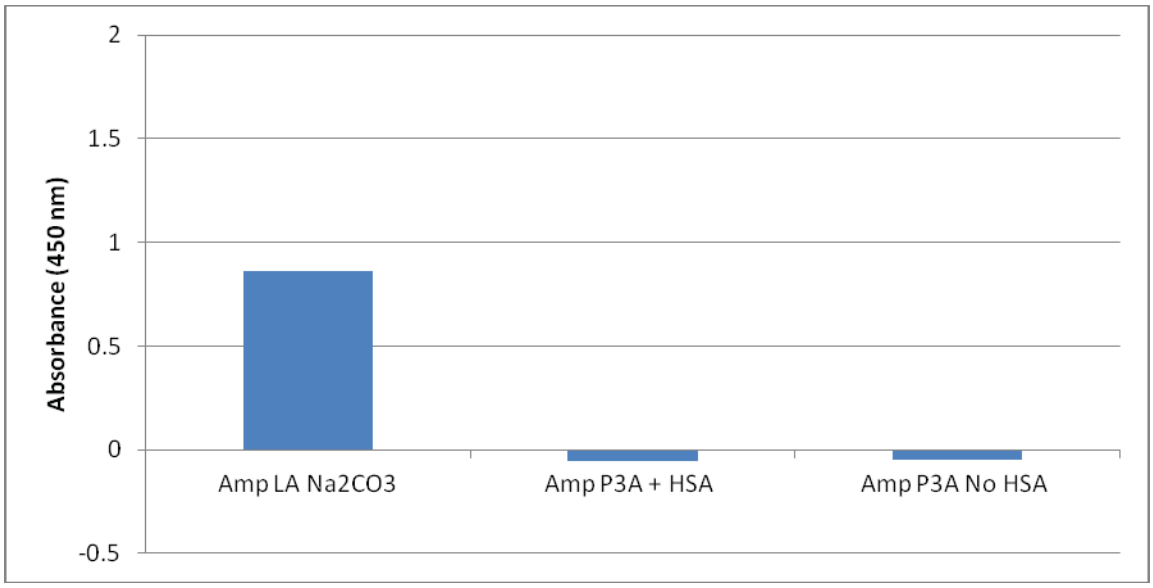

Figure 16: Amplified phage (Amp P3A) M13 ELISA performed on a poly-vinyl chloride plate with OVA block following three rounds of panning against human serum albumin

The amplified phage (Amp LA) following the third round of panning against human serum albumin (Amp P3A), with a concentration of $2.2x10^{11}$ pfu/ml, was tested by coating a poly-vinyl chloride plate with or without human serum albumin in the presence of sodium carbonate buffer. An ELISA was performed as described in Figure 10. Amplified phage library stock (Amp LA) with a concentration $2.1x10^{12}$ pfu/ml was bound to the plate with sodium carbonate buffer and used as a positive control. The no HSA well had no ligand, only amplified phage (Amp P3A). The blank well was coated with human serum albumin and no phage was added. The blank absorbance was subtracted from the test absorbance. HRP/Anti-M13 was added to all the wells. All conditions were performed in triplicate. Absorbance was read at O.D. 450 nm.

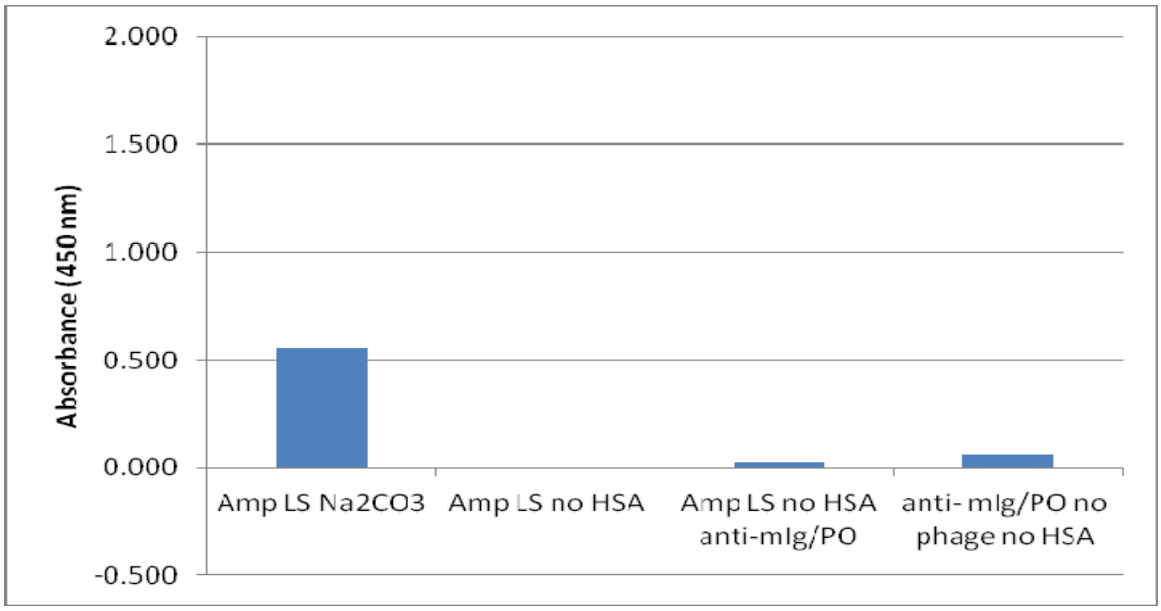

Figure 17: M13 control ELISA using OVA blocking buffer

An ELISA was performed as described in Figure 10 using OVA block. Positive control wells of a poly-vinyl chloride plate were coated with amplified phage library in sodium carbonate buffer (Amp LS, $1.0x10^{10}$). The Amp LS, no HSA well was coated with blocking buffer followed by phage and peroxidase-conjugated anti-M13. The Amp LS, no HSA, anti-mIg well contained no HSA, only phage and peroxidase conjugated anti-mIg. The anti-mIG, no phage, no HSA well was incubated with sodium carbonate (no ligand), followed by peroxidase conjugated anti-mIg. The blank well was coated with human serum albumin, no phage, and then HRP/Anti-M13 monoclonal conjgate. All conditions were performed in triplicate. Absorbance was read at O.D. 450 nm. The blank absorbance was subtracted from all other values.

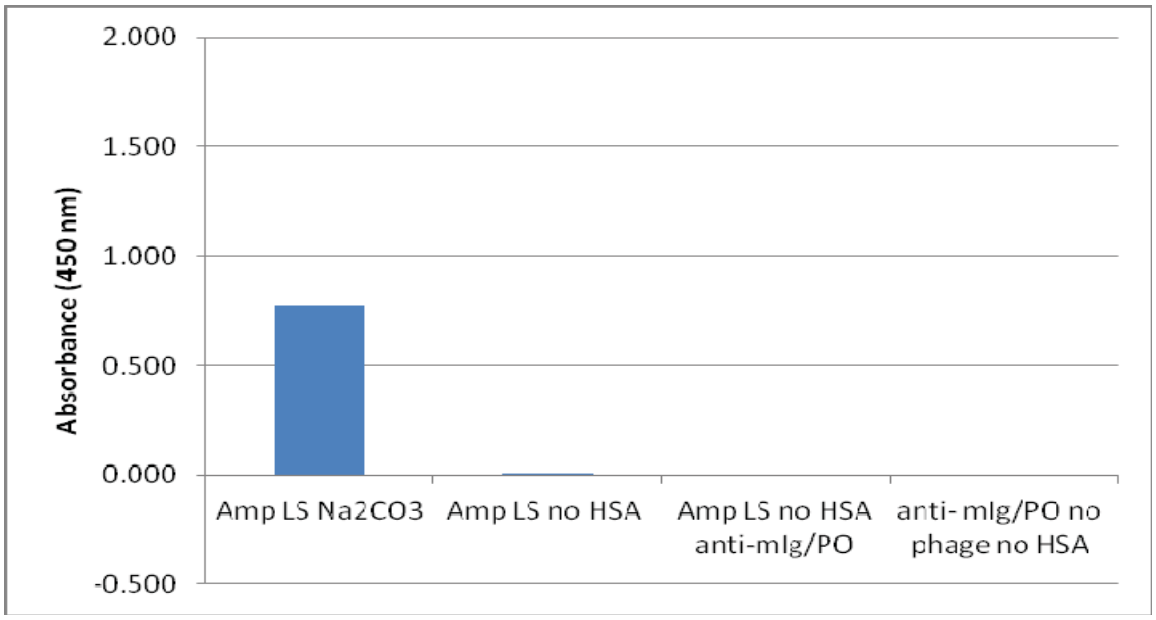

Figure 18: M13 control ELISA using casein blocking buffer

An ELISA was performed as described in Figure 10 using casein block. Positive control wells of a poly-vinyl chloride plate were coated with amplified phage library in sodium carbonate buffer (Amp LS, $1.0x10^{10}$). The Amp LS, no HSA well was coated with blocking buffer followed by phage and peroxidase-conjugated anti-M13. The Amp LS, no HSA, anti-mIg well contained no HSA, only phage and peroxidase conjugated anti-mIg. The anti-mIG, no phage, no HSA well was incubated with sodium carbonate (no ligand), followed by peroxidase conjugated anti-mIg. The blank well was coated with human serum albumin, no phage, and then HRP/Anti-M13 monoclonal conjgate. All conditions were performed in triplicate. Absorbance was read at O.D. 450 nm. The blank absorbance was subtracted from all other values.

negative controls also gave less non-specific signals when casein was used as the block and sample buffer.

CHAPTER VI: DISCUSSION

Phage display is a powerful technique that has played an important role in immunology, drug discovery, cell biology, plant science, pharmacology, veterinary medicine and biochemistry engineering (Sidhu *et al.,* 2000). The random display of molecules on the surface of phage particle is achieved by the fusion of a nucleotide encoding gene to a coat protein gene (Smith, 1985). Some of the molecules that can be expressed on the surface of phage particles are antibodies and peptides. Antibody phage display technology allows for the rapid isolation of monoclonal enriched antibody fragments specific for a given target antigen (Burton, 2001). The advantage of this technique over the use of hybridoma technology is that the antibody production is fast and single chain variable fragment of an antibody (scFv) molecule avidity towards a target can be increased by constructing it into a multivalent polymer. The main disadvantage of antibody phage display is that typically a full length antibody containing the functional constant region is not produced (Azzazy and Highsmith, 2002). Peptide phage display technique is commonly used today in many research facilities. Phage display libraries with random peptides are selected by bio-panning, producing enriched ligand specific peptide molecules. The advantages of peptide phage display are; the isolated specific peptides are stable as they are less likely than scFvs to be dependent upon secondary structure for binding. The disadvantage of this technique is that non-specific peptide binders may be isolated together with the specific target binders (Thomas et al., 2003).

The long-term goal of this project is to isolate specific ligands that bind to human serum albumin so as to couple the peptides to nanofiber detectors. Albumin is the most abundant protein in plasma; it has a molecular mass of approximately 67 kDa. Human serum albumin (HSA) has half-life of 19 days (Theodore, 1996). Albumin has several functions (Dennis et al., 2002), including: maintaining plasma pH, contributing to colloidal blood pressure, serving as a major drug transport protein in plasma and as a carrier of many metabolites and fatty acids. In our future studies we hope to use albumin nanodetectors to monitor injury in military personnel from a distant site and locate the site where aid is needed. To achieve this goal, a phage display peptide library with seven random amino acids (heptapeptide) fused to the coat protein pIII of M13 phage via a flexible linker Gly-Gly-Gly-Ser was used. HSA specific peptides will be isolated, purified and sequenced to isolate the peptides that are stable and can withstand diverse environmental conditions.

The utility of this method has been demonstrated by Wu and coworkers (Wu *et al.,* 2011), who used phage display to obtain peptides specific for the enzyme alanine aminotransferse (ALT) that was used to make an electrochemical peptide-based biosensor. Although they did not use HSA as their ligand for the project, their methodology was the similar to that used for this project. An M13 phage display library was panned for binding to immobilized ALT, allowing the detection of ALT specific phage. After the last round of panning, they titered the phage and blue plaques were randomly picked and amplified. They performed an ELISA assay to test whether or not the selected clones could bind to ALT. These peptide receptors were purified then

coupled to C-terminal cysteine for gold immobilization. This detector was used to detect the concentration of ALT in human blood.

The hypothesis of this project states that phage display can be used to produce peptides that bind specifically to HSA. Similar research projects were conducted by Sato and coworkers (Sato *et al.,* 2002) and by Dennis and coworkers (Dennis *et al*., 2002).. Sato and coworkers constructed M13 disulfide-constrained cyclic peptide phage-display libraries to isolate HSA specific peptides. Dennis and coworkers constructed a phage library that displayed a turn-helix peptide motif to isolate peptide. In both research projects a phage library was constructed and then exposed to human serum albumin during the panning process. The selected peptides were tested for specificity to HSA using an ELISA assay. These papers indicate that phage display can be used to produce HSA specific peptides. We chose to use a library that displayed non-cyclized heptapeptides, as our intention is to have to peptides maintained in a dry state, which would likely disrupt secondary structure required for binding of the cyclized peptides or turn-helix peptides.

In the study by Dennis and coworkers (Dennis *et al*., 2002), the pooled phage library was exposed to HSA (10μg/mL) immobilized on MaxiSorp® plate. The plate was blocked with PBS containing 1% OVA for the three rounds of panning, on the fourth round the plate was blocked with TBS-casein blocker. Phage was added and allowed to bind to HSA for 2 hours. The plate was washed 6 times with PBS-0.05% Tween 20 and then eluted by lowering the pH (500 mM KCl, 10 mM HCl, pH 2). After each round of panning the eluted phage was titered and then amplified. HSA specific phage from the

fourth round of panning was tested for specificity using an ELISA. After performing the ELISA assay they found that the isolated peptide bound to HSA. In our studies, we have used similar methods. Our panning was performed on polyvinyl chloride plates, rather than MaxiSorp®, but the blocking buffers and elution buffers used during panning were identical to those in the study by Dennis and coworkers.

In this project, amplified phage library stock (Amp LA) and (Amp LS) were affinity selected for HSA specific monoclonal phage (Dennis et al., 2002; Sato et al., 2002). This screening technique does not require special instruments, it is easy to perform and can be performed in a relatively short period of time (Kay et al., 2001). Three rounds of panning were carried out in this study. It is recommended that no more than four rounds of panning be performed, to maintain the binding phage sequence diversity and to reduce the number of non-specific binders (Heng et al., 2007). HSA (10μg/ml) in 0.05 M $NaHCO_3$, pH 9.6 was immobilized on a polyvinyl chloride plate. Other investigators used MaxiSorp® plates with HSA (10μg/ml) in PBS (Dennis *et al.,* 2002 and Sato *et al.,* 2002) for biopanning. The uncoated region was blocked with PBS, containing 1% ovalbumin except for the third round of panning where Tris-buffered saline-casein was used. Amplified phage library diluted in PBS was allowed to bind then washed in TBS-0.01% Tween 20 for the first round of panning then the concentration of the wash was increased to TBS-0.05% Tween 20 for the last two rounds. Heng and coworkers (Heng *et al*., 2007) used trypsin (100 μl) to elute bound phage. We we used the method preferred by Sato and coworkers in which elution is accomplished by a change of pH to break the bond between the phage and the target. The bound phage was eluted with 500mM KCl, 10mM HCl, pH 2. Both the eluted and the amplified phage were then titered.

Following isolation of HSA specific phage, an ELISA was developed to determine the concentration of phage needed for a positive ELISA. Amplified phage was coated directly onto a polyvinylchloride plate using sodium carbonate buffer, then PBS containing 1% OVA block was added. Anti-M13/HRP monoclonal antibody was added, then finally the substrate (TMB). The results demonstrated that our ELISA would detect bound phage if the phage concentration was over 10^7 pfu/ml (Figures 8 and 9)

To determine the specificity of the isolated HSA binding phage, an M13 ELISA was developed. This was tested on three different kinds of plates; MaxiSorp® plates, polyvinyl chloride plate and tissue culture plate (Dennis *et al.*, 2002 and Wu *et al.,* 2011). MaxiSorp® plates are a high capacity protein-binding polystyrene plates that have a hydrophilic surface that is optimized for binding to immunoglobulin and other proteins having both hydrophilic and hydrophobic components. Polyvinyl chloride plates are ideal for solution-based assays, while tissue culture plates are coated with poly-L lysine to encourage binding of negatively charged molecules (Dennis *et al.,* 2002).

In experiments comparing ligand bound to polyvinyl chloride and MaxiSorp® plates, positive control phage bound to the plates with sodium carbonate buffer (Figures 10 and 11 Amp LA) produced higher absorbance on the polyvinyl chloride plates. The negative controls (no HSA) were also better in the polyvinyl chloride plates, demonstrating specific binding of amplified library only in the presence of HSA. However, binding to the plates without HSA could be due to binding to ovalbumin used as the blocker, as the libraries had not been selected to bind to any particular protein and could potentially bind to either of these proteins. It is also possible that phage could bind

to exposed plastic (Adey *et al*., 1995). An investigation performed by Adey *et al.,* described phage binding to plastic by a not well understood mechanism. It is believed that it could be because of nonspecific hydrophobic interactions. They found binding to plastic that was preferentially seen with peptides containing Tyr and Trp residues. They recommended that to reduce the number of plastic binding phage, the plate should be coated with high density target, thoroughly covering the non-specific sits on plastic with target followed by block and that an elution method specific for the binding peptides should be used.

Further investigation the three different kinds of plates was done (Figures 12-15) in the presence of diluted HSA sodium carbonate or PBS. Ovalbumin blocker was used and anti-M13/HRP conjugated monoclonal antibody. Shekarchi and coworkers (Shekarchi et al., 1984) evaluated the binding of antigen on various microtiter plates for use in ELISAs. They found that polystyrene and polyvinyl chloride plates bound well to gamma globulin. They recommended that for any ELISA assay plastic type, lot number and the manufacturer are some of the elements that should be considered because any changes can affect the results. In our studies, we found the least non-specific binding when using polyvinyl chloride plates. After testing the plates, polyvinyl chloride was used to test the specificity of Amp LA and Amp P3S (Figure 16), it was found out that our M13 ELISA was working and that phage binds to HSA using OVA blocker.

The next test examined the influence of the block used (Dennis *et al*., 2002) during an ELISA assay (Figures 17 and 18). Vogt et al., 1986 tested dry milk, casein, gelatins from pig and fish skin, serum albumin as blocking agents for ELISA. Each

protein was tested in different ranges of dilution, with direct peroxidase conjugate incubation or incubation with peroxidase conjugate after washing. They found that casein was the best block. In our experiment we used ovalbumin and casein blocks, although both gave the expected results, casein is highly preferred. Casein uses protein-plastic interaction as a way of blocking while ovalbumin blocks through protein-protein interactions (Vogt et al., 1986).

In summary, in this study HSA isolation using biopanning was successfully achieved. The results show that after panning and amplification a higher concentration of phage was achieved. The M13 ELISA assays showed that polyvinyl chloride plate is the most effective plate to use. An advantage of this plate is that it is less costly and does not require special treatment before use. An ELISA was also performed to test the use of different blocks and casein was shown to produce the best results. In these studies we have proven our hypothesis that phage display can be used to produce peptides that bind specifically to HSA. In future work the isolated clones will be analyzed for specificity using the M13 ELISA. Proteins from unique positive clones will be purified. These purified proteins will be coupled to carbon nanotubes and tested for their ability to specifically detect human serum albumin.

CHAPTER VII: REFERENCES

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