8 Development of a Peptide-Liposome Immunoassay for the Detection of Biological Toxins

8.1 Project Summary

The growing threat of bioterrorism in recent years has increased the interest in the development of improved detection methods for hazardous biological agents. These agents include bacteria, viruses, toxins and spores. Their use in a bioterrorist attack is a major concern. They may also occur naturally and pose a threat to food and water supplies. The danger of these biological agents is that their presence may not be known until people start getting sick or dying. Present detection methods, based on culture techniques and bioassays, are extremely sensitive, but can take up to 4 days to complete. Hence, there is an urgent need for real-time detection and environmental monitoring methods that can be done in the field away from a laboratory.[1]

The long term goal of this research is to develop a highly sensitive, immuno-based biosensor for the rapid detection of a variety of different biological agents. The most essential aspect of an immuno-based biosensor is the probe which specifically binds to the agent and, as part of an analytical platform, generates a measurable signal that is caused by this interaction.[1] In most cases, these probes consist of antibodies isolated from immunized animals. However, antibodies can be sensitive to environmental conditions, where denaturing will reduce antigen-binding affinity. Also, they can be very expensive to produce and isolate. Therefore, an alternative probe would be beneficial. *The objective of this project is to determine the feasibility of developing a peptide-tagged liposome probe for the rapid detection of a biological agent.* Here, the liposome surface would be tagged with a short peptide that specifically binds to a target biological agent. This binding event results in some measurable signal (*e.g.* liposome fluorescence quenching, change in liposome mass). Our *rationale* is such a probe will be cheaper and more stable than antibody-based probes, and can be readily applied to real-time detection and environmental monitoring methods.

This project, funded by the Department of Defense, is a joint collaboration with Luna Innovations (Blacksburg, VA). It is a multidisciplinary project that combines biotechnology and analytical chemistry. Roger Van Tassel, of the BioChem Group at

Luna Innovations, is leading this effort with the development of liposome-based assays for the detection of several biological agents. The research group of Dr. Kevin Van Cott and Dr. Richey M. Davis is assisting the effort by using phage display technologies to identify peptide sequences that specifically bind to these agents that can be incorporated into liposomes.

8.1.1 Objective and Specific Aims

The specific objective of this research is to develop a sensitive peptide-liposome immunoassay technique for the rapid detection of biological toxins. We aim to use short peptide sequences that can specifically bind to a target biological toxin instead of antibodies isolated from immunized animals. The main advantage of this technique is that these short peptide sequences are cheaper and more stable than presently used antibody-based methods. To help accomplish this objective, the following specific aim was proposed for this work:

1. ***Search for a peptide sequence that will specifically bind to cholera toxin using phage display technology that will be used in a liposome-based immunoassay***

   We hypothesize that we will be able to identify a short peptide sequence that will specifically bind to a target toxin. To test this hypothesis, we will use phage display to search a library of peptide sequences for a sequence that will bind cholera toxin that is immobilized on a polystyrene plate. Multiple binding sequences will be identified through the DNA sequencing of the gene expressing the displayed peptides. From this, a consensus peptide sequence that strongly binds cholera toxin will be identified for use in further experiments to develop a peptide-liposome immunoassay for the detection of cholera toxin.

With the conclusion of this part of the project, we aim to demonstrate that phage display can be used to identify peptide sequences that can bind biological toxins. These peptide sequences can be incorporated into liposomes to form an immuno-based probe that can then be used as part of a sensitive and rapid detection method. Although cholera toxin is used here, this method should be applicable to developing detection methods for a variety of biological toxins.

8.1.2 Significance of Research

Currently, there are few methods for the real-time monitoring and detection of threat biological agents. Most methods require lengthy culturing experiments or

expensive antibody-based probes. *Therefore, the presented research is significant because we plan to demonstrate an alternative detection method using peptide-liposome immunoassays that is rapid, fieldable and highly sensitive.* Using phage display libraries, peptide sequences binding to a variety of biological toxins can be screened for and identified. Hence, the work presented here can also be applied to the development of new detection methods for a number of biological toxins.

8.2 Background

The purpose of this section is to briefly discuss two topics that are of importance to this work. Section 8.2.1 introduces the biological agent, cholera toxin, which will be used as the target of interest in this research. This section will discuss what this agent is and why it is important to develop a rapid method for detecting its presence. Section 8.2.2 will introduce the technique known as "phage display" and how it can be used to screen vast libraries to search for peptide sequences that bind specifically to a variety of targets.

8.2.1 Cholera Toxin

The serious and potentially deadly disease known as cholera is caused by the gram-negative bacterium *Vibrio cholerae*. When ingested, *V. cholerae* may colonize the small intestine and produce cholera toxin. Cholera toxin is an oligomeric protein of 84,000 Da consisting of 6 subunits: A_1B_5.[2] The 5 B-subunits surround the single A-subunit, and facilitate the binding of the toxin to G_{M1} ganglioside receptors on mammalian cell surfaces.[3,4] Once the toxin is bound, the A-subunit is transferred into the cell, where it acts as an ADP-ribosylating enzyme. ADP-ribosylation of the G_S protein by the A-subunit leads to the activation of adenylate cyclase which then increases cAMP levels and alters the activity of ion transport systems. This leads to the loss of Na^+ and K^+ ions by the cell, as well as water, which can lead to severe diarrhea. [5]

If untreated, the rapid loss of body fluids can lead to dehydration, shock, and even death. The disease continues to be a worldwide biological health threat, killing 120,000 people annually.[6] Since the bacterium is spread through contaminated water and food supplies, the disease is common in developing countries, including those in the Indian subcontinent and sub-Saharan Africa.[7] It is also believed that cholera toxin poses a

threat as a weapon for bioterrorists. For these reasons, there is great interest in developing a rapid detection and/or real-time monitoring method for cholera toxin in water and food supplies.

8.2.2 Phage Display

Over the last decade, phage display technology has developed into a useful technique for the screening of vast peptide libraries to rapidly identify peptide ligands for a variety of target molecules. This technique utilizes recombinant DNA technology to generate a library of random short peptide sequences fused with the coat protein of a bacteriophage.

For this work, the Ph.D.-12™ Phage Display Peptide Library Kit from New England Biolabs®, Inc. (Beverly, MA) was used. This kit contains an M13 phage library of random peptide 12-mers fused to the N-terminus of the minor coat protein, pIII. The library consists of greater than 10^9 different peptide sequences. The M13 phage is a filamentous bacteriophage that consists of a single-stranded, circular DNA molecule (genome) coated with a layer of proteins, giving it a rod-like shape.[5] The virus enters a bacterial cell, uses the cell's own machinery to replicate, and then it exits the cell through the membrane into the surrounding medium, free to infect additional cells. Therefore, infected cells are not lysed, but they will grow at slower rates than usual. As a result, virus particles can be amplified to $>10^{12}$ plaque forming units (pfu) per mL of bacterial culture.[8]

During the screening process, the library is incubated in the presence of a target-of-interest which is immobilized on an immunoassay plate. Phage displaying peptide sequences having no interaction with the target were washed away. The specifically-bound phage were then eluted with an acidic solution. The eluted phage were collected, amplified and subjected to several more rounds of binding to enrich the pool of phage containing peptide sequences that bind to the target strongly. This process is referred to as panning.[9] After the last round, the eluted phage clones are analyzed by DNA sequencing to determine the binding peptide sequence. The process of panning is illustrated in Figure 8.1.

Using this general method, random peptide display libraries have been utilized in a variety of different applications.[10, 11, 12, 13] This technique has already been proven to be a valid method for the identification of a short peptide sequence that will bind specifically to biological threat agents.[14, 15, 16] Here, we use phage display to identify a peptide sequence that will specifically bind to a biological agent, and then incorporate that peptide into a liposome-based immunoassay for the detection of that biological agent.

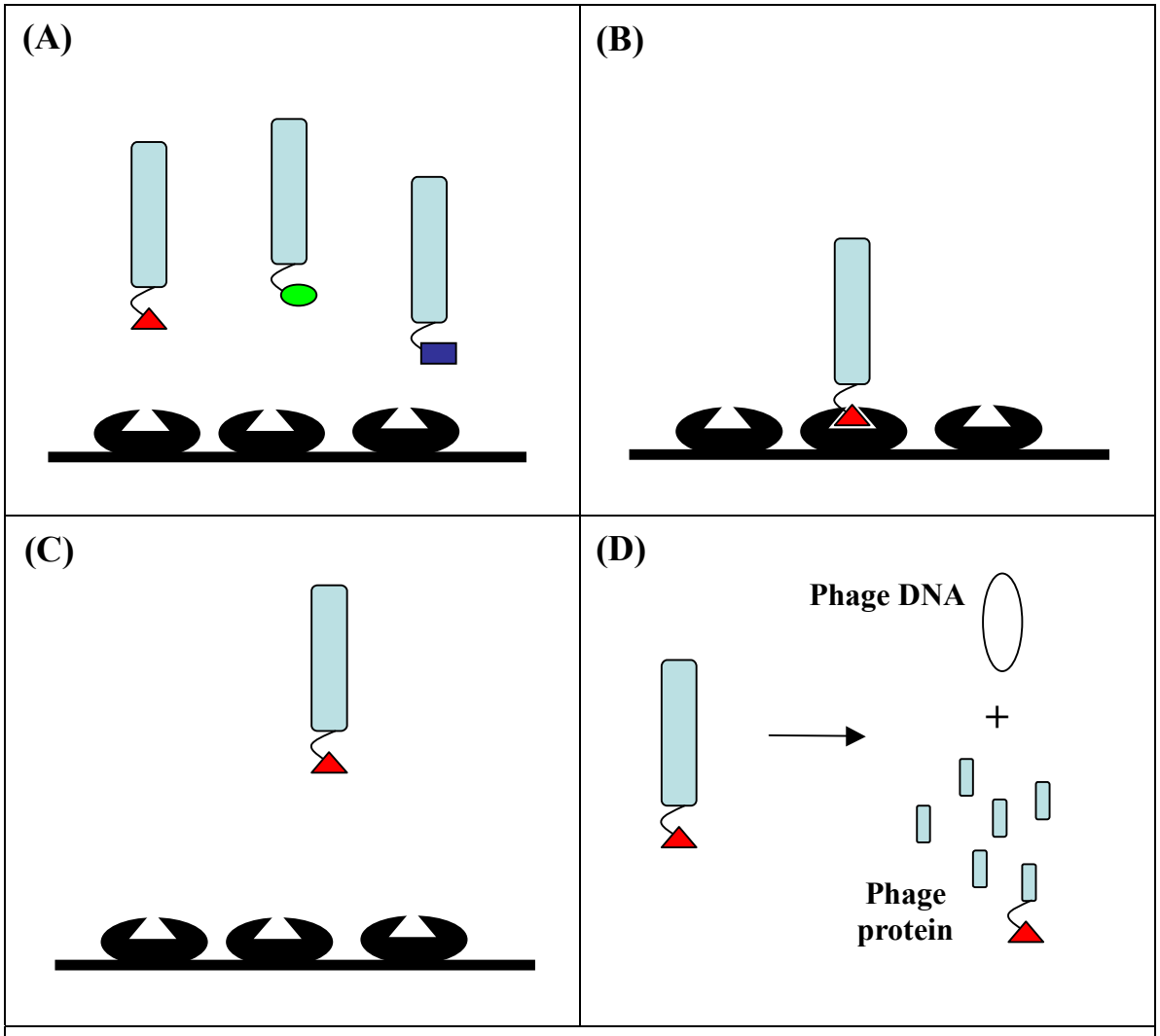

Figure 8.1 Schematic summarizing the process of panning. Each phage in the library displays a different peptide sequence, and they are exposed to an immobilized target (Panel A). Unbound phage are washed away leaving only specifically-bound phage (Panel B). Bound phage are eluted (Panel C). The eluted phage are amplified and the panning process is repeated two more times. Finally, DNA from the binding phage is isolated and sequenced (Panel D).

8.3 Experimental

This section is divided into 3 separate parts. Section 8.3.1 discusses the panning experiments that were conducted to identify peptide sequences specifically binding to cholera toxin immobilized on a plate. Sections 8.3.2 and 8.3.3 cover the experiments used to analyze ability of phage isolated in Section 8.3.1 to bind specifically to cholera toxin.

8.3.1 Panning Using Phage Display

A phage display library was screened for peptide sequences that specifically bind to cholera toxin. The experimental steps used during the panning process are described below.

Cholera Toxin – Cholera toxin from *Vibrio cholerae* type Inaba 569B was purchased from List Biological Laboratories, Inc. (Campbell CA). One vial reconstituted in 1mL of deionized water results in a solution containing 1mg toxin in: 0.05 M Tris, 0.2 M NaCl and 0.001 M Na_2EDTA at pH 7.5. The solution was stored at 4 °C.

Coating of Plate with Cholera Toxin – A solution of 100 μg/mL of cholera toxin in 0.1 M $NaHCO_3$ (pH 8.6) was prepared. 1.5 mL of this solution was added to a 60 x 15 mm sterile polystyrene Nunclon∆ dish, purchased from VWR International (So. Plainfield, NJ). The plate was swirled to completely wet the surface. The plate was incubated overnight at 4 °C with gentle agitation.

Panning – The toxin solution used to coat the plate was decanted and remaining uncoated surface sites were blocked using a sterile BSA solution, or Blocking Buffer (0.1 M $NaHCO_3$, pH 8.6, 5 mg/mL BSA, 0.02% NaN_3). The blocking solution was incubated in the plate for 1 hr at 4 °C and then decanted. The plate was washed 6 times with TBST (50 mM Tris-HCl, pH 7.5, 150 mM NaCl + 0.1%[v/v] Tween-20).

Ph.D.-12™ Phage Display Peptide Library Kit was purchased from New England Biolabs®, Inc. (Beverly, MA). Phage (~4 x 10^{10} virons) were added to the plate in 1 mL of TBST. The plate was incubated with gentle shaking for 10-60 min at 25 °C. Non-binding phage were decanted, and weakly bound phage were washed away in 10 washes of TBST. Bound phage were eluted with 1 mL of 0.2 M Glycine-HCl, pH 2.2, 1 mg/mL BSA by gently shaking for less than 10 min. The eluate was neutralized with 1 M Tris-HCl, pH 9.1. The phage were amplified by adding the eluate directly to a 20 mL ER2738 *E. coli* culture (at early-log phase) and incubated at 37 °C for 4-5 hrs. The phage was isolated by centrifuging out the cells and collecting the supernatant. Phage were precipitated by adding 1/6volume of a PEG/NaCl solution (20%w/v) polyethylene glycol-8000, 2.5 M NaCl) and incubating overnight at 4 °C.

The next day, the phage were pelleted by centrifugation and the supernatant was decanted. The phage pellet was re-suspended in 1 mL TBS and re-precipitated with PEG/NaCl on ice for 1 hr. The phage were pelleted again by centrifugation. After the supernatant was decanted, the phage were re-suspended in 200 μL TBS, 0.02% NaN_3.

This is the *amplified eluate*. The eluate was titered to determine the phage concentration for the next round of panning.

This procedure was repeated 2 more times, selecting for the phage that bound the immobilized cholera toxin the strongest. After titering the amplified eluate, the appropriate volume containing 1-2 x 10^{11} phage forming units (pfu) was used for each subsequent round of panning.

Sequencing of Phage Clones – After the 3rd round of panning, the eluated phage were used to infect 200 µL of an ER2738 *E. coli* culture (at mid-log phase) and plated on pre-warmed LB/IPTG/Xgal plates. The plates were incubated overnight at 37 °C. Phage plaques were identified from their blue color. A sterile pipet tip was used to stab a blue plaque and inoculate 1 mL of diluted (1:100) overnight ER2738 *E. coli* culture. This was repeated for a total of 20 plaques. The cultures were incubated at 37 °C with shaking for 4.5 hrs.

The cultures were transferred to microcentrifuge tubes and centrifuged for 30 sec. The supernatant was collected and re-spun. The upper 80% of the supernatant was removed with a sterile pipet and stored at 4 °C. Samples for long-term storage were diluted 1:1 with sterile glycerol and stored at -80 °C.

For purification of phage DNA, 500 µL of the phage-containing supernatant above was transferred to a fresh tube. Phage were precipitated by adding 200 µL of the PEG/NaCl solution and incubated at 25 °C for 10 min. The phage were pelleted by centrifugation and the supernatant was removed with a pipet. The pellet was re-suspended in 100 µL Iodide Buffer (10 mM Tris-HCl, pH 8.0, 4 M NaI) and 250 µL ethanol and incubated for 10 min at 25 °C to precipitate the single-stranded phage DNA. The mixture was centrifuged for 10 min and the supernatant was discarded. The pellet was washed once in 70% ethanol and dried under a vacuum. The DNA pellet was re-suspended in 30 µL TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA) and stored at -20 °C.

DNA samples from 20 plaques were submitted to Cleveland Genomics, Inc. (Cleveland, OH) for sequencing. 5 µL of the DNA in TE buffer from above from each plaque was submitted along with the -96 sequencing primer that was included in the phage display kit. The DNA sequences obtained corresponded to the *anticodon* strand of the template. The corresponding complimentary strand sequence of the randomized region was determined. The 3rd position of each codon was checked for the presence of a G or T to confirm the phage DNA came from the combinatorial library. Finally the amino acid sequence encoded by this strand was determined using the genetic code.

8.3.2 ELISA: Cholera Toxin-Coated Plates

This experiment was used to analyze the ability of the phage isolated during the panning process to bind cholera toxin. Here, cholera toxin was coated onto a microtiter plate and challenged with each phage clone. To determine if phage bound to the toxin, an antibody against M13 phage was used. The process is shown in Figure 8.2.

Phage Amplification – Phage samples determined to possess possible cholera toxin binding sequences during the panning experiments were amplified once again within ER2738 *E. coli* cultures. Each clone was used to infect 20 mL of culture (at mid-log phase). The culture was incubated at 37 °C with shaking for 4.5 hrs. The cells were pelleted by centrifugation and the supernatant was collected. Phage were precipitated by adding 1/6volume of PEG/NaCl solution and incubated at 4 °C overnight.

The mixture was centrifuged for 15 min to pellet the phage. The supernatant was discarded and the pellet was re-suspended in 1 mL TBS. Phage were re-precipitated with the PEG/NaCl solution on ice for 1 hr. The mixture was centrifuged and the supernatant was discarded. Each phage pellet was re-suspended in 50 µL TBS and titered. The solution was stored at 4 °C

Coating of ELISA plate – Immulon® 2HB 96-well immunoassay microplates purchased from Sigma (St. Louis, MO) were used for the ELISA experiments. One well for each clone to be analyzed was coated with 200 µL of a cholera toxin solution (100 µg/mL in 0.1 M $NaHCO_3$, pH 8.6). The plates were incubated overnight at 4 °C. One additional well was coated with cholera toxin as a negative control. Two wells were also coated with 100 µL of a control antigen (Anti-M13 monoclonal antibody) included in the Recombinant Phage Antibody System (RPAS) Detection Module that was purchased from Amersham Biosciences Corp. (Piscataway, NJ).

ELISA – Once the plates were coated, they were shaken to remove excess coating solution. Each coated well was blocked with Blocking Buffer. For each coated well, a corresponding uncoated well was blocked in the same manner. The blocked plates were incubated for 2 hrs at 4 °C. Excess blocking buffer was removed and the wells were washed 6 times with 1X TBST.

For each phage to be analyzed, ~5 x 10^{11} virons were prepared in 200 µL TBST. A positive control phage (M13K07) included in the RPAS Detection Module was diluted 100-fold in 1X PBS. To 100 µL of diluted positive control phage, 20 µL of Blocking Buffer and 80 µL of 1X PBS was added. The phage solutions were incubated for 30 min at 25 °C to block any non-specific protein-protein interactions that may occur. 200 µL of each phage solution was added to the corresponding coated and uncoated wells. For a negative control, 200 µL of TBST was added to a well coated with cholera toxin and an uncoated well. The plate was incubated at 25 °C for 2 hrs. After the 2 hr incubation, the phage solutions were removed and all the wells were washed 6 times with TBST.

HRP/Anti-M13 monoclonal conjugate included in the RPAS Detection Module was diluted 1:5000 in Blocking Buffer. 200 µL of this conjugate was added to each well and the plate was incubated at 25 °C for 1 hr. The plate was then washed again 6 times with TBST. 200 µL of an ABTS substrate solution (21 mL of a 0.22 mg/mL ABTS in 0.05 M citric acid, pH 4.0 + 36 µL of 30% H_2O_2) was added to each well. The plate was incubated for 60 min at 25 °C. The ABTS-HRP reaction in each well was read using a Labsystems Multiskan RC (Finland) microtitre plate reader set at 405 nm and recorded.

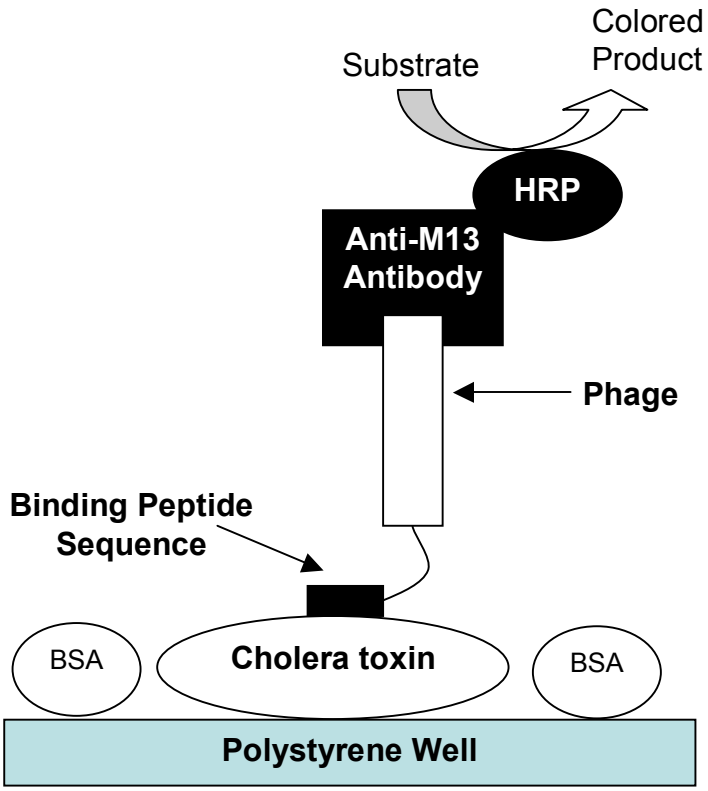

Figure 8.2 Schematic illustrating the ELISA experiments conducted with cholera toxin-coated plates. After coating with toxin, the plates were blocked with BSA and then challenged with each isolated phage clone. Anti-M13 conjugated to HRP was then added. The HRP reaction was monitored by OD @ 405 nm.

8.3.3 ELISA: Phage-Coated Plates

This experiment was used as an alternative method for analyzing the ability of the phage isolated during the panning process to bind cholera toxin. In this experiment, the phage clones were coated onto a microtiter plate and challenged with cholera toxin. To determine if toxin bound to the immobilized phage, an anti-cholera toxin antibody was used. The process is shown in Figure 8.3.

Coating of ELISA plate – Phage samples determined to possess peptide sequences binding to cholera toxin from panning experiments were amplified as before. Two wells for each clone to be analyzed were coated with 40 μL of ~5 x 10^{11} pfu/mL solution in TBS. For a negative control, 40 μL of TBS was added to a separate well. For a positive control, one well was coated with cholera toxin as in Section 8.3.2. The plates were incubated overnight at 4 °C.

ELISA **–** After the plates were coated, they were blocked and washed as previously. 50 µL of cholera toxin at a concentration of 0.01 mg/mL in Blocking Buffer was added to the wells coated with each phage as well as the negative control well. The second set of wells coated with phage and the positive control well were incubated in Blocking Buffer. The plates were incubated at 25 °C for 2 hrs followed by 6 washes with TBST.

Goat anti-choleragenoid for cholera toxin subunit B (purchased from List Labs Biological Laboratories) was diluted 1:1000 in blocking buffer. 200 µL of this solution was added to each well and the plates were incubated 2 hrs at 25 °C. The plates were then washed 6 times with TBST. Mouse monoclonal Anti-Goat IgG-Peroxidase conjugate (purchased from Sigma) was diluted 1:1000 in blocking buffer. 200 µL of this solution was added to each well and the plate was incubated 2hrs at 25°C, followed by 6 washes with TBST.

The HRP substrate solution was prepared by using SigmaFAST™ OPD Tablet Sets. 100 µL of the substrate solution was added to each well and incubated for 2 min at 25°C. 100 µL of 3 N sulfuric acid was added to each well to stop the reaction. The reaction in each well was read using a Labsystems Multiskan RC (Finland) microtitre plate reader set at 492 nm and recorded.

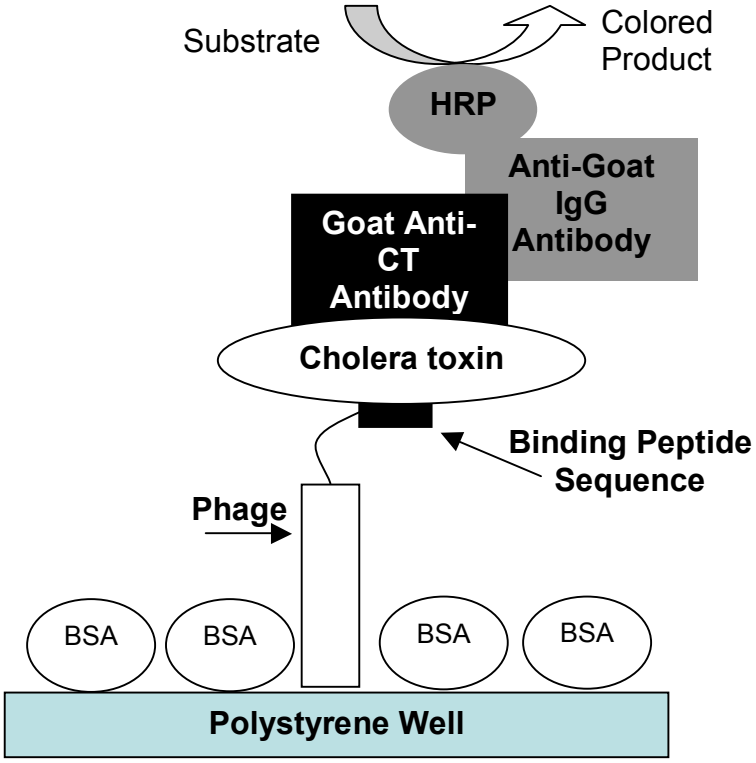

Figure 8.3 Schematic illustrating the ELISA experiments conducted with phage-coated plates. After coating with phage, the plates were blocked with BSA and then challenged with cholera toxin. Anti-cholera toxin was added, followed by anti-Goat IgG-HRP conjugate. The HRP reaction was monitored by OD @ 492 nm.

8.4 Results and Discussion

As described above, we used a phage display library to screen for peptide sequences that bind to cholera toxin. This section discusses the results obtained from the panning experiments, as well as the analysis of the isolated phage clones.

8.4.1 Panning Experiments

After 3 rounds of panning, 20 phage clones were isolated and submitted for DNA sequencing. This allowed for the determination of the DNA sequence encoding for the random peptide sequence fused to the pIII coat protein of each phage clone. Using the genetic code, the 12-peptide sequence was determined. The sequences for all 20 clones are given in Table 8.1.

Clone	Peptide Position											
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
1	H	L	H	K	Q	H	P	R	S	T	H	W
2	L	P	H	H	W	H	S	K	I	R	V	V
3	G	P	H	K	H	L	H	F	M	P	H	R
4	K	L	P	H	H	H	K	N	P	T	F	I
5	W	P	H	Y	H	F	T	R	A	P	S	P
6	Q	Y	S	I	I	F	S	S	V	D	P	L
7	W	H	K	H	R	V	S	P	E	I	E	W
8	Y	P	H	H	T	H	T	A	H	K	P	R
9	W	P	H	H	P	R	H	P	L	P	R	P
10	A	P	H	N	R	S	M	T	P	W	L	V
11	S	L	W	H	G	H	K	H	H	S	P	L
12	H	S	S	S	H	H	H	H	L	S	M	R
13	H	H	R	H	P	P	L	T	P	P	A	I
14	L	P	H	W	K	H	P	H	T	P	R	P
15	Y	W	P	K	P	H	S	H	H	A	R	T
16	F	P	W	W	H	H	Q	T	L	H	N	R
17	K	H	L	H	K	T	H	P	H	L	I	F
18	H	H	H	H	F	R	P	M	P	R	D	W
19	S	L	W	H	G	H	K	H	H	S	P	L
20	I	S	P	K	I	H	H	H	Q	H	K	H

Table 8.1 The 12-amino acid sequences obtained from 20 phage clones isolated after 3 rounds of panning against cholera toxin.

The sequences found in Table 8.1 were analyzed rigorously to identify a consensus sequence. A consensus sequence is likely to be a short sequence having some degree of homology. Homology could be a result of sequences consisting of identical amino acids, charge structure, hydrophobicity or secondary structure. Upon inspection of the sequences, it can be seen that there is an abundance of histidine (H) and proline (P) residues. These residues account for 42% of the residues in the 20 peptide sequences. Also, 38% of the residues are basic, or positively charged at pH~7. Therefore, it was hypothesized that these properties may be required for the peptide sequences to bind to cholera toxin.

We identified 5 phage clones that potentially contained a consensus sequence. The possible 6-residue consensus sequence that we identified was:

Trp/Leu – Pro – His – His – X – His/Lys/Arg

or

W/L – P – H – H – X – H/K/R

Here, the peptide in the 1st position could be Trp or Leu, X could be any residue, and the peptide in the 6th position could be His, Lys or Arg. The phage clones that contained variations of this possible consensus sequence are given in Table 8.2.

	Selected Phage Clones Containing Possible Consensus Binding Sequence					
Clone	**W/L**	**P**	**H**	**H**	**X**	**H/K/R**
2	L	P	H	H	W	H
4	L	P	H	H	H	K
5	W	P	H	Y	H	F
9	Y	P	H	H	T	H
10	W	P	H	H	P	R

Table 8.2 Phage clones containing possible consensus cholera toxin-binding sequence.

The phage clones shown in Table 8.2 were isolated, amplified and stored for further analysis. The following experiments were used to analyze these sequences to confirm their ability to bind cholera toxin.

8.4.2 ELISA: Cholera Toxin-Coated Plates

The first set of experiments used to analyze the possible toxin-binding phage, used the common technique known as ELISA, enzyme-linked immunosorbent assay. Here, microtiter plates were coated with cholera toxin and then challenged with each of the 5 phage clones. Next, anti-M13 antibody conjugated to HRP was added, which binds to any phage that is bound to the immobilized cholera toxin. Finally, the presence of phage bound to cholera toxin was determined using the ABTS-HRP reaction, which can be monitored by OD at 405 nm.

Positive and negative controls were included in the experiment. The positive control consisted of wells coated with a positive control antigen (Anti-M13 monoclonal antibody), which was challenged with a positive control phage (M13KO7) and finally incubated with anti-M13 antibody conjugated to HRP. The negative control consisted of wells that were not challenged with phage. For each sample ran with target-coated wells, an identical set of experiments were run with uncoated wells.

The results from the ELISA experiment using wells coated with the target molecule are tabulated in Table 8.3 and are also shown in Figure 8.4. The positive control sample with target-coated wells showed a much higher signal than without target. This suggests that the positive control phage successfully bound to the antigen and secondary antibody (Anti-M13 antibody conjugated to HRP). The negative control showed relatively no difference between coated and uncoated wells, as expected. Both control experiments suggest that the ELISA procedure was valid.

Unfortunately, the results from the phage clones did not suggest that any appreciable binding to cholera toxin was occurring. The values varied for all 5 clones, but there was not a large difference between the values for coated and uncoated wells, as was seen with the positive control. However, the readings were quite higher than background (OD_{405} ~ 0.06 from negative control). This suggests that some binding to the plate was occurring. This was troubling since the wells were blocked with BSA, which will bind to

any free sites on the polystyrene surface. BSA is used as a blocking agent since it has been shown that very few peptide sequences will bind strongly to BSA because of its extremely hydrophilic nature.[17] Therefore, this leads to the troubling conclusion that the wells are incompletely blocked, possibly leading to the phage binding to the plate.

Phage	OD at 405 nm	
	w/ Target	w/o Target
2	0.583	0.344
4	0.141	0.228
5	0.638	0.463
9	0.263	0.277
10	0.197	0.286
Positive Control	2.142	0.063
Negative Control	0.055	0.059

Table 8.3 OD values at 405 nm obtained from ELISA experiments using cholera toxin-coated wells.

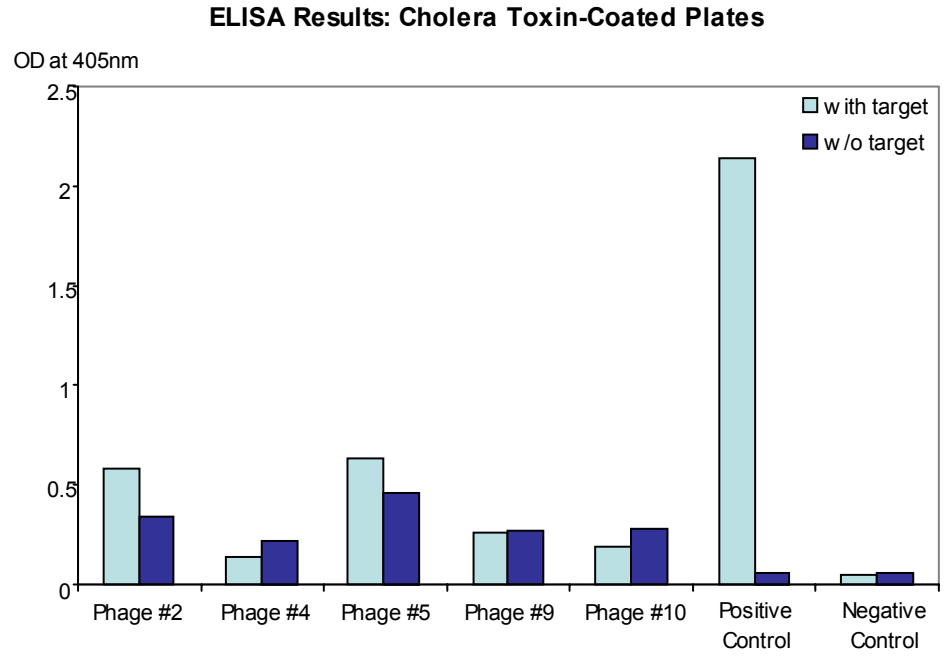

Figure 8.4 Bar graph showing OD values at 405 nm obtained from ELISA experiments using cholera toxin-coated wells.

8.4.3 ELISA: Phage-Coated Plates

Microtiter plates were coated with each phage clone and then challenged with cholera toxin. Next, anti-cholera toxin (produced in goat) was added, which bound to any toxin that will bind to the immobilized phage. Then, anti-Goat IgG-HRP conjugate was added, which bound to anti-cholera toxin. Finally, the presence of cholera toxin bound to phage was determined using the peroxide-HRP reaction, which can be monitored by OD at 492 nm. A positive control was included in the experiment. The positive control consisted of wells coated with a cholera toxin instead of the phage. For each phage control, a separate well was also coated with phage, but was not challenged with toxin.

The results from the ELISA experiment using wells coated with the isolated phage clones are tabulated in Table 8.4 and are also shown in Figure 8.5. The positive control sample with toxin-coated wells showed a much higher signal than those with phage-coated wells. This suggests that the anti-cholera toxin and anti-Goat IgG-HRP conjugate worked properly and that the ELISA procedure was valid.

Phage	**OD at 492 nm**	
	w/ CT	**w/o CT**
2	0.159	0.159
4	0.164	0.124
5	0.148	0.132
9	0.140	0.127
10	0.135	0.130
Positive Control	1.920	---

Table 8.4 OD values at 492 nm obtained from ELISA experiments using phage-coated wells.

The results from the phage clones did not suggest that any appreciable binding to cholera toxin was occurring. There was little difference between the values for samples challenged with cholera toxin and those that were not. This suggests that cholera toxin was not binding at all to the immobilized phage. The values seen could simply be a result of background signals. This indicates that the phage clones were not binding the toxin.

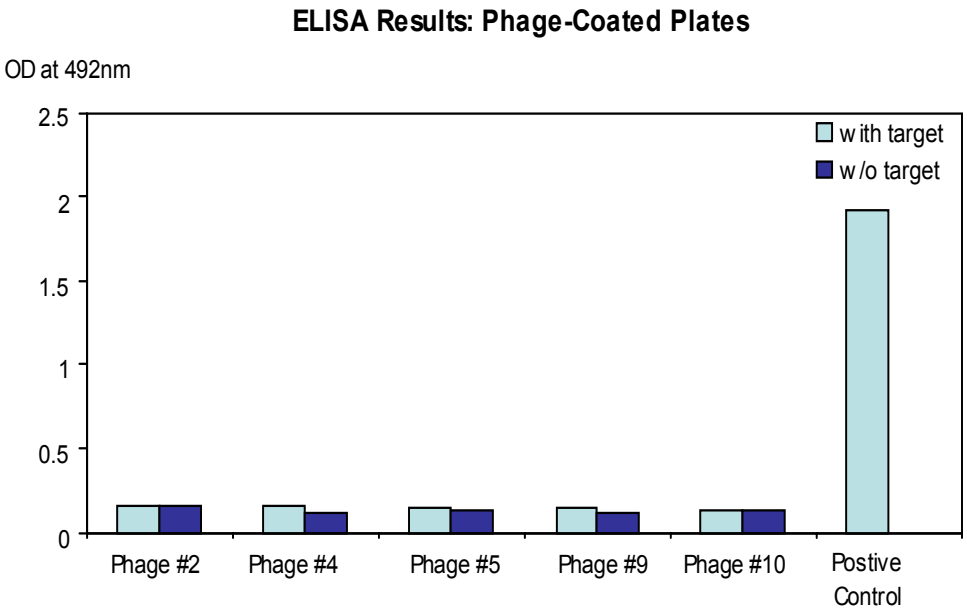

Figure 8.5 Bar graph showing OD values at 492 nm obtained from ELISA experiments using phage-coated wells.

8.4.4 Plastic-Binding Sequences

The results from the ELISA experiments suggest that the clones isolated were not binding to cholera toxin. Therefore, the sequence (W/L-P-H-H-X-H/K/R) is not valid as a binding sequence and the clones isolated were not true cholera toxin binders. However, as mentioned in Section 8.4.1, the peptide sequences did not seem to be random, so they were binding to something during the panning process.

After further analysis, it was hypothesized that it was possible that the phage clones isolated were 'plastic binders'. Adey *et al.* isolated phage from peptide display libraries that bound directly to polystyrene surfaces.[18] They found that peptide sequences rich in Trp and Tyr residues will bind to polystyrene plates, even when blocked with BSA. Only 2% of the residues in sequences isolated during our panning were Trp and Tyr. However, New England Biolabs®, Inc. suggests that sequences rich in aromatic residues will bind to polystyrene. So in addition to the residues found by Adey *et al.*, they suggest His and Phe will also lead to plastic binding. Hence, 37% of the residues in our sequences could be "plastic binders". Therefore, it unfortunately appears that during our

panning experiments, we selected for phage that were binding to the polystyrene plates and not to cholera toxin.

Both Adey *et al.* and New England Biolabs®, Inc. suggest methods to avoid selecting for phage that bind to plastic. Adey *et al.* suggest using a higher density of immobilized target, blocking with non-fat milk instead of BSA, and using an elution scheme that is specific to the target molecule. New England Biolabs, Inc. suggests using a different panning technique where the phage is reacted with the target in solution. The phage-target complex is then captured using beads (crosslinked agarose) that can specifically bind to the target (*e.g.* functionalized with antibodies).

8.5 Conclusions

Recently, interest in new methods for the rapid detection and real-time monitoring of biological toxins has grown. In this work, we hypothesized that a peptide-liposome immunoassay would be successful for this application. To demonstrate this, we used phage display in order to identify a peptide sequence that specifically binds to cholera toxin which could be incorporated into a functionalized liposome where the binding event would result in some measurable signal. After 3 rounds of panning, we selected 20 phage clones that were possible toxin binders. However, after analyzing these clones using ELISA-based formats, we determined that the panning experiments did not yield true cholera toxin binders. There is some evidence that suggests that the clones we isolated were a result of displayed peptides binding directly to the plastic plates and not to the immobilized cholera toxin. Unfortunately, we were unable to investigate additional phage display methods and the project was continued using natural ligands for cholera toxin (*e.g.* G_{M1} ganglioside receptors).

8.6 References

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