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Avram Siegel
West Virginia University

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Peptide Based Molecular Recognition Elements for Binding 2,4,6-Trinitrotoluene

Avram Siegel

Thesis submitted to the College of Engineering and Mineral Resources
at West Virginia University
in partial fulfillment of the requirements
for the degree of

Master of Science
in
Chemical Engineering

Robin Farmer, Ph. D., Chair

David Klinke, Ph. D.

Charter Stinespring, Ph.D.

Department of Chemical Engineering

Morgantown, WV

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Abstract

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Avram W. Siegel

Three peptides labeled AS2, AS3 and AS6 were designed to evaluate the role of secondary structure in a peptide's ability to selectively bind 2,4,6 trinitrotoluene (TNT). The peptides were produced using recombinant DNA methods through *E. coli* expression. It was hypothesized that AS6 would form a random coil, AS2 would form an α -helix and AS3 would form a β -sheet. The secondary structures of the peptides were determined using circular dichroism spectroscopy and the binding of the peptides to TNT was studied using an enzyme linked immunosorbent assay. After expression, the presence of the peptide in the final purified product was tested using a variety of methods including amino acid analysis western blot, and mass spectrometry. It was concluded that the *E. coli* did not consistently produce the desired peptides or produced them at very low levels, which precluded conclusive assessment of the contribution of peptide secondary structure in binding TNT.

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1 Introduction

1.1 TNT Detection

Detection of explosives has been a concern for the purposes of public safety and environmental monitoring. Trinitrotoluene is a known toxin and mutagen which has detrimental affects to various wildlife.^{1, 2} Numerous methods have been developed to detect TNT. One method used microcantilevers to detect TNT through deflagration. The TNT was adsorbed onto the silicon microcantilever. A current was sent through the cantilever to heat the TNT and caused desorption and deflagration. The bending of the microcantilever due to the deflagration of the TNT enabled the detection of the substance. A sample as small as 50 pg can be detected and contaminants such as water vapor, gasoline vapor and solvents do not interfere with detection due to the much slower desorption of the TNT.³

Traditional laboratory detection systems have also been used to detect TNT. Plasma chromatography has been studied to detect TNT vapors. TNT can be detected through its positive and negative mobility spectra. This instrument can detect 10 ng in 100 ml of air. This instrument is also insensitive to possible contaminants in the air such as water vapor or other light gases.⁴

TNT has also been detected in soil using thermal decomposition of explosive within the soil by heating it with a platinum coil and pumping the products into an amperometric nitrogen oxide sensor. This detection system has a lower detection limit (LDL) of 500 ppb-wt within soil samples, although the system requires the soil to be dry before testing. The readings may also be skewed by other naturally occurring volatile chemicals that are similar to TNT decomposition products.⁵ Many of these methods use long sample preparation time and expensive, heavy equipment that can only be used in a laboratory environment. These obstacles limit their use in the field.

Currently TNT and explosives in general are detected using several different methods.^{6, 7} One field detection method is using a gas chromatograph (GC) with a thermal energy analyzer (TEA). The GC-TEA device detects explosives by pyrolyzing the nitro groups to form a nitosyl radical then reducing it to nitrogen dioxide which is in an excited state. This molecule emits a light which is detected by a photomultiplier.

Explosives can also be detected by their ion mobility using a GC connected to an ion mobility spectrometer (GC-IMS). This device creates analyte ions and injects them into a drift region. Different ions are separated and identified by their drift times due to having different masses. The IMS device has been able to detect TNT in the picogram range. In airports, a piece of equipment commonly to assist in the detection of explosives is a portal. A portal is a door frame shaped piece of equipment that blows air across a person as he or she walks through. The puff of air is collected to be further analyzed for any explosive chemicals that may have been dislodge or volatized from the person in the portal. These pieces of equipment are very expensive and require technical expertise as well as annual maintenance. Bomb sniffing dogs are also a common method for detecting and are able to detect trace amounts of a large variety of explosives. Although a dog cannot communicate the type of explosive found and can only work short amounts of time. Dogs also require a handler to perform their job.

Sensors, in general, that employ biological probes are able to bypass some of these logistical problems. Biological probes such as DNA, antibodies or peptides have high selectivity and can be conjugated with elements that fluoresce when the component binds. This quality enables little to no sample preparation time and detection can be read by simply looking for fluorescence of the indicator molecules.

1.2 Biosensing Molecules and Organisms

Biosensors use a biological molecule or organism to detect other biological molecules, viruses, bacteria, or chemicals. Biosensors can be incorporated into portable, fast and relatively inexpensive devices for specific chemical measurement. These sensors have been used in a wide array of fields including measurement of pollutants,⁸ detecting methane,⁹ virus detection for diagnosis of disease^{10, 11} and hormone detection for pregnancy tests.¹²

Whole organisms can be used as biosensors to detect chemicals. Bees have been used as a passive detector of chemical and biological agents due to the tiny hairs collecting chemicals that the bee passes through. A bee has been shown to be able to “scavenge” airborne bacteria and microbeads in a wind tunnel environment. The particles are collected by the electrostatic interaction between the bee and the particles.¹³

A bee can also be trained to actively seek out and fly around a targeted volatile chemical. They are conditioned to do this by injecting trace amounts of the chemical into their feeders. This was tested using vapor plumes of 2,4,6-trinitrotoluene (TNT). After conditioning, the bees were released into a field and were tracked by light detection and ranging. This bee detection system has a detection rate of 97-99% with false negative and false positive probabilities 1-2.5% and less than 1% respectively.^{14, 15} The bees sense the TNT through small proteins located on the antenna called odorant binding proteins (OBPs). These small proteins transport the small odorants to environmental receptors on the antennal olfactory sensilla.¹⁶⁻¹⁸

Much simpler organisms such as algae have been used to detect volatile chemicals. The detection of chemicals by algae can be monitored through the chlorophyll *a* fluorescence of photosystem II (PSII) or oxygen.^{19, 20} These methods measure toxic effect of the chemical being detected on the algae. In the detection of herbicide, quinine binding sites in the PSII are blocked. This toxic effect drastically changes the reaction kinetics of the fluorescence of the chloroplasts.^{21, 22} Other chemicals such as methanol can reduce oxygen production.¹⁹ Algae has also been used to detect TNT at concentrations ranging from 0.5 to 31.3 mg/l. Both wild type and TNT resistant mutants are used to increase the specificity.²⁰

A heavily researched area of biosensors is the use of biological molecules such as antibodies, DNA, proteins or small peptides for fluorescent detection assays. These molecules are commonly used in molecular beacons which fluoresce when they bind to a target molecule. A molecular beacon is made of three main parts: a fluorophore, a quencher and the binding biological molecule. The molecule has a hairpin shape where the quencher and fluorophore are held in close proximity prior to binding. The quencher accepts electrons from the fluorophore to prevent the molecule from fluorescing. Upon binding to the target molecule, the molecular beacon undergoes a conformation change, which causes separation of the quencher and fluorophore. Molecular beacons are commonly used to detect pathogens or other biological molecules. Molecular beacons containing nucleic acid as the binding molecule have been used in conjunction with polymerase chain reaction (PCR) techniques to detect *Vibrio cholera* (*Cholera toxin*) and enterotoxigenic *Escherichia coli* (*E.coli*) from environmental water samples.²³⁻²⁵

Oligonucleotide molecular beacons have also been used to detect HIV DNA or enzymes from clinical serum samples.^{10, 11} This process involves amplifying HIV DNA using PCR. Molecular beacons are added into the amplification reaction to bind to the DNA. As the DNA is amplified the signal from the molecular beacon grows as more and more beacons bind to the target DNA. The enzyme is detected by the cleaving of the quencher on the probe by the enzyme. Multiple strains of HIV can be detected in the same sample due to the specificity of the primer within the molecular beacons. The DNA can be quantified by determining the number of thermal cycles the PCR performs before a significant fluorescence from the molecular beacon is detected.

1.3 Antibodies

Monoclonal antibodies have been developed that bind to TNT for use in immunoassays.²⁶ The most common immunoassay is the Enzyme Linked ImmunoSorbent Assay (ELISA). Two commonly performed ELISA formats are the sandwich assay and the competitive assay. The sandwich assay is performed by immobilizing the antibody on an ELISA plate surface as shown in Figure 1.

Figure 1: Sandwich ELISA

1) An antibody is immobilized on a surface by binding the antibody to the surface through Van der Waal forces and hydrogen bonding, 2) Buffered solution containing the antigen is incubated with bound capture antibody, 3) Unreacted molecules are washed away, 4) A second antigen specific antibody that is enzyme labeled is added, and 5) Unbound labeled antibody is washed away, a substrate is added to the well which is oxidized by the enzyme which is detected by optical density measurements.

A buffered solution containing the antigen of interest is incubated with this bound captured antibody. Unreacted molecules are washed away and a second antigen specific enzyme labeled antibody is added. After unbound labeled antibodies are washed away an enzyme substrate is added. Substrated hydrolysis by the enzyme results in release of chromophore, fluorophore, chemiluminescent light or produces a precipitant. Formation of the hydrolysis product is directly related to the amount of antigen added to the ELISA.^{27, 28}

In a competitive assay an antigen-enzyme complex competes with an unlabeled antigen from a standard or sample shown in Figure 2. The intensity of the signal after binding is inversely related to the concentration of unlabeled antigen.²⁹ A sandwich ELISA developed specifically for detecting TNT in water as a pollutant due to manufacturing and purification of TNT as well as ammunition storage has been reported. The ELISA was able to distinguish TNT from other nitroaromatic compounds and had a detection limit of 20 ng/L. This immune assay was also tested with spiked water samples and was able to quantify the amount of TNT with low variation.³⁰

Figure 2: Competitive ELISA

1. An antigen with a competing molecule is incubated with the antibody which is immobilized on a surface 2. The unbound antigens and the competing molecules are washed away 3. A secondary enzyme conjugated antibody is incubated with the immobilized antibody. 4. The competing molecule results in a reduced signal after the substrate is added inducing the enzyme to fluoresce.

Other studies have been performed to develop technologies to improve the detection capability of immunoassays. An evanescent wave fiber-optic biosensor has been developed to detect TNT pollution in different bodies of water. The detection device uses an optic fiber with a TNT binding antibody attached to the end of it. The antibody is attached to a fluorescently labeled trinitrobenzene (TNB) molecule. The fluorophore is excited by a laser light to create a signal. The fibers are exposed to a sample and if the sample contains TNT it displaces the TNB and reduces the intensity of the signal.³¹

1.4 Peptide based biosensors

There are a large variety of biosensors that use peptides as their biological recognition element³²⁻³⁴. Peptides have certain advantages over antibodies due to peptides requiring less labor and time to produce. The cost of producing peptides is also considerably less than antibodies since they can be produced from simple organisms such as *E. coli* and yeast. Antibodies also may not be producible due to an analyte having non-antigenic properties.

Phage display peptides are commonly used as recognition elements in biosensors. A single phage can display hundreds of copies of a single peptide. The large number of displayed peptides would greatly increase the chance that the displayed peptide would find its target analyte. Phage display peptides have been researched as biomolecular recognition elements for Staphylococcal enterotoxin B (SEB).³³ A phage display library was screened to obtain peptides that would bind to SEB. The phage displayed peptides were tested in an ELISA format. Nine different peptides were selected from a library and tested for binding to SEB and with cross reactivities against other staphylococcal enterotoxins. Finally the peptides were tested in a fluoroimmunoassay and compared to anti-SEB antibodies. Although the phages were not able to produce a signal as great as the antibody which binds to SEB, it was able to produce a detectable signal showing the peptide binding to SEB.

Phage display has also been used for the detection of TNT.³² A phage library displaying 12-mer peptides were screened for TNT binding peptides. The goal of

this research project was to detect TNT in environmental waters. The binding of the phage displayed peptides were tested in an ELISA format using TNB. TNB is used in an ELISA format due to the ability of trinitobenzenesulfonic acid (TNBS) to bind to primary amine groups. The binding immobilized the molecule to the bottom of an ELISA plate via ovalbumin or BSA. The phages were used in ELISAs where TNB was conjugated to ovalbumin and immobilized on the bottom of the plate. The phages binding to TNB was tested in artificial seawater. Competitive ELISAs using TNT were also performed. The phages had a detection limit of 10mg/l of TNT in seawater.

Another format that peptides are used is peptide nucleic acids (PNA). PNAs have been used in a electrochemical biosensor for detecting genetic mutations in the tumor suppressor gene *p53*.³⁴ The PNA molecule hybridizes upon contact with its target complementary DNA sequence. The binding is monitored using constant-current chronopotentiometry. PNAs are desired over DNA aptamers due to their ability to detect single base pair mutations. This biosensor was able to detect the mutation in about 2 minutes using only 0.8 μ M of the PNA.

Recombinant DNA is the ligation of engineered DNA into the natural DNA of an organism. This technique was first published by Cohen, Chang, Boyer and Helling.³⁵ One of the primary uses of recombinant DNA is producing engineered peptides that a particular organism, such as an *E. coli* cell, would not normally produce. Recombinant DNA methods have been widely researched in the pharmaceutical and medical fields. This method has been used for developing antimicrobial³⁶ and antifungal peptides³⁷ for medical treatments and producing elastin-like proteins for drug carriers.³⁸ Recombinant DNA is also common for producing protein and peptide based molecular recognition elements for biosensors. One such study produced proteins for binding to lipidopolysaccharide and lipid A for the detection of gram negative bacteria.³⁹ A green fluorescent protein was designed and attached to a beta sheet hairpin protein. The other end of the hairpin contained one or two strands of a 5 amino acid motif that alternated basic and hydrophobic amino acids. This motif bound to the lipidopolysaccharide. These proteins were tested in mediums of different pH's and ionic strengths. It was shown that the protein with only 1 binding strand had a higher sensitivity with a detection range of 2.5ng to 20ng. The protein with 2 binding strands had a binding range of 7.5ng to 60ng.

1.5 Carbon Materials and Their Detecting Abilities

Although peptides have a high selectivity when binding to an analyte, the binding event needs to be detected to confirm the presence of the analyte. Graphene shows promise as a candidate for use within a sensor device due to its high sensitivity.^{40, 41} Graphene is a single layer of carbon atoms bonded together in a hexagonal pattern. Graphene is mostly made from graphite which is multiple layers of stacked graphene. This material is currently being studied in gas specific detection.⁴⁰ The high electron mobility and structure of graphene gives it the potential for use as a detector for sensing gas molecules in the parts per billion range. The detection principal is based on changes in carrier concentration on the surface that occur when gas molecules are absorbed onto the graphene producing a change in the resistivity of the grapheme, and it is this change that allows analyte quantifications. A graphene sensor device can also be reused; the adsorbed molecules can be removed by annealing the graphene in a vacuum at 150° C. This desorption process can be repeated without poisoning the material. The device can also be cleaned by exposure to ultraviolet light.

Graphene based detectors have been tested to detect explosives and chemical warfare agents.⁴¹ A graphene oxide film was tested to detect dinitrotolene (DNT), hydrogen cyanide (HCN) and other organophosphates. The low frequency noise of the detector is tuned to different analytes by exposing the film to hydrazine hydrate vapor which reduces the film to graphene. This detector was able to detect all analytes tested in the parts per billion range. This device also was significantly more sensitive to HCN than a carbon nanotube detector tested under the same conditions. It is also possible to functionalize this detector by covalently bonding chemicals to chemically active oxygen defects on the surface of the graphene oxide sheet. Functionalization of the graphene would increase the selectivity of the detector for other possible chemical or biological analytes.

Carbon nanotubes, which are cylindrically shaped graphene sheets, have also been studied as gas detectors and biosensors. Two common sensor formats that are used with

carbon nanotubes are chemiresistive and field effect transistor (FET) sensors. Chemiresistive sensors detect analytes by a change of capacitance due to the analyte directly adsorbing on to the carbon nanotube or binding to an aptamer absorbed on to the surface of the carbon nanotube. Chemiresistive carbon nanotubes sensors have been shown to detect NO₂ at ranges of 10-50 ppb.⁴² The selectivity and sensitivity of a carbon nanotube can be significantly increased by coating the surface with a polymer that would make the carbon nanotube insensitive to contaminant molecules. Coating a carbon nanotube with polyethyleneimine (PEI) increases its selectivity towards NO₂ in the presence of NH₃ and lowers the LOD to 100 ppt NO₂. NH₃ can also be detected by changing the coating to Nafion (sulfonated tetrafluoroethylene based fluoropolymer-copolymer). Harmful warfare agents such as sarin gas have also been researched as a possibility of being detected by chemiresistive nanotubes.⁴³ Carbon nanotubes were coated with hexafluoroisopropanol substituted polythiophene (HFIP-PT) to increase the selectivity of the carbon nanotubes. The sensor was tested using dimethyl methylphosphonate (DMMP) due to its similarity in hydrogen bonding and vapor pressure as well as being less toxic than sarin gas. The interaction between the DMMP and the HFIP-PT carbon nanotube is attributed to the hydrogen bonding. This sensor was also shown to have a high selectivity when tested in the presence of other vapors and a high sensitivity with a detection limit of 0.05ppm.

Carbon nanotubes have become a large dominion within the biosensor field. Chemiresistive nanotube biosensors have been developed to detect glucose⁴⁴ and a FET carbon nanotube biosensors can detect thrombin using a oligonucleotides.⁴⁵ The lightweight, high sensitivity and reliability of carbon nanotube biosensors have made it ideal for field detection of explosives. A four amino acid TNT binding peptide has been incorporated into a FET carbon nanotube biosensor by attaching it to a carbon nanotube binding peptide via a tetraglycine linker.¹⁶ The interaction energies of the biosensor with 3 different explosives (TNT, RDX and HPT) were calculated using molecular simulations; TNT had the highest interaction energy. Lab tests were also performed on this biosensor using the 3 explosives. The current between the source and drain were monitored while the biosensor was exposed to the explosives. A bare carbon nanotube was also tested as a control. The current in the bare tube showed a decrease in current

when exposed to any of the explosives. The coated carbon nanotube showed an increase in current when exposed to TNT and a decrease when exposed to the other explosives. The interaction of the TNT molecule with the 4 amino acid peptide was modeled using molecular simulations. The simulations revealed that the TNT creates a hydrogen bond with a Trp amino acid in the peptide and the ring of the TNT stacks on the surface of the nanotube through π - π interactions.

The main drawback of many carbon based sensors is the low selectivity. A bare graphene sheet will absorb many different molecules which will cause a change in the capacitance or resistance of the graphene sheet. Incorporating a biomolecular recognition element such as a peptide onto a graphene sensor would incorporate the selectivity of a biosensor with the sensitivity of a graphene based sensor.

1.6 Problem Statment

The proposed project encompasses the design of a peptide sequence which was designed to bind TNT molecules. The design of the sequence was based on the phage displayed peptides reported by Goldman et al.³² The peptides that were designed as part of the project labeled (AS2p, AS3p, AS4p, AS5p and AS6p) was synthesized by using recombinant DNA methods to produce His tagged peptides. These peptides were used to test the binding mechanism between the peptides and TNT, specifically whether the overall structure or certain amino acids in the sequence determines the strength and specificity of binding to the TNT molecule. ELISA tests were conducted using anti-His, enzyme linked antibodies.

Understanding the binding event between peptides and TNT will facilitate incorporation of this peptide based system into a graphene biosensor. Future work beyond the scope of this project will be to create a novel biosensor that incorporates the sensitivity of graphene gas detectors with the selectivity of peptide based biosensors. The device is expected to operate by measuring the change in the graphene's conductivity due to the acceptance or donation of electrons from the peptides attached to the surface. A solution with TNT will be exposed to the graphene bound peptides and any TNT that binds to peptides will change the conductivity of the graphene enabling the detection and

quantification of TNT in the solution. The schematic of the device can be seen in and how it detects TNT can be seen in Figure 4a and b.

Figure 3: Peptides Inserted Into the Graphene

Figure 4: TNT Binding to Peptides

The potential for the high sensitivity of this device would make it useful for detecting very dilute amounts of TNT in the environment for the purpose of assessing levels of either contamination in various habitats or detecting nearby mines. This ability would make it useful for rapid on site monitoring of environments near areas associated with high production or use of TNT. Other peptides could be adapted to be used with this device expanding its uses beyond TNT detection. The device could also display multiple peptides to detect several different molecules in a solution. This device could expand the field of biosensors beyond the lab for faster more specific detection of chemical agents. Therefore, developing a highly selective peptide that interacts with TNT is pertinent to the future of the biosensor project.

2 Materials and Methods

2.1 Materials

Plasmids pUC-19, pET-19b, and pET-24a were acquired from Invitrogen (Carlsbad, CA). Altered pJ201 and pExpress414 plasmids were purchased from DNA 2.0 (Menlo Park, CA). DNA oligonucleotides were also attained from Invitrogen. The restriction enzymes were purchased from Invitrogen and Fermentas (Glen Burnie, MO). Plasmid purification kits and nickel-chelated sepharose resin were procured from Qiagen (Valencia, CA). Chemicals required for protein expression and purification were purchased from Fisher Scientific (Denver, CO) and Sigma (St. Louis, MO). The TNBS and TMB substrate was purchased from Fisher Scientific. The BSA and ovalbumin was purchased from Sigma. The anti-His antibodies were purchased from Qiagen. The ELISA plates were purchased from ThermoScientific.

2.2 Cloning Strategy

The TNT binding peptides were synthesized using *E. coli* cells which were induced to express certain peptides. This was accomplished through a cloning strategy shown in Figure 5. In this strategy a DNA sequence, sequenced to produce the desired peptide, is designed and inserted into an *E. coli* cloning plasmid. This plasmid is cloned to increase the amount of desired DNA. This DNA insert is extracted out using restriction enzymes and then ligated into an expression plasmid. Finally the plasmid is transformed into chemically competent *E. coli* cells and expressed to make the desired peptide. The strategy uses type II restriction enzymes, and enables accurate production of peptides.

Figure 5: Modified cloning strategy for producing peptides

2.3 DNA Cloning and Plasmid Engineering.

Initial DNA work performed using an oligonucleotide designated RSF34 that was designed to produce a peptide to bind chondroitin sulfate. This peptide was researched because the peptide binding is well characterized. The segment was designed with BamHI and EcoRI restriction sites. These sites enable the ligation of the segment into the pUC19 plasmid. The DNA segment was purchased as two lyophilized complimentary strands. These strands were resuspended in water for a final concentration of $1\mu\text{g/ml}$. Both strands were mixed with 1M MgCl_2 and 2M NaCl and heated in boiling water for 10 minutes. The water and DNA mixture were placed in an insulated vessel to let cool slowly overnight to room temperature to allow the DNA to anneal and create a single double stranded oligonucleotide. The annealed DNA was then purified by using a nucleotide removal kit (Qiagen). Plasmid pUC19 was prepared for ligation by digesting with BamHI and EcoRI restriction enzymes for 4 hours at 37°C . After the digest $0.5\mu\text{l}$ of calf intestinal alkaline phosphatase (CIP) was added and the plasmid was incubated at

37°C for an additional 30 minutes for dephosphorylation which prevents the recombination of partially digested plasmid. Electrophoresis was performed on the digested plasmid using 1% agarose gel to separate the DNA segments from the digested plasmid. The plasmid was extracted from the gel and purified using a gel extraction kit (Qiagen). The pUC19 plasmid was ligated with RSF34 at room temperature for 1 hour using T4 DNA ligase. RSF34 was added in excess at ratios of 1:600 plasmid to RSF34. The increased amount of oligonucleotide would increase the possibility of ligation with the plasmid. The ligated plasmid, pUC19-RSF34, was then transformed into DH5 α strain of chemically competent *E. coli* cells. The transformed cells were agitated in liquid media for 1 hour at 37°C. After the incubation, 50 μ l of the liquid media with cells were plated on solid media containing the antibiotic Ampicillin. The plates were incubated for 16 hours at 37°C. After the growing period, a single colony was selected from the petri dish and incubated at 37°C in a 5 ml liquid culture for 16 hours. A cell stock was made from the 5 ml colony by freezing with 20% glycerol at a 1:1 ratio at -80°C. A 500 ml liquid culture was then made from the cell stock and the DNA was purified from the culture using a plasmid maxi kit (Qiagen).

The modified plasmid pUC19-RSF34 was then digested using restriction enzymes BamHI and NdeI to acquire the RSF34 insert to be ligated into pET19b. There was great difficulty attempting this ligation. It was hypothesized that the NdeI enzyme was not digesting the plasmid as efficiently as the BamHI. Another oligonucleotide, AS1, was designed having BamHI restrictions sites on both ends. Ligation of this oligonucleotide was also unsuccessful after many attempts.

The focus of the research was changed to a project that is more applicable to the biosensor field. The project involves creating and analyzing a peptide that binds to TNT for detection purposes.

2.4 Design of TNT Binding Plasmids and Current Work

DNA segments were designed to produce peptides based from a 12 amino acid, TNT binding peptide designed by Goldman et al.³² Six peptides were listed in the paper: 3 and 9 which were strongly binding, 1 and 2 which were weakly binding, and 14 and 15

which were nonbinding. The authors state that 3 amino acids found in both of the strongly binding peptides were the main contributors to the peptide binding to TNT. The peptides were investigated to ascertain whether the binding properties of the peptide had any relation with the structure of the peptide. It was found that both strongly binding peptides were made up of a majority of amino acids that had a strong propensity for forming a random coil.

In this work 5 oligonucleotides were designed. The oligonucleotides were labeled AS2 through AS6 as shown in Table 1 with their amino acid sequence.

AS2	5' TCCATGTGCGTACATCGGATTATCGTCGTTGTATGGGGAGTGGTAGTACATCGGATTATCGTCGTTGTATGGGGAGTGGTCTAAAGCTT 3'
AS2p	MCV HR IIVVV W GVVV HR IIVVV W GVV
AS3	5' TCCATGTGCGAACATCGGGCCGCAGAGGAAGAGTGGGATGAAGAGGAACATCGGGCCGCAGAGGAAGAGTGGGATGAAGAGTAAAGCTT 3'
AS3p	MCE HR AAEEE W DEEE HR AAEEE W DEE
AS4	5' TCCATGTGCGTATTATTGATTATCGTCGTTGTAGGAGGAGTGGTAGTATTATTGATTATCGTCGTTGTAGGAGGAGTGGTATAAAGCTT 3'
AS4p	MCVLLIIVVVGGVVVLLIIVVVGGVV
AS5	5' TCCATGTGCAAGAAGCGGGCCGCAGAGGAAGAGGACGATGAAGAGAAGAAGCGGGCCGCAGAGGAAGAGGACGATGAAGAGTAAAGCTT 3'
AS5p	MCKKRAAEEEDDEEKRAAEEEDDEE
AS6	5' TCCATGTGCTGGCATCGGACACCCTCTACGTTATGGGGGGTAATTTGGCATCGGACACCCTCTACGTTATGGGGGGTAATTTAAAGCTT 3'
AS6p	MCW HR TPSTL W GVIW HR TPSTL W GVI

Table 1: Design of peptides AS2p, AS3p AS4p, AS5p, and AS6p and their respective DNA inserts AS2, AS3, AS4, AS5, AS6 used for cloning and expression of the peptides

The segments were designed to encode peptides that tested the contribution of the overall structure of peptide versus the 3 binding amino acids histidine, arginine and tryptophan (in bold and highlighted in Table 1) reported to bind TNT. Each peptide is a dimer with 12 amino acids that are repeated. AS2p contains the 3 main binding amino acids with the rest of the 12 amino acids replaced with amino acids with a strong propensity for forming β -sheets. AS3p also has the 3 main binding amino acids although the remaining amino acids are replaced by amino acids with strong propensity for forming α -helices. AS4p is made of amino acids that have a strong propensity for forming β -sheets. This peptide is assumed to be the negative control since it has none of the binding amino acids and is predicted to form a different secondary structure than the original TNT binding peptide. AS5p contains amino acids with a strong propensity to form α -helices. The peptide that is derived from AS6 (AS6p) and is the positive control. This peptide is a dimer of the original 12 amino acid peptide. All peptides were designed to contain a His-tag attached to the N-terminal. The purpose of the tag is to facilitate purification as well as detection of the peptide using an anti-His antibody.

All the DNA segments were ordered in pJ201 plasmids and expression plasmids pJExpress414. These plasmids were received as lyophilized DNA. The plasmid pJExpress414AS6 was transformed into NEBExpress cells and BL21 cells. The NEBExpress were plated onto solid media with 200 ng/ml Ampicillin and the BL21 were plated on to solid media containing antibiotics 30 ng/ml Ampicillin and Chloramphenicol. The reason for two antibiotics is that the strain BL21 has Chloramphenicol resistance and the plasmid pJExpress414 has Ampicillin resistance. A single colony was selected from the plates, grown overnight in 5ml cultures and made into cell stocks by mixing 500 μ l of the culture with 500 μ l of 20% glycerol.

2.5 Protein Expression

pJExpress414AS6 in BL21 cells to express peptides for TNT binding. A 50 ml culture was made with the correct antibiotics and was incubated for 16 hours. 10 ml from

the 50 ml culture is placed into 500 ml of liquid media. The 500 ml culture was incubated and agitated for 2.5 hours after which an optical density measurement was taken using a UV-VIS to estimate cell density of the culture. Measurements were taken, approximately, every hour until an optical density of 0.8 was reached. The culture was then induced to express protein by adding a 500 ml solution of 0.1 g/ml isopropyl- β -D-thiogalactopyranoside (IPTG) in water. Optical density was recorded every hour for an additional 4 hours to determine if cell death was taking place. At the end of the 4 hours, the culture was centrifuged, decanted and placed in the freezer. All samples taken for OD measurements were centrifuged, decanted and resuspended in an urea buffer with pH 8.0 for SDS Page electrophoresis to determine if the desired peptide was produced.

2.6 Protein Purification

After cells were induced to produce the peptides for four hours, the cultures were centrifuged at 6000g for 15 min at 4°C and resuspended in an urea buffer. The cells were then sonicated for 1 minute with 2 seconds pulses and 15 sec breaks between pulses. The cells were next centrifuged at 10,000g for 30min to remove cell debris and the supernatant was collected. To purify the desired peptide from the other cell proteins, the supernatant was incubated with nickel agarose beads for 1 hour while shaking at room temperature. The beads were then separated from the unbound proteins in a column. The beads were washed and the peptide eluted with 8M urea at descending pH's. The wash step used urea at pH 8. The column was filled with 10ml of the wash urea at pH 6.3 and allowed to completely drain. A sample was taken to measure the OD at 280nm. If the OD was above 0.1 the column was filled with another 10ml of urea at pH 6.3. Once the OD was below 0.1 the column was then filled with an elution buffer which is urea at pH 5.9. Again, the OD was monitored until it was below 0.1. Finally a second urea elution buffer at pH 4.5 was added to the column and again the OD is monitored until it was below 0.1. A sample of each fraction was taken for SDS-P electrophoresis. To remove the peptides from the solution the elution fractions were dialyzed in DI water and then lyophilized.

Inclusion body purification was also used on *E. coli* cultures to extract the desired peptide. After expression, the culture was centrifuged at 6000g for 15 min at 4°C. The

pellet was resuspended in a lysis buffer with Triton-X, Dithiothreitol (DTT) and phenylmethanesulfonylfluoride (PMSF). The cells were then sonicated for 1 min pulsing with 2 seconds on and 15 seconds off. MgSO_4 , DNase and lysozyme were added to the sonicated cells and incubated at room temperature for 20min. The cells were centrifuged at 5000g for 15 min at 4°C. The supernatant was removed and the pellet was resuspended in lysis buffer. The resuspended pellet was again incubated with DNase and lysozyme at room temperature for 20 minutes. The pellet was resuspended in lysis buffer and centrifuged an additional 2 times without incubating with lysozyme and DNase. After the final centrifugation the pellet was resuspended in a wash buffer centrifuged for a final time then resuspended in 8M urea at pH 8.

2.7 SDS Page Gel

After each expression and purification sodium dodecyl sulfate polyacrilimide electrophoresis (SDS-P) gels were made to track expression and purification of the peptides. The separating gels were made at 15% with a 6% stacking gel. After loading 5 μl of sample, the electrophoresis of the gel was run at 100v for 1h and 30min. The peptides were stained with either Coomassie Blue for 30min or with silver stain.

2.8 Amino Acid Analysis

After purification of the peptides several methods were used to confirm the presence of the peptides within the lyophilized material. Samples of each peptide were sent to the UC Davis Proteomics Core Facility for amino acid analysis. The amino acid analysis is performed by digesting the peptide material with HCl and reducing the peptides to their amino acid components. The digested material was analyzed using chromatography. The result was reported in nmol of each amino acid. The percent content of each amino acid is compared to the expected percent content based on the structure of the peptide.

2.9 Matrix Assisted Laser Desorption/Ionization (MALDI)

MALDI was also used to determine if there were peptides within the purified and lyophilized mass that contained a molecular weight similar to the expected AS6 peptide. The lyophilized samples were mixed with 5 mg/ml α -cyano-4-hydroxycinnamic acid (CHCA) matrix solution of 50/50 Acetonitrile and 2% Trifluoroacetic acid. An aliquot of 1 μ l of the mixture was placed on a gold chip (Ciphergen Biosystems, Inc., Fremont, CA) and dried to form a film. The MALDI instrument contained a linear time of flight (TOF) mass spectrometer (PBS IIc, Ciphergen Biosystems, Inc.). Biomarker Wizard software (Ciphergen Biosystems, Inc., Fremont, CA) was used to evaluate all of the data.

2.10 Tandem Mass Spectrometry (MS/MS)

Tandem Mass Spectrometry was used to analyze gel bands, lyophilized peptides, unpurified *E. coli* mass and purified material from inclusion body purification. All samples were treated with a tryptic digest over night at 37°C. Aliquots of 10 μ l samples were injected into a 1D high pressure liquid chromatography (HPLC) instrument. The samples were then analyzed using a ESI-qTOF MS SYNAPT™ (Waters Corp., Millford, MA). The samples were injected into the mass spectrometer using a positive-mode electrospray spray at a capillary voltage of 2.8 kV. The peptide material was dissociated using through collision induced dissociation (CID) cell in argon at 15 eV. The peptides were sequenced and queried against a peptide database, www.uniprot.org, using Micromass MassLynx 4.0 software suite (Waters, Millford, MA) for Windows XP. All analyses were performed at NIOSH (Morgantown, WV) by Dr. Justin Hettick.

2.11 Western Blots

Western Blots were used to confirm the presence of the desired peptide. An electrophoresis SDS-P gel was loaded with lyophilized peptide mixed with DI water. The gel ran for 30min at 100v. The peptide was then transferred from the gel onto a membrane using electrophoresis. The protein was transferred for 1h 15min at 45v. The membrane was then washed in Tris buffered saline (TBS) for 5 min and blocked using a blocking buffer purchased from Qiagen. The membrane was then washed 3 times with TBS with tween (TBS-T) for 5 minutes a wash. The membrane was incubated with anti-

HIS antibody at a 1:2000 concentration in blocking buffer overnight. The membrane was washed 3 times with TBS-T. The first wash for 5 minutes, the second and third were for 10 and 15 minutes respectively. The membrane was then developed in 10ml of lumiglow for 1min. To detect the florescence of the antibodies the membrane was placed on a plastic film and, exposed on the film for 2 min.

2.12 Circular Dichroism (CD)

The secondary structure of each peptide was analyzed using Jasco J-810 CD (Easton, MD). The samples were dissolved in a 10mM phosphate solution and were scanned in 1mm path length cuvettes. A baseline scan of the buffer was recorded and subtracted from the measurements. The measurements were recorded at a range of wavelengths from 190 to 250nm at 50nm/min with 2 scans averaged. Samples were made at concentrations of 1, 0.5 and 0.25 mg/ml and at pH's of 2, 7, 8 and 12. Data was taken at nanometer intervals with a bandwidth of 1nm.

2.13 ELISA

Binding between each designed peptide and TNB was accomplished using Enzyme Linked Immunosorbent Assay (ELISA) . The TNB was immobilized on the surface of the ELISA plate by binding it to bovine serum albumin (BSA). The TNB reacts with the BSA through primary amines. TNB is mixed with coating buffer at a ratio of 1:5.68. Coating buffer is 1.59g of Na_2CO_3 and 2.93g of NaHCO_3 in 1 L of water. BSA is also mixed with coating buffer to a concentration of 1mg/ml of BSA. 25 μ l of the TNB solution was mixed with 1 ml of the BSA solution and reacted at room temperature for 30 minutes. The working solution was diluted in coating buffer 1:200. Aliquots 100 μ l was added to each well and incubated for 6hrs at room temperature. The plate was washed 3 times with phosphate buffer saline containing 0.05% Tween 20 (PBS-T). After washing, 200 μ l of blocking buffer was added to each well to prevent nonspecific binding and incubated at room temperature for 2hrs. The blocking buffer consists of PBS-Tween with 3%BSA. The plate was then washed 3 times with PBS-T. Serial dilutions of the

peptide sample were made and 100µl of each dilution was added in duplicate to the ELISA plate. The peptides were incubated overnight at 4°C and then for an additional 2 hrs at room temperature. The plate was washed 3 times with PBS-T. An anti His antibody was diluted in blocking buffer at 1:2000 and then incubated in the well for 2hrs at room temperature. The plate was washed a final 3 times with PBS-T. Tetramethylbenzidine (TMB) is an enzyme which becomes oxidized when it reacts with the horse radish peroxidase (HRP) conjugated to the antibody. 100µl of TMB was added to each well and incubated at room temperature in a dark place for 30min. The HRP-TMB reaction turns the solution a blue color. To stop the reaction, 50µl of 0.18M H₂SO₄ is added to the TMB, which turns the solution from a blue to a yellow color. The absorbance of the solution is read at 450nm on a plate reader (ELX808LBS Biowhittaker, Basel, Switzerland).

3 Results

3.1 DNA Ligation and Transformation

Preliminary work was performed using oligonucleotide RSF 34. This oligonucleotide, once ligated into a plasmid and transformed into a bacterial cell, would code for a protein that would bind the protein chondroitin sulfate. RSF 34 was ordered as 2 strands and annealed together overnight. The insert was later cut out and extracted from the gel. Figure 6 shows the agarose gel of the annealed DNA.

Figure 6: Annealed RSF 3-4, Lanes: 100 Base Pair ladder, Annealed Sample

The band at the bottom of the agarose gel is the annealed DNA which was separated from unannealed DNA by electrophoresis. The band was just under the 100 basepair marker which is the expected position of the DNA, due to its molecular weight.

To create a renewable supply of insert RSF 34, the insert was ligated into the cloning plasmid pUC19. This plasmid was digested with the enzymes BamHI and EcoRI. The concentration of the annealed insert extracted from the gel was determined by analysis of a concentration gel containing both digested plasmid and oligonucleotide. The concentration gel can be seen in Figure 7.

Figure 7: Concentration Gel, Wells: (1)1000 Base Pair Ladder (2)Digested pUC19 (3) Undigested pUC19 (4) 100 Base Pair Ladder (5) RSF 1-2 (6) RSF 3-4

Concentrations of DNA are determined by comparing fluorescence intensity of the DNA with the fluorescence intensity of the closest corresponding DNA marker of known concentration of DNA. It was determined that the cut plasmid had approximately the same relative fluorescence as the 3000 base pair marker and the oligonucleotide had twice the relative fluorescence of the 100 base pair marker. The plasmids were ligated with RSF 34 at ratios of 1:300 and 1:600, plasmid to oligonucleotide. The volumes that were required for these ratios were determined using Equation 1.

$$\frac{\text{Length of insert (kb)}}{\text{length of vector}} * \text{ng of vector} * \text{ratio of insert to vector} = \text{ng of insert needed} \quad (1)$$

This value was compared to the closest molecular weight ladder marker to determine the concentration required for the ligation

The ligated DNA was transformed into DH5 α cells and plated. Colonies grew from the ligated DNA that used a ratio of 1:300. The cells were tested for presence of the oligonucleotide RSF34 by extracting the DNA, digesting the plasmids with EcoRI and BamHI and separating the components using electrophoresis gel. Figure 8 shows the separated components.

Figure 8: Digested pUC19 plasmid with insert Wells: (1) 50 Base Pair Ladder (2) Digested pUC19 with RSF 3-4 (3) Digested pUC19 with RSF 1-2 (4) Digested pUC19 with RSF1-2

Lanes 1-3 are the digested pUC19-RSF1-2 plasmids. Each lane is DNA extracted from cells of three different colonies grown on solid media. A faint fluorescence between the 200 and 300 base pair DNA marker (circled) indicate the presence of RSF34 within pUC19 plasmids. Attempts to extract the RSF 34 insert from pUC19 by digestion with BamHI and NdeI and ligate into the expression vector pET19 were unsuccessful. Technical problems in DNA manipulation have prevented further work on this project.

The current project involves the creation of 3 dimers consisting of a repeated 12 amino acid peptide labeled AS2p, AS3p and AS6p. The focus of the project was reduced to the 3 peptides containing the 3 amino acids due to time constraint. Segments of DNA AS2, AS3 and AS6 were encoded to produce the peptides, AS2p, AS3p and AS6p respectively. The segment corresponding to the AS6p peptide was purchased already ligated into the pJ414Express plasmid.

3.2 Peptide Expression and Purification

The DNA plasmid containing our target sequence was transformed into NEBExpress and BL21 expression strains. During Expression samples were taken every hour after induction of the cells to periodically assess continued growth. Table 2 shows the optical densities (OD) versus time of the two strains.

Table 2: Optical Densities showing the OD values versus time of NEBExpress and BL21 strains of *E. coli* following induction of IPTG

Time(hr)	OD	Time(hr)	OD	Time(hr)	OD
Expression 1 (NEBExpress)		Expression 2 (BL21)		Expression3 (BL21)	
0.0	Started	0	Started	0	Started
2.5	0.639*	2.5	0.95*	2.5	0.546*
3.5	1.148	3.5	0.225**	3	0.781
4.5	0.216**	4.5	0.207	4.5	0.196**
5.5	0.188	5.5	0.249	5.5	0.157
6.5	0.124	6.5	0.272	6.0	0.171
		7	0.248	6.5	0.197
				7.0	0.195

*Induction of *E. coli* Cells

**OD samples from this time on were diluted 1:10 with liquid media

Expression 1 was performed using the NEBExpress strain of *E. coli*. Expression 2 and 3 use the BL21 strain. The BL21 strain produced consistently higher optical

densities than the NEBExpress strains as seen in the higher OD values in Expression 2 and 3, so initial expressions were performed with the BL21 strain.

The samples collected from measuring the optical densities were stored in an 8M urea buffer at pH 8.0 to be analyzed on a SDS-P gel. Figure 9 shows a representative gel of an induction profile for AS6p in BL21 cells.

Figure 9: Induction Profile Expression 3 BL21 strain of *E. coli*, cells were induced after 2.5 hrs of incubation

The boxed area is the desired peptide after induction. There is an increased intensity with increased expression time at the expected molecular weight which would indicate an increase amount of peptide within the cell culture. The peptide being produced has a weight of about 5.3 kDa, however the peptide's high charge density may affect migration through the SDS Page gel. The density charge would explain the appearance of the peptide above the 6kDa ladder marker.

The main culture was centrifuged for 15 min at 6000 rpm and resuspended in urea buffer with pH 8. AS6p was purified from the cell mass using a series of 8M urea buffers at decreasing pH values from 6.3 to 4.5. Samples during each phase of the purification were taken to assess recovery and purity. Figure 10 shows an image of an SDS-P gel of a protein purification of AS6p.

Figure 10: Purification Profile of peptide AS6p. Lysate lane is after the cells have been sonicated. Flow through is the initial flow through the column. Wash 1-6 and 7 indicates the number of times which the column was filled with 10ml of the wash urea buffer

The lysate lane is a sample taken directly after cells have been lysed; therefore contain all cellular protein. The last six lanes contain show the different urea washes and elutions. The number after the labeled buffer is the number of collected washes. A wash consisted of filling the column with approximately 10 ml of the respective buffer. One flow through contains a volume of 10mL of urea buffer. The pHs for the 8M urea wash buffer, and elution buffers were 6.3, 5.9 and 4.5, respectively. The faint lines in the last two lanes indicate the presence of the eluted and purified peptide AS6p. NEBExpress cell line was used in this purification and was the only cell line to show a band near the molecular weight of AS6 peptide, therefore all experimentation was performed with peptide material created from the NEBExpress cell line. The purified peptides were dialyzed, freeze dried and have been sent to an outside lab to be analyzed for confirmation of the primary structure of the peptide. SDS Page gels of purification profiles for AS2p and AS3p have also been produced. Figure 11 and 12 are purification profiles of AS2p and AS3p.

Figure 11: SDS Page gel of a Purification Profile of AS3p, bottom ladder marker is 10kDa

Figure 12: SDS Page Gel of a Purification Profile of AS2p, bottom ladder marker is 10kDa

The protocol for these gels was the same as AS6p with the exception that an extra elution buffer at pH 5 was used in the purification for AS2p and AS3p. This extra buffer was added to wash away more of the undesired peptides before eluting the desired peptide. None of the purification profiles produced contained a band in any of the elution buffers in an appropriate position in the lane indicating the presence of the peptides AS2p or AS3p. A band consistently appeared around 20 kDa which was hypothesized to contain the desired peptide. It was assumed that the desired peptide may be interacting with contaminants which would prevent it from running to the appropriate length.

3.3 Western Blot

Further efforts were taken to ensure that the desired peptides were being produced from the plasmids AS6, AS2 and AS3. Western Blot was preformed on the peptides to confirm their presence in the purified and lyophilized products. Figure 13 shows a Western Blot of AS6 and AS2 from lyophilized samples mixed with DI water.

Figure 13: Western Blot of AS6 and AS2

A faint mark can be seen above the 50 kDa base pair marker (within the overlaid square) which may indicate the presence of peptide AS6. This peptide may be running high than expected due to either its high hydrophobicity or interaction with larger contaminant peptides.

3.4 MALDI

Lyophilized samples of AS6 were also analyzed using Matrix Assisted Laser Desorption/Ionization (MALDI) to discern if there were any peptides within the final product having a mass similar to AS6 which may indicate the presence of AS6. Figure 14 shows the MALDI results for AS6.

Figure 14: MALDI result for lyophilized sample of AS6p

The results show the presences of some species that have a molecular weight around 5 kDa which may indicate the presence of the peptide AS6 having a weight of 5170 Da. Lyophilized samples of AS6p and AS2p were also sent out for amino acid analysis.

None of the amino acid analysis strongly indicated the presence of the peptides. This can be concluded by the high negative deviation of histidine in the sample. A

sample containing the peptide would have a higher histidine content due to the His-tag attached to all of the peptides. The results can be seen in Appendix A.

3.5 Tandem Mass Spectrometry

A SDS-P gel of different elution fractions from different peptides were made to discern if any of the higher running bands visualized contained the desired peptides. Any contaminants that were present in the higher running bands were also sequenced and identified. Figure 15 is the SDS Page gel that was produced from elutions of pH5.9 (D buffer) and pH 4.5 (E buffer) elution fractions for analysis using tandem mass spectrometry (MS/MS).

Figure 15: Gel for Tandem Mass Spectrometry Analysis

The lanes are labeled with the peptide, the elution fraction (D is pH 5.9 and E is pH 4.5) and the batch expression number. The bands were excised from the gel and labeled as sample numbers 1-6 as indicated in Figure 15. The sequences were entered into the software to search the proteins found in the samples. None of the bands analyzed contained the desired peptide. The contaminants sequenced in each sample can be seen in Appendix B. This data also shows that the purification process which used nickel resin bound many undesirable proteins which contaminate the final lyophilized product. Since the desired peptides could not be seen in the lyophilized products or the SDS-P gels it was suspected that the peptides could be loss during the purification process. Crude

samples for AS6, AS2 and AS3 were prepared to be analyzed by MS/MS to determine if the peptides were being produced within the cell. The samples were prepared by dialyzing the supernatant, after sonication, against 0.1M sodium bicarbonate. The dialyzed product was centrifuged for 30min at 2,500G. The supernatant was finally filter through a 10,000 MW filter before analysis. None of the peptides were detected in the crude samples using MS/MS.

3.6 Inclusion Body Purification

Many of the peptides designed are highly charged or highly hydrophobic which may be detrimental to the biology of the *E. coli* which may cause the *E. coli* to produce the peptide within an inclusion body. Inclusion body purification was performed on AS6. Figure 16 shows the gel of the inclusion body purification.

Figure 16: Inclusion Body Purification Gel. Lanes: 1) Supernatant after sonication 2) Supernatant after first digest with DNase and Lysozyme 3) Supernatant after second digest 4) Supernatant after first wash with lyse buffer 5) Supernatant after second wash

A band appears in the gel in the last lane (shown in the box) which may indicate a peptide being released from the inclusion bodies after denaturing from the 8M urea. This can be concluded since the band does not appear in the previous lane. The final elution from the inclusion body purification was incubated with nickel resin beads and placed in a column to attempt to further purify and isolate the AS6 peptide. This was unsuccessful due to the material having a high viscosity that prevented it from flowing through the column. This sample was then analyzed in MS/MS. The sample was incubated with trypsin so it would digest any peptides, which may be present, from the nickel resin beads. The digested product was then filtered through a 10,000 MW filter. The AS6 peptide could not be detected by MS/MS. It is possible that the cells do not consistently express the AS6 insert within the plasmid due to the peptide's high hydrophobicity.

3.7 Circular Dichroism

The paper which this research is based (Goldman et al, 2002), contributes binding of its particular peptide to 3 amino acids within the peptide. One of the main goals of this project is to determine what contribution peptide secondary structure has to TNT-peptide binding.

ASp6 and ASp2 are expected to form α -helix in their native state due to amino acids in the sequence having a high propensity for forming this structure. ASp3 has been designed with amino acids that have a high propensity for making a β -sheet. The structures of these peptides have been analyzed using circular dichroism (CD) to verify that these peptides conform to their predicted structures. The CD signal is measured by dividing the value for the difference in the two circular polarizations and the average total light intensity as shown in Equation 2.

$$CD = \frac{v_{AC}}{v_{DC}} * G \quad (2)$$

v_{AC} is the difference in the two circular polarizations, v_{DC} is the total average light intensity and G is a calibration scaling factor. The peptides were measured in a 10mM phosphate solution at concentrations of 1 mg/ml, 0.5 mg/ml and 0.25 mg/ml and pH's of

2, 7, 8 and 12. The CD readings were scanned at a range of 190nm to 250nm. Figure 17 and 18 shows CD readings at pH 2 and pH 12, respectively.

Figure 17: Circular Dichroism Reading Using a 10mM Phosphate Solution at pH 2 Using a 0.5 mg/ml Concentration

Figure 18: Circular Dichroism Reading Using a 10mM Phosphate Solution at pH 12 Using a 1 mg/ml Concentration

Many of the data from the CD readings do not show any significant results revealing any sort of secondary structure. Readings using a buffer at pH 2 and 12 did show some results that were greater than the background noise, although the same results were seen

for all the peptides. These readings may have been from the change of secondary structure of all the protein material within the sample due to the extreme pH values. Several reasons have been hypothesized for the poor CD signal. The final lyophilized products have been shown to contain contaminants that are much bigger than the peptides and these contaminants could interrupt the reading of the peptides. All of the lyophilized samples were very hydrophobic when mixing it with the phosphate solution and particles have been observed when running the CD experiments. This insolubility could also prevent a proper CD reading from being obtained. Finally the peptides may not have been present in the samples due to the *E. coli* inconsistently expressing the peptides.

3.8 ELISA

ELISA tests were performed upon all peptides to test the binding of the peptide to TNB. These tests were performed using sandwich ELISA. An anti His antibody was used to bind and detect the peptide in the assay. Figure 19 shows the setup for the sandwich ELISA with the anti-His antibody.

Figure 19: Sandwich ELISA experiment design for peptides with His-tag

The bottom of the ELISA plate was coated with 1,3,6-trinitrobenzene (TNB) conjugated with BSA which are molecules 1 and 2 in Figure 12. The peptide was incubated with the TNB-BSA to allow it to bind, indicated by molecule 3. An HRP conjugated anti His antibody (number 4) was added to the plate to bind to the His-tag on the peptide. The literature,³² which these experiments were based on, conjugated TNB to ovalbumin and

then immobilized it on the bottom of the plate. The first ELISA experiment used this method with limited success. Figure 20 shows the ELISA results using TNB-ovalbumin.

Figure 20: First ELISA Experiment using TNB-Ovalbumin

The low absorbance reading and no relationship between peptide concentration and absorbance may be due to a low TNB content. TNB binds to proteins through primary amine groups. The blocking protein used in the ELISA contained more amine groups than the ovalbumin therefore more TNB molecules could be attached to BSA. Using BSA as the immobilizing agent for TNB would also simplify the ELISA experiment. The second ELISA compared the effectiveness of the TNB-BSA to the effectiveness of the TNB-ovalbumin in the ELISA experiment. Figure 21 shows the second ELISA comparing TNB-ovalbumin to TNB-BSA.

Figure 21: The ELISA Showing the Comparison of Using TNB-BSA to TNB-Ovalbumin

The signal from the 1 mg/ml concentration using TNB-BSA is significantly larger than the signal using TNB-Ovalbumin. This is due to BSA being a much larger protein and containing more amine groups which the TNB can bind. The peptide was also bound to the bottom of the plate to test if the antibody would bind only to the peptide material and confirm the presence of the AS6 peptide. BSA was used as the conjugating molecule for TNB for the remaining ELISAs. Figure 22 shows an ELISA of AS6 binding to TNB-BSA at concentrations from 1 mg/ml to 0.0001 mg/ml.

Figure 22: ELISA of AS6 binding to TNB-BSA

The graph shows a decrease in absorbance with a decrease in peptide concentration. This relation may indicate the binding of AS6 to TNB, but there is a high amount of non-specific binding of the peptide to the BSA at 1 mg/ml. It is not known how much of the absorbance read under 1 mg/ml is due to non-specific binding. All the following ELISA's tested the non-specific binding at different concentrations to determine what percentage of the absorbance read was due to non-specific binding. It was not possible to reproduce this data. Many of the following ELISAs did not show a relation between decreasing peptide concentration and an increase in absorbance that was above non-specific binding. Some ELISA's showed a reverse relation between peptide concentration and absorbance. Figure 23 is an ELISA that shows an increase in absorbance with a decrease in peptide concentration.

Figure 23: AS6 and TNB-BSA ELISA

The increase in absorbance may have been from contaminants that may have competed with AS6 by non-specifically binding to TNB or by binding to BSA and blocking binding

sites on TNB. Many other ELISA's also did not show any binding of AS6 to TNB. Figures 24 is an example of an ELISA with negative results.

Figure 24: ELISA of AS6 and TNB-BSA

It has been shown that the E. coli inconsistently express the peptides which would produce negative results for ELISAs. ELISAs were ran for both AS2 and AS3. Both of these peptides did not indicate binding to TNB. Figure 38 is an ELISA for AS2 and Figure 25 is an ELISA for AS3.

Figure 25: ELISA of AS2 binding to TNB-BSA

Figure 26: ELISA of AS3 binding to TNB-BSA

These results could also be caused by inconsistent *E. coli* expression or the peptides having no affinity for binding to TNB. The remaining ELISA results can be seen in Appendix D.

4 Conclusions and Suggestions for Future Work

The peptides AS6 and AS2 are very hydrophobic nature and the peptide AS3 is highly charged. Due to these qualities, their presence within *E. coli* may be detrimental to the biology of the cell. This incompatibility may cause the cells to not express or to only express small quantities of the desired peptides. Inconsistent expression has hindered the research efforts to attain conclusive results. The His-tag purification system has also produced final products with many undesirable contaminant proteins. These proteins have the potential of interfering with ascertaining the secondary structure of the peptides through CD and may also have competed with binding in the ELISA experiments. Many of the lyophilized peptides that were tested may also not have contained the desired peptides due to inconsistent expression which may also produce false or negative results.

The main challenges to overcome in potential future endeavors to continue this work would be to consistently produce the peptides and purify them. The first course of action that is recommended is to retry the inclusion body purification and to denature the inclusion bodies using guanidine hydrochloride. The urea 8M buffer may not have denatured the inclusion bodies adequately to release the peptides inside. Another avenue that could be pursued is to use different vectors and *E. coli* strains that may be more tolerant of the peptides and which may produce and store them within the periplasmic space. Once the peptides are produced a better purification method may be needed. Using a different tag such as a strep tag may lead to better purification results. If the peptides cannot be produced within *E. coli* then synthetic production could also be an option for future research.

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6 Appendix

6.1 Appendix A

The following data is the results for the Amino Acid Analysis.

Table 1.A : Amino Acid Analysis of AS6

Expected residue	Expected mole%	Observed Mole %
ASX	2.27	10.23
Thr	9	4.52
Ser	11.36	4.7
Glx	0	11.43
Pro	4.55	2.43
Gly	11.36	12.11
Ala	0	8.96
Cys	2.27	0
Val	4.55	6.55
Met	4.55	2.58
Ile	4.55	4.52
Leu	4.55	7.52
Tyr	0	6.99
Phe	0	1.42
His	27.27	4.13
Lys	0	5.81
Trp	9.09	1.05
Arg	4.55	5.05

Table 2.A: Amino Acid Analysis of AS2
AS2

Expected residue	Expected mole%	Observed Mole %
ASX	2.27	10.8
Thr	0	4.33
Ser	6.82	4.37
Glx	0	11.21
Pro	0	3.36
Gly	11.36	12.42
Ala	0	10.66
Cys	2.27	0
Val	27.27	8.19
Met	4.55	1.81
Ile	9.09	4.06
Leu	0	7.68
Tyr	0	1.09
Phe	0	3.41
His	27.27	5.29
Lys	0	5.24
Trp	4.55	1.27
Arg	4.55	4.35

6.2 Appenix B

The following Tables are the theoretical cleaving of peptides AS6, AS2 and AS3 by trypsin.

Table 1.B: Segments of AS6 of digest with trypsin

AS6		
mass	position	peptide sequence
2673	1-23	MGHHHHHHHHSSGHGSGSMCWHR
1453	24-35	TPSTLWGVIWHR
973	36-44	TPSTLWGVI

Table 2.B: Segments of AS2 of digest with trypsin

AS2		
mass	position	peptide sequence
2586	1-23	MGHHHHHHHHSSGHGSGSMCVHR
1376	24-35	IIVVVWGVVHR
983	36-44	IIVVVWGVV

Table 3.B: Segments of AS6 of digest with trypsin

AS3		
mass	position	peptide sequence
2616	1-23	MGHHHHHHHHSSGHGSGSMCEHR
1530	24-35	AAEEEWDEEEHR
1107	36-44	AAEEEWDEE

This data was collected from the tandem mass spectrometry analysis of the gel in Figure 15. The first column in each table contains the name of the protein found and the reference number of the peptide as it is found in the online database queried (www.uniprot.org). The second column is the false positive rate where the results are queried randomly against other databases to see if the segments match other protein fragment sequences. The third column is the average mass of the protein found. The fifth column is the number of peptide fragments that were found and match parts of the greater protein. The sixth column is the number of different fragments found from the protein and the last column indicate the percent of the protein all the different fragments cover.

Table 3: Species Found in Sample 4.B

protein.name	protein.score	falsePositiveRate	avgMass	MatchedProducts	MatchedPeptides	seqCover(%)
SLYD_ECOLI (P30856) FKBP-type peptidyl-prolyl cis-trans isomerase slyD	1151.61	0	20852	204	14	67.35
YADF_ECOLI (P36857) Protein yadF.	197.37	0	25096	19	2	11.36
GCH1_ECOLI (P27511) GTP cyclohydrolase I (EC 3.5.4.16) (GTP-CH-I).	90.58	0	24699	14	3	21.27

Table 4: Species Found in Sample 5.B

name	score	falsePositiveRate	avgMass	MatchedProducts	MatchedPeptides	seqCover(%)
EFTU_ECOLI (P02990) Elongation factor Tu (EF-Tu) (P-43).	3193.85	0	43182	285	17	46.56
SLYD_ECOLI (P30856) FKBP-type peptidyl-prolyl cis-trans isomerase slyD	811.65	0	20852	106	7	40.82
ALF_ECOLI (P11604) Fructose-bisphosphate aldolase class II (EC 4.1.2.	1000.55	0	39016	135	15	35.75
ACKA_ECOLI (P15046) Acetate kinase (EC 2.7.2.1) (Acetokinase).	634.92	0	43290	72	12	33
CARA_ECOLI (P00907) Carbamoyl-phosphate synthase small chain (EC 6.3.5	298.19	0	41431	32	4	14.92
RLUD_ECOLI (P33643) Ribosomal large subunit pseudouridine synthase D (	133.61	0	37121	15	3	21.47
ARGD_ECOLI (P18335) Acetylornithine aminotransferase (EC 2.6.1.11) (AC	391.28	0	43635	47	7	29.38
MTOX_ECOLI (P40874) N-methyl-L-tryptophan oxidase (EC 1.5.3.-) (MTOX).	203.72	0	40902	25	4	20.16
GLYA_ECOLI (P00477) Serine hydroxymethyltransferase (EC 2.1.2.1) (Seri	322.18	0	45316	33	4	13.91

Table 5: Species Found in Sample 6.B

name	score	falsePositiveRate	avgMass	MatchedProducts	MatchedPeptides	seqCover(%)
EFTU_ECOLI (P02990) Elongation factor Tu (EF-Tu) (P-43).	3009.62	0	43182	290	20	49.36
ALF_ECOLI (P11604) Fructose-bisphosphate aldolase class II (EC 4.1.2.	1745.7	0	39016	266	25	40.22
ACKA_ECOLI (P15046) Acetate kinase (EC 2.7.2.1) (Acetokinase).	855.29	0	43290	118	14	37.5
ARGD_ECOLI (P18335) Acetylornithine aminotransferase (EC 2.6.1.11) (AC	849.98	0	43635	89	12	39.75
CARA_ECOLI (P00907) Carbamoyl-phosphate synthase small chain (EC 6.3.5	385.38	0	41431	43	6	30.63
ASNA_ECOLI (P00963) Aspartate--ammonia ligase (EC 6.3.1.1) (Asparagine	275.3	0	36650	27	5	26.06
YFBE_ECOLI (P77690) Hypothetical protein yfbE.	413.19	0	41649	48	5	20.58
TDH_ECOLI (P07913) Threonine 3-dehydrogenase (EC 1.1.1.103).	147.75	0	37239	13	2	8.5
MTOX_ECOLI (P40874) N-methyl-L-tryptophan oxidase (EC 1.5.3.-) (MTOX).	236.45	0	40902	29	5	24.73
GATD_ECOLI (P37190) Galactitol-1-phosphate 5-dehydrogenase (EC 1.1.1.2	351.86	0	37390	38	7	27.75
SLYD_ECOLI (P30856) FKBP-type peptidyl-prolyl cis-trans isomerase slyD	318.53	0	20852	80	6	57.14
YBIC_ECOLI (P30178) Hypothetical oxidoreductase ybiC (EC 1.1.1.-).	223.71	0	38897	27	3	18.56
MRP_ECOLI (P21590) Mrp protein.	217.73	0	39938	21	3	13.82
ADD_ECOLI (P22333) Adenosine deaminase (EC 3.5.4.4) (Adenosine aminoh	129.87	0	36397	19	3	20.12
ODO1_ECOLI (P07015) 2-oxoglutarate dehydrogenase E1 component (EC 1.2.	299.3	0	105061	35	8	13.72
LACI_ECOLI (P03023) Lactose operon repressor.	237.8	0	38564	38	6	19.72
SUCC_ECOLI (P07460) Succinyl-CoA synthetase beta chain (EC 6.2.1.5) (S	266.59	0	41392	39	7	22.94

ISCS_ECOLI (P39171) Cysteine desulfurase (EC 4.4.1.-) (Thil transpersu	105.69	0	45089	10	2	5.94
TKRA_ECOLI (P37666) 2-ketogluconate reductase (EC 1.1.1.215) (2KR) (2-	96.91	0	35395	16	3	23.77
ADD_ECOLI (P22333) Adenosine deaminase (EC 3.5.4.4) (Adenosine aminoh	129.87	0	36397	19	3	20.12
ODO1_ECOLI (P07015) 2-oxoglutarate dehydrogenase E1 component (EC 1.2.	299.3	0	105061	35	8	13.72
LACI_ECOLI (P03023) Lactose operon repressor.	237.8	0	38564	38	6	19.72
SUCC_ECOLI (P07460) Succinyl-CoA synthetase beta chain (EC 6.2.1.5) (S	266.59	0	41392	39	7	22.94
ISCS_ECOLI (P39171) Cysteine desulfurase (EC 4.4.1.-) (Thil transpersu	105.69	0	45089	10	2	5.94
TKRA_ECOLI (P37666) 2-ketogluconate reductase (EC 1.1.1.215) (2KR) (2-	96.91	0	35395	16	3	23.77

Table 6: Species Found in Sample 7.B

name	score	falsePositiveRate	avgMass	MatchedProducts	MatchedPeptides	seqCover(%)
EFTU_ECOLI (P02990) Elongation factor Tu (EF-Tu) (P-43).	2989.3	0	43182	242	17	49.36
SYE_ECOLI (P04805) Glutamyl-tRNA synthetase (EC 6.1.1.17) (Glutamate-	2177.45	0	53815	215	22	46.5
TNAA_ECOLI (P00913) Tryptophanase (EC 4.1.99.1) (L-tryptophan indole-l	921.28	0	52773	87	13	28.24
STHA_ECOLI (P27306) Soluble pyridine nucleotide transhydrogenase (EC 1	871.75	0	51429	89	10	40.65
GLNA_ECOLI (P06711) Glutamine synthetase (EC 6.3.1.2) (Glutamate--ammo	1302.91	0	51772	132	14	49.15
GATZ_ECOLI (P37191) Putative tagatose 6-phosphate kinase gatZ (EC 2.7.	623.96	0	47108	66	10	35
DLDH_ECOLI (P00391) Dihydroliipoamide dehydrogenase (EC 1.8.1.4) (E3 co	693.56	0	50557	68	8	26.43
SLYD_ECOLI (P30856) FKBP-type peptidyl-prolyl cis-trans isomerase slyD	166.73	0	20852	40	3	57.14
AGAL_ECOLI (P06720) Alpha-galactosidase (EC 3.2.1.22) (Melibiase).	522.21	0	50657	82	9	24.61
UXUB_ECOLI (P39160) D-mannonate oxidoreductase (EC 1.1.1.57) (Fructuro	330.66	0	53580	42	6	16.87
PEPQ_ECOLI (P21165) Xaa-Pro dipeptidase (EC 3.4.13.9) (X-Pro dipeptida	210.95	0	50176	31	5	19.19
GLYA_ECOLI (P00477) Serine hydroxymethyltransferase (EC 2.1.2.1) (Seri	504.34	0	45316	56	7	24.7
GUAA_ECOLI (P04079) GMP synthase [glutamine-hydrolyzing] (EC 6.3.5.2)	128.86	0	58679	17	4	15.05
CARA_ECOLI (P00907) Carbamoyl-phosphate synthase small chain (EC 6.3.5	178.57	0	41431	20	4	18.59
CISY_ECOLI (P00891) Citrate synthase (EC 4.1.3.7).	114.31	0	48063	10	2	5.15
ACKA_ECOLI (P15046) Acetate kinase (EC 2.7.2.1) (Acetokinase).	99.72	0	43290	18	3	14.25
G6PI_ECOLI (P11537) Glucose-6-phosphate isomerase (EC 5.3.1.9) (GPI) (	109.67	0	61529	14	3	8.2
MURC_ECOLI (P17952) UDP-N-acetylmuramate--alanine ligase (EC 6.3.2.8)	110.77	0	53626	18	3	13.65
ALF_ECOLI (P11604) Fructose-bisphosphate aldolase class II (EC 4.1.2.	152.24	0	39016	28	5	20.95
MURE_ECOLI (P22188) UDP-N-acetylmuramoylalanyl-D-glutamate--2	99.73	0	53212	14	3	13.16
PEPD_ECOLI (P15288) Aminoacyl-histidine dipeptidase (EC 3.4.13.3) (Xaa	117.27	0	52784	16	3	7.44

Table 7: Species Found in Sample 8.B

name	score	falsePositiveRate	avgMass	MatchedProducts	MatchedPeptides	seqCover(%)
EFTU_ECOLI (P02990) Elongation factor Tu (EF-Tu) (P-43).	3123.63	0	43182	294	22	44.53
ALF_ECOLI (P11604) Fructose-bisphosphate aldolase class II (EC 4.1.2.	2022.18	0	39016	294	26	40.22
ACKA_ECOLI (P15046) Acetate kinase (EC 2.7.2.1) (Acetokinase).	1060.17	0	43290	153	15	47
GLYA_ECOLI (P00477) Serine hydroxymethyltransferase (EC 2.1.2.1) (Seri	594.45	0	45316	67	8	27.1
ARGD_ECOLI (P18335) Acetylorithine aminotransferase (EC 2.6.1.11) (AC	1016.08	0	43635	125	17	49.63
ARGE_ECOLI (P23908) Acetylorithine deacetylase (EC 3.5.1.16) (Acetylo	395.99	0	42347	46	6	28.72
CARA_ECOLI (P00907) Carbamoyl-phosphate synthase small chain (EC 6.3.5	831.49	0	41431	95	13	35.6
YBIC_ECOLI (P30178) Hypothetical oxidoreductase ybiC (EC 1.1.1.-).	267.2	0	38897	31	3	18.56
MTOX_ECOLI (P40874) N-methyl-L-tryptophan oxidase (EC 1.5.3.-) (MTOX).	428.82	0	40902	51	9	33.06
GATD_ECOLI (P37190) Galactitol-1-phosphate 5-dehydrogenase (EC 1.1.1.2	281.22	0	37390	23	4	10.98
TDH_ECOLI (P07913) Threonine 3-dehydrogenase (EC 1.1.1.103).	117.75	0	37239	11	2	8.5
RLUD_ECOLI (P33643) Ribosomal large subunit pseudouridine synthase D (	219.17	0	37121	28	5	30.37
YFBE_ECOLI (P77690) Hypothetical protein yfBE.	354.25	0	41649	35	5	20.58
ISCS_ECOLI (P39171) Cysteine desulfurase (EC 4.4.1.-) (Thil transpersu	139.26	0	45089	15	3	10.64

Table 8: Species Found in Sample 9.B

name	score	falsePositiveRate	avgMass	MatchedProducts	MatchedPeptides	seqCover(%)
EFTU_ECOLI (P02990) Elongation factor Tu (EF-Tu) (P-43).	1636.91	0	43182	128	12	38.93
SYE_ECOLI (P04805) Glutamyl-tRNA synthetase (EC 6.1.1.17) (Glutamate-	2639.4	0	53815	262	27	52.02
GLNA_ECOLI (P06711) Glutamine synthetase (EC 6.3.1.2) (Glutamate--ammo	1626.86	0	51772	157	17	45.09
AGAL_ECOLI (P06720) Alpha-galactosidase (EC 3.2.1.22) (Melibiase).	433.77	0	50657	44	5	17.07
STHA_ECOLI (P27306) Soluble pyridine nucleotide transhydrogenase (EC 1	653.58	0	51429	64	8	35.48
TNAA_ECOLI (P00913) Tryptophanase (EC 4.1.99.1) (L-tryptophan indole-l	375.05	0	52773	31	7	17.2
GATZ_ECOLI (P37191) Putative tagatose 6-phosphate kinase gatZ (EC 2.7.	509.06	0	47108	43	6	22.86
PEPD_ECOLI (P15288) Aminoacyl-histidine dipeptidase (EC 3.4.13.3) (Xaa	197.14	0	52784	23	5	14.88
GLYA_ECOLI (P00477) Serine hydroxymethyltransferase (EC 2.1.2.1) (Seri	409.75	0	45316	49	6	20.86
DCDA_ECOLI (P00861) Diaminopimelate decarboxylase (EC 4.1.1.20) (DAP d	151.1	0	46177	19	4	13.33
YACK_ECOLI (P36649) Probable blue-copper protein yack precursor.	298.19	0	56556	28	4	12.98
ALF_ECOLI (P11604) Fructose-bisphosphate aldolase class II (EC 4.1.2.	334.05	0	39016	46	7	34.08
DLDH_ECOLI (P00391) Dihydrolipoamide dehydrogenase (EC 1.8.1.4) (E3 co	236.12	0	50557	24	4	12.9
PEPQ_ECOLI (P21165) Xaa-Pro dipeptidase (EC 3.4.13.9) (X-Pro dipeptida	153.64	0	50176	15	3	10.16
CODA_ECOLI (P25524) Cytosine deaminase (EC 3.5.4.1) (Cytosine aminohyd	107.4	0	47459	8	2	6.57
G6PI_ECOLI (P11537) Glucose-6-phosphate isomerase (EC 5.3.1.9) (GPI) (	100.2	0	61529	11	3	7.47
YLIG_ECOLI (P75802) Hypothetical protein yliG.	104.23	0	49581	13	3	11.56
ACCC_ECOLI (P24182) Biotin carboxylase (EC 6.3.4.14) (A subunit of ace	102.95	0	49320	10	3	10.02
ACKA_ECOLI (P15046) Acetate kinase (EC 2.7.2.1) (Acetokinase).	136.08	0	43290	17	3	14.25
CARA_ECOLI (P00907) Carbamoyl-phosphate synthase small chain (EC 6.3.5	80.19	0	41431	8	1	6.28

6.3 Appendix C

The following data is the CD readings for AS2, 3 and 6 at concentrations of 1 mg/ml, 0.5 mg/ml and 0.25 mg/ml at pH values 2, 7, 8 and 12.

Figure 27: CD result for AS6 at pH 2 in a 10mM Phosphate Solution

Figure 28: CD Result for AS6 at pH 7 in a 10mM Phosphate Solution

Figure 29: CD Results for AS6 at pH 8 in a 10mM Phosphate Solution

Figure 30: CD Results for AS6 at pH 12 in a 10mM Phosphate Solution

Figure 31: CD Results for AS2 at pH 12 in a 10mM Phosphate Solution

Figure 32: CD Results for AS2 at pH 7 in a 10mM Phosphate Solution

Figure 35: CD result for AS3 at pH 2 in a 10mM Phosphate Solution

Figure 36: CD result for AS3 at pH7 in a 10mM Phosphate Solution

Figure 37: CD result for AS3 at pH 8 in a 10mM Phosphate Solution

Figure 38: CD result for AS3 at pH 12 in a 10mM Phosphate Solution

6.4 Appendix D

This data shows ELISA's for AS6p, AS2p, and AS3p.

Figure 39: AS6 and TNB-BSA ELISA

Figure 40: ELISA of AS6 and TNB-BSA

Figure 41: ELISA of AS6 and TNB-BSA

Figure 42: ELISA of AS6 and TNB-BSA

Figure 43: ELISA of AS6p and TNB-BSA

Figure 44: ELISA of AS2p and TNB-BSA

Figure 45: ELISA of AS2p and TNB-BSA

Figure 46: ELISA of AS2p and TNB-BSA

Figure 47: ELISA of AS3p and TNB-BSA

Figure 48: ELISA of AS3p and TNB-BSA