# Effect of Polyethylene Oxide (PEO) Modification on the Structure and Antimicrobial Activity of the Antimicrobial Peptide WLBU2

by
Miranda A. Raper

A THESIS

submitted to

Oregon State University

University Honors College

in partial fulfillment of
the requirements for the
degree of

Honors Baccalaureate of Science in Bioengineering
(Honors Scholar)

Presented May 6, 2016
Commencement June 2016

# AN ABSTRACT OF THE THESIS OF

Miranda A. Raper for the degree of Honors Baccalaureate of Science in Bioengineering presented on May 6, 2016. Title: Effect of Polyethylene Oxide (PEO) Modification on the Structure and Antimicrobial Activity of the Antimicrobial Peptide WLBU2 .

Abstract approved:

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Karl. F. Schilke

Sepsis is a blood infection that is caused by circulating bacteria cells and cell fragments, which contain immunogenic lipopolysaccharides (LPS) or endotoxin. The circulating endotoxin results in a severe dysregulation of the inflammatory response that ultimately leads to organ failure. One promising treatment method is removal of endotoxin by passing blood through a high-throughput, microfluidic device coated with polyethylene oxide (PEO) and antimicrobial cationic amphiphilic peptide WLBU2. A critical step in the development of this microfluidic device is to demonstrate that PEGylation of WLBU2 at the N-terminal group does not negatively affect WLBU2's LPS-binding and antimicrobial activity. Verification of successful PEGylation was performed using gel permeation chromatography (GPC). Secondary structure changes were monitored using circular dichroism (CD), which concluded that the WLBU2 in all PEO-WLBU2 conjugates (5, 10, 20, and 30 kDa) was still able to adopt an α-helical structure in the presence of LPS. Antimicrobial activity was assessed using a liquid kinetic assay against *E. coli*, which illustrated that WLBU2 in the conjugates was able to maintain some antimicrobial activity after PEO-

modification. The results of this project suggest that attachment of a PEO tether to WLBU2 is a feasible method for further attachment to the proposed microfluidic device surface.

Key Words: Cationic amphiphilic peptides (CAPs), antimicrobial peptides, polyethylene oxide (PEO), PEGylation, peptide structure, circular dichroism (CD), gel permeation chromatography (GPC)

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I understand that my project will become part of the permanent collection of Oregon State University, University Honors College. My signature below authorizes release of my project to any reader upon request.

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Miranda A. Raper, Author

## ACKNOWLEDGMENTS

Thanks to my mentor, Dr. Karl Schilke, for providing the opportunity to work in his lab, providing this project and guiding me through the research process. A special thanks to Dr. Matthew Ryder for teaching me invaluable research skills and providing guidance through experimental obstacles as well as creating a fun and friendly lab environment. Thanks to Dr. Joe McGuire and Dr. Adam Z. Higgins for serving on my committee and offering helpful advice and inspiration throughout my undergraduate career. Thanks to Dr. Kain Wu and Dr. Kerry McPhail for teaching me how to use and troubleshoot the Circular Dichroism (CD) instrument. Thanks to Fallon Fumasi for teaching me how to perform liquid kinetic assays using the plate reader. Thanks to Kat Walker and Dr. Skip Rochefort for teaching me how to use the gel permeation chromatography instrument and helping analyze my data. Thanks to the Schilke lab group for helping with various lab techniques and providing friendly support. I would like to thank my parents for believing in me and encouraging me to reach for excellence. Lastly, I would like to acknowledge the University Honors College for providing a well-rounded undergraduate education.

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# INTRODUCTION

## Sepsis

Sepsis is a blood infection that affects over one million people annually in the United States alone, with a mortality rate of 28-50% (more than prostate cancer, breast cancer, and AIDS combined).[1] Sepsis was listed as the most expensive hospital condition by the Agency for Healthcare Research and Quality in 2011 with more than $20 billion spent on treatment each year.[1] The number of sepsis-related cases has been increasing due to various factors, including heightened awareness of the condition, increased longevity of people with chronic diseases, the spread of antibiotic-resistant organisms, and an increase in the number of invasive procedures. Many different types of microbes can cause sepsis, including bacteria, fungi and viruses, but bacterial infections are the most common source. Sepsis is caused by circulating bacteria and bacterial cell wall fragments, which contain immunogenic lipopolysaccharides (LPS) or endotoxin from the cell membrane of Gram-negative bacteria. The circulating cells or endotoxin result in a severe dysregulation of the inflammatory response, and leads to systemic inflammation and organ failure.[2]

The current standard of treatment for sepsis is intravenous (IV) fluids combined with early administration of a broad spectrum of antibiotics which are effective against a variety of bacteria.[3] However, use of antibiotics is problematic for a number of reasons, particularly because indiscriminate use can result in the rise of antibiotic resistance in bacteria, limited efficacy against resistant bacteria, and undesirable side-effects. These undesirable side-effects include hypersensitivity immunological reactions, tissue toxicity, damage to the neurosensory system, and interference with metabolism and drug interactions.[4] In addition, these antibiotics only kill the live bacterial cells, and are generally ineffective at removing the resulting dead cells and cell fragments (endotoxin) in the blood from circulation. The presence of the endotoxin (which is largely unaffected by antibiotics), not the live cells is a

primary cause of the massive inflammatory response and toxic effects which are characteristic of acute sepsis.

One promising treatment method being developed at Oregon State University is removal of endotoxin by passing blood through a high-throughput, microfluidic device which is coated with polyethylene oxide (PEO) and antimicrobial cationic amphiphilic peptides

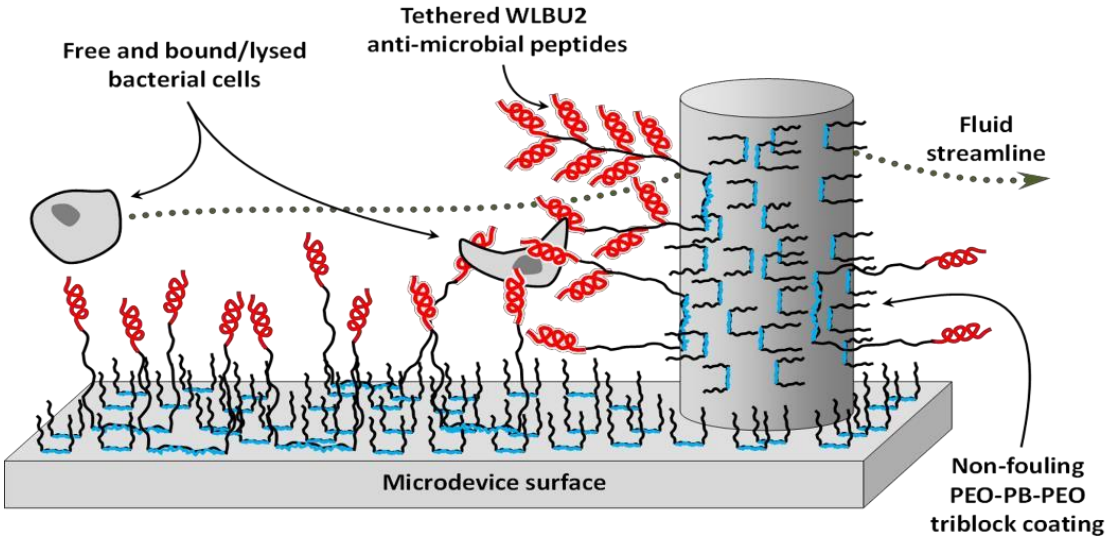


**Figure 1.** Cartoon of proposed microfluidic device to treat sepsis. The surface consists of polyethylene oxide (PEO)-polybutadiene (PBD) - PEO tri-blocks that tether the peptide of interest, WLBU2 (shown as red helices). WLBU2 is designed to capture bacteria cells and cell fragments from flowing solution/blood. Courtesy of Dr. Karl Schilke.

(CAPs) (Figure 1). The device is thought to operate similar to dialysis in which the patient's blood would exit the body, flow through the device, where the bacterial cells and cell fragments are removed, and the 'clean' blood would then flow back into the patient's body. In this research, the N-terminal amine groups of the CAPs are chemically attached to the ends of pendant polyethylene oxide (PEO) chains (PEGylation). The pendant PEO chains are part of PEO-polybutadiene (PBD)-PEO triblocks in which the PBD portion is hydrophobic and the PEO portions are hydrophilic.[5] Tethering of the CAPs to the hydrophilic PEO chains allows the peptides to have more contact with the bacteria-containing solution, resulting in greater bacteria cell and cell fragment capture. The PBD part of the triblock is chemically

bound to the device surface allowing the PEO chains to form a brush layer.[5] The PEO brush provides steric hindrance, which prevents protein aggregation that can initiate clotting and platelet activation, and also makes the device surface biocompatible.[6]

PEGylation is the process of attaching a PEO tether to the end of a peptide or protein. Previous research with antimicrobial peptide nisin A found that it was difficult to access the carboxylic group for tethering purposes despite the flexibility of the C-terminal portion of the peptide and had much better yields when tethering to the N-terminal group.[7] Guitto et al. also found that nisin A lost all antimicrobial activity upon PEGylation, which could only be explained by the PEO chains altering the mobility of the peptide.[7] The effects of PEGylation on WLBU2 activity are of interest in this study because PEGylation could inactivate the WLBU2 as was seen with nisin A, which is undesirable in the proposed microfluidic device. It is not uncommon for PEGylated peptides and proteins to retain only fractions of their original activity. This is due to interaction of the PEO chains with active sites, modification of the protein structure and folding, or a change in the protein or peptide's solubility.[7] This same phenomena could occur when PEGylating the peptide of interest, WLBU2, to the device surface with the proposed microfluidic device to treat sepsis.

The use of immobilized antimicrobial peptides in a blood filtration device is not a new concept. Toraymyxin columns have been used in Japan since 1994 to remove endotoxin from the bloodstream.[8] These adsorption columns contain immobilized Polymyxin B, an antimicrobial peptide that also binds endotoxin. Polymyxin B is not being investigated in this study because previous results produced by the Schilke Lab group suggest that Polymyxin B is less effective at capturing endotoxin compared to WLBU2.

## Liposomes and Lipopolysaccharides (LPS)

A liposome is a spherical vesicle consisting of at least one lipid bilayer. Liposomes are used as a vehicle of administration for drugs and nutrients because of their high degree of

biocompatibility and their ability to encapsulate a large amount of material.[9]

Lipopolysaccharides (LPS) are characteristic components of the cell wall in Gram-negative bacteria and form vesicles in solution (Figure 2) (endotoxin).[10] LPS consists of three main regions: Lipid A, core sugars, and O-antigen, and is present in either the smooth or rough form. The O-antigen region is the outer region of the LPS molecule and is highly variable within a single organism. Some Gram-negative bacteria carry out mutations within the synthesis of the O-antigen region that results in the lack of expression. The truncated form of LPS is known as "rough" LPS and the O-antigen-containing wild-type is known as "smooth" LPS.[12] The Lipid A region is the hydrophobic, membrane-anchoring component of LPS and is responsible for the toxic activity of the whole LPS molecule. The detailed structure of LPS varies between bacterium and the Lipid A region is highly conserved among most Gram-negative bacteria.[11-13]

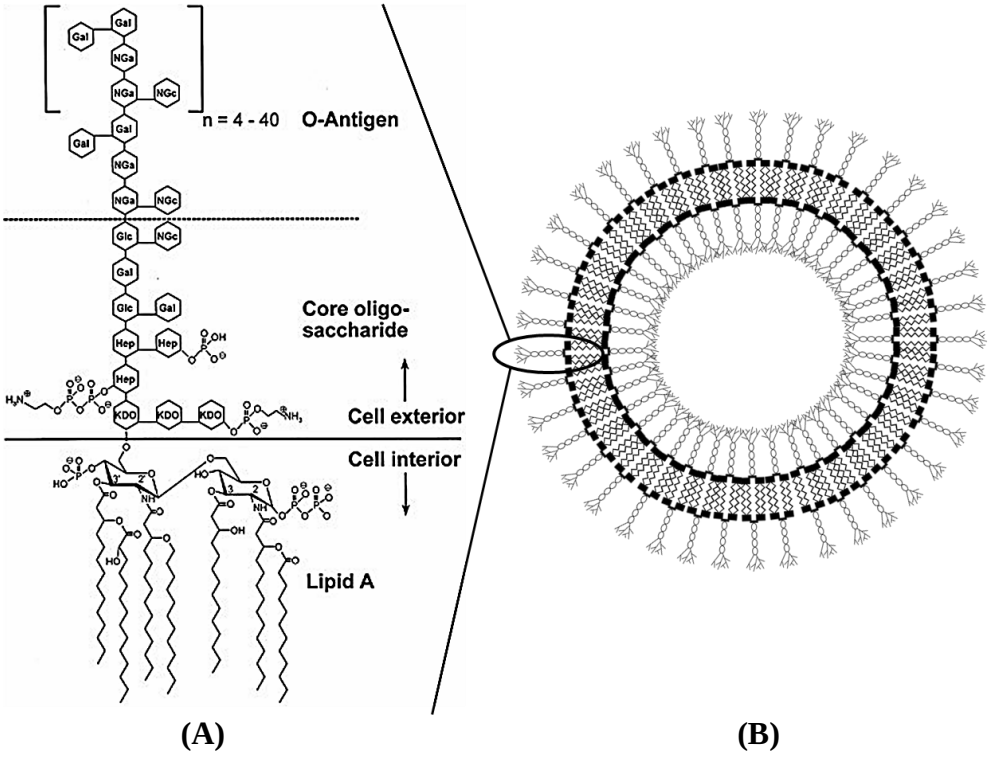


**Figure 2. (A)** Components of lipopolysaccharide (LPS) bilayer consisting of Lipid A, core sugars, and O-antigen.[14] **(B)** Cartoon of LPS vesicle in aqueous solution. Courtesy of Dr. Matthew P. Ryder.

## Cationic Amphiphilic Peptides and WLBU2

Antimicrobial peptides (AMP) are ~10-40 amino acids long and are key components of the innate immune system.[15] AMPs disrupt bacterial membranes using detergency-like packing disruption or formation of pores mechanisms (Figure 3).[15] These interactions are initially believed to occur through electrostatic bonding between the positively charged AMP and the negatively charged bacterial membrane.[16] Therapeutic applications of AMPs utilize the selectivity of AMPs to efficiently disrupt bacteria and other pathogens without causing significant damage to human cell membranes.[15]

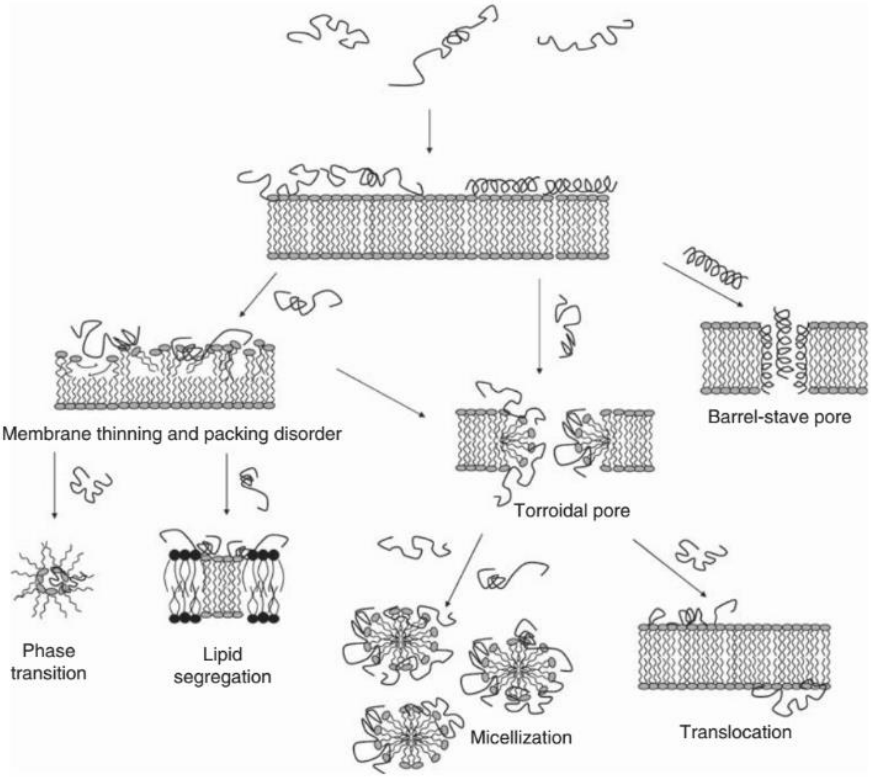


**Figure 3.** Schematic illustration of antimicrobial peptide (AMP) interaction with lipid membranes. The lines represent AMPs that can be disordered (squiggles) or α-helical (helices).[15]

There are many subgroups of AMPs based on their amino acid compositions and structure.[16] Cationic peptides are short AMPs (<40 amino acid residues) that are disordered in aqueous solution but form α-helices in the presence of phospholipid vesicles, liposomes, or

Lipid A.[16] The extent of α-helicity correlates to the antibacterial activity against Gram-negative and Gram-positive bacteria; the greater the α-helical content, the stronger the antimicrobial activity.[16] Cationic amphiphilic peptides (CAPs) are peptides with positive charges lining one side of the helical molecule and hydrophobic residues lining the opposite side.

WLBU2 (RRWVRRVRRWVRRVVRVVRRWVRR) is a synthetic, 24-residue *de novo* engineered CAP with 13 positively charged arginine residues and 11 nonpolar valine or tryptophan residues (Figure 4, left).[17] WLBU2 stands for "Tryptophan-substituted lytic base unit 2" meaning it is a dimer of lytic base units with tryptophan residues substituted along the sequence. In membranes (or membrane mimicking solvent, e.g. TFE), the separation of the charged and hydrophobic residues (Figure 4, right) illustrates the amphiphilic nature of WLBU2. WLBU2 was specifically designed to penetrate and bind bacterial cell membranes and endotoxin in the presence of human blood cells and proteins.[18] The exact mechanism of WLBU2 cell penetration is unknown but is suspected to be the formation of Barrel-stave pores (Figure 3).

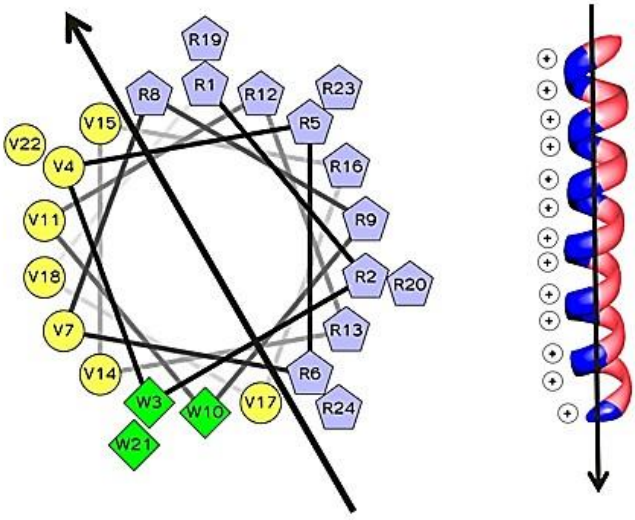


**Figure 4.** (Left) WLBU2 helix wheel diagram. Blue residues represent the positively-charged arginine residues, yellow and green residues represent hydrophobic valine and tryptophan residues, respectively. (Right) Helical configuration of WLBU2 showing face-segregated charged and hydrophobic residues. Courtesy of Dr. Karl Schilke.

Previous research suggests that WLBU2 can capture bacteria and endotoxin without rupturing the bacteria cell membrane. Tethering the peptide to the surface of a microfluidics hemoperfusion device could allow it to capture and remove bacteria and endotoxin from circulating blood.[19] The proposed microfluidic hemoperfusion device allows for precise control and a high surface area to volume of flowing blood ratio. Arrangement of the channels allows form margination of the flow that causes the red blood cells to migrate towards the center of the channel and the remaining blood components, including endotoxin, push out towards the channel walls. This margination is expected to increase the exposure of WLBU2 on the channel surface to the circulating, endotoxin-containing solution, yielding better endotoxin capture.

## PEGylation

As suggested by Guitto et al., PEGylation of nisin A was more successful when PEGylating the N-terminal group as opposed to the C-terminal group.[7] Guitto et al. also found that PEGylation resulted in an inactive nisin A because its mobility was decreased upon PEGylation.[7] Therefore, it is hypothesized that PEGylating WLBU2 at the N-terminal group will result in a structure change that could result in a loss of antimicrobial activity because WLBU2 is also hypothesized to be a pore-former (Figure 3, right), similar to nisin A. The PEGylation reaction used in this study is a single step, $S_N2$ reaction using NHS- activated PEO polymer and WLBU2 (Figure 5) with dimethyl sulfoxide (DMSO) as the solvent. DMSO, a non-aqueous solvent, was used for the PEGylation of WLBU2 to prevent hydrolysis of the NHS-ester on the NHS-activated PEO polymer.

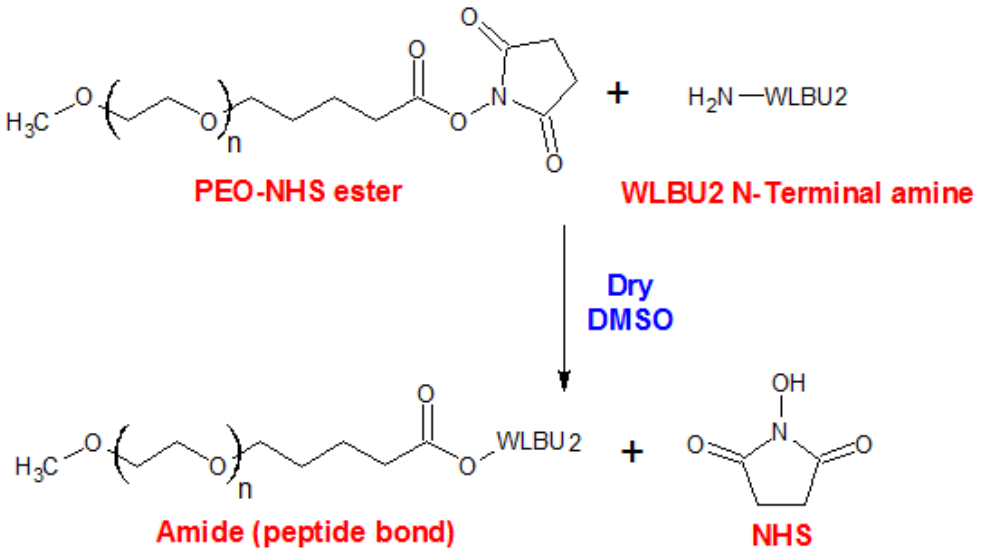


**Figure 5.** WLBU2 PEGylation SN2 reaction scheme using NHS-activated PEO polymer and DMSO as the solvent.

## Circular Dichroism (CD)

Circular dichroism (CD) is an instrumental technique used to measure differences of left-handed and right-handed circularly polarized light absorption attributed to protein/peptide confirmation.[20] When two electromagnetic plain light waves are of equal magnitude and perpendicular to each other they can create a left- or right-handed circularly polarized light wave (Figure 6).[21]

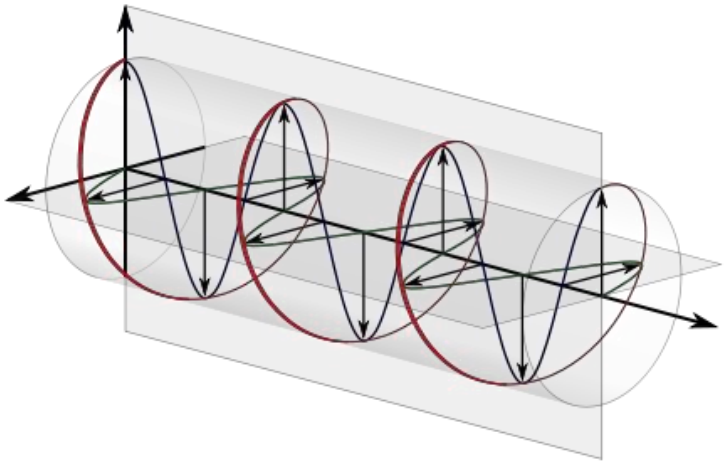

**Figure 6.** Cartoon of a circularly polarized light wave (red line) made from two plain light waves 90° out of phase with each other.[21]

When proteins and other chiral molecules interact with far UV light (190-250 nm), they absorb left- or right-handed circularly polarized light differently.[21] The shape of the absorbance spectrum gives an indication of the molecule's secondary structure (Figure 7).

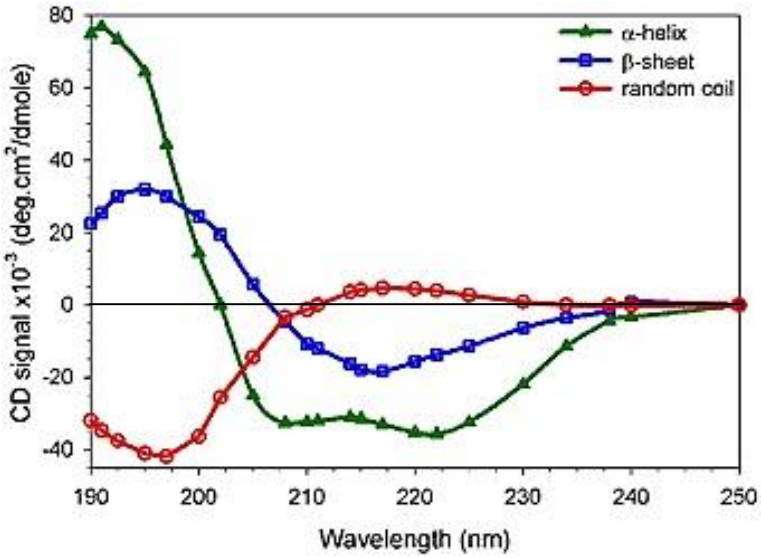


**Figure 7**. Characteristic circular dichroism (CD) spectra for α-helical, β-sheet, and 'random coil'/disordered molecules.[23]

The CD spectra for a disordered peptide, such as cationic AMPs in aqueous solution, is usually characterized as 'random coil' by a single band below 200 nm. 'Random coil' is not actually a random structure but is termed 'random' because no other types of secondary structures are apparent. Alpha-helical structures are usually characterized by two negative bands at 208 and 222 nm along with a positive band at 195 nm.[22] Further analysis can be performed on the spectra using software to determine the degree of α-helicity of the protein. One potential caveat to CD analysis of peptides, absolute structural content is more difficult to analyze and is prone to over-interpretation when using CD for this purpose.

## Gel Permeation Chromatography (GPC)

Gel permeation chromatography (GPC), also known as size-exclusion chromatography (SEC), is a liquid chromatography technique that separates molecules based on their hydrodynamic radius in solution.[24] GPC was used in this study to verify successful

PEGylation of WLBU2. GPC was used instead of a more direct analysis, such as NMR, for conjugation verification because an amide bond is formed through the PEGylation of WLBU2. This additional peptide bond would be very difficult to detect among the other 24 peptide bonds already present in WLBU2. Therefore, it was easier to verify successful conjugation using GPC by analyzing the change in effective hydrodynamic radius with the addition of a PEO tether.

In GPC, the column is filled with rigid, porous gel beads that may consist of polyacrylamides or porous glass.[25] The pore size of the gel beads determines the molecular size range across which the separation occurs.[24] The sample is injected into the column and is chased with the mobile phase solvent, typically phosphate buffered saline (PBS) for polymers, which is pumped through the column.[25] Smaller molecules are able to travel into the pores of the gel beads while larger molecules are excluded from the pores. Thus, larger molecules travel around the gel beads and elute from the column first, followed by the smaller molecules and analytes. Figure 8 shows a schematic of the GPC process and illustrates the separation of differently sized molecules. Solvent parameters, such as pH, ionic strength, and concentrations of additives, can influence sample separation.[26] Charge of the species can also influence separation and can lead to ion exclusion or irreversible adsorption to the gel beads.[26]

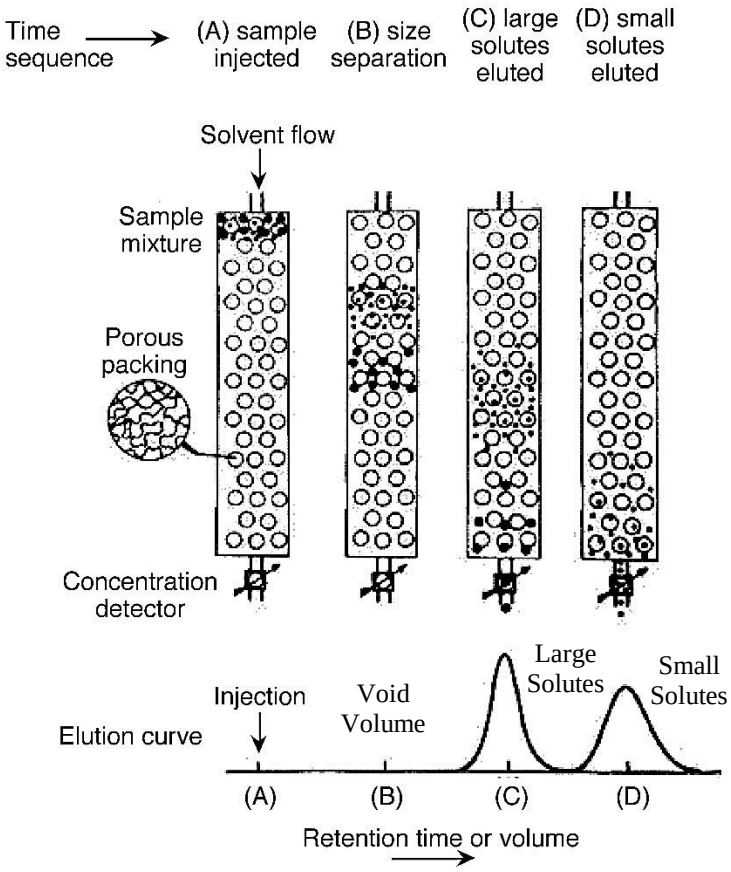


**Figure 8**. Schematic of gel permeation chromatography (GPC) process and elution curve output. The larger molecules are represented by the larger spheres and the smaller molecules are represented by the smaller spheres. [27]

### *E. coli* Liquid Kinetic Assay

Bacterial growth was monitored with a liquid kinetic assay using Gram- negative bacteria, which allowed investigation of the growth inhibition properties of PEGylated WLBU2 versus unmodified WLBU2. *Escherichia coli* (*E. coli*) was used as a model Gram-negative bacteria, because it is a widely studied strain of bacteria, is easily accessible and is clinically relevant. In this assay, *E. coli* growth is monitored using the optical density at 600 nm ($OD_{600}$), after introduction of PEGylated WLBU2 during the exponential growth phase (Figure 9), where cell division occurs most

rapidly. Inhibition of cell growth would result in a reduced $OD_{600}$ when compared to the negative control. WLBU2 exhibits antimicrobial activity against Gram-negative bacteria, and this assay was used to determine if WLBU2 retained this activity after N-terminal modification with various lengths of PEO chains.

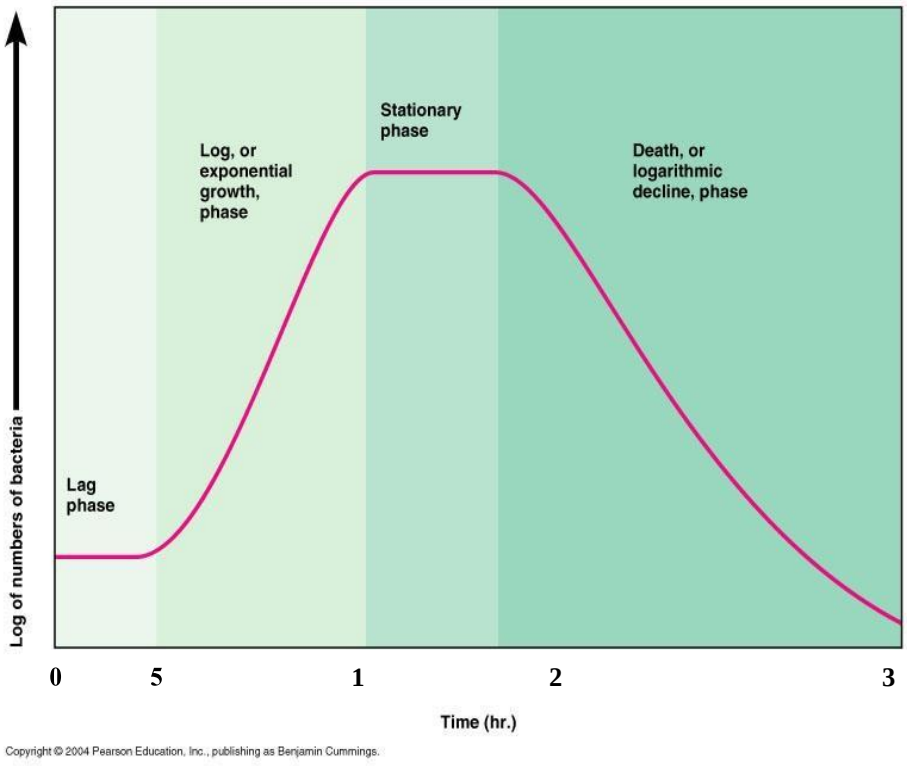


**Figure 9.** Graphical representation of the four E. coli growth cycle phases. Addition of PEGylated or control (PEO only, unmodified WLBU2, and ampicillin) occurs approximately at the end of the lag phase based on experimental design.[28]

## STATEMENT OF PURPOSE

A critical step in the development of a microfluidic hemoperfusion device to treat sepsis is to demonstrate that PEGylation of WLBU2 at the N-terminal group does not negatively affect WLBU2's LPS-binding and antimicrobial activity. My *central hypothesis* is that PEGylation of WLBU2 at the N-terminal will cause minimal secondary structural changes, and WLBU2 will remain active for LPS capture by retaining its antimicrobial

activity. Verification of successful PEGylation was performed using gel permeation chromatography (GPC). Secondary structure changes were monitored using circular dichroism (CD). Antimicrobial activity was assessed using an *E. coli* liquid kinetic assay.

## MATERIALS AND METHODS

*Reagents and Solvents*

WLBU2 (lot # P16321408), molecular weight 3.4 kDa, was purchased from GenScript (Piscataway, NJ). NHS-activated PEO polymers (2-30 kDa) [lots # 121-43, 128- 83, 120-41, 123-151, and 121-68] were purchased from Laysan Bio (Arab, AL). DriSolv anhydrous DMSO (CAS: 67-68-5, lot # 53312) was from EMD Millipore. Ethanol (200 Proof, CAS: 64-17-5, lot # V1016), methanol (anhydrous, lot # H15753), and HPLC-grade water (CAS: 7732-18-5, lot # MFCD00011332) were purchased from J.T. Baker. PEO, molecular weight 6 kDa, (Fluka, lot # 122446614305349), ampicillin sodium salt (Sigma, lot # 16H0383), and powdered LB broth (Amresco, lot # 3513C054) were used for liquid kinetic assay tests. *Escherichia coli* cells (2011 ATCC 23922) were used in this study.

Lipopolysaccharides from *Pseudomonas aeruginosa* purified by phenol extraction, were obtained from Sigma (St. Louis, MO). PBS (137 mM NaCl, 2.68 mM KCl, 10.1 mM $Na_2HPO_4$, 1.76 mM $KH_2PO_4$, 7.69 mM $NaN_3$, pH 7.4) (1L) was filtered and used as the mobile phase for GPC experiments.

*PEO-WLBU2 Conjugation Protocol (PEGylation)*

PEO-WLBU2 conjugation was performed using commercially available NHS-activated PEO polymers (Figure 5). Each vial of WLBU2 was quoted by the manufacturer to contain 5 mg, but upon further analysis using UV spectrometry to detect the tryptophan residues at 280 nm, it was determined that each vial only contained ~3 mg of peptide.

Therefore it was assumed that each new vial contained ~3 mg of peptide. A 100 mg/mL solution of each NHS-PEO polymer was made by adding 1 mL of dry DMSO to each original vial containing 100 mg of NHS-PEO. A 50% molar excess of the respective NHS-PEO polymer stock was added to a 3 mg vial of WLBU2 (Table 1) then filled to a final volume of 1 mL using dry DMSO.

**Table 1.** Volumes of 100 mg/mL NHS-PEO polymer required for 50% molar excess of NHS-PEO to WLBU2 (3 mg) in each reaction vial.

| NHS-PEO polymer MW (kDa) | Percent Mass of WLBU2 (%) | Volume of 100 mg/mL Stock Added (µL) | Volume of DMSO Added for Final Volume of 1 mL (µL) |
|---|---|---|---|
| 5 | 31.3 | 66 | 934 |
| 10 | 18.5 | 132 | 868 |
| 20 | 10.2 | 265 | 735 |
| 30 | 7.0 | 397 | 603 |

After addition of the NHS-PEO and dry DMSO, the WLBU2 vial was flushed with argon gas after mixing to prevent introduction of atmospheric water that would promote hydrolysis of the NHS ester portion of the NHS-PEO. The solutions were then placed on a rotisserie at 37°C for 24 hours to allow the reaction to occur with constant mixing.

*PEO-WLBU2 Purification Protocol*

The PEO-WLBU2 conjugates were purified by precipitation with cold methanol (Figure 10). Each reaction mix (1 mL) was added to a 15 mL Falcon tube, after which 10 mL of methanol was added and the tube was vortexed. Ethyl ether, hexanes, and ethanol were each investigated as the precipitation solvent. These solvents were investigated because WLBU2 is insoluble in them and would therefore precipitate out of solution. Ethyl ether and hexanes were discarded because they produced a two-phase mixture due to DMSO being mostly immiscible with those solvents. Various volumes of precipitation solvent were added to each

1 mL reaction solution (10-50 mL). The greatest precipitation yield occurred with methanol in a 1:10 ratio of DMSO: methanol (for the reaction solution to precipitation solvent volume).

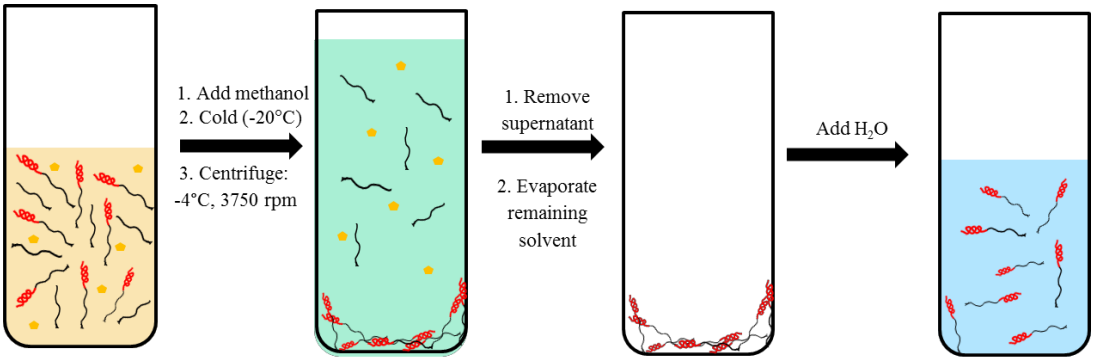


**Figure 10.** Schematic of precipitation protocol used in purification of WLBU2-PEO conjugates. The red curly q's represent WLBU2, the black squiggle lines represent PEO and the yellow pentagons represent the NHS byproduct. Removal of the NHS byproduct was not verified.

The Falcon tubes were held at -20 °C overnight to maximize precipitation. The Falcon tubes were then centrifuged at -4°C (the lowest temperature possible with the centrifuge) at 3750 rpm for 15 minutes to concentrate the precipitate. The supernatant was discarded, and the precipitate was then suspended in 10 mL of cold methanol. The precipitation process consisting of the supernatant removal and subsequent addition of methanol was repeated for a total of 3 times. The remaining solvent was evaporated under vacuum. The resulting 'dry' conjugate was dissolved in 1 mL of HPLC-grade water and transferred to a 2 mL centrifuge tube. PEO-WLBU2 conjugate concentration was determined by the absorbance of the tryptophan residues of WLBU2 at 280 nm ($A_{280}$) in a UV spectrometer (WLBU2 $\varepsilon_{280}$=16,500 $M^{-1}cm^{-1}$). It was initially assumed (and later verified using GPC) that all WLBU2 in the purified sample was in PEO-WLBU2 conjugates.

*Semi-Quantitative Analysis of the Extent of Conjugation- Gel Permeation Chromatography (GPC)*

The formation of stable bubbles when an aqueous solution of the PEO-WLBU2 product was agitated indicated that the polar PEO and partially nonpolar WLBU2 were in fact chemically bonded, forming a large amphiphilic conjugate with evident surfactant properties. This phenomena did not happen with WLBU2 alone in aqueous solution. However, it was necessary to obtain more direct evidence of successful conjugation. Semi-quantitative analysis of the extent of conjugation was performed using gel permeation chromatography (GPC). GPC requires sample concentration to be 0.5-1 mg/mL with a sample volume of 200 µL. Due to limited amounts of each conjugate, 500 µL of 0.25g/L PEO-WLBU2 (5kDa) and 0.5 g/L PEO-WLBU2 (10 and 20 kDa) were made by diluting the samples with HPLC-grade water. This sample volume allowed for duplicates of each conjugate. Each sample was treated with sodium dodecyl sulfate (SDS) in a 20X molar excess in an attempt to ensure all positive arginine residues on the WLBU2 were neutralized, to prevent interactions with the anionic GPC column. The samples were degassed and pumped through the column at 0.5 mL/min at room temperature. Post-detector column effluents were discarded.

*Analysis of Antimicrobial Activity- Liquid E. coli Kinetic Assay*

The residual antimicrobial activity of the PEO-modified WLBU2 was analyzed using a liquid Gram-negative *Escherichia coli* (2011 ATCC 23922) bacteria liquid kinetic assay. One culture loop of thawed stock *E. coli* was added to 20 mL of autoclaved LB broth and incubated for 4 hours at 37 °C with shaking prior to running the assay. Sterile LB broth and LB broth with *E. coli* cells were each added to 3 wells of a clear, 96-well microplate in a semi-randomized fashion. Triplicates of WLBU2, with and without conjugated PEO chains of various lengths, were added to the liquid suspensions of *E. coli* for a final concentration of 10

μM. Positive and negative controls of the experiment were ampicillin (0.1 mg/mL stock), unmodified WLBU2, and PEO alone (respectively), which were added to respective wells to a final concentration of 10 μM. All samples were run in triplicate at 37 °C. The plate was placed in a Victor $V_3$ plate reader without a lid, and the optical density at 600 nm ($OD_{600}$) was measured every fifteen minutes up to 24 hours to determine the extent of inhibition of bacteria growth. An OD600 of 1.0 is equivalent to $3x10^9$ cfu/mL.[28]

*Analysis of Peptide Secondary Structure- Circular Dichroism (CD)*

The effect of PEGylation on secondary structure of WLBU2 was analyzed using circular dichroism (CD), which measures absorbance of left- and right-handed circularly polarized light in the far UV spectra when it is passed through a solution of a peptide or protein. A total of 1.5 mL of 0.2 mg/mL solutions of each of the 5, 10, 20, 30 kDa PEO-WLBU2 conjugates in HPLC-grade water allowed for triplicates, except for the 5 kDa PEO-WLBU2 sample which only allowed duplicates. Each sample was measured with a scanning speed of 200 nm/min from 260 to 180 nm with a total of 5 accumulations. After measuring the conjugate alone, 4 μL of 10 mg/mL *P. aeruginosa* lipopolysaccharide (LPS) in HPLC water was added, to result in a final concentration of 0.1 mg/mL LPS per sample. This solution was mixed by inversion ~5 times, then measured under the same operating parameters to observe any changes in WLBU2 secondary structure caused by the presence of LPS.

## RESULTS AND DISCUSSION

*Semi-Quantitative Analysis of the Extent of Conjugation- Gel Permeation Chromatography (GPC)*

Figure 11 shows the results of GPC analysis of the PEO-WLBU2 conjugates compared to unmodified WLBU2. Because of the high positive charge of WLBU2, each sample was

treated with SDS to prevent interaction with the anionic column resin. The first elution peak for each conjugate appears to align with the elution peak for WLBU2. The second elution peak represents the WLBU2-PEO conjugate for each respective sample. Elution peaks corresponding to SDS are not shown because they appear at a later time and are not of interest.

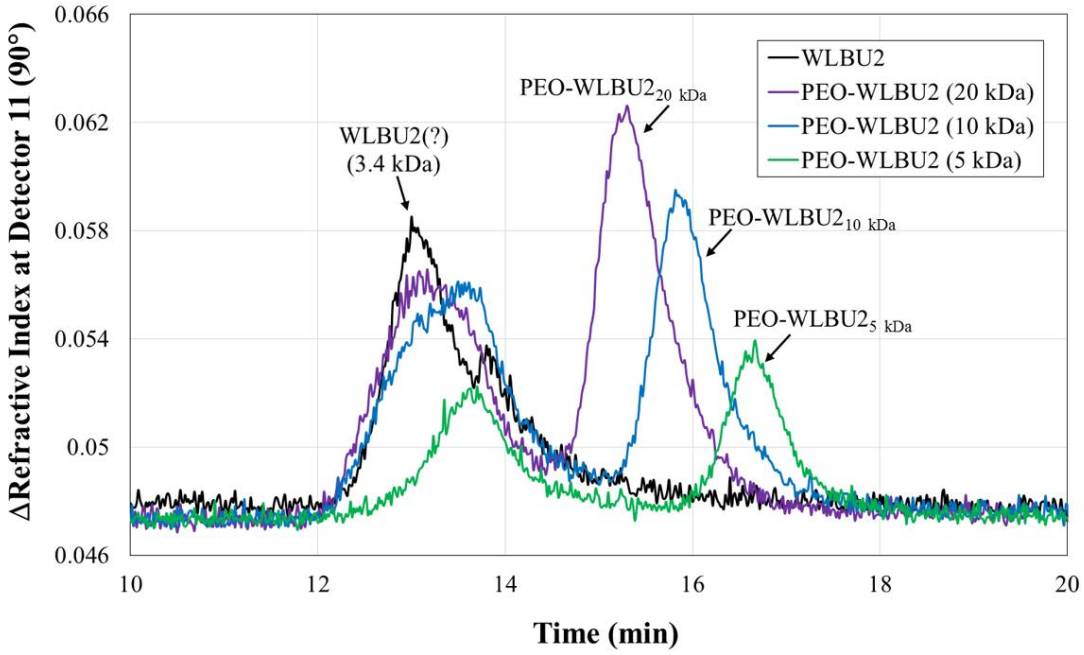


**Figure 11.** Gel permeation chromatography (GPC) data for unmodified WLBU2 and PEO-WLBU2 (5, 10, and 20 kDa) using detector 11 (90°). This data represents the average of each sample's duplicates.

Larger compounds elute from the column first, and this trend was observed when comparing the PEO-WLBU2 conjugates because the elution peak order for thePEO-WLBU2 conjugates was 20, 10, and then 5 kDa. However, the presence of the WLBU2 peak before each conjugate was unexpected, and may represent unreacted WLBU2 from each conjugation reaction. This observed phenomena may be the result of strong electrostatic interactions between unmodified WLBU2 versus PEO-modified WLBU2 and the column resin. Each sample was treated with sodium dodecyl sulfide (SDS), which acted to ion pair with the positive residues on WLBU2 to give the molecule an overall neutral charge to prevent

interaction with the anionic column. When treated with SDS, unmodified WLBU2 has a lesser charge-to-mass ratio compared to PEO-WLBU2 and would be more strongly repelled by the resin than PEO-WLBU2 would be. This stronger repulsion may be the reason that WLBU2 eluted from the column before the PEO-WLBU2 conjugates. The importance of these electrostatic interactions should be investigated further.

A second theory is that the SDS molecules could be forming micelles with an overall negative charge on the outer surface (Figure 12)[30]. WLBU2 could be forming electrostatic interactions with the SDS micelles resulting in a bulkier molecule with a larger hydrodynamic radius. This may explain why the apparent WLBU2 elution peak occurs before the PEO-WLBU2 elution peaks. SDS alone needs to be analyzed using GPC to determine the elution time for an SDS micelle before any conclusions can be drawn regarding the 'WLBU2' elution peaks.

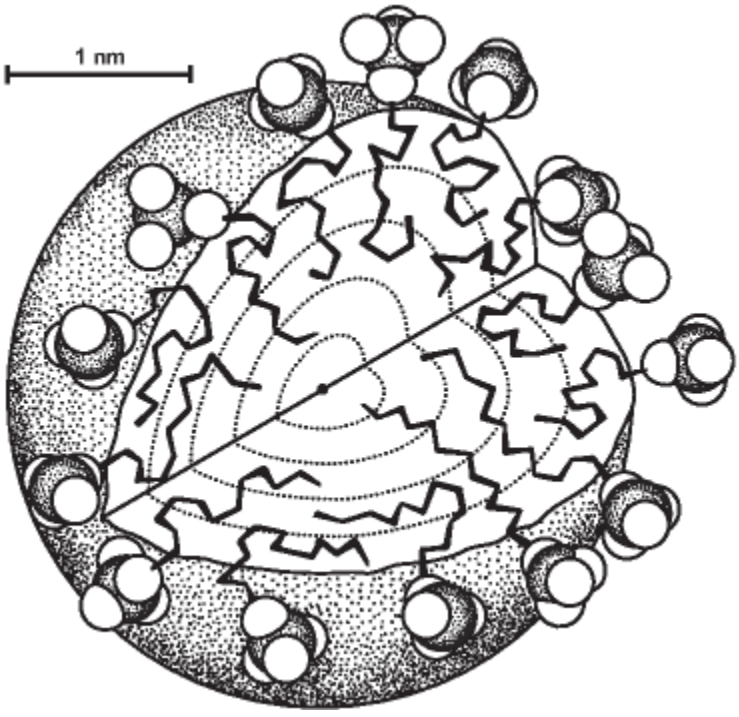


**Figure 12.** Sodium dodecylsuphate (SDS) micelle drawn to scale. The micelle contains 60 SDS molecules[30].

A third theory is that PEO in the PEO-WLBU2 conjugate could be anchoring the entire conjugate in the pores of the gel beads, thus impeding elution. Anchoring is where particular parts of a branched molecule can behave as separate molecules, penetrate into the gel bead pores, and anchor the entire molecule.[31] PEO can adopt a loose helix structure[32] in water that is smaller than the disordered WLBU2 and is able to fit into the pores of the gel particles. As the PEO-WLBU2 conjugates move through the column, the PEO could be entering the pores, thus impeding elution. Unmodified WLBU2 does not have the PEO tether and is not slowed as it moves through the column.

*Analysis of Peptide Secondary Structure- Circular Dichroism (CD): Controls*

The controls used for CD were PEO and LPS alone, WLBU2 mixed with PEO (non-conjugated), and WLBU2 with LPS (Figure 13). Neither PEO nor LPS alone exhibited a significant different in absorbance of polarized UV light, because they are achiral molecules. This result was as expected because PEO has a simple, repeating molecular formula. The presence of PEO did not change the CD spectra of WLBU2 so PEO is thought to not interact with WLBU2 to give rise to the CD signal (peptide's secondary structure). LPS does not itself significantly contribute to the CD signal.

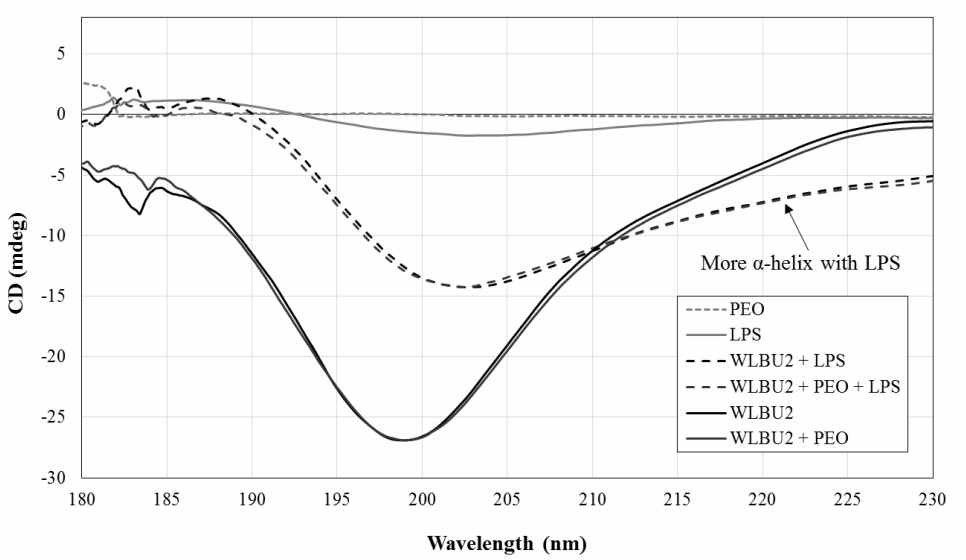


**Figure 13**. Circular dichroism (CD) spectra of PEO alone, LPS alone, and mixtures of WLBU2 with LPS and/or PEO.

CD spectra for unmodified WLBU2 and the PEO-WLBU2 conjugates (5, 10, 20, and 30 kDa) alone in solution are shown in Figure 14. All samples had a final total concentration of 0.2 mg/mL in HPLC-grade water.

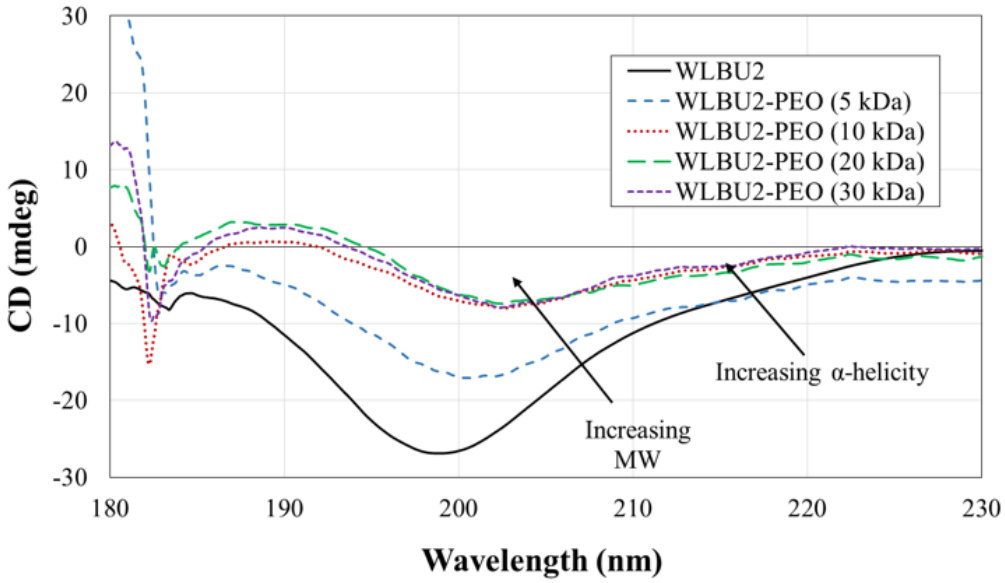


**Figure 14.** Circular dichroism (CD) spectra of unmodified WLBU2 and PEO-WLBU2 conjugates (5, 10, 20, and 30 kDa). All samples had a concentration of 0.2 mg/mL with HPLC-grade water as the solvent. The CD signal for the WLBU2-PEO conjugates was multiplied by a factor of 10 to facilitate comparison with WLBU2.

The signal for the WLBU2-PEO conjugates was low, so the spectra were multiplied by a factor of 10 to facilitate comparison to that of WLBU2. This was done because each conjugate sample contained the same total mass but the percent of peptide present in each sample was different. All conjugate and WLBU2 samples had a 'random coil' secondary structure when alone in solution but the conjugates with higher molecular weight PEO tethers had increased α-helicity (less 'random coil').

*Analysis of Peptide Secondary Structure- Circular Dichroism (CD): LPS Studies*

CD spectra for the same unmodified WLBU2 and PEO-WLBU2 conjugates (5, 10, 20, and 30 kDa) samples were collected in the presence of added LPS, as shown in Figure 15. The final WLBU2/WLBU2-PEO conjugates and LPS concentrations were 0.2 mg/mL and 0.1 mg/mL, respectively. The signal for the PEO-WLBU2 conjugates was low and was increased by a factor of 5 to facilitate comparison with WLBU2.

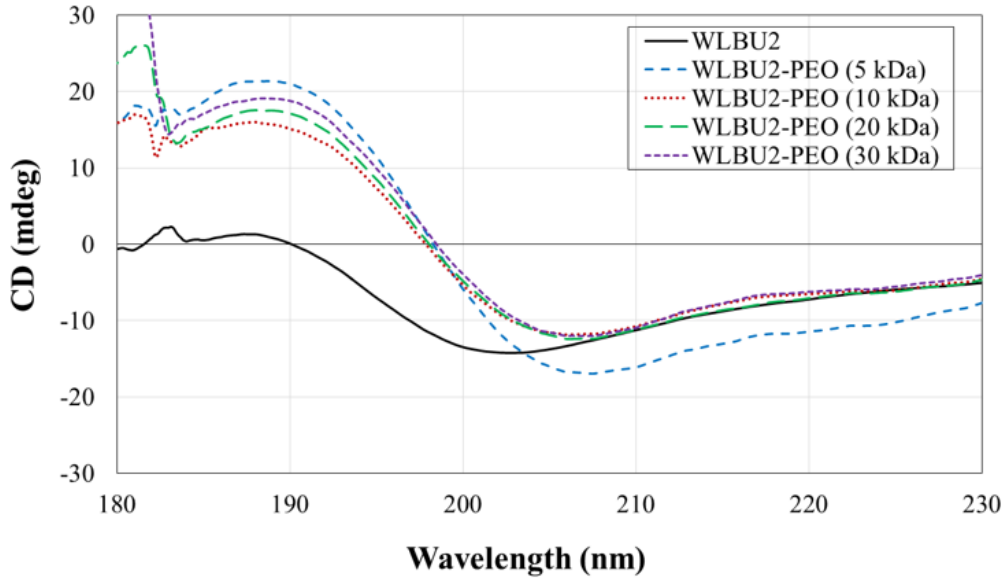


**Figure 15.** Circular dichroism (CD) spectra of unmodified WLBU2 and PEO-WLBU2 conjugates (5, 10, 20, and 30 kDa) with LPS. All samples had a peptide/conjugate concentration of 0.2 mg/mL and an LPS concentration of 0.1 mg/mL. The CD signal for the WLBU2-PEO conjugates was multiplied by a factor of 5 to facilitate comparison with WLBU2.

The presence of LPS led to a stronger CD signal requiring a smaller normalization factor than that of the conjugates alone in solution (5 instead of 10). All conjugate samples had a similar degree of α-helicity with the PEO-WLBU2 5 kDa sample exhibiting the most α-helicity. This structure is similar to that of WLBU2 indicating that the structure of WLBU2 in the presence of LPS is unchanged by PEGylation. It was proposed that WLBU2's mechanism of action is to 'corkscrew' itself into the bacteria cell membrane, and so WLBU2 is able to capture bacteria cells and endotoxin.

The ability of PEO-modified WLBU2 to assume an α-helical structure suggests that tethering of WLBU2 to the proposed microfluidic hemoperfusion device surface will still allow it to function in a similar manner as unmodified WLBU2.

*Antimicrobial Activity- Liquid E. coli Kinetic Assay*

Assay controls were *E. coli* cells only, ampicillin (10 μM), and PEO (10 μM) (Figure 16). The positive control, ampicillin (dashed), resulted in almost complete growth inhibition, as expected. The negative control, PEO alone (6 kDa, dotted), resulted in minimal growth inhibition and a growth curve similar to that of untreated *E. coli* cells. Any growth inhibition observed with the PEO-WLBU2 conjugates is therefore the result of the WLBU2 portion of the compound and not the PEO.

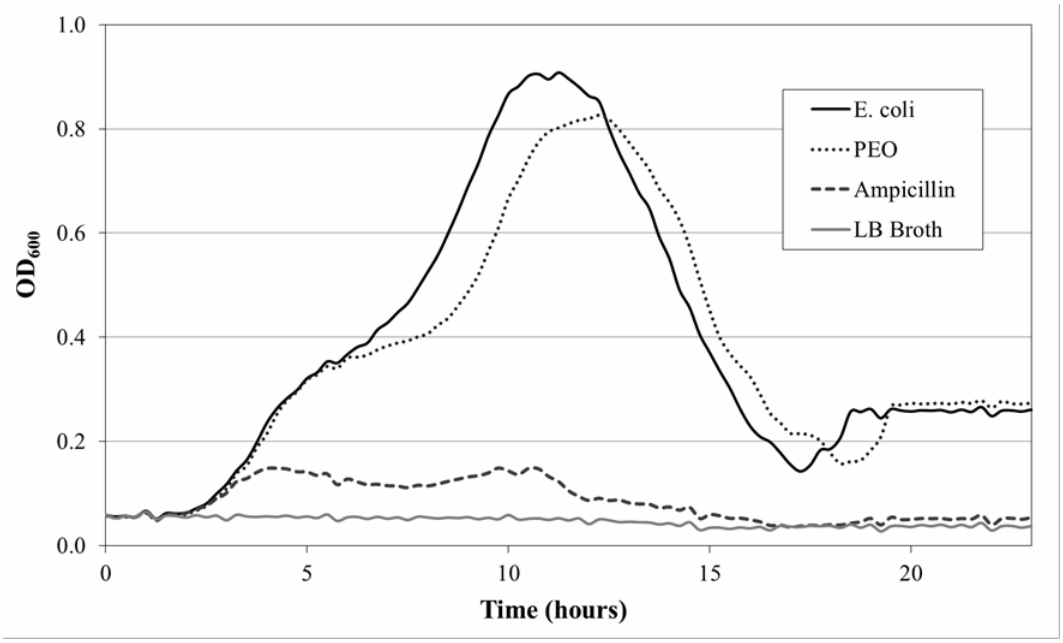


**Figure 16**. Liquid kinetic assay of *E. coli* cells, ampicillin (10 µM), and PEO (6 kDa, 10 µM) controls.

The sharp decline in absorbance after ~10 hours is most likely the result of evaporation which reduced the amount of water available. As the solvent evaporated, the cells became more concentrated and began dying due to lack of nutrients and high ionic strength/ hyperosmotic solution conditions. Upon death, the cells may have lysed, which is consistent with high ionic strength solutions, releasing their contents. This resulted in a higher constant absorbance reading at the end of the experiment (>20 hours). Efforts were made to reduce evaporation by maintaining a saturated environment using a wet paper towel and covering the wells with clear packing tape. While these efforts were successful at reducing solvent evaporation, they created undesireable anaerobic growth conditions (packing tape) and required constant monitoring of saturation levels (wet paper towel).

Assay results obtained for each PEO-WLBU2 conjugate and WLBU2 are shown in Figure 17. Unmodified WLBU2 nearly completely inhibited bacterial growth, while the PEO-WLBU2 conjugates exhibited lesser inhibitory activity. The trend was that increasing PEO chain length resulted in inhibitory activity that approached that of unmodified WLBU2.

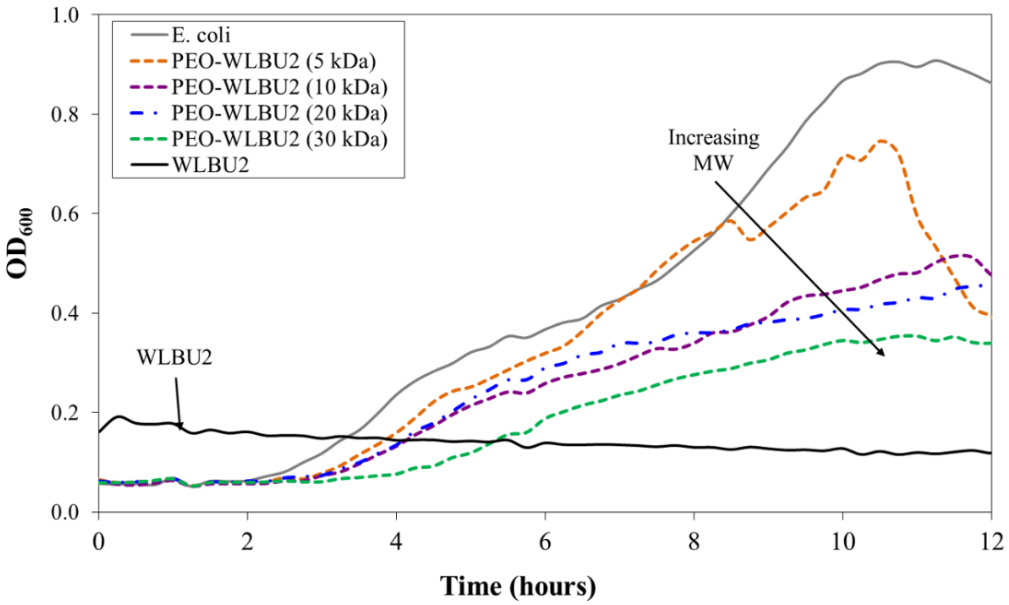


**Figure 17.** Liquid E. coli kinetic assay of PEO-WLBU2 conjugates (5, 10, 20, 30 kDa) and unmodified WLBU2. All samples had a final concentration of 10 µM. An $OD_{600}$ of 1.0 is equivalent to $3x10^9$ cfu/mL.[28]

This suggests that a longer PEO chain possible provides more bacterial interaction than short PEO. The mechanism for why this occurs requires further investigation. An alternative interpretation is that long-chain PEO reacts at lower yields, leaving more unmodified WLBU2 and inert PEO in the reaction mixture. This is supported by larger WLBU2 elution peaks for the larger PEO-WLBU2 in the GPC experiments (Figure 11) and smaller CD signals for the larger PEO-WLBU2 conjugates alone and in the presence of LPS (Figure 13-Figure **14**). This test should be replicated with a solution known to contain pure PEO-WLBU2 conjugates and reduced solvent evaporation to see of the same trend is observed.

## CONCLUSIONS

The overarching goal is to develop microfluidic devices that utilize cationic amphiphilic peptides (e.g. WLBU2) to capture bacteria cells and cell fragments (endotoxin) from circulating blood to treat sepsis. The general approach is to tether WLBU2 to the surface of the device using pendant polyethylene oxide (PEO) polymer chains, to allow for greater mobility in solution/blood and provide hemocompatibility. The objective of this project was to modify the peptide of interest, WLBU2, with a N-terminal PEO chain (PEGylation), to demonstrate that PEGylation does not negatively affect WLBU2's LPS-binding and antimicrobial activity. Successful conjugation was verified with gel permeation chromatography. The effects of PEGylation on peptide secondary structure and antimicrobial activity were analyzed using circular dichroism (CD), and a liquid kinetic assay against *E. coli*, respectively. Circular dichroism (CD) concluded that the WLBU2 in all PEO-WLBU2 conjugates (5, 10, 20, and 30 kDa) had a 'random coil' structure when alone in solution, but was still able to adopt an α-helical structure in the presence of LPS. This is the same behavior as observed with unmodified WLBU2.

*E. coli* liquid kinetic assays illustrated that WLBU2 in the conjugates was able to maintain some antimicrobial activity after PEO-modification. It was also observed that the larger PEO-WLBU2 conjugates behaved more like unmodified WLBU2. This suggests that a longer PEO chain provides more bacterial interaction than short PEO. Alternatively, the longer PEO chains react at lower yields, leaving more unmodified WLBU2 and inert PEO in the reaction mixture. The results of this project suggest that it is feasible to attach a PEO tether to WLBU2 for later attachment to the proposed microfluidic hemoperfusion device surface to increase peptide mobility in solution. Further tests should be performed with tethered WLBU2 to a surface to prove that it is effective at capturing LPS (and eventually bacteria cells and cell fragments) from a circulating solution.

## FUTURE WORK

Future work involves repeating the above experiments to replicate the results. The next step would be to covalently attach the non-conjugated end of the PEO chain to a surface and flow a solution containing LPS over the surface using quartz crystal microbalance with dissipation (QCM-D) or optical waveguide lightmode spectroscopy (OWLS). Both of these techniques would measure the amount of LPS that bound to the surface. This would be a way to test whether the WLBU2 in the conjugates is still able to effectively capture LPS. This test would also allow comparison of the extent of LPS capture with increasing PEO chain length. It is speculated that longer PEO chains would allow for greater peptide mobility and greater interaction with the flowing solution. This would be beneficial in the final microfluidic hemoperfusion device to treat sepsis because it would allow WLBU2 to capture bacteria cells and cell fragments (endotoxin) from a few nanometers away from the outer edges of the flow channels of the device instead of only what actually contacts the channel surface.

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