AN ABSTRACT OF THE THESIS OF

Anthony C. Amsberry for the degree of Honors Baccalaureate of Science in Bioengineering presented on Dec 2, 2013. Title: Developing a membrane leakage assay for the binding of bacterial lipopolysaccharide by the cationic amphiphilic peptide WLBU2

Abstract approved:

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Dr. Karl F. Schilke

Sepsis, caused by bacterial release of endotoxin, is a serious blood infection that causes uncontrolled systemic immune response. In North America alone, sepsis kills between 220,000 and 380,000 people each year, more than prostate cancer, breast cancer, and HIV/AIDS combined. Sepsis treatment is complicated and often requires prolonged hospital stays, costing $17 billion annually. There is a need for an external hemoperfusion to device bind to endotoxin molecules, thereby removing them efficiently from the bloodstream without having to add additional cleaning agents to blood. Cationic amphiphilic peptides (CAPs) tethered to non-fouling surfaces in hemoperfusion microchannels hold promise for effective endotoxin removal. WLBU2, an engineered amphipathic CAP, is expected to bind endotoxin in a way that leaves endotoxin vesicles largely intact. This project aimed to develop a membrane-leakage assay to quantitatively measure endotoxin membrane permeabilization in the presence of CAPs. Calcein, self-quenched at 60 mM, was loaded vesicles of lipopolysaccharides (LPS), an analogue for endotoxin. LPS vesicles containing calcein were isolated from unencapsulated dye via dialysis, and stored at 4^{o}C. Successful isolation was confirmed by a measured increase in fluorescence upon the addition of Triton X-100. Percent leakage was calculated by comparing leakage from WLBU2 addition to leakage from Triton X-100 addition (full

permeabilization). Dye-loaded liposomes provide great potential for future investigation with other engineered peptides and surface-binding experiments.

Key words: Lipopolysaccharide, LPS, calcein leakage, membrane permeabilization, WLBU2, polymyxin B, sepsis

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Developing a membrane leakage assay for the binding of bacterial lipopolysaccharide by the cationic amphiphilic peptide WLBU2

by

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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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Introduction

Sepsis Treatment

Severe sepsis is a blood infection that affects approximately 750,000 Americans each year, and 28-50% of these cases end in death.[1] Sepsis kills more Americans than prostate cancer, breast cancer, and HIV/AIDS combined, and $17 billion is spent on sepsis treatment each year.[2] Sepsis-related deaths continue to rise, which may be due to current disease treatment practice, increased longevity of people with chronic diseases, the spread of antibiotic-resistant disease strains, and various other factors. Most cases of septic shock are triggered by endotoxin-producing Gram-negative bacteria. Lipopolysaccharide (LPS), or endotoxin, is released from the bacterial cell wall during bacterial growth or due to antibacterial immune response. This triggers dysregulated release of proinflammatory cytokines, which leads to blood vessel damage and organ failure in septic-patients, often leading to death.

Current sepsis treatment is a very long process. Common treatments are IV fluids, antibiotics, and other medications that require an average hospital stay of approximately three weeks.[1] There is a need for an external device that uses hemoperfusion to bind to target molecules (*i.e.* endotoxin), thereby removing them efficiently from the bloodstream without having to add additional drug molecules to the blood. It is possible that cationic amphiphilic peptides (CAPs) can be used to selectively bind blood pathogens during a hemoperfusion process. Previous studies with hemoperfusion have shown significant depletion of platelets and white blood cells.[3,4] Peptides with high affinity for target molecules can be anchored to a surface by a polymer chain and adsorb endotoxin effectively. A schematic of such a device is provided in Figure 1. Blood

travels from the vein of a septic patient to a microfluidic device, contaminated blood enters an array of capillary numerous capillary tubes coated with immobilized polyethylene oxide (PEO) chains to which are tethered with CAPs, endotoxin is bound by active CAPs, and "clean" blood re-enters the body.

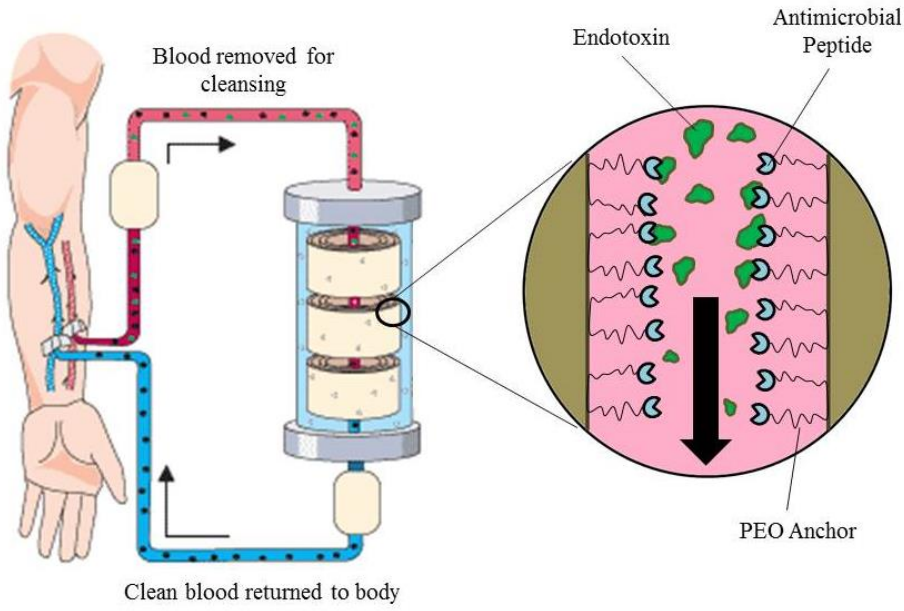


Fig. 1. Schematic of future hemoperfusion device to filter endotoxin from blood of sepsis patients, adapted from (Kurz 2013).[5] Note: Antimicrobial peptides in schematic do not show accurate helical structure.

Liposomes and LPS

A liposome is an artificially created vesicle comprised of a lipid bilayer (*see Fig. 2B*). Liposomes are commonly used for drug delivery and can be used to mimic cells that contain the same lipid membrane components. Phospholipids such as dimyristoyl-phosphatidylcholine (DMPC) and dimyristoylphosphatidylglycerol (DMPG) are commonly used to form unilamellar vesicles that mimic eukaryotic and prokaryotic cells. Lipopolysaccharides (LPS) are characteristic components of cell walls in Gram-negative bacteria, forming vesicles when placed in solution (Fig. 2). LPS is made of Lipid A

(most conserved component among species), a central core (less conserved), and an O-antigen (least conserved). LPS vesicles can be formed by disruption of biological membranes (e.g. lysis or sonication) and allowing individuals molecules to spontaneously assemble vesicles, even at low concentrations. LPS is a pyrogenic compound that can induce fever, and should be handled carefully in the laboratory setting.

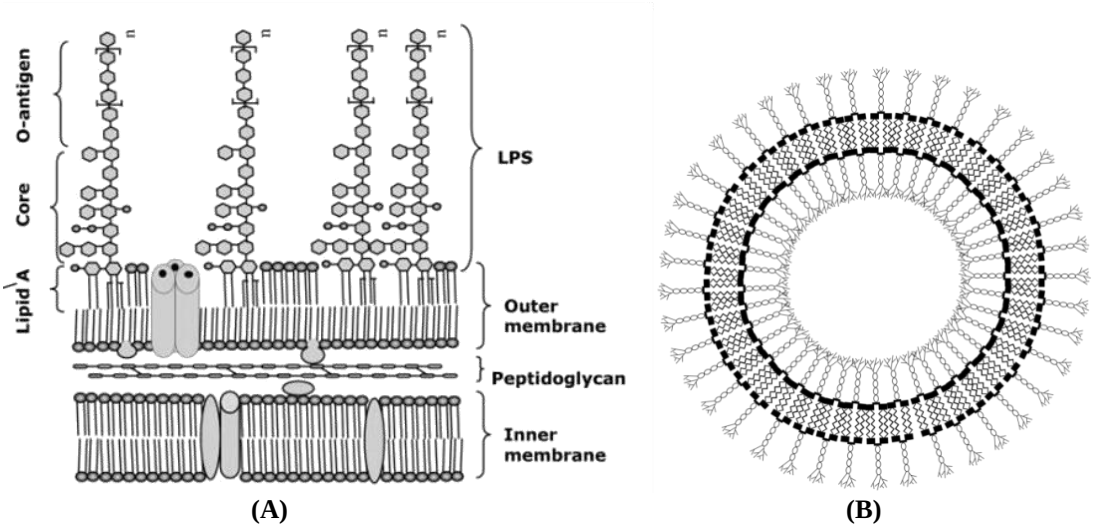
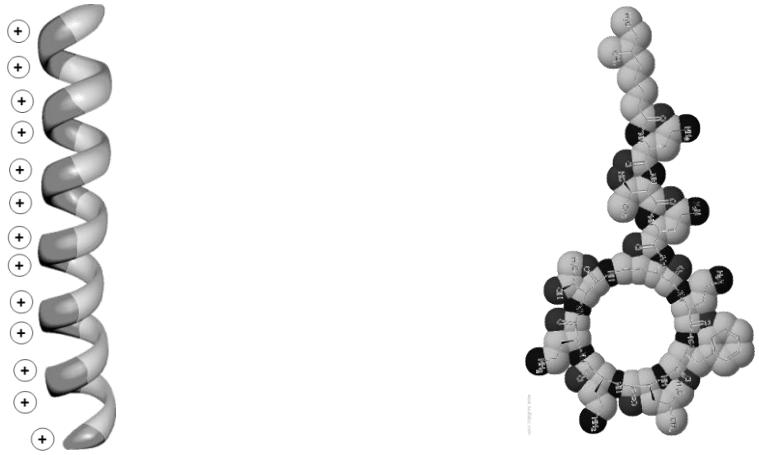


Fig. 2. (A) LPS (attached to outer membrane of Gram-negative bacteria) consisting of Lipid A, core, and O-antigen. Free LPS molecules will self-assemble and form **(B)** LPS vesicle.

Cationic Amphiphilic Peptides and WLBU2

A cationic amphiphilic peptide (CAP) is a peptide with positive charges and hydrophobic side-chains on opposite sides of an alpha-helix, which bind and disrupt negatively-charged bacterial membranes. This physical, non-metabolic mechanism kills pathogens has limited effect on antibiotic resistance.[6] WLBU2 (Fig. 3) is an engineered, amphipathic CAP that tightly binds to LPS in a way that leaves vesicles largely intact, in contrast to polymyxin B (a common antimicrobial peptide that is hereby referred to as PMB) which has shown to disrupt vesicles[7] (Fig. 3). Immobilized PMB has been used in

Toraymyxin columns to treat septic patients in Japan and Eastern Europe, yet PMB has also been known to disrupt endotoxin membranes and release endotoxin fragments into the bloodstream. PMB is also neurotoxic and nephrotoxic, meaning it can only be used in external applications. The WLBU2 sequence has been engineered for better antimicrobial activity with greater helicity and tryptophan content. The peptide has been shown to bind and kill *Staphylococcus aureus* and *Pseudomonas aeruginosa* in human serum without damaging mammalian cells.[8] These attributes make WLBU2 promising to replace PMB for blood cleansing applications.



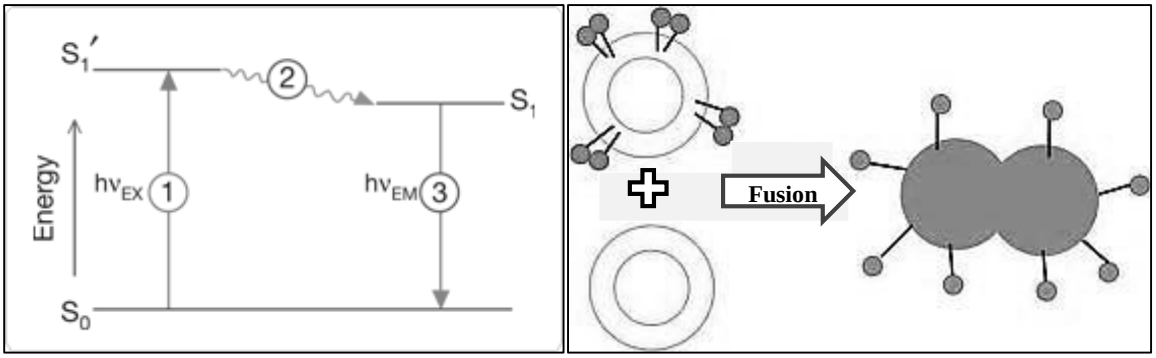
(A) **(B)**

Fig. 3. (A) Helical form of WLBU2 shown with charges and **(B)** polymyxin B.

Mechanism of Fluorescence Quenching

Fluorescence of a molecule can be summarized in three steps (Fig. 4). First, a high-energy photon of light excites a singlet state electron. Then, by several possible molecular interactions, energy is partially dissipated and yields a relaxed singlet state. Finally, a photon (with energy equal to the difference between the relaxed singlet and

ground state) is emitted, causing fluorescence. This is different than phosphorescence, which is a much slower process.



(A) **(B)**

Fig. 4. (A) Electronic-state illustration of fluorescence[9], caused by (1) excitation of an electron to singlet state (S_1'), (2) excited-state lifetime to relaxed singlet excited state (S_1), and (3) fluorescence emission of energy $h\nu_{EM}$. **(B)** Self-quenching occurs when an excited fluorophore interacts with one in the ground state.[10]

Self-quenching occurs when a fluorophore quenches another fluorophore, leading to a loss of fluorescence signal. Self-quenching occurs at high molecule concentrations, because fluorophores need to be physically close to each other for resonant energy transfer and release of photons. This is useful because self-quenched dye that leaks out of vesicles will be diluted and fluorophores will be able to release lower-energy photons.

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Calcein Leakage Assay

Calcein (Fig. 5) is water-soluble and membrane-impermeable[11,12,13] and is self-quenched at concentrations of 60-100 mM.[14] Calcein is very pH sensitive, because there are 4 functional groups that can donate protons to solution (circled on Fig. 5A). These functional groups are important because they ionize at higher pH, preventing calcein molecules from indiscriminately traveling across the nonpolar membrane. Calcein is an example of a fluorescent probe that can self-quench, and its excitation and emission spectra is given in Figure 5. The observed right-shift is known as the Stokes shift.

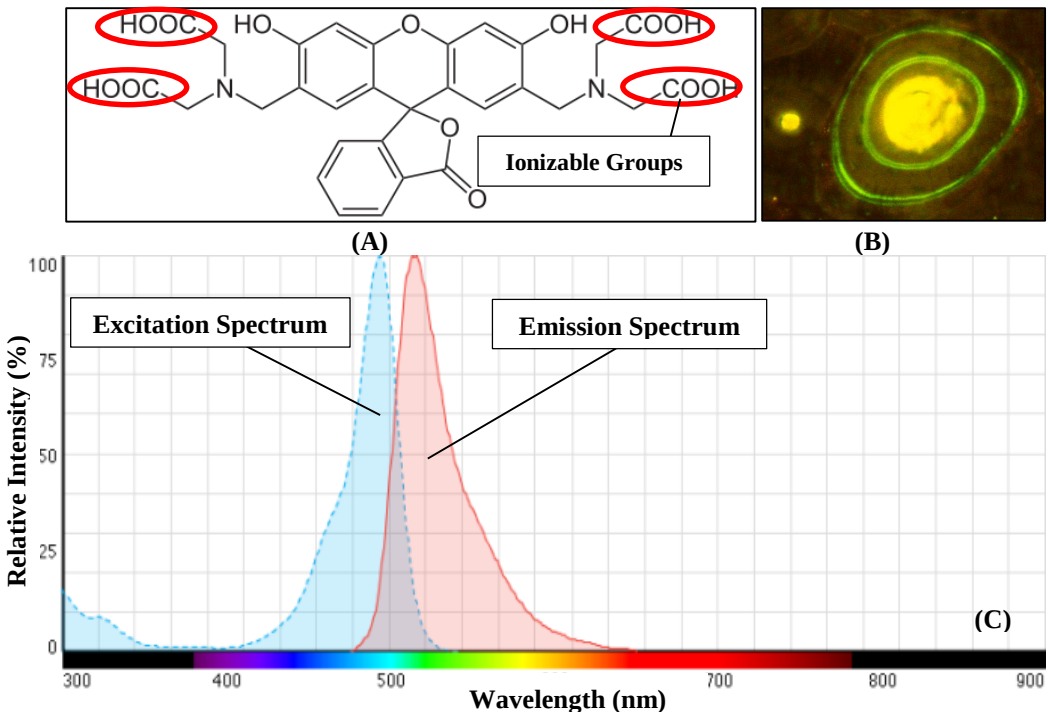


Fig. 5. **(A)** Protonated calcein molecule. **(B)** Calcein dye binds to actively mineralizing tissues, including new bone matrix formation in osteons.[15] **(C)** Excitation and emission spectra for calcein. Highest intensity response occurs when excited at 495 nm and emission is read at 515 nm.[16]

Calcein leakage is a technique used to investigate vesicle membrane permeabilization.[11] This method measures fluorescence change of calcein-loaded vesicles as encapsulated calcein leaks to external buffer. This relatively simple technique is used to specifically investigate membrane dynamics because it uses vesicles instead of live cells. This simplifies analysis because there is no interference with cellular components of live cells.[17]

If vesicle membranes are permeabilized, calcein molecules leak out of vesicles, become diluted by the extra-vesicular buffer (EXB), and fluorophores are no longer quenched by neighbouring fluorophores. Calcein fluoresces at a bright green color (515 nm emission). The extent of calcein quenching is then compared to a positive control that represents 100% leakage, typically marked by the addition of a detergent like Triton X-

100 (1-10% v/v) to solubilize the lipid membrane.[18,19,20] Lorin *et. al.* (2011) used a calcein leakage assay to characterize membrane integrity of DMPC and DMPG (vesicles mimicking eukaryotic and prokaryotic cells) in the presence of synthetic amphiphilic peptides.[18] Membrane studies with LPS vesicles often use Tris EXB, pH 7.4-8.5, with 100 mM salt.[17,19,21,22] Size-exclusion chromatography with Sephadex resin (G50 or G75) is commonly done to isolate vesicles from nonencapsulated dye.[18,19,22,23] Duzgunes (2004) used dialysis (4ºC; 3 changes of 4.0 L EXB) to remove free calcein from dye-loaded vesicles.[24]

Factors affecting Membrane Permeability of LPS vesicles

Vesicle size, incubation time, and incubation temperature are factors that affect liposome permeability. Small unilamellar vesicles (SUVs, ~50 nm diameter) are an order-of-magnitude more permeable than large unilamellar vesicles (LUVs, ~1000 nm diameter).[25] An incubation time of 24 hours at 37ºC causes leakage 4 times greater than incubation for 2 hours.[25] The permeability of vesicles (100 nm diameter) at 50ºC is nearly one order-of-magnitude larger than at 25ºC.[25]

The effect of pH on membrane permeability is significant. A low pH environment can prevent vesicles from forming because calcein will be fully protonated and non-ionic, preventing dye enclosure inside vesicles. Also, low pH may affect stability of membrane lipids. Duzgunes (2004) performed a leakage assay with calcein-containing liposomes held in buffers of various acidities, finding that significant leakage occurs even at pH 5.[24] Buffer solutions should have a pH of at least 7.4.[17,19,21,22,25]

Statement of Purpose

There is a need to understand the fate of entrapped LPS in the presence of antimicrobial peptides, namely WLBU2. If endotoxin is released or ruptured, the problem must be identified and managed. This project aims to develop a simple (low cost, minimal equipment usage) and effective membrane-leakage assay to quantitatively measure endotoxin (LPS) membrane permeabilization in the presence of antimicrobial peptides (AMPs). Previous experiments have shown WLBU2 to bind to LPS and induce minimal vesicle permeabilization. It is hypothesized that calcein fluorescence quenching can be used to determine membrane permeability of vesicles, and that the addition of WLBU2 will confirm prior experiments by inducing minimal membrane permeabilization. This will help determine if endotoxin "shedding" may be an issue in a target sepsis-treatment device using WLBU2 or other AMPs.

Materials and Methods

Methods were adapted from the protocol outlined by Chongsiriwatana and Barron (2010). Self-quenched calcein (60 mM) was encapsulated in LPS vesicles. Dialysis (10,000 MWCO) was used to isolate vesicles from un-encapsulated dye and fluorescence change due to vesicle permeabilization was measured for vesicles exposed to WLUB2, PMB, Triton X-100 surfactant (positive control), and buffer (negative control).

I. Preparation of Assay Components

Preparation of Self-Quenched Calcein Dye

Calcein (C0875-5G, Lot # SLBH2476V) was purchased from Sigma-Aldrich (St. Louis, MO). A 50 mL beaker was filled with 8.0 mL RO water and set on a plate with stirring. Calcein (0.436 g) and 0.0182 g Tris-HCl (Sigma, Trizma T-1535, Lot #97H5426) was added; dark-orange powder remained undissolved. Aqueous NaOH (1N) was added in 250 µL aliquots to reach a pH of 7.4. Solution pH was monitored constantly with a digital pH analyzer (Corning 350), and pH strips (Precision Labs, Cottonwood AZ) to indicate pH at low volumes. A final pH of 7.4 was reached, and dye solution was brought to 15 mL. Solution color and solubility changed upon addition of base (Fig. 9).

Preparation of Extravesicular Buffer (EXB)

A clean 1.0 L beaker was filled with 900 mL RO water, and set on a plate with stirring. To this was added 1.212 g Tris-HCl (Sigma, Trizma T-1535, Lot #97H5426) and 5.84 g NaCl (JT Baker, Lot # 050536). Solutes were dissolved in solution and pH was adjusted to 7.4 by dropwise addition of NaOH. Approximately 1 mL NaOH (1 N) was

needed per liter EXB to reach a pH of 7.4. Water was added to bring solution to 1.0 L. Tris EXB (6.0 L) was prepared and stored in a 2-gallon Nalgene reservoir.

Dye-Loading of LPS Vesicles

Purified lipopolysaccharide (Lot # 9143) from *Pseudomonas aeruginosa* was purchased from Sigma-Aldrich (St. Louis, MO) and prepared at 10 mg/mL in HPLC water. LPS solution, stored at 4°C, was removed from the refrigerator and transferred (10-200 µL) to concentrated calcein (0.5-2.0 mL), forming a final LPS concentration of 0.1-1 mg/mL. Pre-formed LPS vesicles were ruptured by vortexing (30 seconds) and sonication (15 minutes). The solution was left to stand still for 5-10 minutes, allowing vesicles to re-form around concentrated calcein.

Preparation of Triton X-100 Solution

Ten microliters of Triton X-100 (FisherBiotech, Lot # 033078) was combined with 990 µL RO water in a microfuge tube, forming 1% Triton X-100 (v/v). Solution was shaken for 20 seconds.

Preparation of WLBU2 and PMB

Lyophilized WLBU2 was purchased from Genscript (Piscataway, NJ) and combined with HPLC-grade water and deuterated water (D_2O) to a final stock concentration 5 mg/mL. One-hundred microliters of WLBU2 were prepared in Tris EXB at 50 µM. Stock PBS (545 µM) was removed from the freezer, thawed, and prepared at 50 µM (1.5 mL total) in Tris EXB.

I. Purification of Vesicles

Centrifugation, dialysis, and size-exclusion chromatography were used in attempt to separate dye-loaded LPS vesicles from concentrated, unencapsulated calcein dye.

Isolation by Centrifugation

Calcein (30 mM) was dissolved in HEPES EXB. LPS (100 µL) was combined with 0.90 mL dye in a 15 mL Falcon® tube, and sonicated for 20 minutes. This sample was transferred to a centrifuge tube and spun in a benchtop centrifuge (Eppendorf 5415) for 10 min at 13,000 rpm, and then for an additional 20 min. Centrifugation also was done with a 1.0 mL sample of LPS (0.1 mg/mL) in brown calcein dye (60 mM) at pH 7.4.

Isolation by Size-Exclusion Chromatography

Approximately 4 mL Sephadex G-10 resin (Sigma, Lot # 074K1033, MWCO 700 Da) was hydrated in 6 mL Tris EXB. A 9" Pasteur Pipet (VWR, No. 14673-043) was used as a chromatography column. The narrow end of the glass pipette was cut, leaving a tapered opening at the bottom of the column (approximately 4 inches in length). Sterile cotton was placed at the column base. A twist-tie was wrapped around the indented ring at the top of the column to provide grip and prevent it from slipping downward between the ring-clamp fingers. The swollen resin was carefully added to the column at a slanted angle to prevent air bubble entrapment. The column was packed to above the twist-tie line. Cotton was packed tightly above resin (to prevent resin upflow and provide a distributor for injected sample). Appendix C shows a photograph of the system setup. The column was washed with 2 bed volumes EXB, and effluent collected in a waste

beaker. Twenty-four clean microfuge tubes were placed on a sample collection rack. Once the buffer line was just above the top cotton wad, 100 µL sample (1 mg/mL LPS, 60 mM calcein, 10 mM Tris-HCl) was injected to the top of the column and allowed to drain into the bed. The plastic tube, one end attached 2' above the column to provide hydrostatic pressure for the flow, was connected to the top of the column and EXB added dropwise to the top end of the tube. Care was taken to prevent air bubble entrapment in the plastic tube. Sample collection took place keeping a constant fluid pressure head (approximately 3 cm of fluid above the top cotton wad). Aliquots were collected every 4 drops. Methods for chromatography in a plastic column are provided in Appendix I.

Isolation by Dialysis

10,000 MWCO: Fifty microliters of LPS were combined with 450 µL calcein dye (60 mM calcein, 10 mM Tris-HCl) in a 15 mL Falcon® tube, vortexed for 30 seconds on a VWR LabDancer, and sonicated for 15 min (VWR - Model 50D). The vortex and sonication steps were done to rupture previously-formed LPS vesicles and allow vesicles to re-form around concentrated dye. The solution was left to stand for 5 minutes to ensure that vesicles had re-formed.

Approximately 1.5 L Tris EXB was transferred to a large 3.5 L beaker. A Slide-A-Lyzer 10K dialysis cassette (10,000 MWCO, 0.1-0.5 mL, Pierce # 66415) was inserted into the groove of a foam float, with cassette port #1above the surface of the water.[27] The float and cassette were placed in the EXB solution for 2 minutes to allow the dialysis membrane to hydrate. A 1.0 mL syringe (BD, connected to a BD 18G1 PrecisionGlide Needle) was inserted into the 15 mL Falcon® tube containing LPS solution, and was filled

with 0.5 mL solution. LPS solution was transferred through port #1 of the dialysis cassette, taking care not to penetrate the dialysis membrane with the tip of the inserted needle. Excess air was removed by the syringe, and the dialysis cassette was placed back into the groove of the foam float. Port #1 was in the groove of the foam to prevent leakage through the inlet port). The float and cassette were placed in the 3.5 L beaker and set on a stir plate with constant stirring. Photographs of dialysis setup and process is shown in Figure 6.

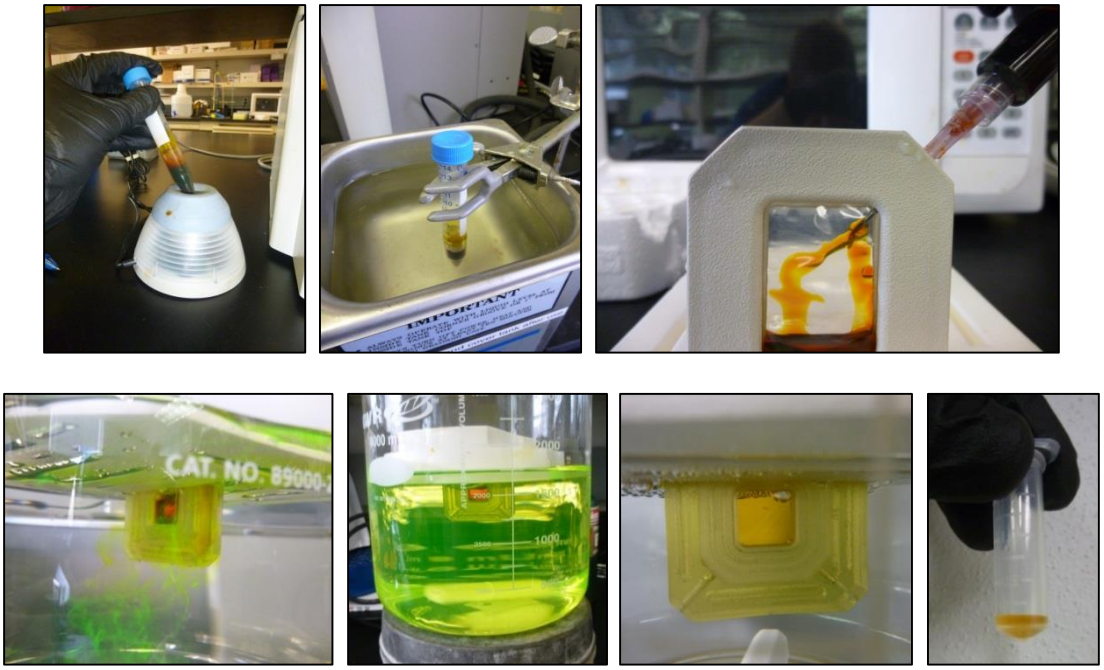


Fig. 6. (Left to right from top left). Vortexing of LPS and calcein dye; sonication; loading of vesicles and un-encapsulated dye in dialysis cassette; un-encapsulated dye escaping dialysis bag; external buffer turns green with time; vesicles were completely isolated when EXB remained clear; final LPS vesicles.

Buffer was exchanged for fresh EXB (1.5 L) after 2 hours, then again after 4 hours, then after 8 hours, and then after 20 hours. A total of four buffer changes were done until external buffer in the 3.5 L beaker remained clear. Purified sample was removed from the dialysis cassette via syringe and transferred to a microfuge tube. A 96-

well plate was filled with duplicates of [30 µL vesicles:110 µL EXB], [10 µL vesicles:130 µL EXB], and [50 µL vesicles] (6 wells total). Samples were tested for initial fluorescence and absorbance. Ten µL EXB and 10 µL Triton X-100 (1.0% v/v) were added to one well for each dilution, and the sample was pipetted 4 times up and down, gently. The plate was read for $F_{485/535}$ immediately following addition of Triton/EXB, 10 minutes after addition, and 10 hours after the addition (plate was stored at 4^{o}C overnight and read after 15 minutes of acclimation to room temperature). Results are provided in Figure 11. The procedure was repeated with 2,000 MWCO cassettes (methods provided in Appendix A), though unencapsulated dye was never fully removed even after 10 days.

II. Analytical Methods

Addition of Peptides to Isolated Vesicles

Calcein-loaded vesicles were added to 12 wells in a 96-well plate (140 µL vesicles each), and initial fluorescence was measured. WLBU2 (10 µL) was added to 4 wells, PMB (10 µL) was added to 4 wells, and 10 µL Tris extracellular buffer (EXB) was added to 4 wells. Each aliquot was pipetted up and down four times. Triton X-100 (10 µL) was added to each aliquot 30 minutes after the addition of peptide. Fluorescence measurements were made prior to peptide addition, 30 minutes after peptide addition, and 10 hours after Triton X-100 addition.

Fluorescence ($F_{485/535}$) and Absorbance Measurement

Fluorescence and absorbance were measured with a PerkinElmer Victor^{3}V 1420 Multi-label Counter (Gleeson Hall, Room 202) using a stored protocol (entitled "Bearded Anthony", Fluorescein 485/535) for excitation at 485 nm and emission at 535 nm. Data files were saved to a USB drive. Absorbance was measured with the protocol for photometry at 490 nm (excitation wavelength).

Limulus amoebocyte lysate test

A *Limulus* amoebocyte lysate (LAL) test makes use of an aqueous extract of amoebocytes from the horseshoe crab (*Limulus polyphemus*) to detect endotoxin present in a sample of interest. LAL reacts with bacterial endotoxin or LPS present in a sample, causing a clotting cascade to occur, and turbidity increases within the sample vial.[28] This reaction can be monitored through continuous absorbance measurement.

A kinetic and quantitative LAL assay was used to detect and quantify the Gram-negative bacterial endotoxin (LPS) in collected chromatography aliquots. A Lonza PYROGENT™-5000 Assay was used. General methods followed standard protocol provided in the assay kit.[29] A total of 5.0 mL LAL was available in the lab, meaning fifty 100-µL samples could be tested. A total of 48 samples were run. LAL was extracted from the freezer and re-suspended in Lonza re-suspension buffer.[29] One-hundred microliters of LPS (1 mg/mL) in 25 mM calcein (60 mM HEPES, pH 1.5) was loaded to the top of a plastic column (10 cm length, 1 cm ID, ~5 mL Bio-Rad P60 gel swollen in HEPES buffer), and flowed at 0.030 mL/min by syringe pump (Harvard Apparatus, PhD 2000). Chromatography aliquots were collected every 4 drops. Each aliquot was diluted 100X

by combining 10 µL of eluted aliquot with 990 µL HEPES EXB in a 2.0 mL microfuge tube, and gently pipetted up and down 4 times. One-hundred microliters of each diluted aliquot was transferred to a 96-well plate (Fig. 7) in duplicate. Then, 100 µL Lonza PYROGENT™-5000 LAL was transferred simultaneously to all sample wells using a 12-tip transfer pipette. The plate was read in a PerkinElmer Victor^{3}V 1420 plate reader (37^{o}C incubation, set to kinetic assay measuring absorbance at 340 nm every 30 seconds, 1 hour in duration). The kinetic assay measured turbidity change over time, representative of endotoxin concentration. A standard curve[29] was developed (Fig 18) to determine endotoxin concentration (Fig 19).

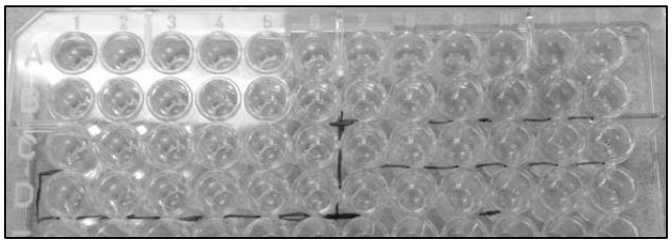


Fig. 7. Plate loading scheme following the addition of LAL. The top two rows are the same diluted aliquots from the chromatography column (as seen by color shade transition).

Results and Discussion

Formation of Self-Quenched Calcein

A cloudy precipitate formed in 30 mM calcein dye (Fig. 8) following heating (in the microwave and 10 minutes in a 40°C bath). The solution formed into a gel after cooling to room temperature for an hour. Calcein cannot be heated to effectively increase solubility. Instead, base (e.g. NaOH) should be added to increase proton acceptors and solubility, allowing calcein to reach self-quenched concentrations.

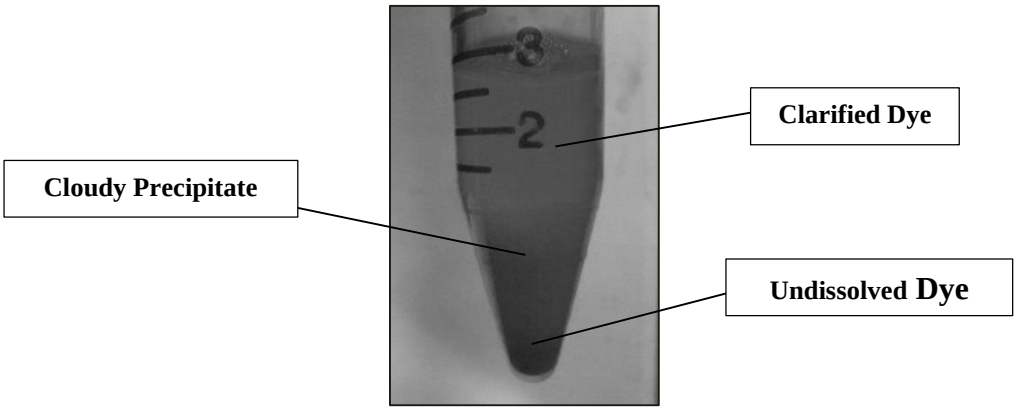
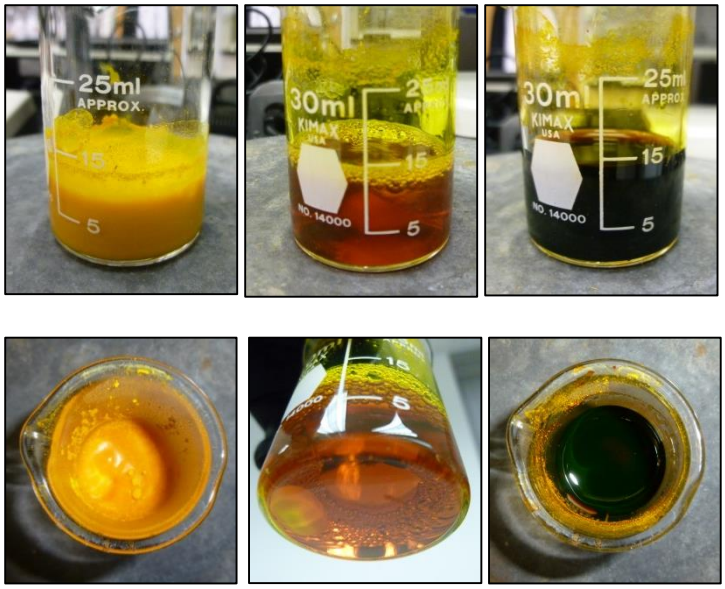


Fig. 8. Formation of precipitate upon heating of calcein (30 mM, no NaOH added). Both microwave and heat bath methods were used in attempt to help dissolve calcein.

Calcein was successfully dissolved at a self-quenching concentration of 60 mM, following the addition of NaOH. Notable color change was observed as solution pH increased (Fig. 9). This calcein dye stock solution was stored and used for dye-loading experiments.



(A) **(B)** **(C)**

Fig. 9. Observed color change and transparency change of calcein dissolved in 10 mM Tris-HCl. **(A)** Calcein at pH 1.5, prior to the addition of NaOH. Significant calcein powder remained undissolved in the opaque orange solution. **(B)** Calcein at pH 6.0, following the addition of approximately 3 mL NaOH (1 N). Solution suddenly turned semi-transparent and dark orange at this point. **(C)** Calcein (15 mL) at pH 7.4, after the addition of approximtely 4 mL NaOH (total). Calcein became dark and less transparent than (B).

Centrifugation

Centrifugation with 1.0 mg/mL LPS and 30 mM calcein (in 60 mM HEPES, 5 mM EDTA, and 150 mM NaCl, pH ~1.5) produced a visible pellet (Fig. 10).

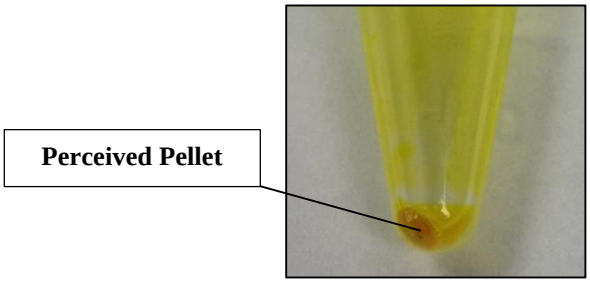


Fig. 10. Visible pellet formed after centrifugation of 1.5 mL LPS (1.0 mg/mL) in 30 mM calcein (in 60 mM HEPES, 5mM EDTA, 150 mM NaCl).

The pellet was resuspended in fresh HEPES buffer, and spun at 13,000 rpm for 30 minutes. The pellet never reformed. Fluorescence increase was observed at various

dilutions of the supernatant (Fig. 11). Due to the fact that calcein is very acidic and insoluble at pH 1.5, it is likely that the visible pellet was simply suspended dye that had not fully dissolved.

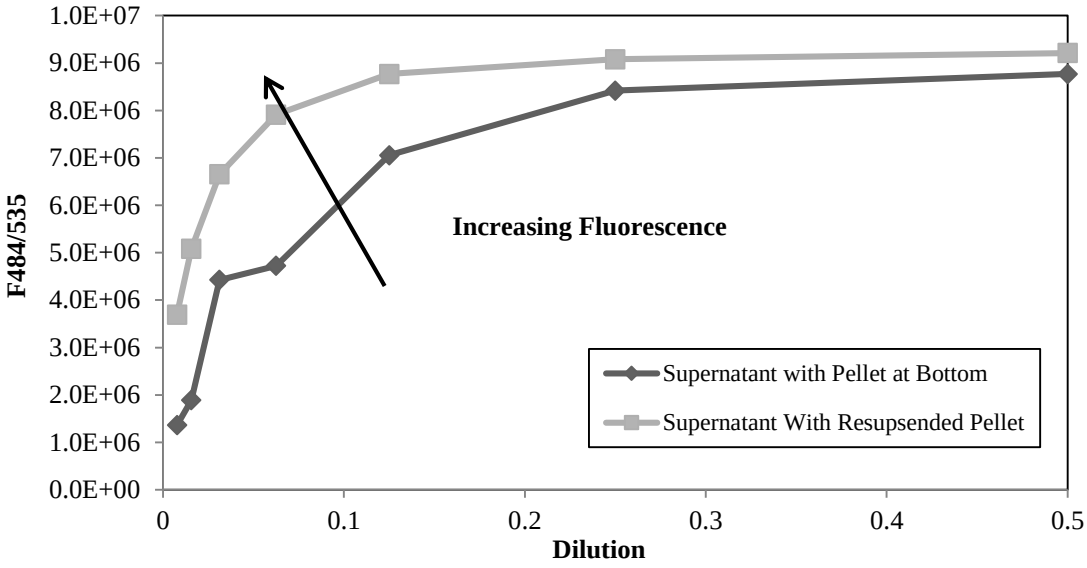


Fig. 11. Fluorescence intensity vs. dilution factor of the supernatant with the original pellet and with the pellet resuspended in solution. Dilution was necessary to unsaturated the fluorescence detector. *Example: 0.25 means ¼ of original concentration.*

Figure 11 shows that the fluorescence detector is saturated above 6.0E+06. Also, centrifugation with LPS (0.1 mg/mL) in pH 7.4 calcein (30 mM) produced no visible pellet. The forces associated with a centrifuge may cause vesicle rupture, similar to the vesicle rupture induced by the vortex and sonicator. Centrifugation was unsuccessful in isolating vesicles, and size-exclusion chromatography was the next attempted method.

Size Exclusion Chromatography

A glass column was loaded with 100 µL sample (1 mg/mL LPS, 60 mM calcein, 10 mM Tris-HCl), aliquots were collected every 4 drops (Appendix III), and fluorescence (485/535 nm) and absorbance (540 nm, 0.1 sec) were measured on aliquot dilutions.

Absorbance was measured at 540 nm (emission wavelength) instead of at the calcein excitation wavelength (485 nm) before it was suggested to measure absorbance at 485 nm. Bubbles, from the added surfactant, were observed to form in the samples that had added Triton X-100. Figure 12 displays A_{540} 10 minutes after addition of Triton or EXB, and A_{540} after 5 days of storage at 4°C. Fluorescence did not change considerably between addition of EXB and Triton X-100.

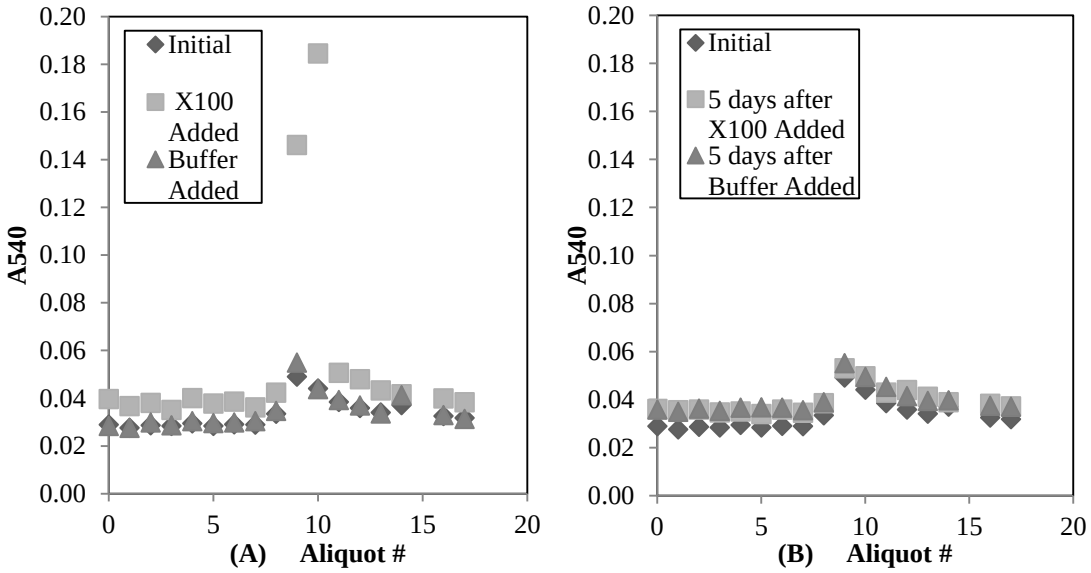


Fig. 12. Absorbance at 540 nm (emission wavelength) for chromatography aliquots of LPS (1 mg/mL) and calcein dye (pH 1.5) for **(A)** 10 min after addition of EXB or X100 (Triton X-100) and **(B)** 5 days after addition of EXB or X100.

No visible color changes were observed upon addition of Triton X-100, implying that the absorbance spike in aliquots 9 and 10 (Fig. 12A) after addition of detergent was a spurious. Bubbles were observed to form in aliquots combined with surfactant. Five days later, no bubbles were present in the wells, and the absorbance readings were significantly lower in aliquots 9 and 10.

Figures 13 and 14 were created from chromatography aliquots with LPS vesicles (1 mg/mL) in calcein dye (pH 7.4, dissolved in Tris EXB) following the addition of Triton

X-100 and EXB. Photographs show the elution color profile, matching each aliquot number on the x-axis. In Figure 13, samples were 100 µL and non-diluted when read in the wells of the plate reader.

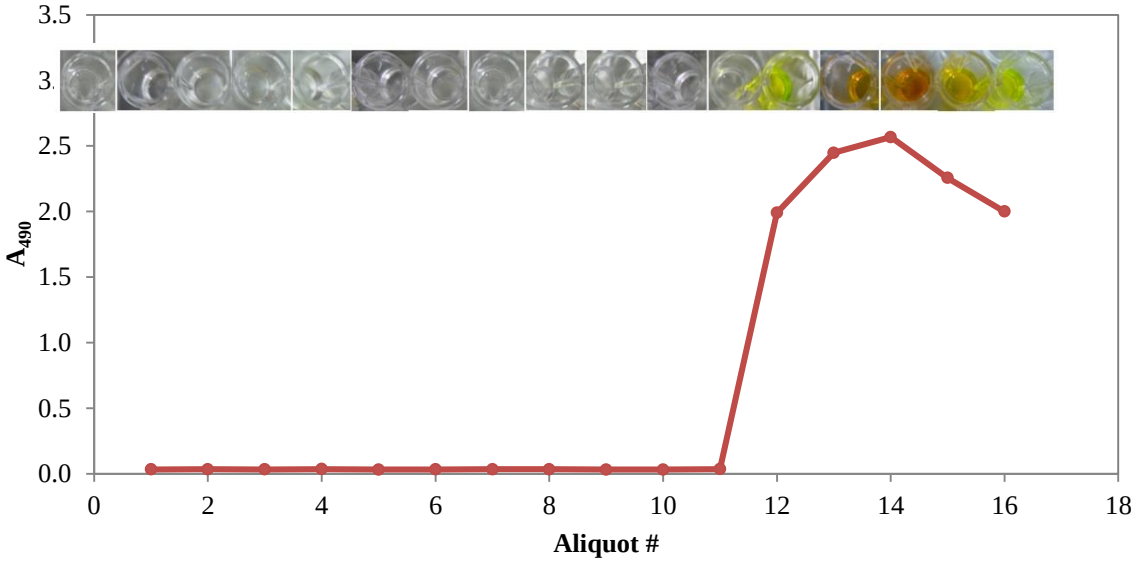


Fig. 13. Absorbance (490 nm) for eluted chromatography aliquots following the addition of Triton X-100 (10 µL). Aliquot photographs match the x-axis. Samples were 100 µL, non-diluted, done in duplicate.

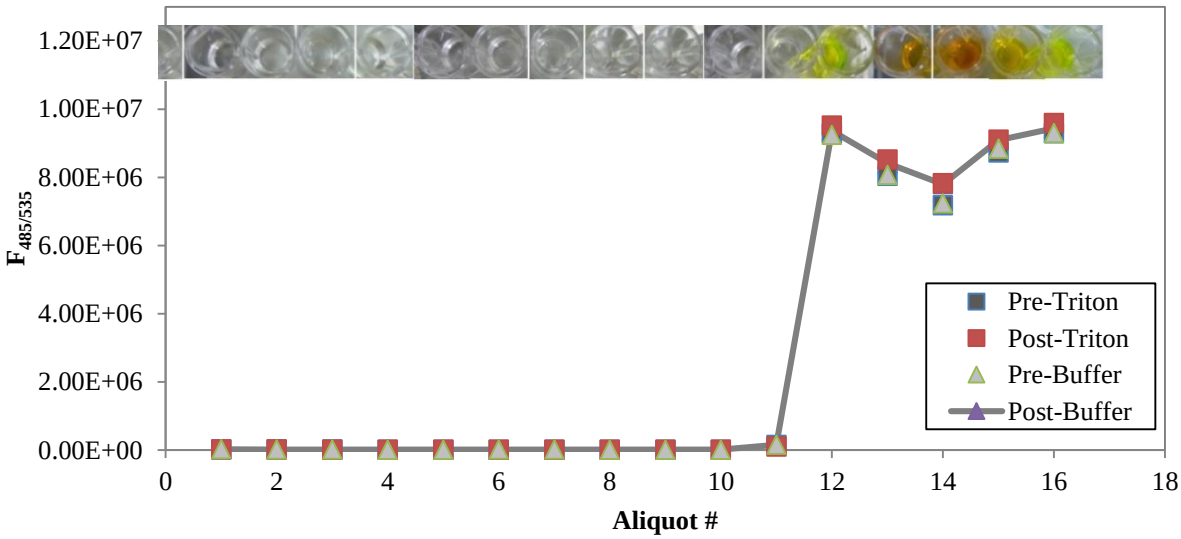


Fig. 14. Fluorescence (excitation 485 nm, emission 535 nm). Samples were read prior to addition of 10 µL Triton X-100 (1% v/v) or EXB, and 20 minutes following the addition of Triton/EXB.

Figure 13 suggests that absorbance is indicative of sample color, and shows that the plastic column was unsuccessful in separating vesicles from un-encapsulated dye.

Dialysis

Dialysis was successful in isolating calcein-loaded vesicles (see physical set-up and annotated photographs in Appendix III). Several qualitative factors confirmed the isolation of dye-loaded vesicles from un-encapsulated dye. First dark-orange dye remained in the dialysis membrane despite clear EXB. This indicates that dye molecules (approximately 623 Da) entrapped in vesicles were unable to exit the dialysis membrane while free dye transferred to dialysate during purification, despite a very large concentration gradient. Second, the color of the remaining dialysis sample was "dirty brown", as described by Chongsiriwatana & Barron (2010).[17] Third, upon the addition of EXB to dilute vesicle samples, the sample did not turn fluorescent green, as it would if free dye was present. In previous samples, un-encapsulated self-quenched calcein (orange-brown color) became green when diluted to less-quenched concentrations. The percent increase in $F_{485/535}$ can be seen in Fig. 15, and percent change in A_{490} readings in Fig. 16. Concentrated vesicles (no dilution) started with only 50 µL vesicles per well, whereas dilutions held 150 µL per well. Percent increase was found by

$$\%\ Increase = 100\left(\frac{F_t - F_{initial}}{F_{initial}}\right),$$

where F_t is fluorescence intensity at a given time, and F_{inital} is fluorescence prior to addition of surfactant or EXB. Raw data is provided in Appendix II.

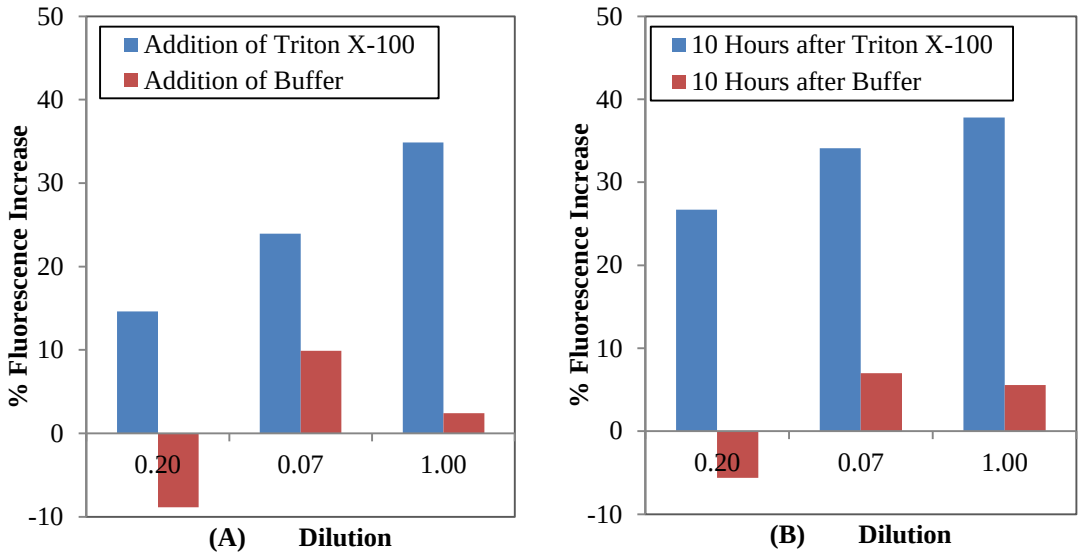


Fig. 15. Percent increase $F_{485/535}$ upon the addition of Triton X-100 and EXB when read **(A)** 10 minutes after addition and **(B)** 10 hours after initial addition and refrigeration overnight.

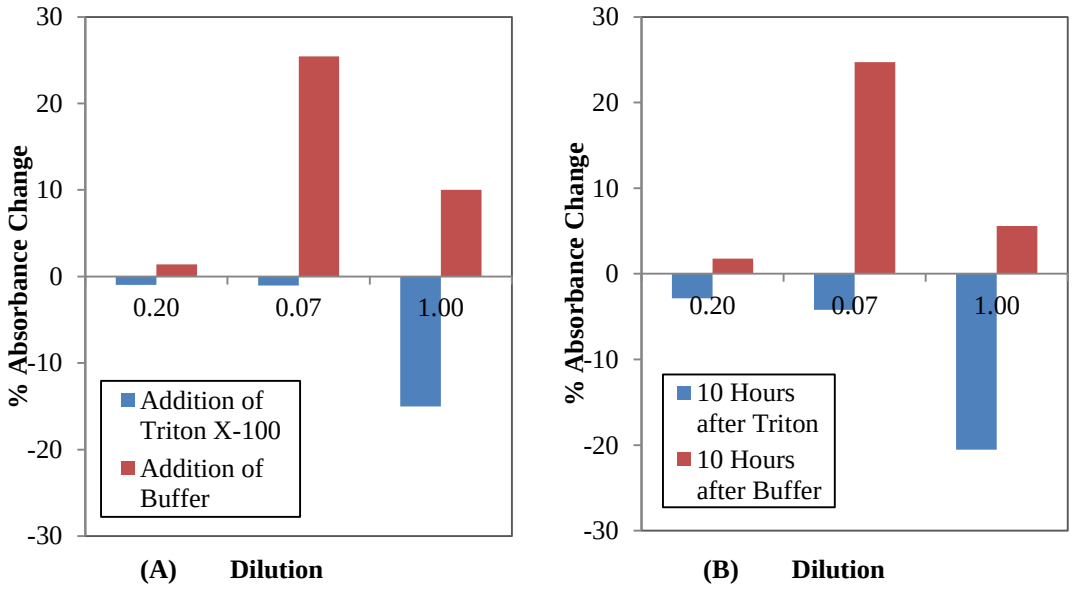


Fig. 16. Percent change in absorbance (at 490 nm) in dye-loaded vesicle upon the addition of 10 µL of X-100 or EXB **(A)** 10 min after addition and **(B)** 10 hours after addition.

Figure 15 shows a change in fluorescence intensity, supporting the claim that vesicles were isolated. From Figure 16, it appears that absorbance changes positively for added buffer, and negatively when vesicles are broken with detergent. Fluorescence

increased slightly more after 10 hours of storage at 4ºC (covered with Parafilm wax and aluminium foil). It appears that cold storage of vesicles with Parafilm wax and aluminum foil cover has minimal effect on absorbance readings, but longer time in presence of a surfactant or similar agent will promote more vesicle rupture[25] and fluorescence, to a certain extent, will continue to increase.

Addition of Peptides

Fluorescence intensity was measured upon the addition of peptides WLBU2 and polymyxin B (Fig. 17). Fluorescence measurements were made prior to peptide addition, 30 minutes after peptide addition, and 10 hours after Triton X-100 addition.

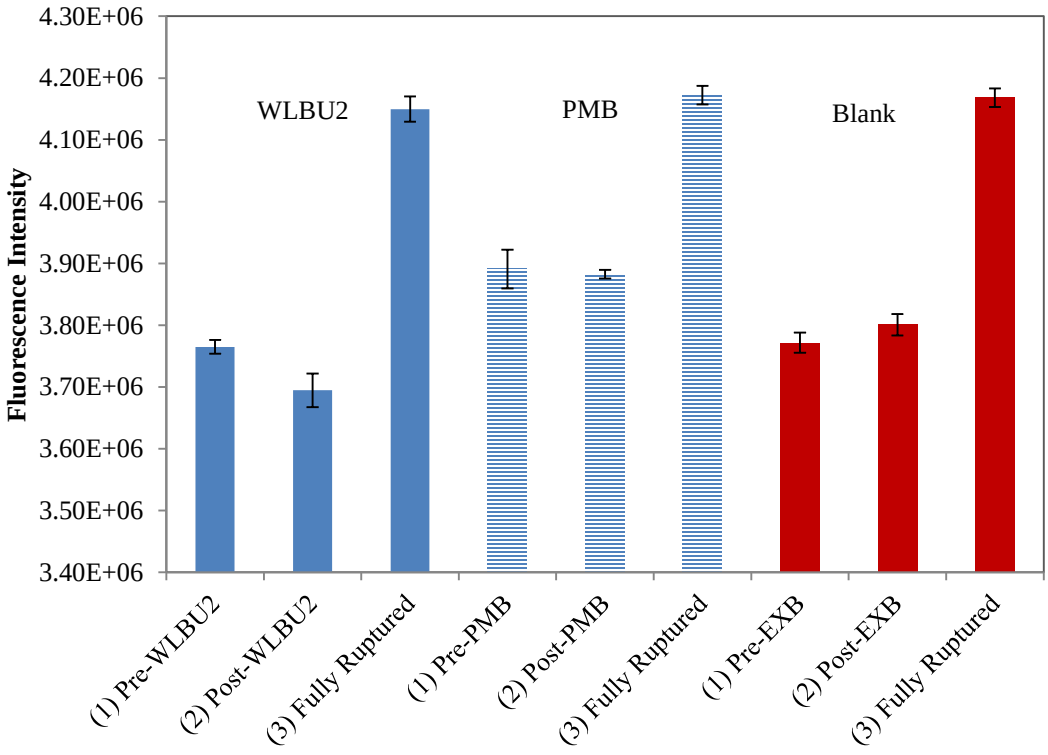


Fig. 17. Fluorescence intensity for LPS vesicles (1) prior to peptide addition, (2) following peptide addition, and (3) following addition of Triton X-100. Error bars represent standard deviation (N=4).

Figure 17 shows that the positive control (Triton X-100) worked, causing permeabilization of vesicles to an assay endpoint (approximately $4.15x10^6$ units). It appears that some vesicles exhibited unprecedented leakage or that un-encapsulated dye was not completely removed, for when buffer was added (negative control, blank) to vesicles, fluorescence increased. This is likely because free calcein molecules became more dilute upon the addition of blank buffer, and adjacent fluorophores did not quench each other. Also, Figure 17 shows an apparent decrease in fluorescence upon the addition of WLBU2. This may be due to molecular interference caused by WLBU2 with free dye or with other buffer components (e.g. NaCl or Tris).

Future trials with calcein at higher-self quenched concentrations (100 mM) can be done to show more definite fluorescence upon the leakage of vesicle membranes.

Effect of Concentration and Time on Unencapsulated Calcein

Chromatography run was done on the plastic column with 25 mM calcein dye (no LPS). Collected samples were diluted 1/16 and 1/128, and fluorescence was measured (Fig. 18). The plate was stored at room temperature for 7 days, covered with foil. The sample was re-run and compared with initial readings (Fig 19).

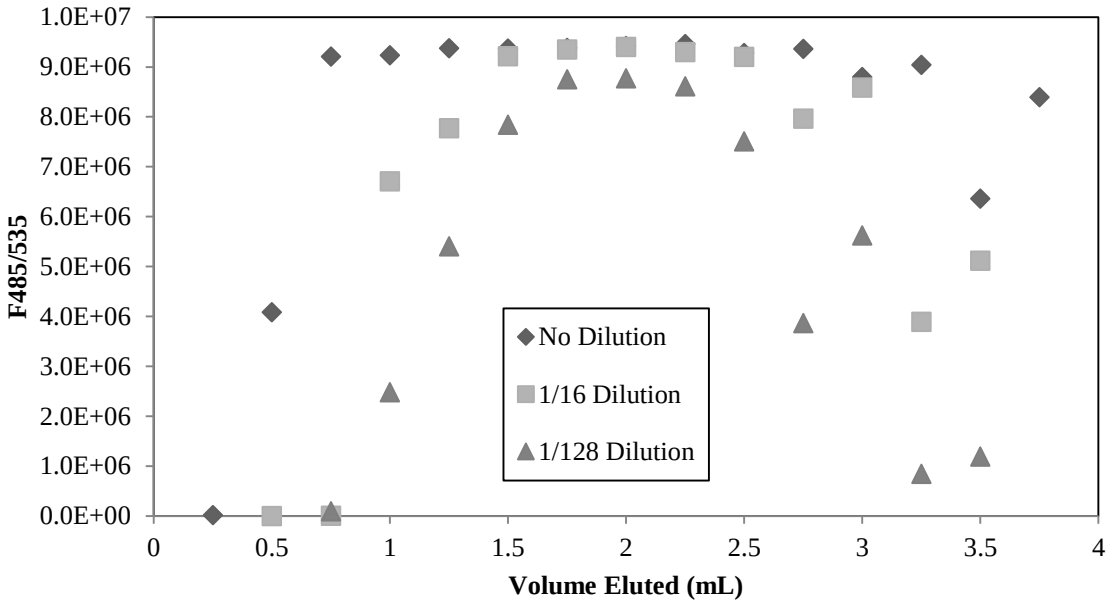


Fig. 18. Fluorescence for various dilutions of calcein dye, collected as chromatography aliquots.

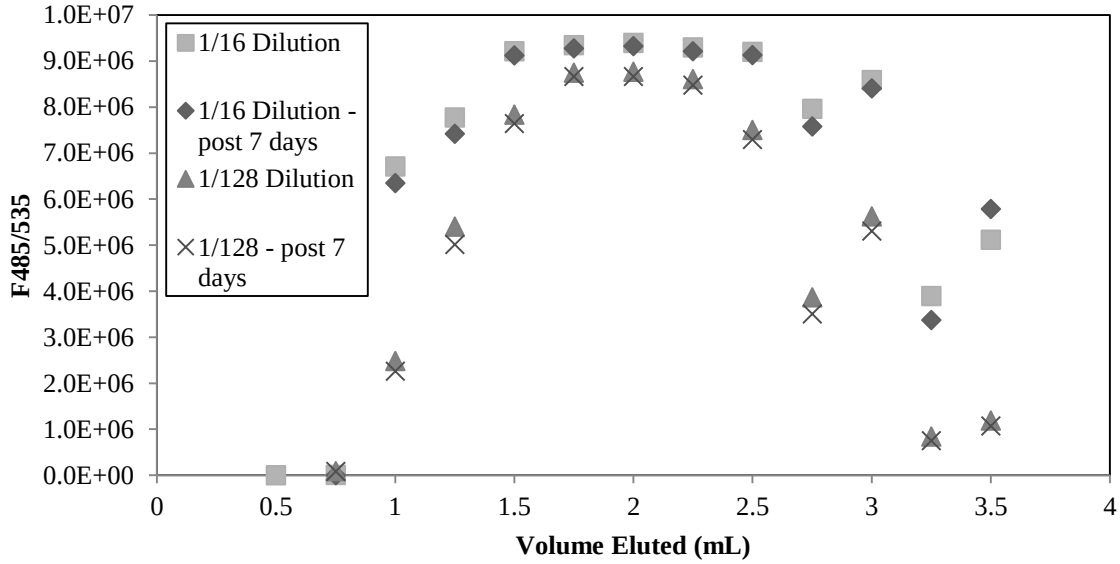


Fig. 19. Fluorescence change after 7 days of storage.

Figure 18 shows that free calcein dye (approximately 0.1-25.0 mM) will saturate the detector of a Victor^{3}V 1420 Multi-label Counter (Gleeson Hall, Room 202) if not first diluted or self-quenched. Figure 19 shows that fluorescence doesn't change considerably

with respect to time, as long as the plate is covered tightly. This is likely because evaporation is negligible if each well is fully covered.

LAL Assay Results

A LAL standard curve was prepared by with a standard from a Lonza LAL kinetic assay kit (Fig. 20). Aliquots were collected from a SEC chromatography (200 µL loaded sample of 25 mM calcein and 1.0 mg/mL LPS), and individual aliquots were diluted 100X. Aliquot endotoxin concentration was determined from the standard (Fig. 20) and chromatography elution profile was plotted (Fig. 21).

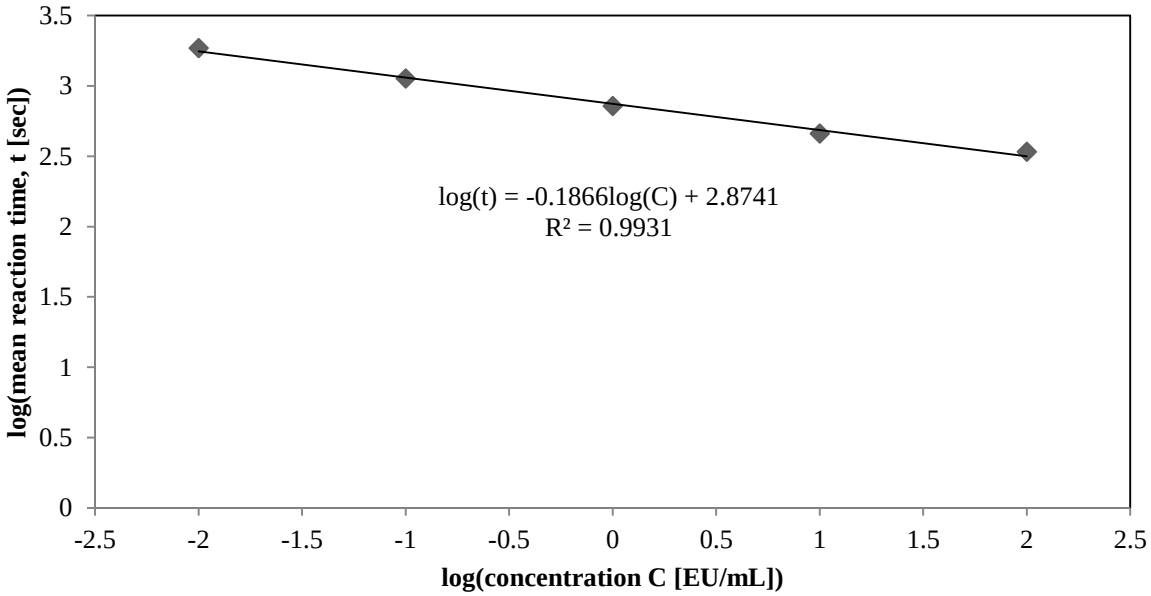


Fig. 20. Log (mean reaction time) in log(sec) vs. log(endotoxin concentration) in log(EU/mL). The standard was run in triplicate.

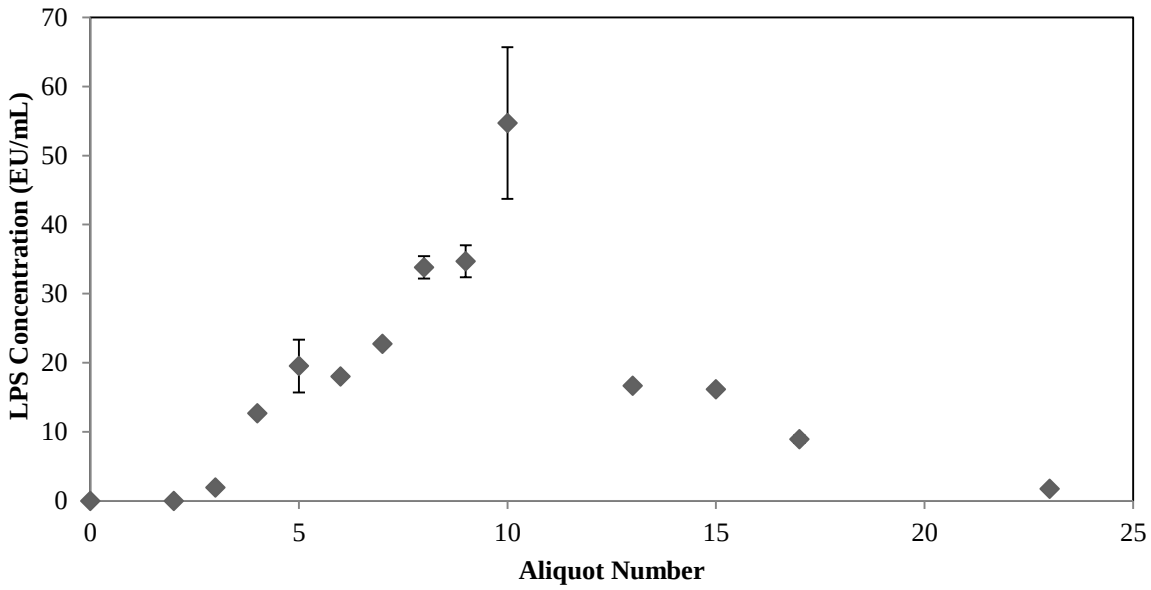


Fig. 21. Endotoxin (LPS) concentration vs. chromatography aliquot #. All collected aliquots were diluted 100X. Sample #0 denotes buffer solution. Duplicates were run for each aliquot. Error bars represent standard deviation (N=2).

In order to limit the amount of background interference caused by the colorful fluorescent dye, the sample was loaded in the chromatography column at very high concentration (1 mg/mL LPS) and 25 mM calcein, and then diluted 100X after aliquots were collected. Dilution was done to reach a target concentration of 10 endotoxin units (EU). The linear range of the LAL test is 0.01-100 EU/mL (0.002-20.0 ng/mL LPS).

Figure 21 shows that endotoxin is eluted along with the band of colorful dye (aliquots 8-12 were green). LPS did not elute in the first aliquots, as expected. This has several implications. First, it may mean that LPS vesicles are not forming, otherwise large LPS vesicles should elute first. Calcein, at low pH, was likely never fully enclosed in vesicles, and the smaller endotoxin fragments are then eluted with the smaller dye molecules. A likely possibility is that the resin pore size (700 Da) does not allow calcein (622 Da) to separate from LPS.

Conclusions

Calcein can donate four protons to solution, and is very acidic (pH 1.5) and insoluble when placed in low volumes of water. Self-quenched calcein dye can be made by addition of base. To dissolve calcein aqueous, heat should not be used because a cloudy suspension will form in the solution and cause it to gel. Aqueous NaOH should be added to bring to physiological pH (7.4).

Centrifugation (20 min, 13,000 rpm) proved ineffective in isolating vesicles. Also, vesicles were not succesfully isolated using size-exclusion chromatography with Sephadex G-10 resin in both plastic and glass columns.

Results of the LAL test show that endotoxin is eluting, just not where it is expected to elute. It is expected that large, un-ruptured vesicles would elute prior to small, calcein molecules. Rather, LPS was eluting with the colored dye. The LAL test confirms that either LPS eluted in fragments (vesicles were never formed originally), or resin and column were not effective in separating vesicles and dye.

Dialysis successfully separated unencapsulated dye from calcein-loaded vesicles. The dialysis cassette with larger pore size (10,000 MWCO) was much more efficient in removing EXB than the smaller pore size (2,000 MWCO). Larger pore size will expedite dialysis, saving time and amount of buffer used. If purified more rapidly, samples can be refrigerated sooner, which may help prevent long-term vesicle rupture if vesicles are stored at 4^{o}C.[25] However, room temperature is sufficiently cool to keep vesicles intact; after dialysis for 10 days at room temperature, vesicles were still contained in the dialysis cassette.

Calcein dye was kept in plate wells for an extended period of time (7 days, covered in Parafilm wax and foil) and did not lose fluorescence. This is useful for long-term calcein storage, providing flexibility to be able to measure fluorescence of ruptured vesicle contents over a longer period of time. Colder storage (e.g. refrigeration with Parafilm and foil) will promote better preservation of vesicles. Literature reports that liposomes, stored for 12 weeks at 2-8^{o}C, maintain physical integrity with respect to vesicle size and encapsulation ability.[30]

The addition of Triton-X-100 may add bubbles, similar to bubbles formed with household detergents. These bubbles may affect absorbance readings. Non-dilute or quenched calcein dye will saturate the fluorescence detector.

Future Work

Improvement of Vesicle Isolation

Dialysis with a 10,000 MWCO cassette proved effective in isolating vesicles with minimal buffer change. Dialysis could be improved by using cassettes that allow for larger samples (3 mL volume, 7,000 MWCO). A larger pore size of the dialysis membrane will allow dialysis to proceed quickly and limit random permeabilization due to long-term exposure to elevated temperature. Also, even though using chilled buffer (to minimize vesicle rupture due to high temperature). However, chilled buffer may also negatively affect mass transfer of un-encapsulated calcein and slow down dialysis.

Size-exclusion chromatography is still a possible method of vesicle isolation. Sephadex G-75 and G-50 (exclusion limits above 30 kDa) are commonly used for separating unilamellar vesicles[31], and should be more succesful in separating calcein (622 Da) from LPS than with Sephadex G-10 (exclusion limit of 700 Da).

Fluorescence change will be more pronounced if the initial concentration of calcein was greater. Future experiments should calcein at 100 mM or greater.

Previous experiments that have employed calcein leakage assays have used heat-shock cycles (to reform vesicles around self-quenched calcein) and vesicle extruders (to create vesicles of uniform size).[18,19,21,22] These techniques would be useful to better control physical properties of LPS vesicles.

Additional Peptide Experiments

The scope of this investigation is to quantitatively measure how peptides will bind to endotoxin (LPS vesicles), to determine if WLBU2 will bind molecules irreversibly

without inducing membrane rupture and causing bacterial contents to leak into the surrounding fluid. A calcein leakage assay can be used to quantify membrane integrity. A sample protocol for replicate experiments with peptides are as follows:

- Portion out six 140 µL aliquots of LPS vesicles in wells on a 96-well plate, using wide-mouth pipette tips that are less likely to rupture vesicles.
- Measure initial fluorescence at 485 nm excitation and 525 nm emission for 0.1 sec (settings saved as "Bearded Anthony" in Fluoremetry protocols on the Wallac plate reader, GLSN 202). Export results to a USB drive.
- Add 10 µL of a desired peptide (WLBU2 or polymixin B) to 3 samples, and add 10 µL buffer (blank) to three samples. Measure fluorescence until no more change happens. This process may take time; consider re-measuring after 1, 5, and 10 hours. Export data.
- Add10 uL Triton X-100 (1% v/v) to each of the wells to determine assay endpoint (full leakage). This process will also take time (fluorescence was greater 10 hours after addition than 10 minutes after addition). Export data.
- The percent leakage can be determined by [L]

$$\%\ Leakage = 100\left(\frac{F_{pep} - F_o}{F_{100} - F_o}\right),$$

where F_{pep} is fluorescence intensity after the addition of a peptide, F_o is the initial fluorescence, and F_{100} is fluorescence after the addition of Triton X-100.

- A control of added EXB should also be done to identify any background noise associated with a change in sample volume (free calcein will be less-quenched and more fluorescent at dilute concentrations).

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Appendices

Appendix I – Additional Methods

Chromatography in Plastic Column

A longer column (from plastic transfer pipette, approx. 6 inches length, 1 cm OD) was also used in attempt to gain better separation of LPS and un-encapsulated dye. The plastic column was prepared as with the glass column, using approximately 5 mL Sephadex G-10 resin. Two bed volumes Tris EXB passed through the column to equilibrate the resin. Once the buffer line was just above the top cotton wad, 100 µL sample (1 mg/mL LPS, 60 mM calcein, 10 mM Tris-HCl) was injected to the top of the column. The plastic tube, fixed on one end to the wall 2 ft above the column, was connected to the top of the column and buffer was added dropwise to the top end of the tube. Sample collection took place keeping a constant fluid pressure head (approximately 6 cm of fluid above the top cotton wad). Aliquots were collected every 4 drops.

Dialysis with 2,000 MWCO Cassettes

2,000 MWCO: Three-hundred microliters of LPS was combined with 2.7 mL calcein dye (60 mM calcein, 10 mM Tris-HCl) in a 15 mL Falcon® tube, vortexed for 30 seconds on a VWR LabDancer, and sonicated for 15 min (VWR - Model 50D), and let stand for 5 minutes to ensure that vesicles re-formed.

Two liters EXB was transferred to a large 3.5 L beaker. A Slide-A-Lyzer 2K dialysis cassette (2,000 MWCO, 0.5-3.0 mL, Pierce # 66225) was inserted into the groove of a foam float, with cassette port #1above the surface of the water [K]. The float and cassette were placed in the EXB solution for 2 minutes to equilibrate the membrane. A 5 mL syringe (BD, connected to a BD 18G1 PrecisionGlide Needle) was inserted into

the 15 mL Falcon® tube containing LPS solution, and was filled with 3 mL solution. The syringe was used to transfer 3 mL of the prepared LPS solution through port #1 of the dialysis cassette, taking care not to penetrate the dialysis membrane with the tip of the inserted needle. Excess air was removed by the syringe, and the dialysis cassette was placed back into the groove of the foam float (port #1 in the groove). The float and cassette were placed in the EXB beaker, set on a stir plate with constant stirring.

Buffer (in 2.0 L amounts) was prepared fresh for every exchange, taking place every 8 hours. A total of 14 buffer changes of 2.0 L were used to remove unencapsulated dye, then vesicles were transferred to two 3,500 MWCO cassettes (Slide-A-Lyzer, 0.5-3.0 mL) for complete removal of external dye.

Optical Density Scan of LPS

An optical density reading at 600 nm (OD600) is commonly used to detect the presence of bacteria and monitor cellular changes [E]. An OD600 test as done to see if LPS vesicles dissolved in concentrated calcein dye could be detected by a simple spectrophotometer reading. A Thermo Genesys 6 spectrophotometer was used to perform an optical density scan at of LPS at 600 nm (OD600). One-hundred microliters LPS (10 mg/mL) was transferred by pipette to a 2.0 mL plastic cuvette containing 0.9 mL calcein (30 mM in HEPES buffer, pH 1.5), with a final LPS concentration of 1.0 mg/mL in calcein. A second plastic cuvette was filled with 1.0 EXB). Both the sample and blank were run on a scanning spectrophotometer for wavelengths of 200-700 nm, increasing at intervals of 10-50 nm. Results were recorded, displayed in Fig. A3.

The same wavelength step size (10-50 nm) was done for each sample, and the scatter plot was connected with a line to show the continuous trend. Figure A3 shows

that absorbance is zero for both LPS-free dye and LPS-loaded dye at 600 nm. This indicates that small LPS molecules (10-1000 kDa) cannot be detected by the OD600 test on a spectrophotometer in the presence of opaque calcein dye.

Appendix II – Fluorescence & Absorbance Data

Table A1. Raw data showing fluorescence increase upon vesicle rupture. Absorbance raw data is also provided.

		Fluoresence (485/535 nm) Reading					
		Before Addition		After 10 min		After 10 hours	
Dilution Factor (Vsamp/Vtotal)	Volume Sample in well (uL)	Triton Added	Buffer added	Triton Added	Buffer added	Triton Added	Buffer added
0.20	*150*	312487	324804	357438	295569	395850	306564
0.07	*150*	125960	122344	154870	133651	168917	130895
1.00	*50*	291041	291884	386717	295985	401012	308078

		Absorbance (490 nm) Reading					
		Before Addition		After 10 min		After 10 hours	
Dilution Factor (Vsamp/Vtotal)	Volume Sample in well (uL)	Triton Added	Buffer added	Triton Added	Buffer added	Triton Added	Buffer added
0.20	*150*	0.401	0.435	0.397	0.441	0.390	0.443
0.07	*150*	0.140	0.112	0.139	0.141	0.134	0.140
1.00	*50*	0.475	0.457	0.404	0.503	0.377	0.483

Appendix III – Additional Figures

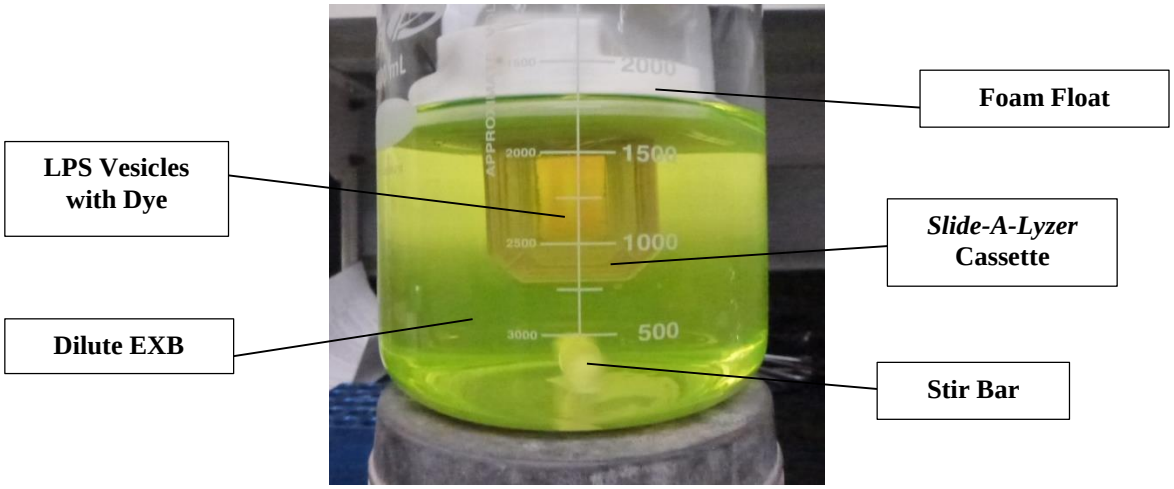


Fig. A1. Dialysis setup, using a Slide-A-Lyzer 2K Dialysis Cassette (3,500 MWCO, 0.5-3.0 mL, Pierce # 66225) filled with 1 mg/mL LPS and, with 1.5 L (approx.) EXB.

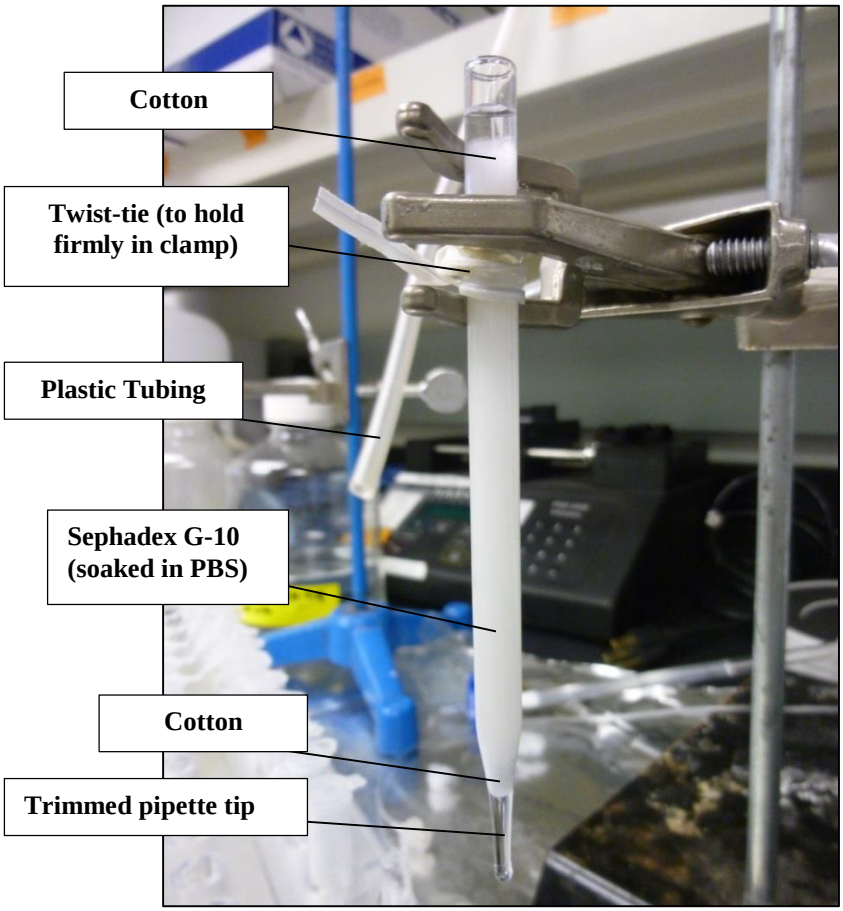


Fig. A2. Photograph of glass column. Resin (Sephadex G-10) was held in column by cotton on the ends. Plastic tubing (background) was elevated above the column, attached at the top, and filled with small increments of EXB until fluid head formed.

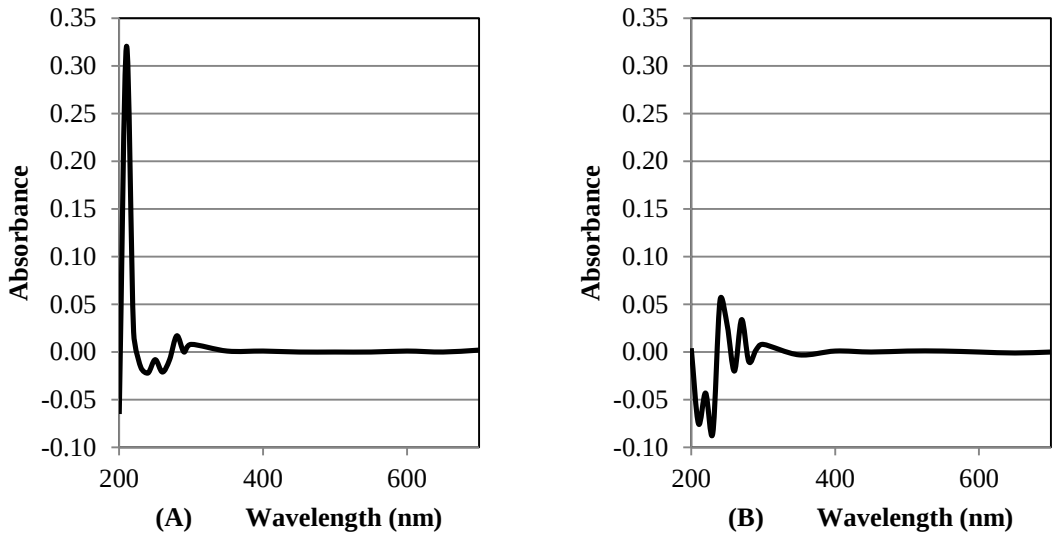


Fig. A3. Measured optical density vs. emission wavelength (nm) for **(A)** calcein dye (30 mM) with no LPS and **(B)** calcein dye (30 mM) with LPS (1.0 mg/mL).

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