

**MAXIMIN 3 BINDING AND LEAKAGE OF MEMBRANES MADE WITH  
ESCHERICHIA COLI POLAR LIPID EXTRACT**

by

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## Abstract

Due to the overwhelming amount of antibiotic resistance developing in bacteria, a new method for rupturing bacterial membranes is being investigated. Antimicrobial peptides (AMPs) are short peptides that produce a leak in a bacterial cell by binding to the bacteria's phospholipid bilayer membrane and creating a hole in the membrane. Currently, most AMPs are too cytotoxic for the human body and are not ready to be used as treatment for bacterial infections. The main goal for this research is to further study AMPs' behavior with *Escherichia coli* vesicles, as well as their degree of leakage. This project focused on membranes made with *E. coli* polar lipid extract and used the AMP Maximin 3 to induce leakage. A leakage assay was used to calculate the percent vesicle leakage at different peptide concentrations. The leakage assay technique uses a vesicle prepared by the addition of *E. coli* lipids in a mixture of buffer and dye or dye-quencher. The lipid was passed through a microscale extrusion membrane to create dye-filled vesicles. After the extrusion was completed, the vesicles were dialyzed overnight. After dialysis, three fluorimeter measurements were taken: first of the vesicles alone; then of the vesicle with a peptide; and lastly of the vesicle, peptide, and detergent. Through measurement and quantification of the degree of leakage induced by Maximin 3 in *E. coli* membrane, an enhanced understanding of the membrane disrupting mechanism of AMPs can be produced.

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## **Introduction**

Antibiotics are amongst the most overprescribed medications today. Antibiotics either destroy (bactericidal) or slow down the growth of bacteria (bacteriostatic). They work in five mechanism of actions: they can disrupt cell membrane function, inhibit cell wall synthesis, inhibit DNA/ RNA synthesis, inhibit protein synthesis, or inhibit folic acid metabolism. If antibiotics disrupt the cell membrane function, they will bind to the cell membrane and alter its' structure and making it more permeable which would disrupt the osmotic pressure and cause leakage of the cell contents. If they inhibit cell wall synthesis, they are considered bactericidal because they kill the bacterial cell. These types of antibiotics won't affect human cells because human cells do not have peptidoglycans that make up the cell wall and therefore these antibiotics won't bind to the cell wall. If the antibiotics inhibit DNA and RNA synthesis, this could lead to lack of protein production. If the antibiotics inhibit protein synthesis, then the bacterium is alive but useless. Since proteins are essential for function, causing a disruption in the protein synthesis would qualify these antibiotics as bacteriostatic. For example, if an antibiotic inhibits folic acid metabolism, that means that the bacteria would not be able to produce adenine or thymine— two amino acids that are essential for DNA production.

Due to the overwhelming amount of antibiotic resistance developing in bacteria, a new method for rupturing bacterial membranes is being investigated. Antimicrobial peptides (AMPs) are short peptides found in all living creatures as part of their innate immune system. This same immune system is responsible for producing tears when a foreign object enters the eye or inducing a sneeze to export any foreign creatures entering the nose. AMPs have demonstrated the cytolytic ability to kill gram-negative and gram-positive bacteria by producing a leak in a bacterial cell via binding to the bacteria's phospholipid bilayer membrane and creating a hole in

the membrane. This reduces the chance of bacteria developing resistance against AMPs because AMPs target the exterior and not the interior of the cell. Currently, most AMPs that are being investigated for medicinal purposes are too cytotoxic for the human body and are not ready to be used as treatment for bacterial infections.

Maximin 3 is an AMP that is 27 amino acids long, it is composed of alpha helices and is isolated from the skin secretion and brain of the toad *Bombina maxima*. The Maximin 3 is part of the toad's epithelial defenses; it is non-selective and works against many different species of bacteria. The concentration of Maximin 3 required to destroy organisms is in the micromolar ( $10^{-6} M$ ). Figure 1 shows the helical structure of the Maximin 3 peptide from an overhead point of view. G1 (on top) is the N- terminus and H27 (to the right) is the C- terminus. (DeLuca, 2013). Figure 2 shows a 3-D structure of the most stable confirmation of Maximin 3 in aqueous solvents. This is a simulation by I-TASSER (iterative Threading Assembly Refinement), a program that simulates the 3-D structure of a protein molecule.

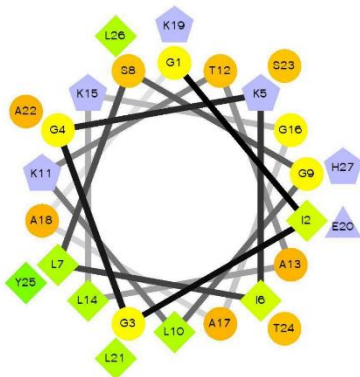


Figure 1- Helical wheel model of Maximin 3, The start (N-terminus) is G1 and the end (C-terminus) is H27. (DeLuca, 2013).

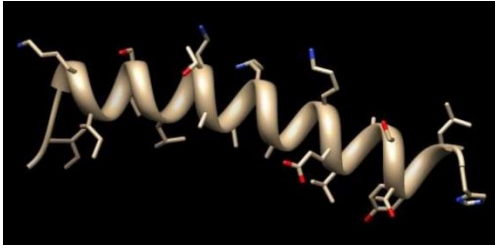


Figure 1a- Maximin 3 structure in aqueous solution, simulated by I-TASSER. (Paulson, 2013).

The main goal for this research is to study the degree of leakage induced by Maximin 3 in vesicles made with *E. coli* polar lipid extract. A leakage assay was used to calculate the percent vesicle leakage at different peptide concentrations. The leakage assay uses vesicles prepared by the addition of *E. coli* lipids to a mixture of buffer, dye, and quencher. The lipid was passed through a microscale extrusion membrane to create dye-filled vesicles.

Performing this experiment using *E. coli* vesicles is useful for several reasons. *E. coli* is a gut bacterium; hence its ideal temperature is human body temperature ( $\sim 37^{\circ}\text{C}$ ). This is excellent because this simulates a body cell and shows how the peptide is going to act on bacteria cell in the human body. *E. coli* can cause life-threatening infections, the antibiotic resistance in *E. coli* is rapidly increasing, leaving the human body without any protection against such difficult symptoms is not favorable (Collignon). *E. coli* can survive both aerobically and anaerobically, and lastly, *E. coli* can replicate every 20 minutes; although these don't improve our study, they are reasons that make *E. coli* a frequently used model organism.

When the lipid solution undergoes microextrusion, it passes through a small polycarbonate membrane which form vesicles around negatively charged ANTS dye and positively charged DPX quencher. These vesicles were made to be used in the fluorometer. The dye is added to ensure that the fluorometer would record the fluorescence levels, and the leakage of the dye and quencher and subsequent separation of the two is what causes the change in the

fluorescence being measured. When the quencher separates from the dye it the dye to show up in the fluorometer uninterrupted, and the fluorescence goes up. When the vesicles are broken in the fluorometer, the dye will depart from the quencher, and more of the absorbance intensity would increase.

After the extrusion was completed, the vesicles were dialyzed overnight to get rid of any dye or quencher particles that after the microextrusion process weren't in a vesicle. Through measurement and quantification of the degree of leakage induced by varying concentration of Maximin 3 in *E. coli* membranes, an enhanced understanding of the concentration needed to disrupt the membrane and efficacy of Maximin 3 peptide can be produced.

The leakage assay tests the effect of the Maximin 3 peptide on the leakage of the *E. coli* vesicles. Three fluorescence measurements were taken: first the buffer and vesicle solution, then the peptide, and lastly a triton detergent. When measurements were taken using the fluorometer of the vesicle and buffer alone, no leakage is expected to be seen so a low baseline is expected to be detected by the fluorometer machine. When the peptide is added to the vesicles over a range of concentrations, this causes varied degrees of leakage of the membrane. This degree of leakage is what is being measured in this experiment. When the Triton detergent is added to the vesicle-buffer-peptide solution, it causes the vesicle to burst open and completely ruptures the membrane. By measuring the vesicle-buffer solution we are setting the minimum absorbance levels that are in the solution. And by measuring the leakage that triton induces we see the maximum leakage that could happen to the vesicles. When the peptide is being measured, we see an increase in the absorbance with an increase in peptide concentration as more peptides would bind to the membrane and cause more disruption in the membrane.

Maximin 3 might be the solution to the problematic antibiotic resistance increasing at an alarming rate, in this research project, we hope to gain more information on how the concentration and temperature of the peptide would affect the leakage.

## **Materials and Methods**

### *Reagents*

A phosphate buffer solution containing 140mM NaCl and 50 mM Na<sub>2</sub>HPO<sub>4</sub>, was prepared by weighing out 32.726 grams of NaCl, and 28.39 grams of Na<sub>2</sub>HPO<sub>4</sub> and dissolving them in 4 liters of deionized water. A stir bar was placed in the beaker, and the solution was stirred until the solutes dissolved. A pH meter was calibrated for basic conditions using pH standards of 7 and 10 and the solution was brought down to a pH of 7.4 using 10% HCl in water. The solution was transferred to an empty storage bottle and labeled and dated. This phosphate buffer was used for all subsequent experiments except for the preparation of ANTS dye and DPX quencher stock solutions.

A different phosphate buffer solution containing 50mM NaCl and 50 mM Na<sub>2</sub>HPO<sub>4</sub>, pH 7.4 was produced by weighing out 0.2922g of NaCl and 0.7098g of Na<sub>2</sub>HPO<sub>4</sub>. Both were placed in a 100mL beaker, a graduated cylinder was filled with deionized water to the line indicating 100 mL. The pH was adjusted, the solution was stirred, and transferred to a labeled bottle as described in the previous paragraph.

A stock solution of 60mM ANTS was prepared by weighing out 0.1282g of ANTS, recording the exact mass, and placing in a labeled vial (named and dated accordingly). 5 mL of 50 mM NaCl in phosphate buffer was poured into the vial. The vial was closed and vortexed until the solid dissolved.

A stock solution of 60mM DPX was prepared by weighing out 0.1266g of DPX powder, recording the exact mass, and placing in a labeled vial (named and dated accordingly). 5 mL of 50 mM NaCl in phosphate buffer was poured into the vial. The vial was closed and vortexed until the solid dissolved. Vials containing the ANTS and DPX stock solutions were covered with parafilm and aluminum foil and stored in the refrigerator.

A stock solution of the *E. coli* lipid solution containing 10mg/mL of *E. coli* in chloroform solvent was prepared by carefully breaking open the powder of *E. coli* polar lipid head produce by Avanti Polar Lipids © with a razor, and adding 100 mg of the *E. coli* lipid and add about 6.0 mL of the chloroform and once the *E. coli* lipid was dissolved, 1 mL of chloroform was added until a total of 10.0 mL is obtained. This stock solution was then aliquoted into several small glass vials with Teflon- lined caps because chloroform dissolves most plastic.

A stock solution of the detergent Triton was made by dissolving 10 mL of Triton X-100 liquid in 90mL of deionized water. This was placed in a glass vial labeled “Triton 1:10” as a 1:10 dilution of Triton has occurred.

### Peptide Preparation

To prepare the peptide solution, a very small amount of frozen Maximin 3 powder supplied by NEO BioLab (Lot No. P140404-MJ394584) was dissolved in 1 mL of buffer in a sample vial. A stir bar was placed in the vial, and stirred until the solution was dissolved. A Cary Win UV- Spectrophotometer program was setup as follows: speed: medium, start: 400 nm, stop: 200 nm. A 1 cm cuvette with 140mM NaCl and 50 mM Na<sub>2</sub>HPO<sub>4</sub> phosphate buffer was used to zero out the background noise. The cuvette with buffer solution was then measured and the absorbance value at wavelength 280 nm was recorded. Another 1 cm cuvette was filled with the

peptide solution diluted 1:10 with the buffer solution. The absorbance at 280 nm of the buffer solution was then subtracted from the absorbance value of the peptide solution. Using Beer's law which states that the absorbance is the product of the extinction coefficient ( $\epsilon$ ), the concentration ( $c$ ) and the path length ( $l$ ) of the cuvette

$$A = \epsilon * c * l$$

It is known that the absorbance is equal to the absorbance of the peptide – the absorbance of the buffer solution. The extinction coefficient is  $1490 \text{ cm}^{-1} * \text{M}^{-1}$ . The concentration is the variable we are solving for. And the path length of the cuvette is 1 cm. This can be rearranged to solve for the concentration of the peptide solution:

$$\text{concentration} = \frac{\epsilon * L}{(Abs_{\text{peptide solution}} - Abs_{\text{buffer solution}})}$$

Since the peptide solution was diluted 1:10 with buffer we multiply the calculated concentration by 10 to get the actual concentration that is in the peptide solution.

### Vesicle preparation

Vesicles in this project were made by *E. coli* polar lipid extract with ANTS dye and DPX quencher dissolved in buffer and extruded.

A 250  $\mu\text{L}$  gas-tight glass syringe was cleaned by filling the syringe with chloroform and dispensing the chloroform into a labeled waste container. In preliminary experiments, 96.3  $\mu\text{L}$  of POPG and 284.7  $\mu\text{L}$  of POPC were dispensed into a vial to prepare a lipid suspension containing 1.25 mM POPG and 3.75 mM POPC.

In later experiments, *E. coli* polar head lipid sample was used. According to the Avanti Polar Lipids © website, this lipid is composed of 67% PE, 23.2% PG, and 9.8% CA. Using the actual molecular masses of PE, PG, and CA, the overall molecular mass of the compound was calculated to be 800.0623 g/mol. The stock solution of the lipid is  $\frac{10mg}{mL}$ , the concentration in this solution is  $\frac{10mg}{mL} * \frac{1mmol}{800.0623mg} = 12.499 mM$ . The total desired concentration of lipid is 5mM, to achieve this the total volume of lipid that would be required from the stock solution is:

$$5mM * 1mL = 12.499mM * V_{desired}$$

$$V_{desired} = 0.4 mL$$

400  $\mu$ L of *E. coli* polar head lipid sample was placed into a vial to prepare a lipid suspension at a concentration of 5 mM. The chloroform in the vial was evaporated (slowly and gently to not cause any splashes) with nitrogen gas in the fume hood until a lipid film formed on the bottom of the vial. The syringe was cleaned again with chloroform and left disassembled in the fume hood. The vial was loosely capped and left in a vacuum desiccator overnight to ensure true evaporation of the chloroform.

The vial containing the lipid film was removed from the vacuum desiccator. 75  $\mu$ L of DPX and 25  $\mu$ L of ANTS from the 60mM stock solutions were mixed together in a 1.5 mL plastic tube. The mixture was added to the vial containing the dry lipid film, the cap was closed, and the solution was vortexed for up to 1 minute, or until no lipid film was visible. The vial was covered with parafilm and then with aluminum foil and placed in a dark location. Two hours later, when the ANTS/DPX solution had fully dissolved and no lipid film was visible, the vial was vortexed again. 900  $\mu$ L of buffer was added and the mixture was vortexed again briefly.

This produced a mixture containing 3.12mM Lipids, 7.5 mM ANTS and 2.5mM DPX that was then passed through a microextrusion apparatus.

The microextrusion apparatus was prepared by placing two Internal Membrane Supports (shown in Figure 1) on a flat surface with the O-rings facing up. One Filter Support was pre-wet with DI water or buffer and placed over an orifice on the internal membrane support. The Filter Supports (10mm filter support from Avanti Polar Lipids) adhered to the Teflon orifice inside the O-ring inner diameter.

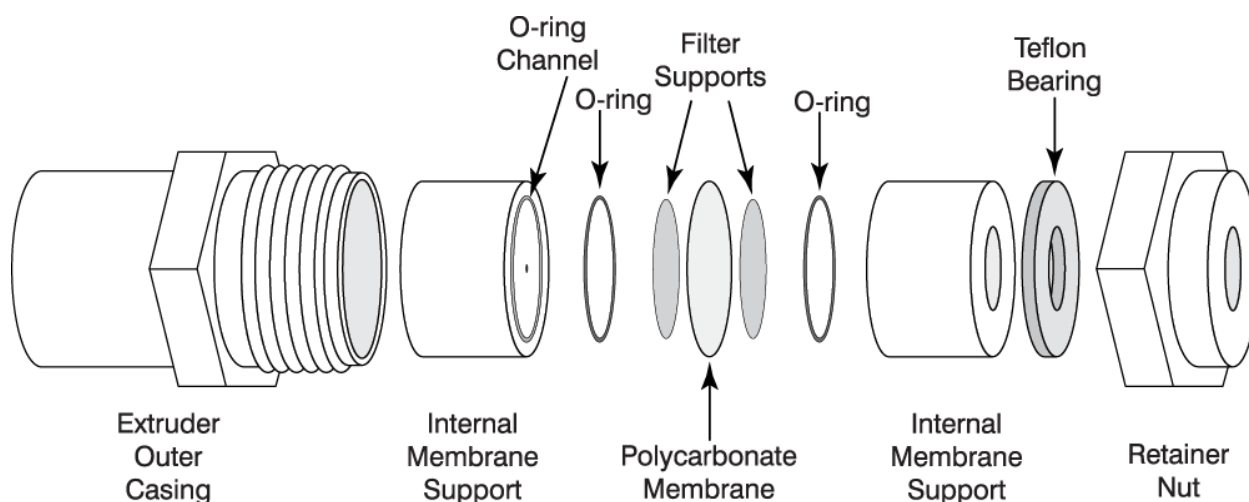


Figure 2- Micro extruder set up [1]

The Internal Membrane Support, with the Filter Support, was inserted into the Extruder Outer Casing with the O-ring facing up. One Polycarbonate membrane from Whatman® (19mm in diameter, with holes 0.08 µm in diameter) was placed on the Extruder Outer Casing over the Filter Support and O-ring. A second polycarbonate membrane was placed on top of the first, ensuring the edges were finely lined up. Only the very edges of the polycarbonate membrane were touched with tweezers to avoid damaging the membrane.

Another Internal membrane support with a Filter Supports was placed into the extruder outer casing (this time with the O-ring facing down) carefully so the Membrane Support was not twisted when came in contact with the membrane. The Teflon bearing was placed into the Retainer Nut, and the Retainer Nut was placed on the threaded end of the Extruder Outer Casing and hand tightened.

One buffer-filled syringe was attached to the extrusion apparatus with an empty syringe on the other end. The buffer was passed back and forth between the two syringes three times to wet the membrane and filter supports and to ensure no leaks. If any leaks were present, the extruder and syringes were tightened or reassembled. The syringes were removed by pulling straight out, not twisting.

The lipid suspension containing DPX and ANTS were then loaded into one of the syringes and carefully placed into one end of the micro extruder. The initial volume of the lipid solution was recorded. The empty gas-tight syringe was placed into the other end of the mini-extruder. The empty syringe plunger was initially set to zero; the syringe filled automatically as the lipid was extruded through the membrane. Pressure was gently applied on the syringe with the lipid until the empty syringe was filled with the lipid solution such that it was completely transferred to the alternate syringe. The volume of the lipid solution was recorded after every pass. The plunger of the alternate syringe was used to transfer the solution back to the original syringe; this was repeated for a total of 11 passes. The vesicles should become more clear as they pass through the extrusion apparatus, because smaller suspended particles scatter less light, therefore, as the vesicles are extruded and get smaller, the solution appears more clear. To ensure that large particles that didn't make it into the vesicle don't contaminate the vesicle solution 11 passes were done. As a result, the lipid solution ended up in the opposite syringe from the one it

started in. The final volume of the lipid solution was recorded and used in the following equation to calculate the total concentration present in the solution after dialysis.

$$M_{before\ dialysis} * L_{before\ dialysis} = M_{after\ dialysis} * L_{after\ dialysis}$$

The filled syringe was removed from the extruder and the lipid suspension was injected into a clean sample vial.

The extruder was cleaned by removing the syringes and rinsing them with DI water 3 times. The extruder was disassembled, and the membranes and filter supports were removed and discarded. Any visible liquid was wiped with a Kimwipe. The extruder was reassembled, and three portions of deionized water were passed through, three times each. Ethanol was also passed through the extrusion apparatus three times. The extruder was disassembled, and the syringes were all rinsed with ethanol over the sink. The disassembled items were left on the benchtop to dry.

The vesicle solution was dialyzed to remove any unencapsulated ANTS/DPX. Dialysate tubing was left in deionized water or buffer for 10 minutes prior to using it to hydrate it and ensure flexibility. A beaker was filled with 500 mL of 140 mM NaCl Phosphate buffer. A knot was tied on one end of the dialysis tubing. The entire vesicle solution was transferred into the dialysis tubing. As much air as possible was squeezed out of the tubing, then a knot was tied at the other end of the tubing. A stir bar was placed in the beaker, the beaker was placed on a stir plate, and the solution was covered with tinfoil and left overnight at room temperature with gentle stirring.

The dialysis tubing was removed from buffer solution and the outside was carefully dried with a Kimwipe. The knot was cut off at one end of the tubing and the vesicle was transferred into a 1.5 mL plastic tube. The dialysis tubing was squeezed to achieve complete volume transfer. A pipetman was used to estimate the volume of the vesicle solution and the volume was recorded. The vesicle was stored in the dark at 4°C and used within 3-4 days.

### Leakage Assay

Three fluorescence measurements were collected as the raw data: vesicles and buffer alone; the vesicle, buffer, and peptide; and vesicle, buffer, peptide, and 1:10 Triton X-100. A Fluorescence Spectrophotometer was used to record this data from each sample with the following settings: Temperature: 25°C, Data mode: fluorescence, scan type: emission, X mode: wavelength, excitation wavelength: 355 nm, excitation/ emission slit: 20 nm, between 475 nm and 525 nm, with a slow scan control. The initial measurement of the vesicle and buffer alone indicates the fluorescence intensity with 0% vesicle leakage. The second measurement of the buffer, vesicle, and peptide shows the percent leakage the peptide induces. The third measurement of the buffer, vesicle, peptide, and Triton-100 shows 100 percent leakage. Some data correlation is detected with the naked eye, but a more analytical approach was taken with a Microsoft Excel program set up by Dr. Elizabeth R. Middleton. This program was able to graph the present leakage vs. peptide concentration, as well as generate a Michealis-Menten plot of the data which is used to calculate the rate of reaction for each peptide concentration with the  $V_{\max}$  and  $K_m$  values.

These measurements were repeated with different concentration of peptides ranging from 2 $\mu$ M-10 $\mu$ M in increments of 2 $\mu$ M and from 25 $\mu$ M -100 $\mu$ M in increments of 25 $\mu$ M. To calculate the total peptide volume needed in each measurement (400 $\mu$ L), the peptide stock concentration

(413.4 $\mu$ M) and target concentration (*in this example* 2 $\mu$ M) are used in the following equation to provide the volume of peptide needed:

$$400\mu L * 2\mu M = X\mu L * 413.4\mu M$$

$$X = 1.96 \mu L$$

Based on the volume of the lipid solution after dialysis, the vesicle concentration is calculated. Using the vesicle concentration, the concentration of vesicle needed in each measurement and the total volume in each measurement, the volume from the vesicle stock solution is calculated.

$$M_{vesicle\ in\ measurment} * L_{total\ of\ measurement} = M_{vesicle\ after\ dialysis} * L_{needed\ per\ measurement}$$

For example, the volume before dialysis was 0.70 mL and the concentration of lipid solution was 5.0mM, and after dialysis the volume was 0.764mL and the calculated concentration was 4.58mM. If each measurement takes 400 $\mu$ L and the desired concentration of vesicle is 300mM, the total volume needed is:

$$400\mu L * 300mM = X\mu L * 4580mM$$

$$X = 26.2 \mu L\ of\ vesicle$$

To calculate the volume of buffer required, subtract the volume of vesicle from the total volume

$$400\mu L - 26.2 \mu L = 373.8\mu L\ of\ Buffer$$

Table 1 shows a typical fluorometer measurement volume data.

[Peptide] ( $\mu\text{M}$ )	Peptide Volume ( $\mu\text{L}$ )	Dilution factor	Buffer volume ( $\mu\text{L}$ )	Vesicle volume ( $\mu\text{L}$ )	[Vesicle] ( $\mu\text{M}$ )
2	1.96	1	373.8	26.2	4.58
4	3.87	1	373.8	26.2	4.58
6	5.81	1	373.8	26.2	4.58
8	7.44	1	373.8	26.2	4.58
10	9.67	1	373.8	26.2	4.58
25	24.2	1	373.8	26.2	4.58
50	48.4	1	373.8	26.2	4.58
75	72.6	1	373.8	26.2	4.58
100	96.8	1	373.8	26.2	4.58

*Table 1- sample fluorometer measurement volume setup*

Fluorometer measurements were taken at 20°C, 25°C, 30°C, and 37°C. First the buffer was added to a 1 cm, 500  $\mu\text{L}$  cuvette. Then the vesicle solution was inverted to ensure proper mixing, and added to the cuvette. The whole solution was mixed for 20-30 seconds using a pipetman with a gel loading tip by pressing down on the plunger to the first stop, holding the pipetman vertically and immersing the tip into the sample in the cuvette, allowing the plunger to return to its original position slowly, and repeating these steps while moving the pipetman around in the cuvette. The first measurement was then taken with the fluorometer and labeled

“μM Peptide V” with the correct concentration. Next, the peptide solution was mixed, measured and added to the cuvette. The whole solution was mixed for 20-30 seconds, another measurement was then taken and labeled “μM Peptide VP” with the same concentration as previously. Lastly, 20μL of Triton X-100 solution was added to the cuvette, the whole solution was mixed for 10 seconds, and the final measurement was taken and labeled “μM Peptide VPT” with the same concentration as previously.

### Data Analysis

As mentioned previously, the data analysis was done using an excel file created by Dr. Elizabeth R. Middleton. The most reproducible measurements for each peptide concentration were copied from the spectrophotometer output file and pasted in the Excel file into the "Selected traces" tab (V, VP, VPT). Once all the data is in the excel- the peptide concentration, peptide volume, and dilution factor is put into the excel program. The peptide concentration for each run is calculated. When the intensity of the peptide at a specific concentration is divided by the leakage induced by complete lysis of the membrane, the percent leakage is calculated. The percent leakage is calculated for every peptide concentration using the sum of all the intensities from the full wavelength measurement (from 474.92 nm until 525.88 nm) for each variable using the following equation:

$$\% \text{ Leakage} = \frac{\sum(\text{Vesicle} + \text{Peptide}) - \sum(\text{Vesicle})}{\sum(\text{Vesicle} + \text{Peptide} + \text{Triton}) - \sum(\text{Vesicle})} * 100\%$$

The percent leakage with the volume correction correct for the total volume of the cuvette as when the peptide and triton are added, the concentrations in the cuvette change. The volume of the buffer and vesicle always add up to 400 μL. To calculate the new concentration after

peptide and after triton addition, a ratio of the new volume with the solutions and the 400  $\mu\text{L}$  is being multiplied by the original fluorescence intensity.

$$\% \text{ Leakage corrected} = \frac{\frac{[\sum(\text{Vesicle} + \text{Peptide})] * (400 \mu\text{L} + \text{volume of peptide})}{400 \mu\text{L} - \sum(\text{Vesicle})}}{\frac{[\sum(\text{Vesicle} + \text{Peptide} + \text{Triton})] * (400 \mu\text{L} + \text{volume of peptide} + 20 \mu\text{L})}{400 \mu\text{L} - \sum(\text{Vesicle})}} * 100\%$$

Then, using the sum of all the absorbance intensities, the % leakage Sum is calculated. This calculation is repeated using the measurements from the intensity at wavelength 515 nm, and the maximum intensity. Using the absorbance intensity at wavelength of 515 nm the % leakage 515 is calculated. And finally, using the maximum intensity value, the % leakage max is calculated.

After all of the data has been calculated, the website <https://mycurvefit.com/> is used to fit the data from the 515 nm wavelength into three equations:

Equation 1: which fits the data into a hyperbolic curve,  $\frac{A*x}{B+x}$ , equation 2: which also fits the data into a hyperbolic curve but sets the maximum % leakage to  $100\% \frac{100*x}{B+x}$ , and equation 3: Thich fits the data into sigmoidal fit to test for any cooperativity amongst the peptides  $\frac{100*x^n}{B^n+x^n}$ .

Equation 1 introduces the parameters A, and B. A represents the maximum leakage, this can be seen on the raw data by the horizontal asymptote the data is approaching. B represents the peptide concentration required to achieve half of the maximum leakage. Equation 2 does the same calculation that equation 1 does, only in this calculation, the maximum percent leakage is set to be 100 % leakage. Equation 3 introduces a 3<sup>rd</sup> variable- n. introducing a third variable makes the shape of the curve more sigmoidal.

## Results and Discussion

The raw data from the fluorometer measurements can be seen in Figure 3. These are nice visualizations of how the peptide impacts the leakage of the vesicle. The blue line labeled “V” represents the leakage that occurs from the vesicle and buffer alone. This line indicates the fluorescence intensity with 0% vesicle leakage. The green line labeled “VP” shows the leakage caused by the addition of the peptide to the buffer and vesicle solution. indicates the fluorescence intensity with the respective vesicle leakage. The black line labeled “VPT” shows the leakage caused by the complete lysis of the membrane from the addition of the Triton X-100. This indicates the fluorescence intensity with 100% vesicle leakage. As the leakage increases the data shows more hyperbolic characteristics. This measurement was performed at 25°C.

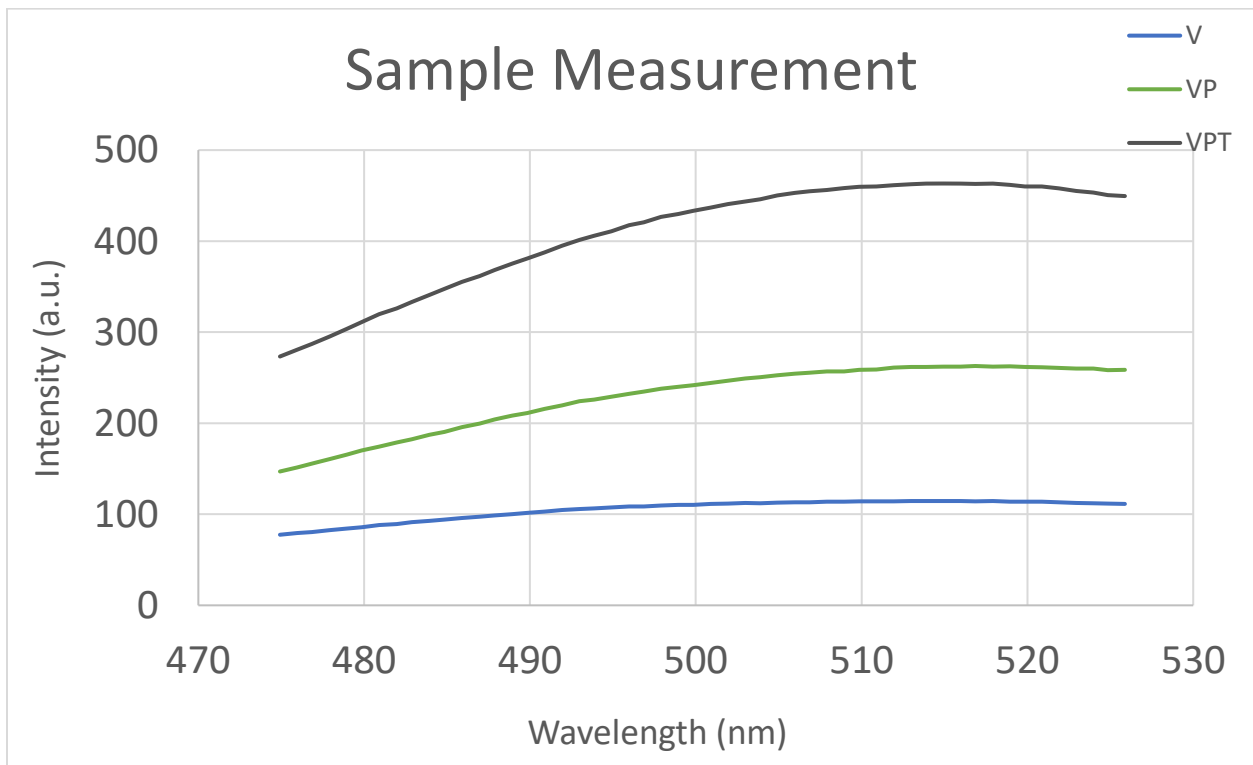


Figure 3- raw data from a sample measurement

### Measurement 1 – 10/02/18-10/03/18

In order to test the leakage induced by Maximin 3 on POPG and POPC, a set of measurements were taken. This measurement was done on a peptide with a concentration of 697.3  $\mu\text{M}$ . With a lipid composition of 1.25mM POPG and 3.75mM POPC. The solution was left in dialysis overnight, the volume decreased from 0.98mL to 0.95mL. Table 2 shows the volume and concentration setup for the fluorometer measurements.

Sample	vol peptide ( $\mu\text{L}$ )	dilution	[peptide] ( $\mu\text{M}$ )	% leakage sum	% leakage max	% leakage 515
2uM peptide V2	11.48	10	2.00	18.20	16.03	18.01
4uM peptide V1	22.96	10	4.00	32.73	30.99	34.40
6uM Peptide V3	34.43	10	6.00	37.20	37.38	41.61
8uM Peptide V2	45.91	10	8.00	39.80	38.80	41.22
1uM Peptide V1	5.74	10	1.00	14.29	13.50	13.44
10uM Peptide V	5.74	1	10.01	67.27	71.46	73.05

*Table 2- Volume and concentration setup for the fluorometer measurements for measurement 1*

The data collected in the fluorometer measurements was graphed showing the percent leakage against peptide concentration. Ideally, all three % leakage types should line up perfectly. If these are not the same then that indicates that the intensities were different depending on the wavelength, that means some other factor is influencing the fluorescence besides the leakage. Therefore an error has been introduced into the measurement. This is shown in Figure 4.

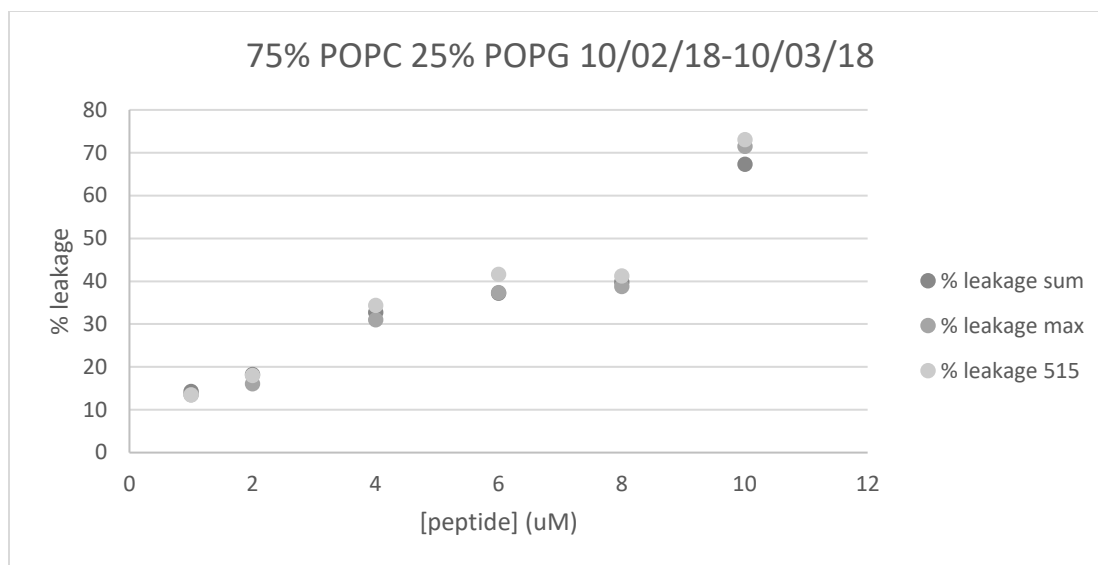


Figure 4- Percent leakage Vs. Peptide Concentration

This data was fitted into the following equation:

$$\frac{A * x}{B + x}$$

During the first data fitting, parameter ‘A’ shows the calculated maximum percent leakage and parameter ‘B’ shows the peptide concentration that produced half of the maximum leakage. The calculated parameters, errors, and R squared values that shows how well the data fits the linear fit are shown in Table 3 below.

A	266.8801
error	374.7
B	31.40841
error	54.91
R squared	0.8707

Table 3- A and B values based on the first data fitting

Using the values of ‘A’ and ‘B’ in Table 3, a hyperbolic fit was overlaid on the data that was collected at 515 nm. This can be seen in Figure 5 below.

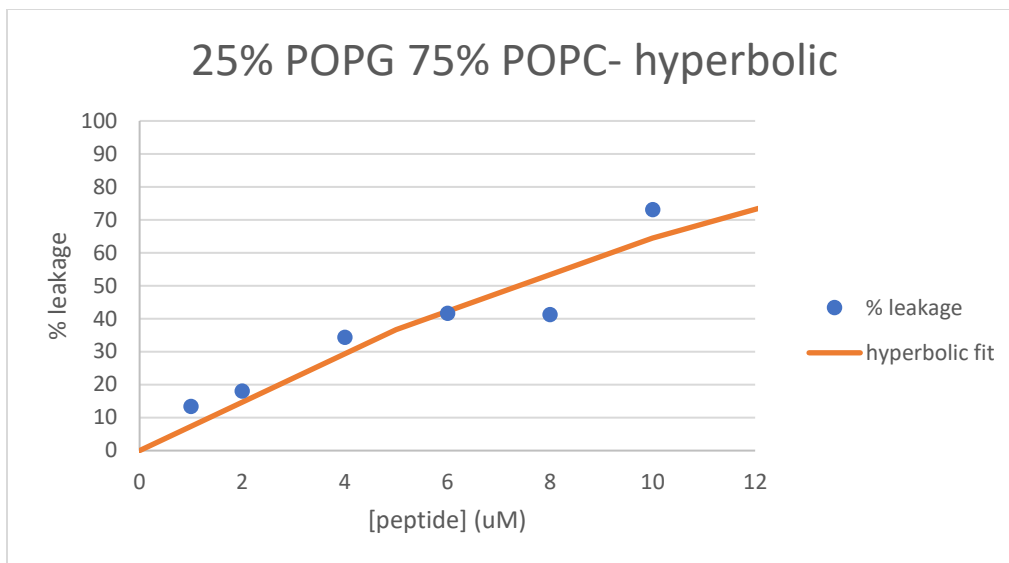


Figure 5- hyperbolic fit of measurement 1

Based on the first data fitting, the maximum percent leakage is 266 percent; any percent over 100 is not a realistic percent. This fit was performed again, during the second data fitting however, 'A' is now set to equal 100 as shown here:

$$\frac{100 * x}{B + x}$$

This should shift 'B' since 'A' wasn't equal to 100 in the first data fitting.

The calculated parameters, errors, and R squared values that shows how well the data fits the linear fit are shown in Table 4 below.

A	100
B	7.456328
error	1.244
R squared	0.8315

Table 4- A and B values based on second data fitting

Using the values of 'A' and 'B' in Table 4, a hyperbolic fit was overlaid on the data that was collected at 515 nm. This can be seen in Figure 6 below.

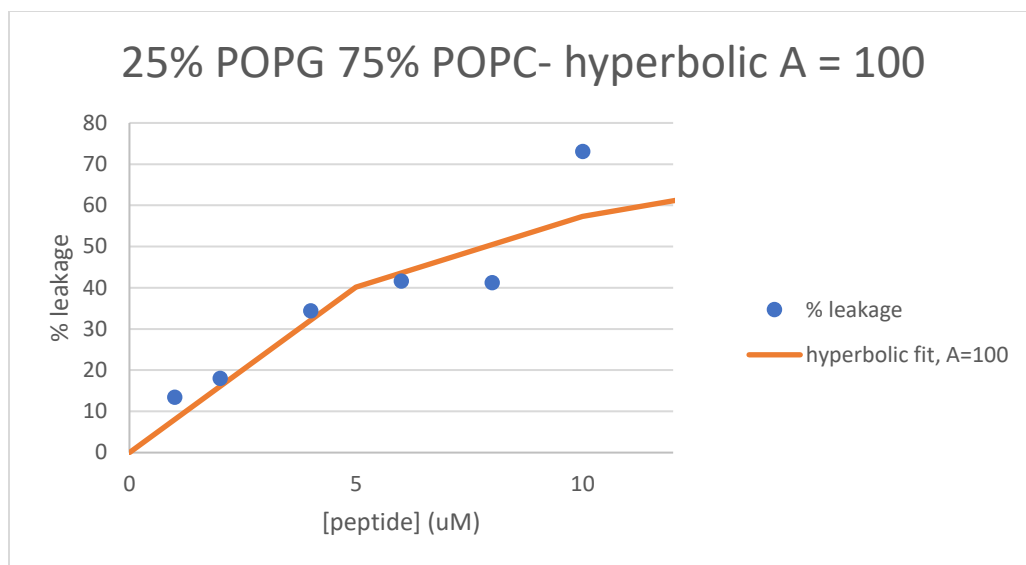


Figure 6- hyperbolic fit for second data fitting

Lastly, an extra parameter ‘n’ was added such that the new equation is:

$$\frac{100 * x^n}{B^n + x^n}$$

Using this equation, ‘A’ is set to 100. ‘B’ and ‘n’ parameters, their error values, and R squared value are calculated from the data and are shown in Table 5 below.

A	100
B	7.1612875
error	1.195
n	1.188864
error	0.3434
R squared	0.8435

Table 5- A and B values based on third data fitting

Using the values of ‘A’, ‘B’, and ‘n’ in Table 5, a sigmoidal fit was overlaid on the data that was collected at 515 nm. This can be seen in Figure 7 below. Since the value of n in Table 5 is 1 that is equivalent to having the same equation in the second data fitting. This means that the fit isn’t sigmoidal and is actually hyperbolic. This shows that there is no binding cooperation

between the peptides. That means that initial binding and leakage does not facilitate further binding and leakage.

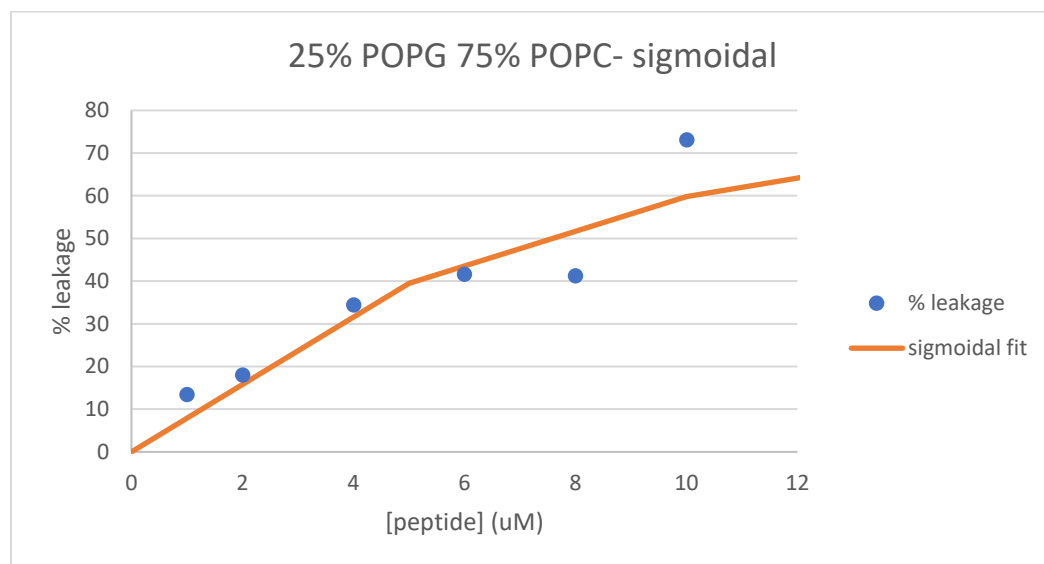


Figure 7- sigmoidal fit based on third data fitting

### **Measurement 2 – 11/15/18-10/16/18**

In order to test the leakage induced by Maximin 3 on *E. coli* vesicles, a set of measurements were taken. This measurement was done on a peptide with a concentration of 267 $\mu$ M. With a lipid composition of 5.19 mM *E. coli* Polar lipid. The solution was left in dialysis overnight, the volume decreased from 0.82mL to 0.79mL. Table 2 shows the volume and concentration setup for the fluorometer measurements.

Sample	vol peptide ( $\mu$ L)	dilution	[peptide] ( $\mu$ M)	% leakage sum	% leakage max	% leakage 515
1uM Peptide V	1.50	1	1.00	-0.41	-1.29	-3.80
2uM Peptide V1	3.00	1	2.00	10.03	6.39	21.81
4uM Peptide V1	6.00	1	4.01	12.99	8.59	25.73
6uM Peptide V1	9.00	1	6.01	10.59	7.39	20.59
8uM Peptide V2	12.00	1	8.01	0.32	-1.73	13.18
10uM Peptide V1	15.98	1	10.67	9.48	7.12	18.25

Table 6- Volume and concentration setup for the fluorometer measurements for measurement 2

The data collected in the fluorometer measurements was graphed showing the percent leakage graphed against peptide concentration. Ideally, all three % leakage types should line up perfectly. If these are not the same then that indicates that the intensities were different depending on the wavelength, that means some other factor is influencing the fluorescence besides the leakage. Therefore an error has been introduced into the measurement. This is shown in Figure 7.

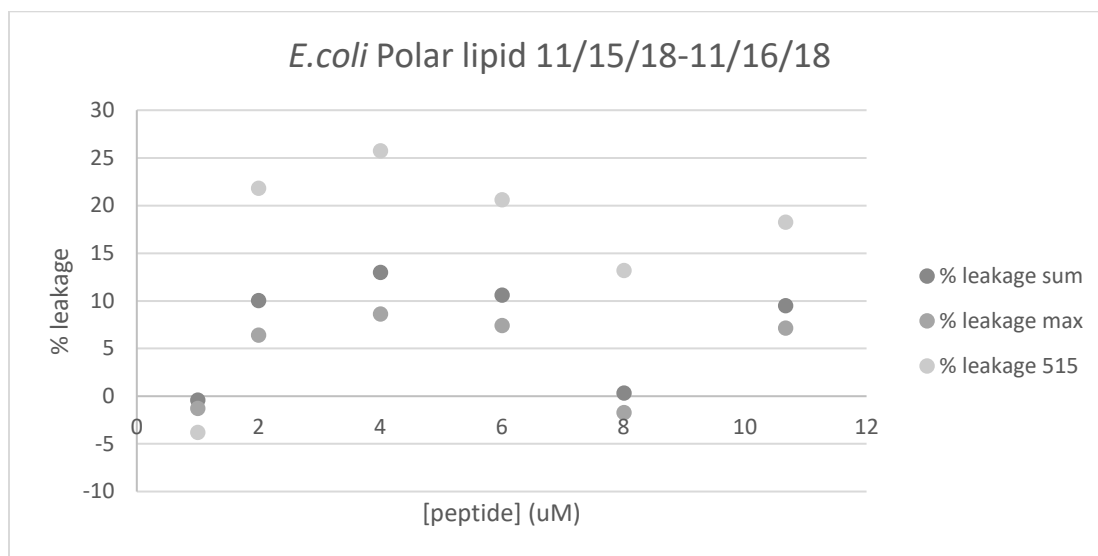


Figure 7- Percent leakage Vs. Peptide Concentration

As mentioned in measurement 1, this data was fitted into the following equation:

$$\frac{A * x}{B + x}$$

During the first data fitting, parameter 'A' shows the calculated maximum percent leakage and parameter 'B' shows the peptide concentration that produced half of the maximum leakage. The calculated parameters, errors, and R squared values that shows how well the data fits the linear fit. These are found in Table 7 below. The maximum percent leakage for this measurement is 23%. That is not a high percent leakage. This shows some fault either in the preparation of the vesicle or in the measurement. Better mixing of the solutions in the cuvette and retaking these

measurements would probably produce better results.

A	23.76231
error	11.82
B	1.611533
error	2.981
R squared	0.3305

Table 7- A and B values based on the first data fitting

Using the values of 'A' and 'B' in Table 7, a hyperbolic fit was overlaid on the data that was collected at 515 nm. This can be seen in Figure 8 below. This shows that the data doesn't show any strong correlation between peptide concentration and percent leakage. This could have either been caused by a bad peptide sample or bad vesicle solution, or by faulty measurements. Since the vesicle solution was discarded after the measurement was performed and before the data was analyzed, this vesicle sample cannot be remeasured. The same peptide sample was used for multiple measurements and the fluorometer machine was not malfunctioning, therefore, it is safe to assume the vesicle sample was the cause for this faulty data.

For completion, further analysis of this data will be done, however, our confidence in this data is very low and it should not be used for any conclusions

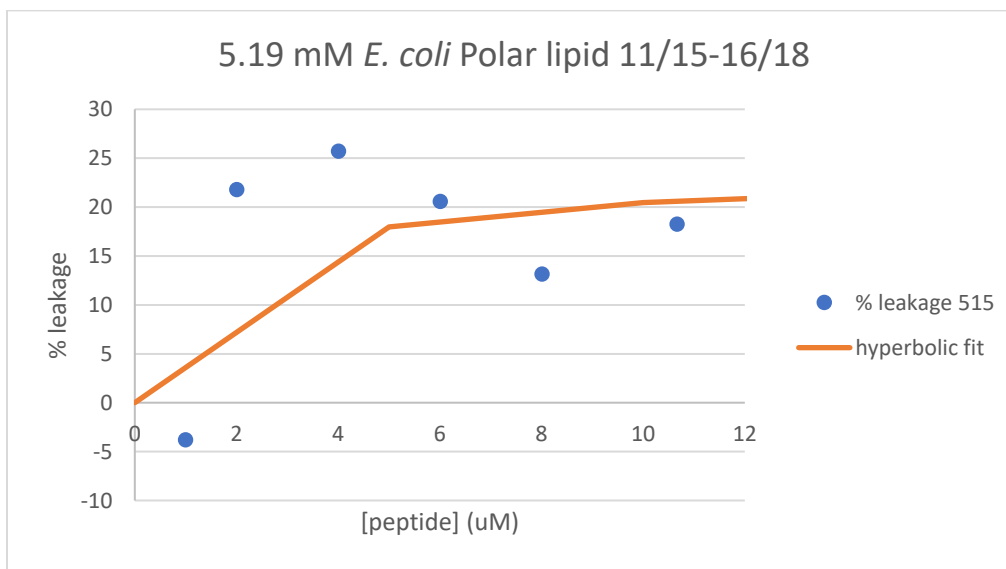


Figure 8- hyperbolic fit for first data fitting

Based on the first data fitting, the maximum percent leakage is 23.8%; that is too low of a percent. This fit was performed again, during the second data fitting, 'A' still shows the calculated maximum percent leakage and 'B' still shows the peptide concentration that produced half of the maximum leakage. However, now A is set to equal 100 as shown here:

$$\frac{100 * x}{B + x}$$

This should shift B since A wasn't equal to 100 in the first data fitting.

This is shown in Table 8. The correlation between the data and hyperbolic fit was so insignificant the actual value could not be calculated by our program.

A	100
B	30.55915
error	10.96
R squared	N/A

Table 8- A and B values based on the second data fitting

Using the values of 'A' and 'B' in Table 8, a hyperbolic fit was overlaid on the data that was collected at 515 nm. This can be seen in Figure 9 below.

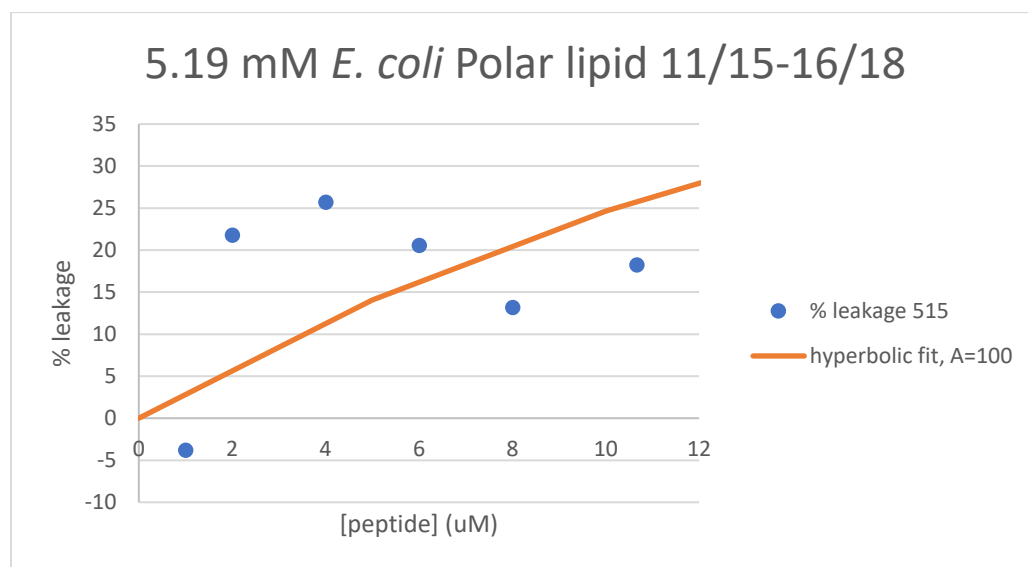


Figure 9-hyperbolic fit based on second data fitting

Lastly, an extra parameter ‘n’ was added such that the new equation is:

$$\frac{100 * x^n}{B^n + x^n}$$

Using this equation, ‘A’ is set to 100. ‘B’ and ‘n’ parameters, their error values, and R squared value are calculated from the data and are shown in Table 9 below.

Based on Table 9, the peptide concentration that would be required to achieve 50% leakage is 359 µM. But with an error value greater than the value we calculated itself, our confidence in this data is very little.

A	100
B	359.7248
error	1775
n	0.37499
error	0.4488
R squared	0.2218

*Table 9- A and B values for the third data fitting*

Using the values of ‘A’, ‘B’, and ‘n’ in Table 9, a sigmoidal fit was overlaid on the data that was collected at 515 nm, as seen in Figure 10. Since the value of n is much less than 1 that is equivalent to having the square root of the second data fitting equation. This means that the fit isn’t sigmoidal and is actually hyperbolic. This shows that there is no binding cooperation between the peptides. That means that initial binding and leakage does not facilitate further binding and leakage.

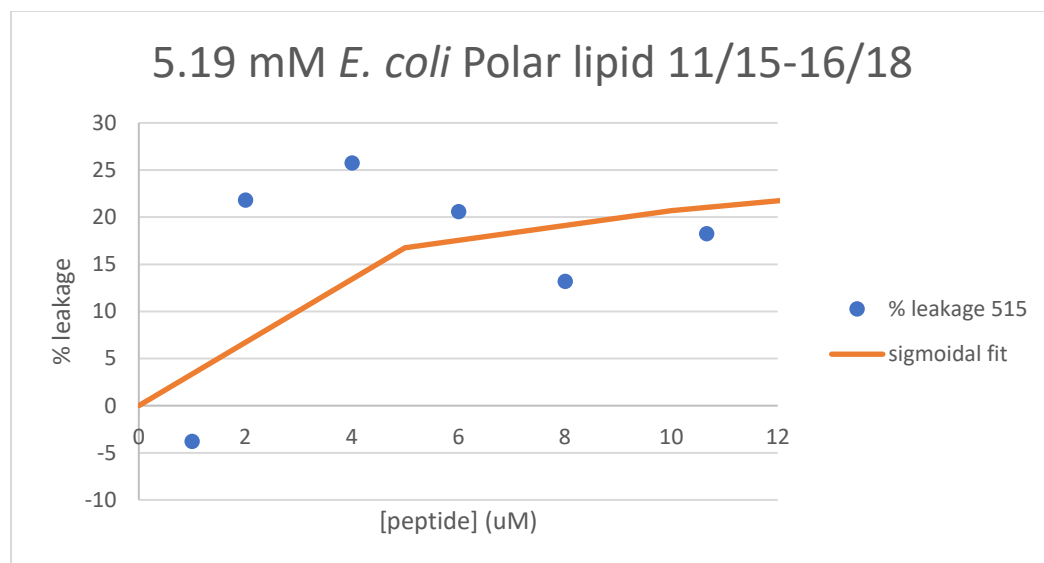


Figure 10- sigmoidal fit based on third data fitting

**Measurement 3 – 11/29/18-11/30/18**

In order to increase our reproducibility, another set of measurements were taken. This measurement was done on a peptide with a concentration of 697.3 µM. with a lipid composition of 5.12 mM *E. coli* Polar lipid. The solution was left in dialysis overnight, and the volume decreased from 0.82mL to 0.79mL. Table 10 shows the volume and concentration setup for the fluorometer measurements.

Sample	vol peptide (µL)	dilution	[peptide] (µM)	% leakage sum	% leakage max	% leakage 515
1uM Peptide V4	1.5	1	1.00	13.64	14.28	14.46
2uM Peptide V3	3	1	2.00	30.77	31.92	32.55
4uM Peptide V2	6	1	4.01	45.48	47.17	47.47
6uM Peptide V	9	1	6.01	44.13	46.60	46.19
8uM Peptide V1	12	1	8.01	55.46	58.31	57.98
10uM Peptide V	15.98	1	10.67	49.78	51.31	51.38

Table 10- Volume and concentration setup for the fluorometer measurement for measurement 3

The data collected in the fluorometer measurements was graphed showing the percent leakage against peptide concentration. Ideally, all three % leakage types should line up perfectly. If these are not the same then that indicates that the intensities were different depending on the

wavelength, that means some other factor is influencing the fluorescence besides the leakage. Therefore an error has been introduced into the measurement. This is shown in Figure 11.

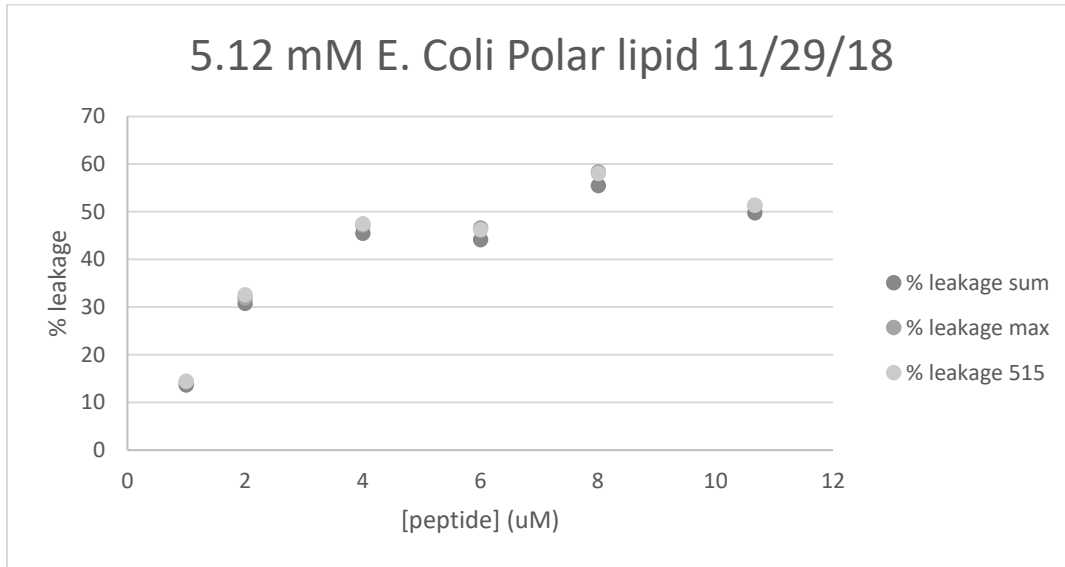


Figure 11- Percent leakage Vs. Peptide Concentration

As mentioned in measurement 1, parameter 'A' shows the calculated maximum percent leakage and parameter 'B' shows the peptide concentration that produced half of the maximum leakage. This is shown in Table 11. The maximum percent leakage for this measurement is 69.4% and the peptide concentration required to get to half of the maximum leakage is about 2.5

A	69.4219	μM
error	8.691	
B	2.497621	
error	0.9525	
R squared	0.9098	

Table 11- A and B values based on the first data fitting

Using the values of 'A' and 'B' in Table 11, a hyperbolic fit was overlaid on the data that was collected at 515 nm. This is seen in Figure 12 below. This curve does look somewhat

hyperbolic, this means that our data is reaching a horizontal asymptote of the maximum percent leakage.

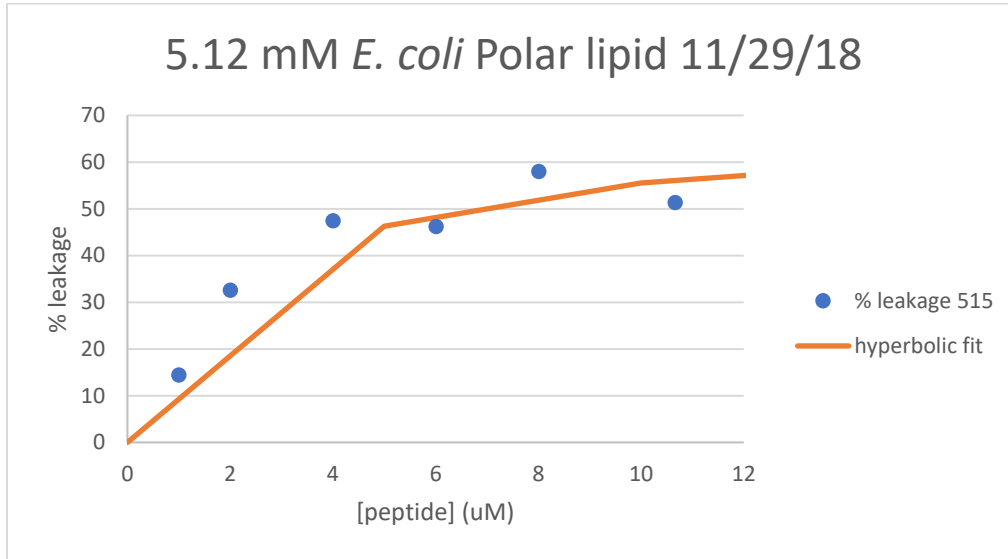


Figure 12- hyperbolic fit of measurement 3

To see if there could be more hyperbolic curve that fits this data, a second data fitting was performed. The parameter ‘A’ still shows the calculated maximum percent leakage and the parameter ‘B’ still shows the peptide concentration that produced half of the maximum leakage. However, now ‘A’ is set to equal 100. This should shift ‘B’ if ‘A’ wasn’t equal to 100 in the first data fitting. These calculated parameters, errors, and R squared values that shows how well the data fits the linear fit are shown in Table 12 below.

A	100
B	6.10314
error	0.868
R squared	0.769

Table 12- A and B values based on second data fitting

Lastly, an extra variable ‘n’ was added such that the new equation is:

$$\frac{100 * x^n}{B^n + x^n}$$

Using this equation, 'A' is set to 100. 'B' and 'n' parameters, their error values, and R squared value are calculated from the data and are shown in Table 17 below. Using the values of 'A' and 'B' in Table 12, a hyperbolic fit was overlaid on the data that was collected at 515 nm. This can be seen in Figure 13 below. This curve also shows hyperbolic characteristics, this means that our data is reaching a horizontal asymptote of the maximum percent leakage.

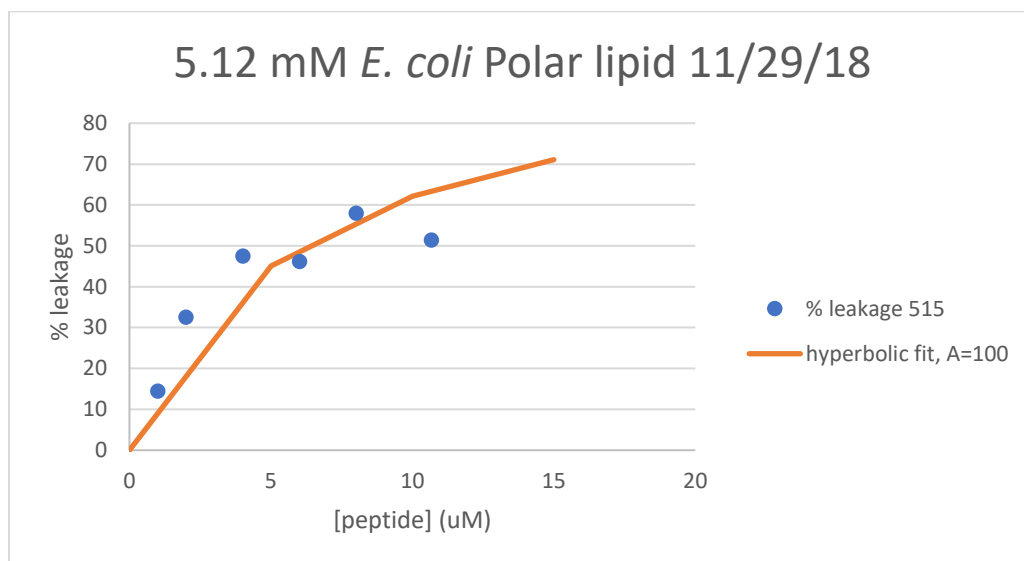


Figure 12- hyperbolic fit for the second data fitting

Using Table 13, the peptide concentration that would be required to achieve 50% leakage is 6.6  $\mu$ M. The sigmoidal fit has very little resemblance to a sigmoidal curve, this is a good indication that the peptide is not cooperative. This can be seen in Figure 14.

A	100
B	6.6367
error	1.162
n	0.708498
error	0.1588
R squared	0.8704

Table 13- A and B values Using third data fitting

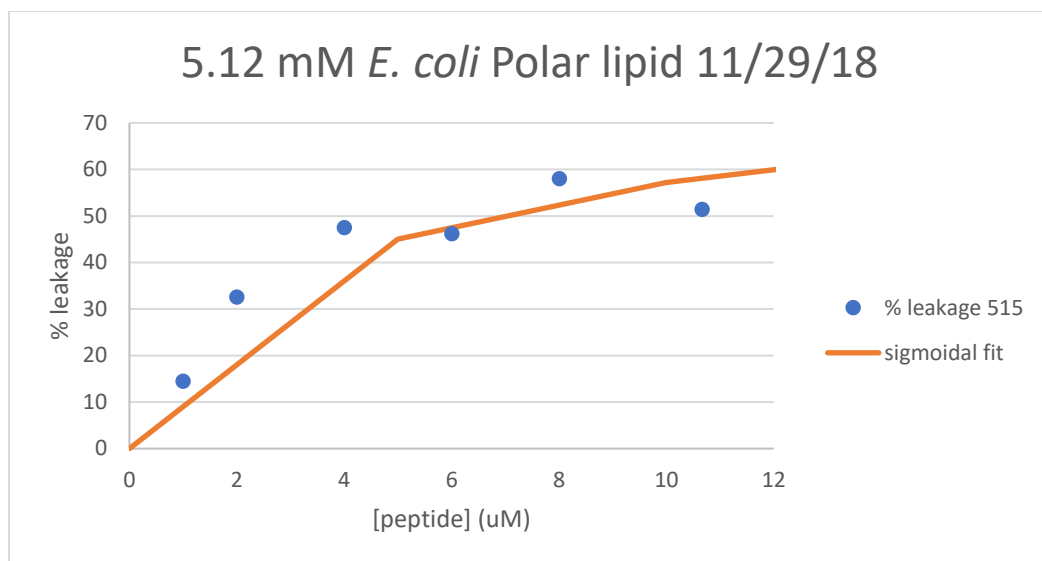


Figure 14- Sigmoidal Fit Using third data fitting

#### Measurements 4 – 1/30/19- 1/31/19

In order to increase our confidence in the reproducibility of our results, another set of measurements were taken. This measurement was done on a peptide with a concentration of 267  $\mu\text{M}$ . With a lipid composition of 5.18 mM *E. coli* Polar lipid. The solution was left in dialysis overnight, and the volume decreased from 0.97mL to 0.40mL. Table 14 shows the volume and concentration setup for the fluorometer measurements.

Sample	vol peptide ( $\mu\text{L}$ )	dilution	[peptide] ( $\mu\text{M}$ )	% leakage sum	% leakage max	% leakage 515
0uL Peptide V1	0	1	0.00	0.00	0.00	0.00
10uL Peptide V	15.98	1	10.67	67.15	68.18	67.45
25uL Peptide V1	37.45	1	25.00	73.72	74.57	74.91
50uL Peptide V	74.91	1	50.00	72.75	75.81	76.33
75uL Peptide V	112.36	1	75.00	80.86	82.25	80.88
100uL Peptide V	149.81	1	100.00	81.94	82.87	82.40

Table 14- Volume and concentration setup for the fluorometer measurements for measurement 4

The data collected in the fluorometer measurements was graphed showing the percent leakage against peptide concentration. Ideally, all three % leakage types should line up perfectly. If these are not the same then that indicates that the intensities were different depending on the

wavelength, that means some other factor is influencing the fluorescence besides the leakage. Therefore an error has been introduced into the measurement. This is shown in Figure 15.

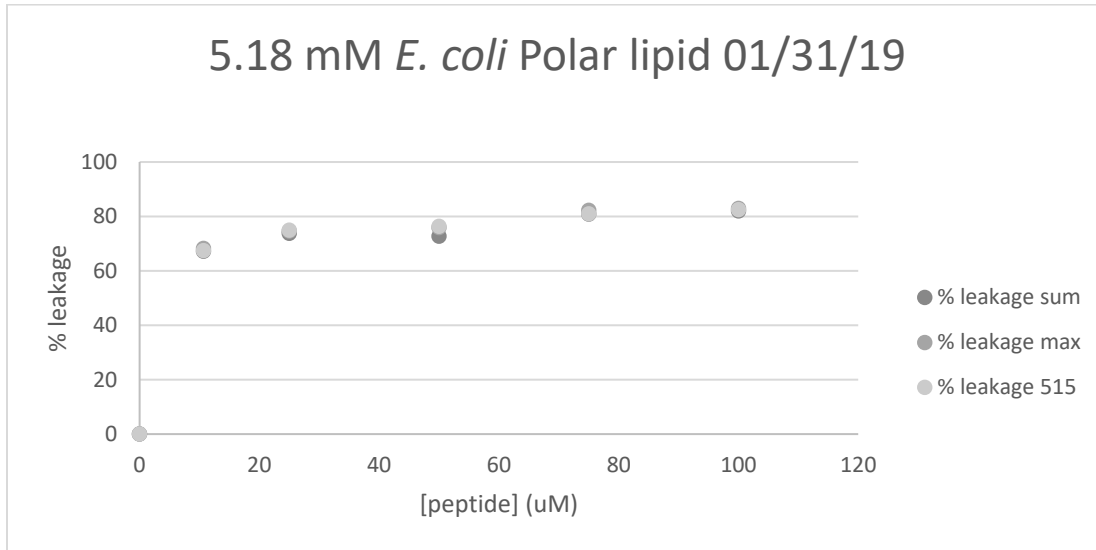


Figure 15- Percent leakage Vs. Peptide Concentration

As mentioned in measurement 1, this data was fitted into the following equation:

$$\frac{A * x}{B + x}$$

Where parameter 'A' shows the calculated maximum percent leakage and parameter 'B' shows the peptide concentration that produced half of the maximum leakage.

The calculated parameters, errors, and R squared values that shows how well the data fits the linear fit are shown in Table 15 below.

A	82.85911
error	1.205
B	2.53848
error	0.4088
R squared	0.998

Table 15- A and B values Using the first data fitting

Using the values of parameters ‘A’ and ‘B’ in Table 15, a hyperbolic fit was overlaid on the data that was collected at 515 nm. The maximum percent leakage for this measurement is 82.9%. That is a high percent leakage. This shows that the data and measurements are good as the maximum percent leakage that was achieved in this run is close to 100% leakage. This can be seen in Figure 16 below. This data showed an almost perfect hyperbolic fit. However, the data jumps from 0 to 60% leakage, this means the steepest and very important part of the hyperbolic curve is missing.

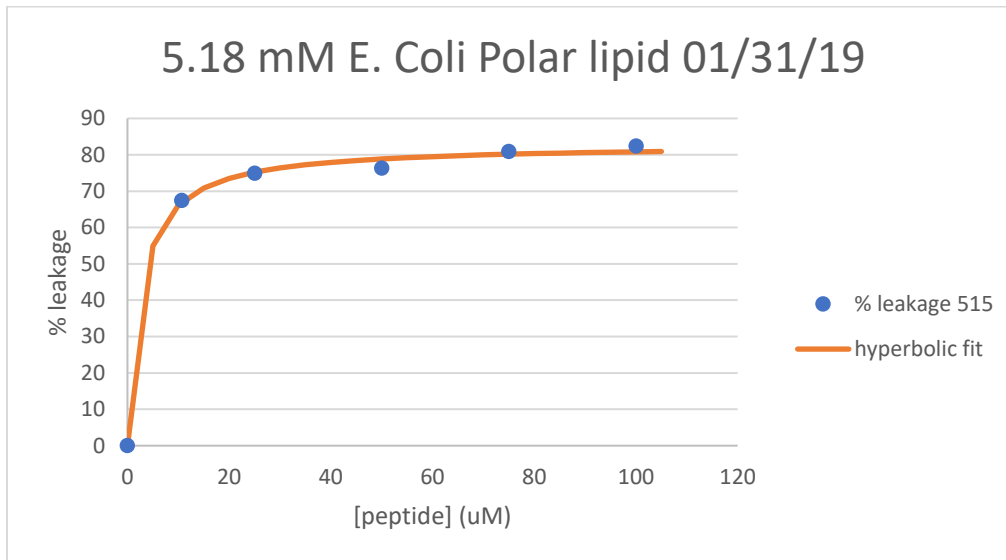


Figure 16- hyperbolic fit for first data fitting

Now ‘A’ is set to equal 100 as shown here:

$$\frac{100 * x}{B + x}$$

This should shift ‘B’ if ‘A’ wasn’t equal to 100 in the first data fitting.

The calculated parameters, errors, and R squared values that shows how well the data fits the linear fit are shown in Table 16 below. The correlation between the data and hyperbolic fit was so strong the value of R<sup>2</sup> is very close to 1.

A	100
B	8.259086
error	2.098
R squared	0.9206

Table 16 - A and B values Using the second data fitting

Using the values of parameter ‘A’ and ‘B’ in Table 16, a hyperbolic fit was overlaid on the data that was collected at 515 nm. This can be seen in Figure 17 below. This hyperbolic relationship is less obvious than the first data fitting. This could be because there were less parameters (because parameter ‘A’ is preset to 100).

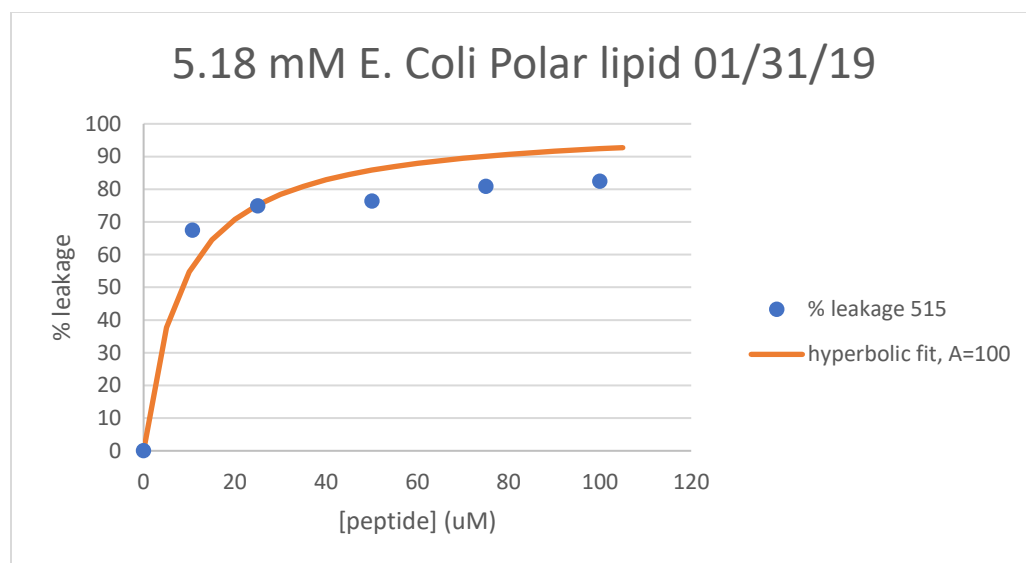


Figure 17- hyperbolic fit Using second data fitting

Lastly, an extra variable ‘n’ was added such that the new equation is:

$$\frac{100 * x^n}{B^n + x^n}$$

Using this equation, ‘A’ is set to 100. ‘B’ and ‘n’ parameters, their error values, and R squared value are calculated from the data and are shown in Table 17 below. The peptide concentration that would be required to achieve 50 percent leakage is 1.26 μM. With such a low error value and an almost perfect R<sup>2</sup> value, we are very confident in these results.

A	100
B	1.258023
error	0.4049
n	0.345811
error	0.03347
R squared	0.999

Table 17- A and B values for the third data fitting

Using the values of 'A', 'B', and 'n' in Table 17, a sigmoidal fit was overlaid on the data that was collected at 515 nm. This can be seen in Figure 18 below. Since the value of n in Table 17 is less than 1 that is equivalent to having the square root of the equation in the second data fitting. This means that the fit isn't sigmoidal and is actually hyperbolic. This shows that there is no binding cooperation between the peptides. That means that initial binding and leakage does not facilitate further binding and leakage.

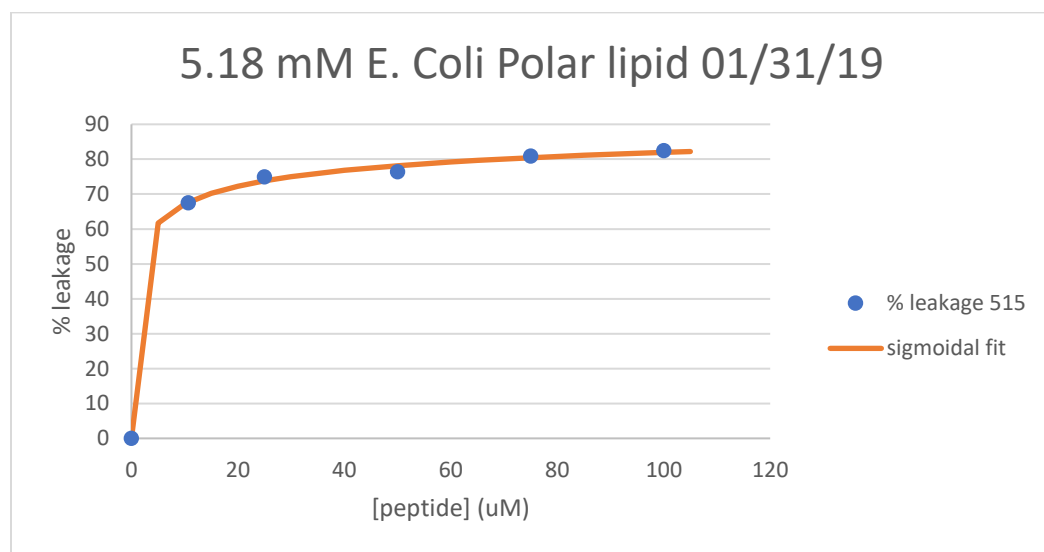


Figure 18- Sigmoidal Fit Using third data fitting

### **Measurement 5 – 2/19/19-2/20/19**

In order to test the leakage induced by Maximin 3 with a concentration of 413.4  $\mu$ M on *E. coli* vesicles, a set of measurements were taken. With a lipid composition of 5.16 mM *E. coli*

Polar lipid. The solution was left in dialysis overnight, the volume decreased from 0.90mL to 0.76mL. Table 18 shows the volume and concentration setup for the fluorometer measurements.

Sample	vol peptide (μL)	dilution	[peptide] (μM)	% leakage sum	% leakage max	% leakage 515
10uM Peptide V4	9.67	1	9.99	82.93	84.71	84.61
25uM Peptide V	24.19	1	25.00	85.09	86.21	85.94
50uM Peptide V	48.38	1	50.00	88.55	89.39	89.11
75uM Peptide V1	72.57	1	75.00	89.07	89.77	89.54
100uM Peptide V	96.76	1	100.00	90.71	91.69	91.55

Table 18- Volume and concentration setup for the fluorometer measurements for measurement 5

The data collected in the fluorometer measurements was graphed showing the percent leakage against peptide concentration. Ideally, all three % leakage types should line up perfectly. If these are not the same ten that indicates that the intensities were different depending on the wavelength, that means some other factor is influencing the fluorescence besides the leakage. Therefor an error has been introduced into the measurement. This is shown in Figure 19.

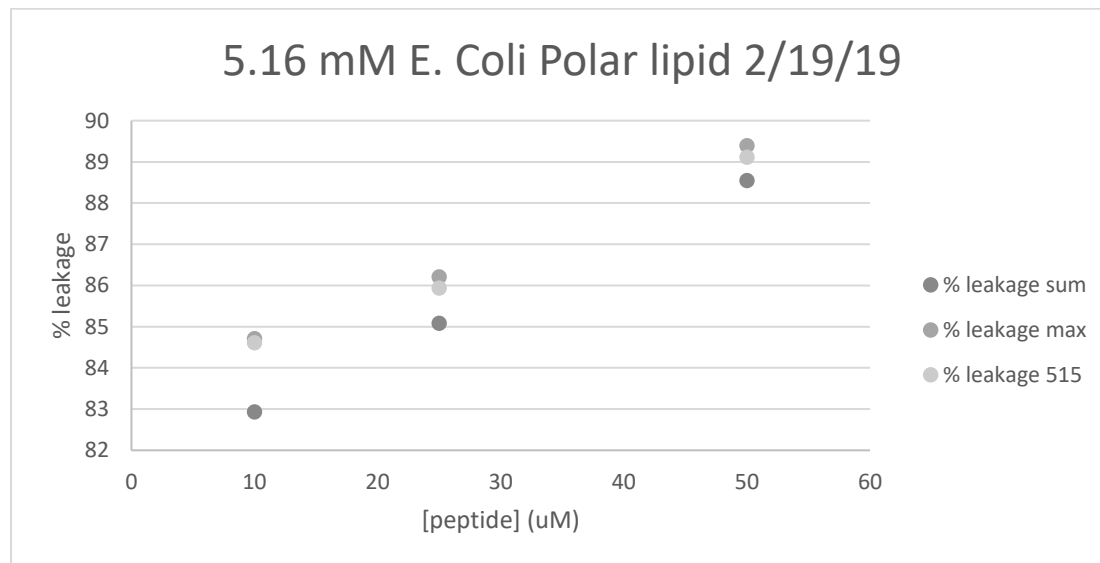


Figure 19- Percent leakage Vs. Peptide Concentration

As mentioned in measurement 1, this data was fitted into the following equation:

$$\frac{A * x}{B + x}$$

Where parameter ‘A’ shows the calculated maximum percent leakage and B shows the peptide concentration that produced half of the maximum leakage. As shown in Table 19, the maximum percent leakage for this measurement is 90.7%. That is a high percent leakage. This might indicate good data, however, when examining the B value, it is seen that this is not the case. A peptide concentration of 0.8  $\mu\text{M}$  probably doesn’t achieve half of the 90% leakage.

A	90.72639
error	1.037
B	0.816361
error	0.2568
R squared	0.7888

Table 19- A and B values Using the first data fitting

Using the values of parameter ‘A’ and ‘B’ in Table 19, a hyperbolic fit was overlaid on the data that was collected at 515 nm. This can be seen in Figure 20 below. This data showed an almost perfect hyperbolic fit. However, the data starts at 60% leakage, this means the steepest and very important part of the hyperbolic curve is missing.

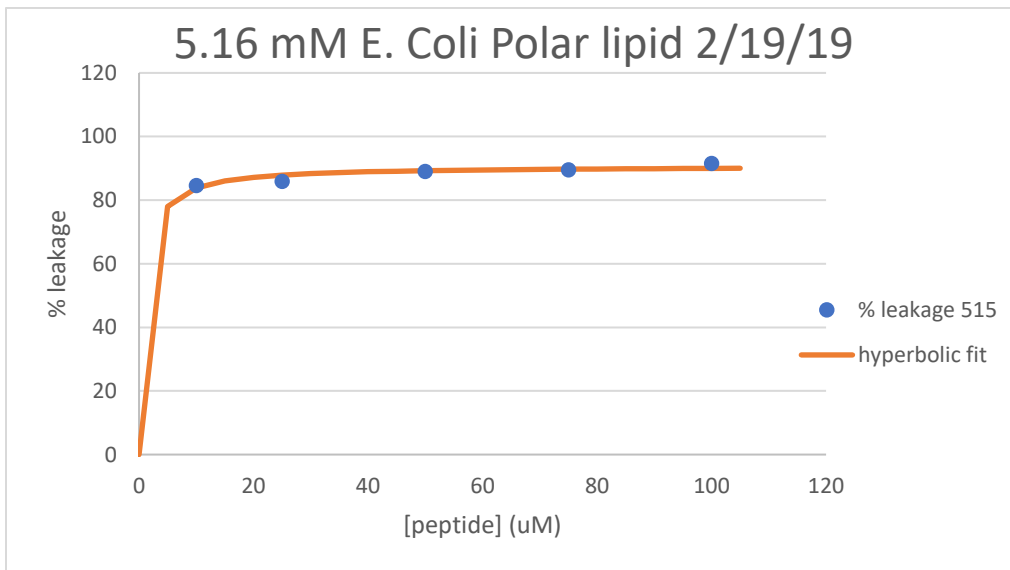


Figure 20- hyperbolic fit for the data fitting

Now parameter 'A' is set to equal 100. This should shift 'B' if parameter 'A' wasn't equal to 100 in the first data fitting. This can be shown in Table 20. The correlation between the data and hyperbolic fit was so insignificant the actual value could not be calculated by our program.

A	100
B	2.743619
error	0.8832
R squared	N/A

Table 20- A and B values Using the second data fitting

Using the values of parameter 'A' and 'B' in Table 20, a hyperbolic fit was overlaid on the data that was collected at 515 nm. This can be seen in Figure 21 below.

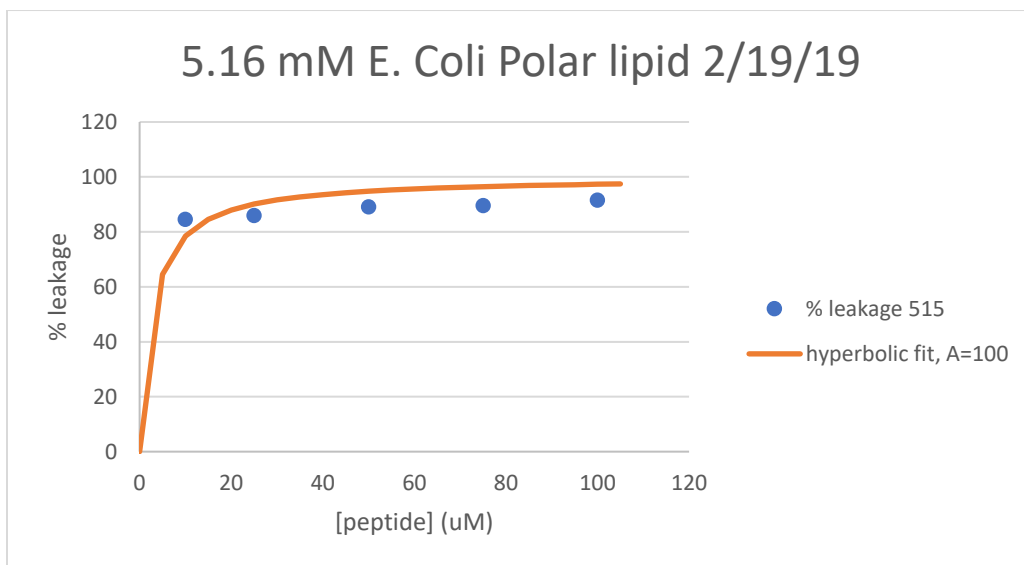


Figure 21- hyperbolic fit Using second data fitting

Lastly, an extra variable 'n' was added such that the new equation is:

$$\frac{100 * x^n}{B^n + x^n}$$

Using this equation, 'A' is set to 100. 'B' and 'n' parameters, their error values, and R squared value are calculated from the data and are shown in Table 21 below.

Using Table 21, the peptide concentration that would be required to achieve half of 100 percent leakage is 0.019  $\mu\text{M}$ . Based on the small error rate and high  $R^2$  value, we are confident in this data.

A	100
B	0.019123
error	0.02406
n	0.265404
error	0.0452
R squared	0.9196

Table 21- A and B values for the third data fitting

Using the values of ‘A’, ‘B’, and ‘n’ in Table 21, a sigmoidal fit was overlaid on the data that was collected at 515 nm. This can be seen in Figure 22 below. Since the value of ‘n’ in Table 21 is less than 1, this is equivalent to having the same equation as the square root of the second data fitting equation. This means that the fit isn’t sigmoidal and is actually hyperbolic. This shows that there is no binding cooperation between the peptides. That means that initial binding and leakage does not facilitate further binding and leakage.

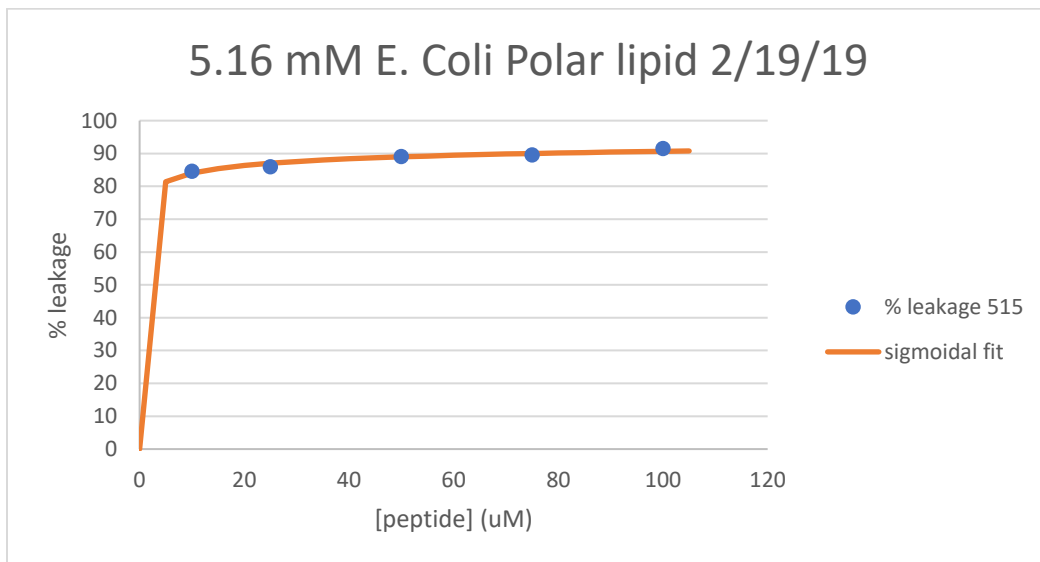


Figure 22- sigmoidal fit Using third data fitting

**Measurement 6- 3/08/19-3/09/19**

In order to increase our confidence in the reproducibility, another set of measurements were taken. This measurement was done on a peptide with a concentration of 413.4  $\mu\text{M}$ . With a lipid composition of 4.21 mM *E. coli* Polar lipid. The solution was left in dialysis overnight, and the volume increased from 0.80mL to 0.95mL. Table 22 shows the volume and concentration setup for the fluorometer measurements.

Sample	vol peptide ( $\mu\text{L}$ )	dilution	[peptide] ( $\mu\text{M}$ )	% leakage sum	% leakage max	% leakage 515
0uM Peptide V1	0	1	0.00	0.00	0.00	0.00
2uM Peptide V	1.96	1	2.03	34.29	36.19	36.20
4uM Peptide V	3.87	1	4.00	42.64	44.60	45.12
6uM Peptide V	5.81	1	6.00	52.22	55.13	55.18
8uM Peptide V	7.74	1	8.00	56.44	59.20	59.02
10uM Peptide V	9.67	1	9.99	73.90	77.00	77.50
25uM Peptide V	24.2	1	25.01	66.68	69.41	69.66
50uM Peptide V	48.4	1	50.02	83.68	85.71	84.89
75uM Peptide V	72.6	1	75.03	84.50	86.53	86.86
100uM Peptide V3	96.8	1	100.04	85.25	86.61	86.91

Table 22- Volume and concentration setup for the fluorometer measurements for measurement 6

The data collected in the fluorometer measurements was graphed showing the percent leakage against peptide concentration. Ideally, all three % leakage types should line up perfectly. If these are not the same then that indicates that the intensities were different depending on the wavelength, that means some other factor is influencing the fluorescence besides the leakage. Therefore an error has been introduced into the measurement. This is shown in Figure 23.

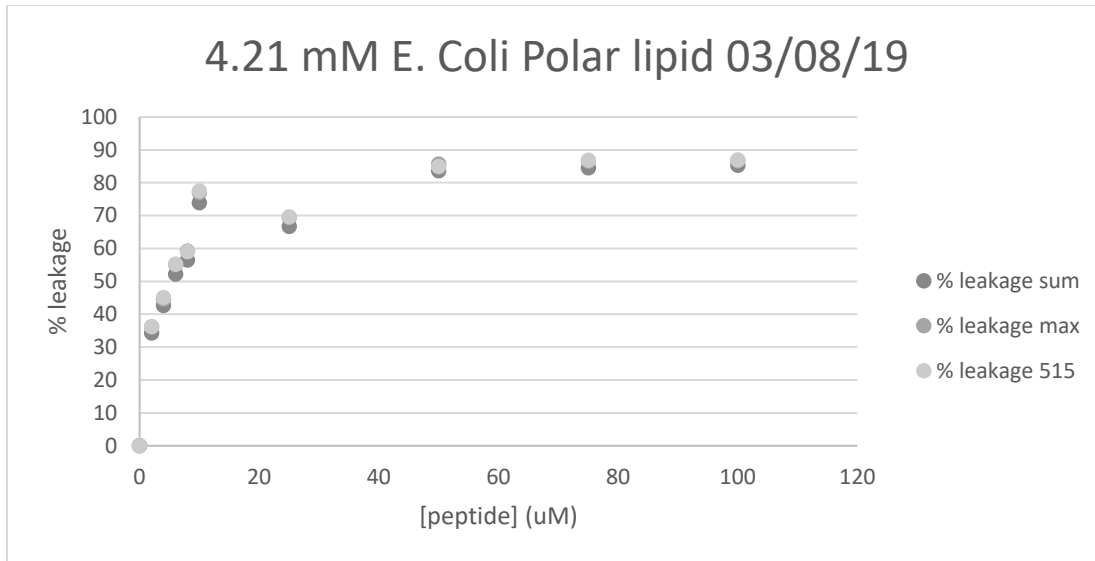


Figure 23- Percent leakage Vs. Peptide Concentration

As mentioned in measurement 1, this data was fitted into the following equation:

$$\frac{A * x}{B + x}$$

Where parameter ‘A’ shows the calculated maximum percent leakage and parameter ‘B’ shows the peptide concentration that produced half of the maximum leakage. The calculated parameters, errors, and R squared values that shows how well the data fits the linear fit are shown in Table 23 below.

As shown in Table 23, the maximum percent leakage for this measurement is 88.9%. That is a high percent leakage. And the peptide concentration required to get to half of that percent leakage is about 3.36 μM.

A	88.92285
error	3.422
B	3.357408
error	0.5835
R squared	0.9656

Table 23- A and B values Using the first data fitting

Using the values of parameter ‘A’ and ‘B’ in Table 23, a hyperbolic fit was overlaid on the data that was collected at 515 nm. This can be seen in Figure 24 below. The hyperbolic relationship is pretty strong, although there are some faulty data around 10 and 25  $\mu\text{M}$  peptide concentrations, overall the data has a hyperbolic shape.

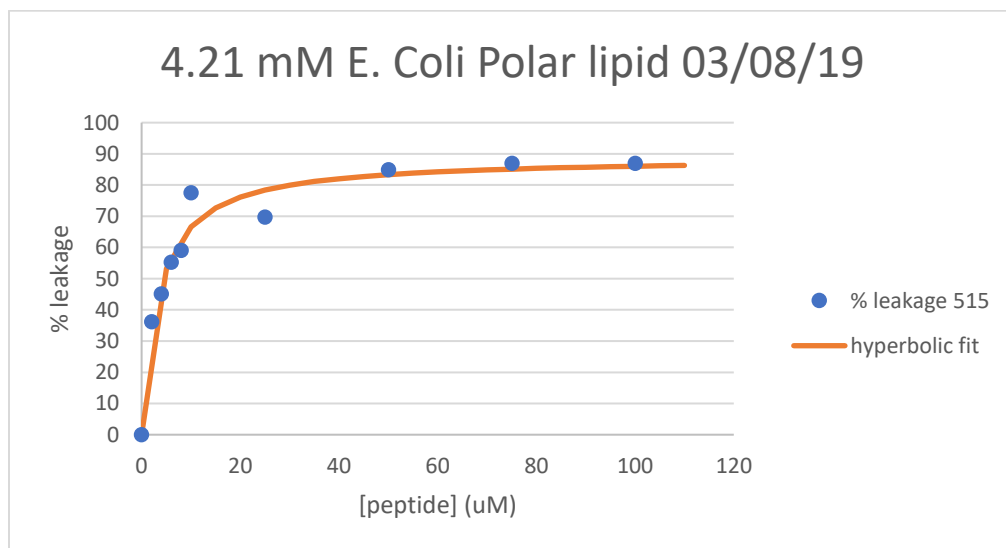


Figure 23- hyperbolic fit for first data fitting

Now parameter ‘A’ is set to equal 100. This should and did shift the B value if parameter ‘A’ wasn’t equal to 100 in the first data fitting. The calculated parameters, errors, and R squared values that shows how well the data fits the linear fit are shown in Table 24.

A	100
B	4.91662
error	0.6809
R squared	0.9246

Table 24- A and B values Using the second data fitting

Using the values of parameter ‘A’ and B in Table 24, a hyperbolic fit was overlaid on the data that was collected at 515 nm. This can be seen in Figure 25 below.

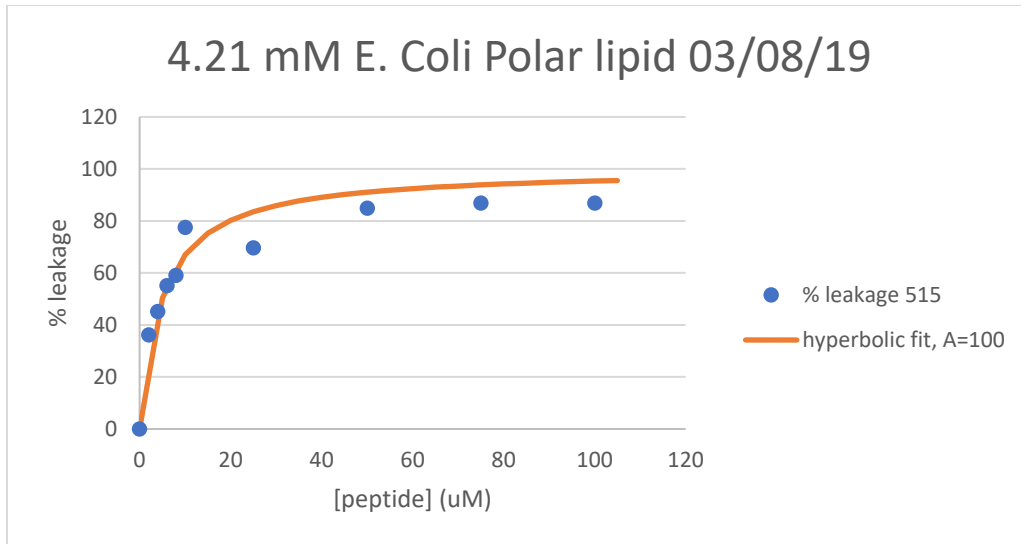


Figure 24- hyperbolic fit Using second data fitting

Lastly, an extra variable 'n' was added such that the new equation is:

$$\frac{100 * x^n}{B^n + x^n}$$

Using this equation, 'A' is set to 100. 'B' and 'n' parameters, their error values, and R squared value are calculated from the data and are shown in Table 25 below. The peptide concentration that would be required to achieve half of 100 percent leakage is 4.26 μM. Based on the large error rate we are not confident in this data.

A	100
B	4.275874
error	0.706
n	0.067248
error	0.09422
R squared	0.9624

Table 25- A and B values for the third data fitting

Using the values of 'A', 'B', and 'n' in Table 25, a sigmoidal fit was overlaid on the data that was collected at 515 nm. This can be seen in Figure 26 below. Since the value of 'n' in Table 25 is less than 1, this is equivalent to having the square root of the second data fitting. This

means that the fit isn't sigmoidal and is actually hyperbolic. This shows that there is no binding cooperation between the peptides. That means that initial binding and leakage does not facilitate further binding and leakage.

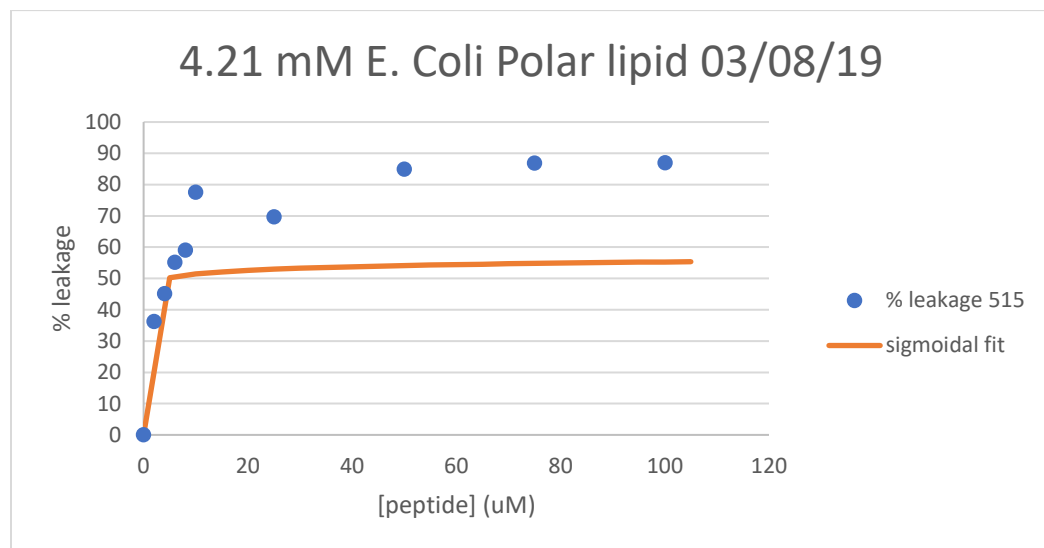


Figure 25- Sigmoidal fit Using third data fitting

**Measurement 7- 3/21/19-3/22/19**

In order to increase our confidence in the reproducibility, another set of measurements were taken. This measurement was done on a peptide with a concentration of 413.4  $\mu\text{M}$ . With a lipid composition of 5.50 mM *E. coli* Polar lipid. The solution was left in dialysis overnight, and the volume decreased from 0.82mL to 0.74mL. Table 26 shows the volume and concentration setup for the fluorometer measurements.

Sample	vol peptide ( $\mu\text{L}$ )	dilution	[peptide] ( $\mu\text{M}$ )	% leakage sum	% leakage max	% leakage 515
0uM Peptide V1	0	1	0.00	0.00	0.00	0.00
2uM Peptide V	1.96	1	2.03	15.78	17.33	16.95
4uM Peptide V1	3.87	1	4.00	38.38	40.08	39.95
6uM Peptide V	5.81	1	6.00	52.77	54.70	54.63
8uM Peptide V1	7.74	1	8.00	64.47	67.30	67.05
10uM Peptide V	9.67	1	9.99	68.54	70.61	70.46
25uM Peptide V	24.2	1	25.01	78.26	79.47	79.38

50uM Peptide V	48.4	1	50.02	83.68	84.34	84.08
75uM Peptide V	72.6	1	75.03	88.15	89.69	89.31
100uM Peptide V	96.8	1	100.04	86.29	87.82	88.07

Table 26- Volume and concentration setup for the fluorometer measurements for measurement 6

The data collected in the fluorometer measurements was graphed showing the percent leakage against peptide concentration. Ideally, all three % leakage types should line up perfectly. If these are not the same then that indicates that the intensities were different depending on the wavelength, that means some other factor is influencing the fluorescence besides the leakage. Therefore an error has been introduced into the measurement. This is shown in Figure 27.

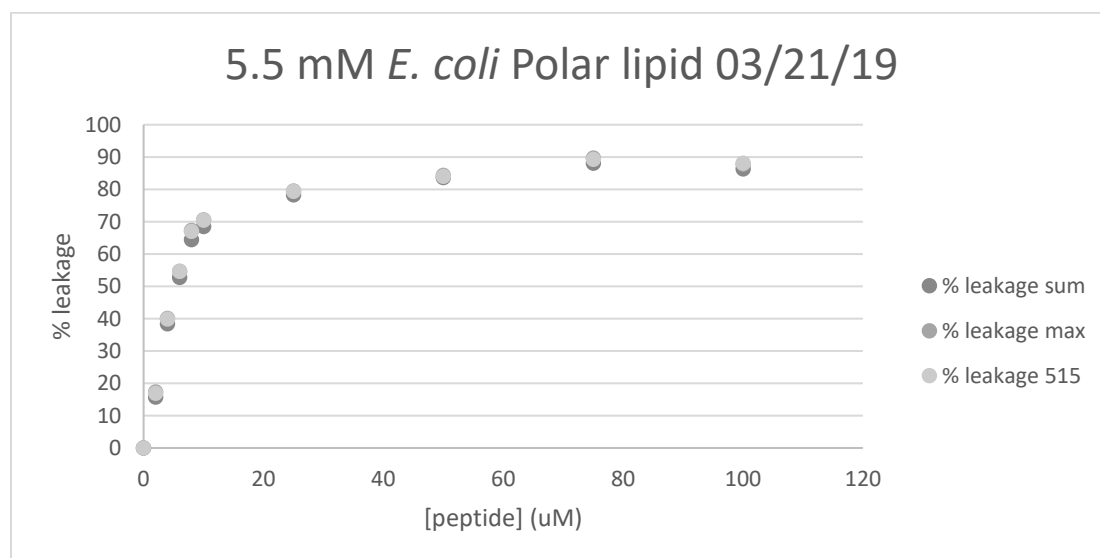


Figure 27- Percent leakage Vs. Peptide Concentration

As mentioned in measurement 1, this data was fitted into the following equation:

$$\frac{A * x}{B + x}$$

Where parameter 'A' shows the calculated maximum percent leakage and parameter 'B' shows the peptide concentration that produced half of the maximum leakage. As shown in Table 27, the maximum percent leakage for this measurement is 94.9%. That is a high percent leakage. And the peptide concentration required to get to half of that percent leakage is about 4.77  $\mu$ M.

A	94.9295
error	3.828
B	4.771
error	0.7711
R squared	0.9708

Table 27- A and B values Using the first data fitting

Using the values of parameter ‘A’ and ‘B’ in Table 27, a hyperbolic fit was overlaid on the data that was collected at 515 nm. This can be seen in Figure 28 below. The hyperbolic relationship is strong, overall the data has a hyperbolic shape.

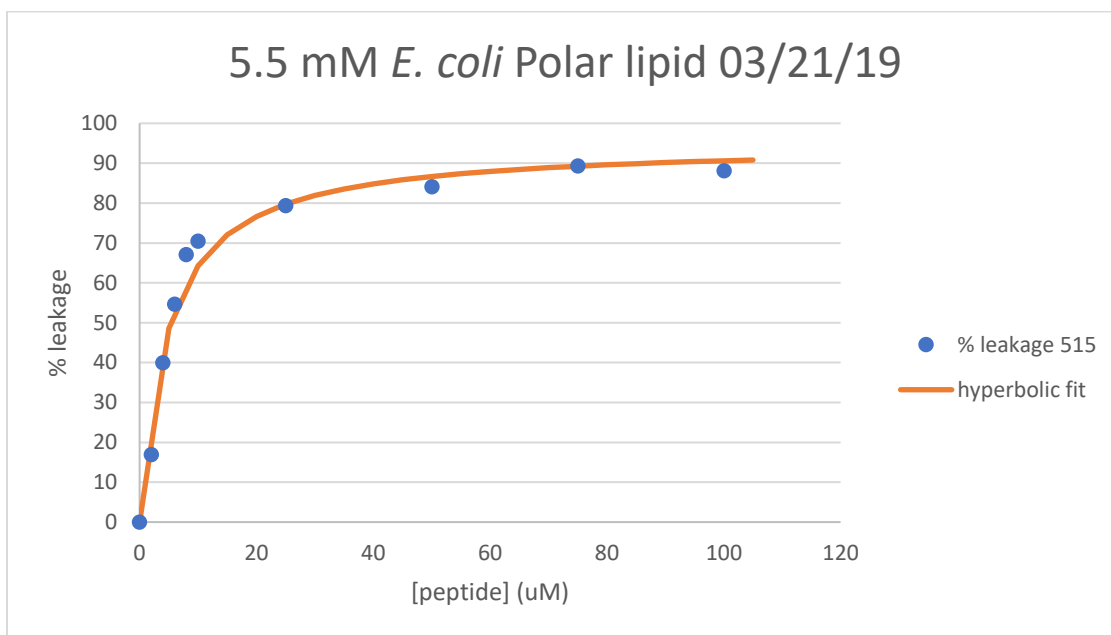


Figure 28- hyperbolic fit for first data fitting

Now parameter ‘A’ is set to equal 100. This should and did shift the ‘B’ value if parameter ‘A’ wasn’t equal to 100 in the first data fitting. The calculated parameters, errors, and R squared values that shows how well the data fits the linear fit are shown in Table 28.

A	100
B	5.52267
error	0.5834
R squared	0.9642

Table 28- A and B values Using the second data fitting

Using the values of parameter ‘A’ and ‘B’ in Table 28, a hyperbolic fit was overlaid on the data that was collected at 515 nm. This can be seen in Figure 29 below.

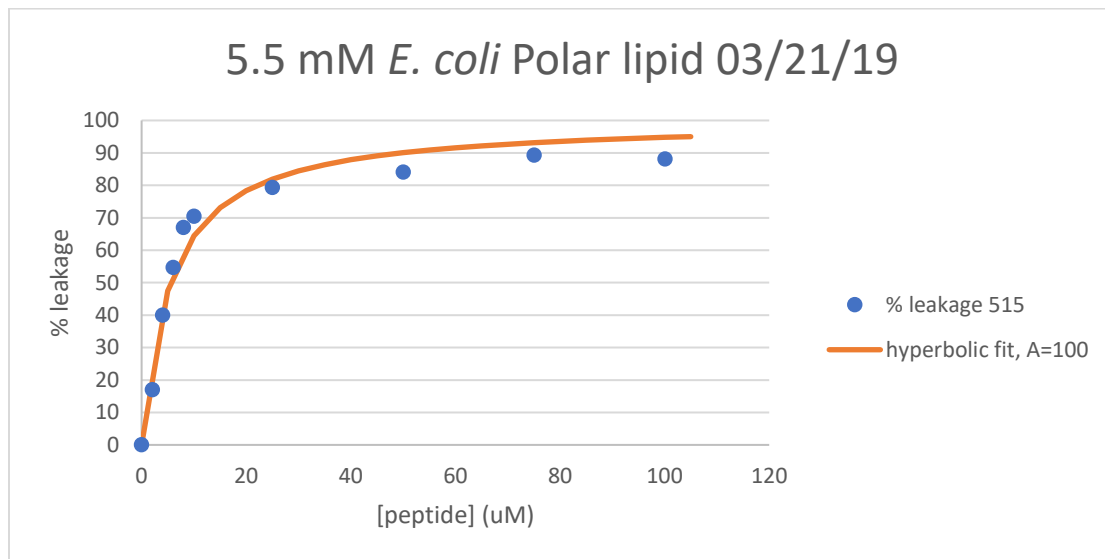


Figure 29- hyperbolic fit Using second data fitting

Lastly, an extra variable ‘n’ was added such that the new equation is:

$$\frac{100 * x^n}{B^n + x^n}$$

Using this equation, ‘A’ is set to 100. ‘B’ and ‘n’ parameters, their error values, and R squared value are calculated from the data and are shown in Table 29 below.

Using Table 29, the peptide concentration that would be required to achieve half of 100 percent leakage is 5.54 μM. Based on the small error rate and high R<sup>2</sup> value, we are confident in this data.

A	100
B	5.536615
error	0.631
n	1.023919
error	0.1447
R squared	0.9643

Table 29- A and B values for the third data fitting

Using the values of ‘A’, ‘B’, and ‘n’ in Table 29, a sigmoidal fit was overlaid on the data that was collected at 515 nm. This can be seen in Figure 30 below. Since the value of ‘n’ in Table 29 is 1 that is equivalent to having the same equation in the second data fitting. This means that the fit isn’t sigmoidal and is actually hyperbolic. This shows that there is no binding cooperation between the peptides. That means that initial binding and leakage does not facilitate further binding and leakage.

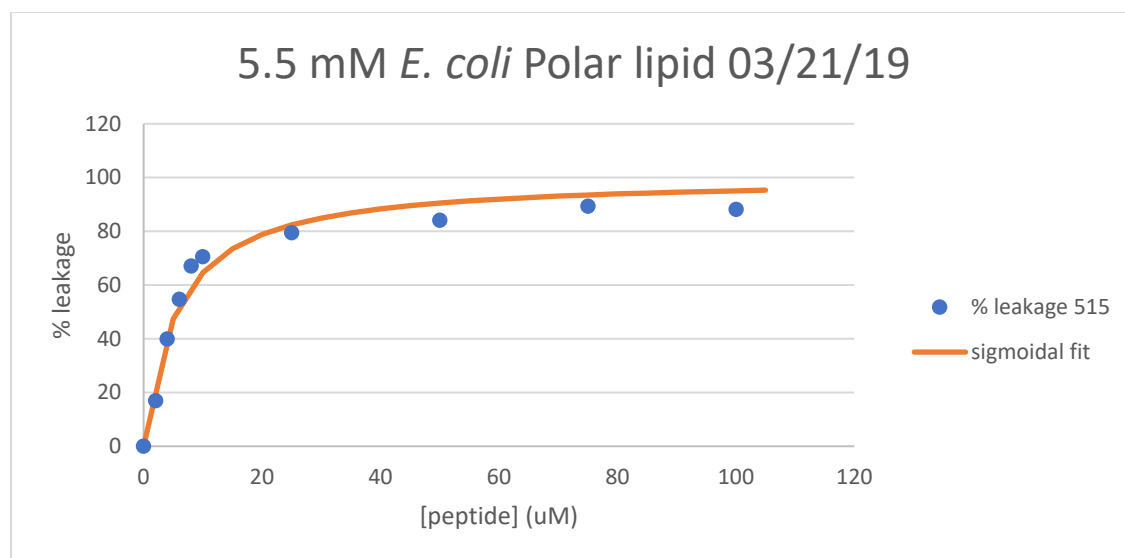


Figure 30- Sigmoidal fit Using third data fitting

**Measurement 8- 4/14/19-4/15/19**

In order to increase our confidence in the reproducibility, another set of measurements were taken. This measurement was also done on a peptide with a concentration of 413.4  $\mu\text{M}$ . With a lipid composition of 5.23 mM *E. coli* Polar lipid. The solution was left in dialysis overnight, the volume decreased from 0.96 mL to 0.918mL. Table 30 shows the volume and concentration setup for the fluorometer measurements.

Sample	vol peptide ( $\mu\text{L}$ )	dilution	[peptide] ( $\mu\text{M}$ )	% leakage sum	% leakage max	% leakage 515
2mM Peptide V	1.96	1	2.03	16.35	18.14	18.43

4mM Peptide V	3.87	1	4.00	28.60	31.27	30.80
6mM Peptide V	5.81	1	6.00	37.63	40.79	41.84
8mM Peptide V	7.74	1	8.00	48.31	52.66	52.75
10mM Peptide V	9.67	1	9.99	55.89	58.26	58.11
25mM Peptide V1	24.2	1	25.01	65.87	71.11	71.09
50mM Peptide V	48.4	1	50.02	93.98	92.22	92.14
75mM Peptide V	72.6	1	75.03	82.37	83.53	83.34
100mM Peptide V	96.8	1	100.04	83.96	86.01	86.04

Table 30- Volume and concentration setup for the fluorometer measurements for measurement 8

The data collected in the fluorometer measurements was graphed showing the percent leakage against peptide concentration. Ideally, all three % leakage types should line up perfectly. If these are not the same then that indicates that the intensities were different depending on the wavelength, that means some other factor is influencing the fluorescence besides the leakage. Therefore an error has been introduced into the measurement. This is shown in Figure 31.

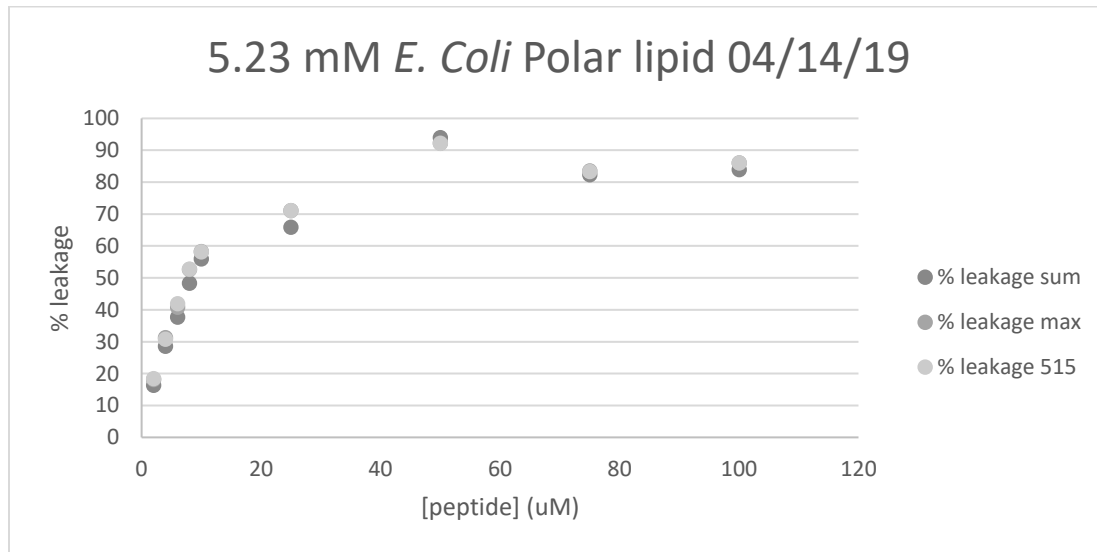


Figure 31- Percent leakage Vs. Peptide Concentration

As mentioned in measurement 1, this data was fitted into the following equation:

$$\frac{A * x}{B + x}$$

While parameter ‘A’ shows the calculated maximum percent leakage and parameter ‘B’ shows the peptide concentration that produced half of the maximum leakage. As shown in Table 31, the maximum percent leakage for this measurement is 95.7%. That is a high percent leakage. And the peptide concentration required to get to half of that percent leakage is about 7.33  $\mu\text{M}$ . The calculated parameters, errors, and R squared values that shows how well the data fits the linear fit are shown in Table 31 below.

A	95.69297
error	3.457
B	7.334836
error	0.9441
R squared	0.9737

Table 31- A and B values Using the first data fitting

Using the values of parameter ‘A’ and ‘B’ in Table 31, a hyperbolic fit was overlaid on the data that was collected at 515 nm. This can be seen in Figure 32 below. The hyperbolic relationship is pretty strong, although there are some faulty data around 50  $\mu\text{M}$  peptide concentrations, overall the data has a hyperbolic shape.

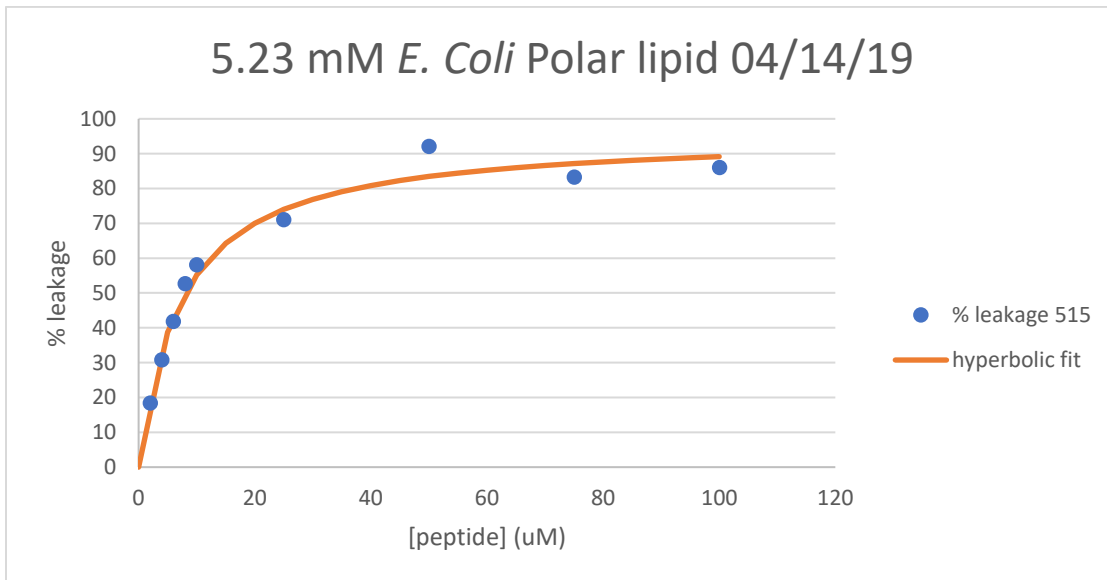


Figure 32- hyperbolic fit for first data fitting

Now parameter 'A' is set to equal 100 as shown here:

$$\frac{100 * x}{B + x}$$

This should shift 'B' if 'A' wasn't equal to 100 in the first data fitting.

The calculated parameters, errors, and R squared values that shows how well the data fits the linear fit are shown in Table 32.

A	100
B	8.256229
error	0.6757
R squared	0.968

Table 32- A and B values Using the second data fitting

Using the values of parameter 'A' and 'B' in Table 32, a hyperbolic fit was overlaid on the data that was collected at 515 nm. This can be seen in Figure 33 below.

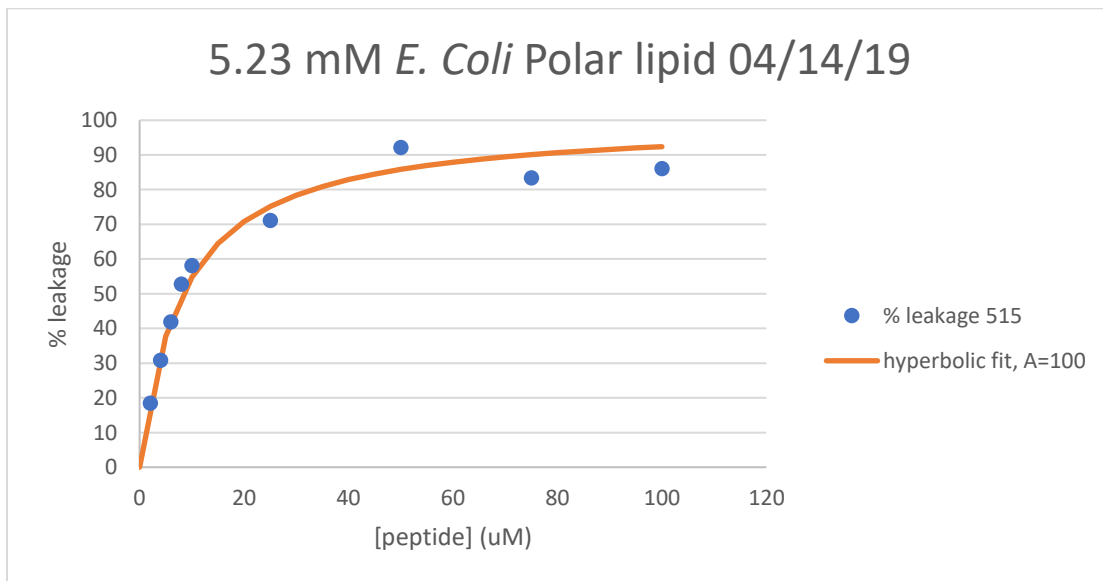


Figure 33- hyperbolic fit Using second data fitting

Lastly, an extra variable 'n' was added such that the new equation is:

$$\frac{100 * x^n}{B^n + x^n}$$

Using this equation, ‘A’ is set to 100. ‘B’ and ‘n’ parameters, their error values, and R squared value are calculated from the data and are shown in Table 33 below.

Using Table 33, the peptide concentration that would be required to achieve half of 100 percent leakage is 8.25 μM. Based on the small error rate and high R<sup>2</sup> value, we are confident in this data.

A	100
B	8.250601
error	0.7369
n	0.949171
error	0.09326
R squared	0.9691

*Table 33- A and B values for the third data fitting*

Using the values of ‘A’, ‘B’, and ‘n’ in Table 33, a sigmoidal fit was overlaid on the data that was collected at 515 nm. This can be seen in Figure 34 below. Since the value of ‘n’ in Table 33 is 1 that is equivalent to having the same equation in the second data fitting. This means that the fit isn’t sigmoidal and is actually hyperbolic. This shows that there is no binding cooperation between the peptides. That means that initial binding and leakage does not facilitate further binding and leakage.

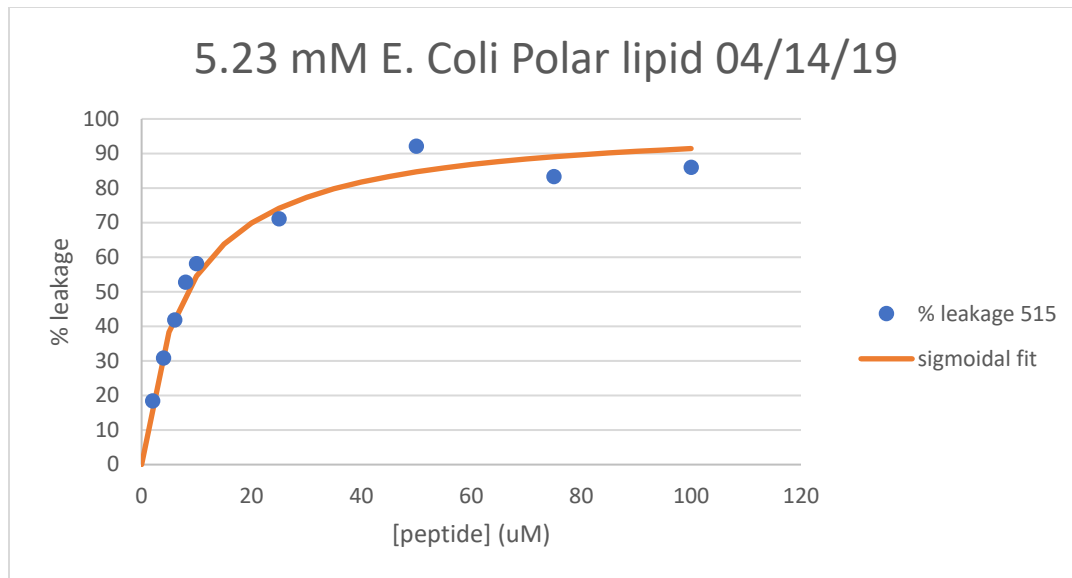


Figure 34- Sigmoidal fit Using third data fitting

**Measurements at various temperatures:**

A third variable was introduced to the data analysis, temperature. The new temperatures that were introduced are 20°C and 30°C. The goal was to widen the range of temperatures measured to include 20-42°C, the low temperatures are important because *Bombina maxima* is a cold blooded toad, thus it is important to know if the peptide will work as well as it does in its original temperature. The high temperatures (37-42°C) are important because 37°C is normal body temperature and 40-42°C are fever temperatures, it is important to test fever temperatures as well as normal body temperatures because if these AMP's replace antibiotics they would be given to some patients with a fever.

**Measurement 9- 4/17/19-3/18/19 - 20°C**

In order to test the leakage induced by Maximin 3 on *E. coli* vesicles at 20°C, a set of measurements were taken. This measurement was done on a peptide with a concentration of 413.4 μM. with a lipid composition of 4.58 mM *E. coli* Polar lipid. The solution was left in

dialysis overnight, the volume increased from 0.70mL to 0.764mL. Table 34 shows the volume and concentration setup for the fluorometer measurements.

Sample	vol peptide (μL)	dilution	[peptide] (μM)	% leakage sum	% leakage max	% leakage 515
0mM Peptide V	0	1	0.00	2.39	2.82	2.62
2mM Peptide V1	1.96	1	2.03	22.11	24.89	24.54
4mM Peptide V	3.87	1	4.00	35.51	38.57	38.65
6mM Peptide V	5.81	1	6.00	43.59	46.76	46.96
8mM Peptide V	7.74	1	8.00	50.85	54.26	54.33
10mM Peptide V1	9.67	1	9.99	59.69	62.93	62.75
25mM Peptide V	24.2	1	25.01	75.86	78.19	78.18
50mM Peptide V	48.4	1	50.02	79.65	82.03	81.72
75mM Peptide V	72.6	1	75.03	86.40	88.84	88.45
100mM Peptide V	96.8	1	0.00	2.39	2.82	2.62

Table 34- Volume and concentration setup for the fluorometer measurements for measurement 8

The data collected in the fluorometer measurements was graphed showing the percent leakage against peptide concentration. Ideally, all three % leakage types should line up perfectly. If these are not the same then that indicates that the intensities were different depending on the wavelength, that means some other factor is influencing the fluorescence besides the leakage. Therefore an error has been introduced into the measurement. This is shown in Figure 35.

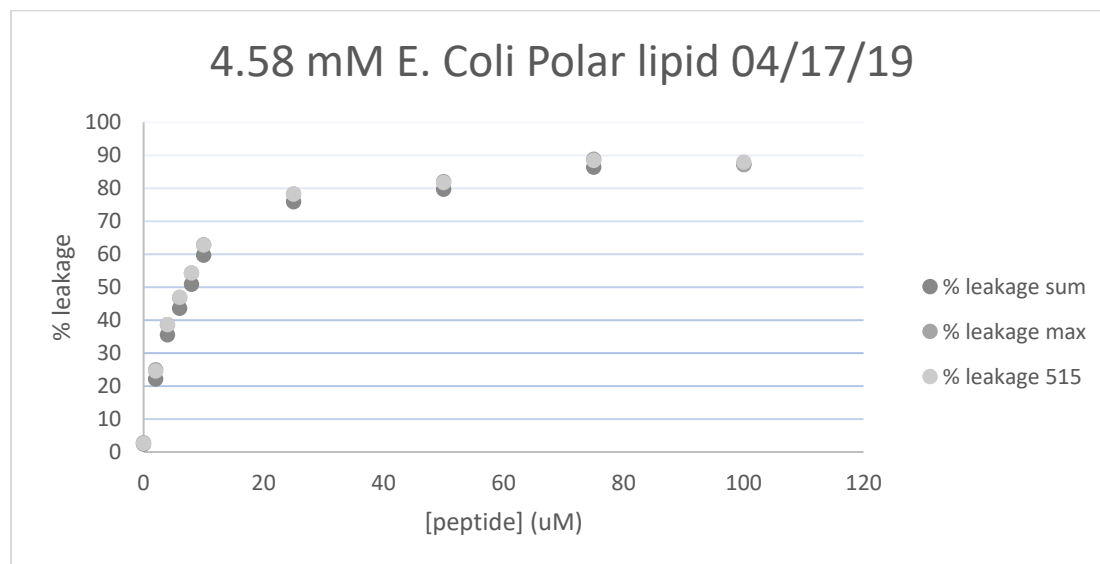


Figure 35- Percent leakage Vs. Peptide Concentration

As mentioned in measurement 1, this data was fitted into the following equation:

$$\frac{A * x}{B + x}$$

While parameter ‘A’ shows the calculated maximum percent leakage and parameter ‘B’ shows the peptide concentration that produced half of the maximum leakage. As shown in Table 35, the maximum percent leakage for this measurement is 93.6%. That is a high percent leakage. And the peptide concentration required to get to half of that percent leakage is about 5.60  $\mu\text{M}$ .

A	93.60716
error	1.348
B	5.596607
error	0.3082
R squared	0.9962

*Table 35- A and B values Using the first data fitting*

Using the values of ‘A’ and ‘B’ in Table 35, a hyperbolic fit was overlaid on the data that was collected at 515 nm. This can be seen in Figure 36 below. The hyperbolic relationship is pretty strong, although there are some faulty data around 10 and 25  $\mu\text{M}$  peptide concentrations, overall the data has a hyperbolic shape.

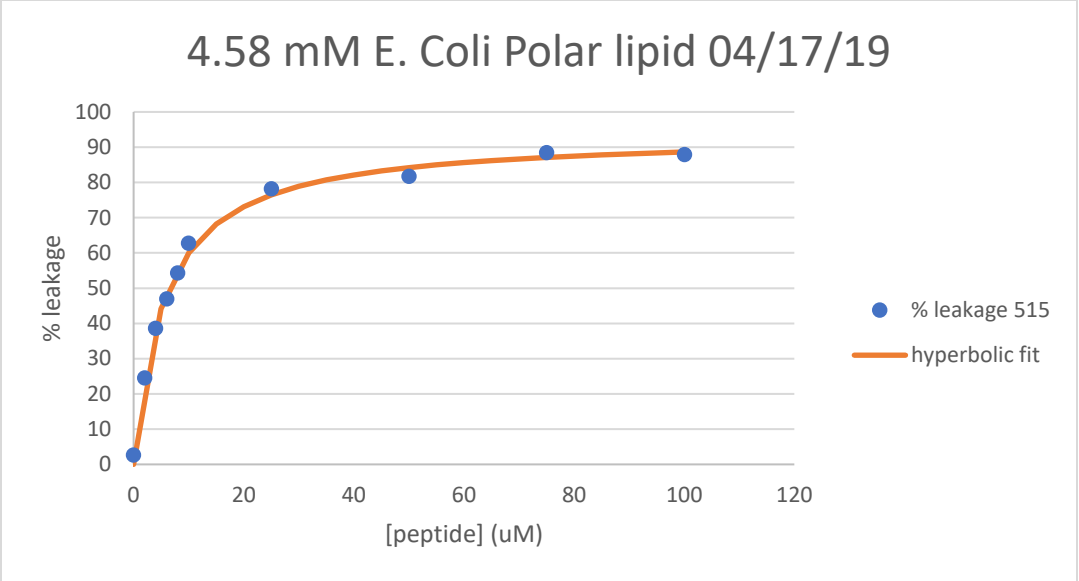


Figure 36- hyperbolic fit for first data fitting

Now 'A' is set to equal 100 as shown here:

$$\frac{100 * x}{B + x}$$

This should shift 'B' if 'A' wasn't equal to 100 in the first data fitting. The calculated parameters, errors, and R squared values that shows how well the data fits the linear fit are shown in Table 36.

A	100
B	6.757181
error	0.4124
R squared	0.9859

Table 36- A and B values Using the second data fitting

Using the values of A and B in Table 36, a hyperbolic fit was overlaid on the data that was collected at 515 nm. This can be seen in Figure 37 below.

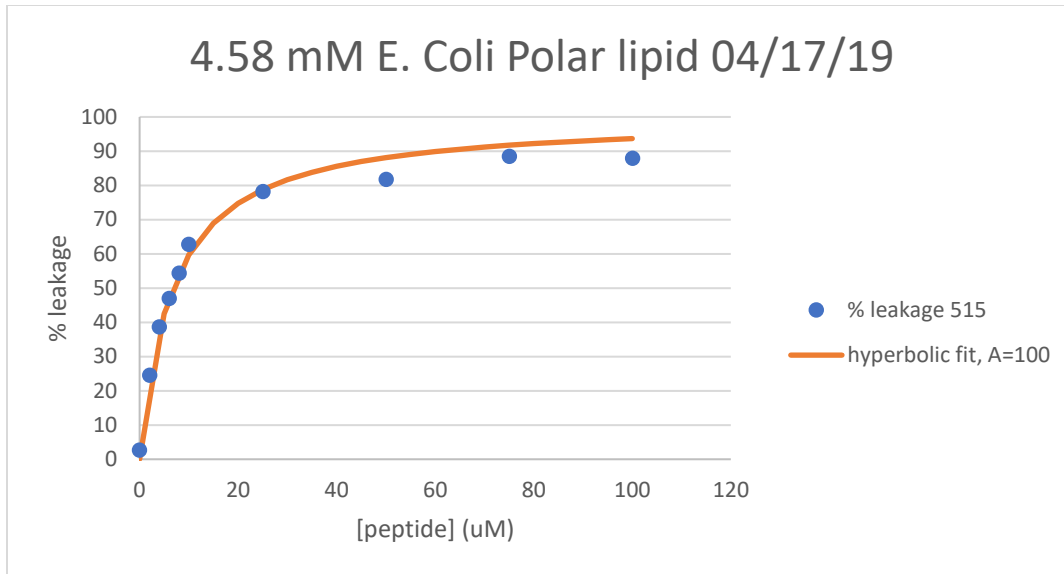


Figure 37- hyperbolic fit Using second data fitting

Lastly, an extra variable 'n' was added such that the new equation is:

$$\frac{100 * x^n}{B^n + x^n}$$

Using this equation, 'A' is set to 100. 'B' and 'n' parameters, their error values, and R squared value are calculated from the data and are shown in Table 37 below. Using Table 37, the peptide concentration that would be required to achieve half of 100 percent leakage is 6.63 μM. Based on the small error rate and high R<sup>2</sup> value, we are confident in this data.

A	100
B	6.627511
error	0.3583
n	0.8550772
error	0.0484
R squared	0.9926

Table 37- A and B values for the third data fitting

Using the values of 'A', 'B', and 'n' in Table 37, a sigmoidal fit was overlaid on the data that was collected at 515 nm. This can be seen in Figure 38 below. Since the value of 'n' in Table 37 is 1 that is equivalent to having the same equation in the second data fitting. This means

that the fit isn't sigmoidal and is actually hyperbolic. This shows that there is no binding cooperation between the peptides. That means that initial binding and leakage does not facilitate further binding and leakage.

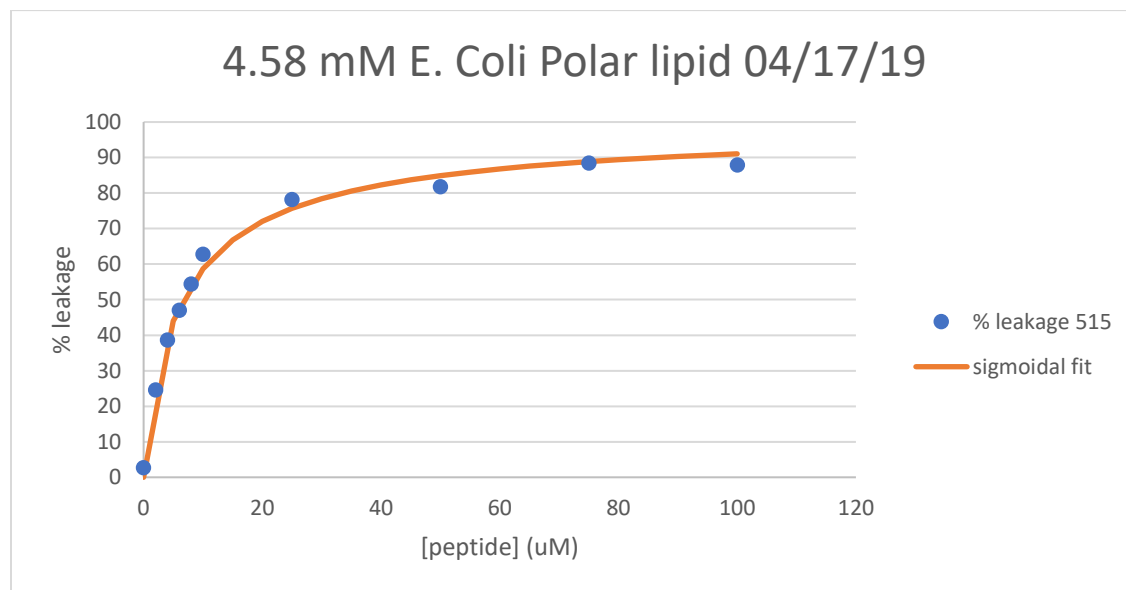


Figure 38- Sigmoidal fit Using third data fitting

**Measurement 10- 4/18/19- 30°C**

In order to test the leakage induced by Maximin 3 on *E. coli* vesicles at 30°C, a set of measurements were taken. This measurement was done on a peptide with a concentration of 413.4 μM. With a lipid composition of 4.58 mM *E. coli* Polar lipid. The solution was left in dialysis overnight, the volume increased from 0.70mL to 0.764mL. Table 38 shows the volume and concentration setup for the fluorometer measurements.

Sample	vol peptide (μL)	dilution	[peptide] (μM)	% leakage sum	% leakage max	% leakage 515
2mM Peptide V	1.96	1	2.03	16.35	18.14	18.43
4mM Peptide V	3.87	1	4.00	28.60	31.27	30.80
6mM Peptide V	5.81	1	6.00	37.63	40.79	41.84
8mM Peptide V	7.74	1	8.00	48.31	52.66	52.75
10mM Peptide V	9.67	1	9.99	55.89	58.26	58.11
25mM Peptide V1	24.2	1	25.01	65.87	71.11	71.09

50mM Peptide V	48.4	1	50.02	93.98	92.22	92.14
75mM Peptide V	72.6	1	75.03	82.37	83.53	83.34
100mM Peptide V	96.8	1	100.04	83.96	86.01	86.04

Table 38- Volume and concentration setup for the fluorometer measurements for measurement 6

The data collected in the fluorometer measurements was graphed showing the percent leakage against peptide concentration. Ideally, all three % leakage types should line up perfectly. If these are not the same then that indicates that the intensities were different depending on the wavelength, that means some other factor is influencing the fluorescence besides the leakage. Therefore an error has been introduced into the measurement. This is shown in Figure 39.

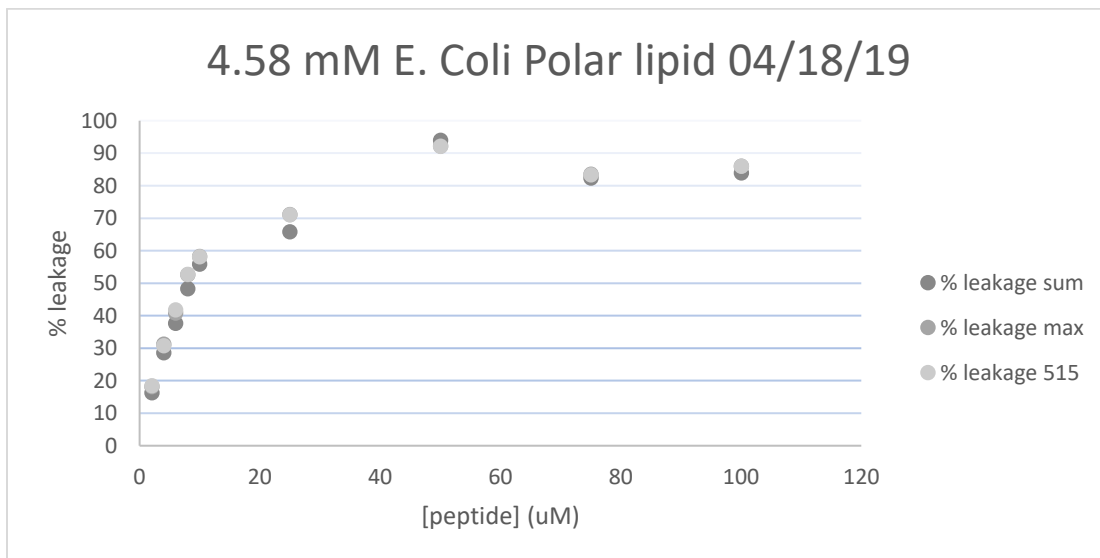


Figure 39- Percent leakage Vs. Peptide Concentration

As mentioned in measurement 1, this data was fitted into the following equation:

$$\frac{A * x}{B + x}$$

While parameter 'A' shows the calculated maximum percent leakage and parameter 'B' shows the peptide concentration that produced half of the maximum leakage. As shown in Table 39, the maximum percent leakage for this measurement is 95.7%. That is a high percent leakage. And the peptide concentration required to get to half of that percent leakage is about 7.33 μM.

A	95.69297
error	3.457
B	7.334836
error	0.9441
R squared	0.9737

Table 39- A and B values Using the first data fitting

Using the values of ‘A’ and ‘B’ in Table 39, a hyperbolic fit was overlaid on the data that was collected at 515 nm. This can be seen in Figure 40 below. The hyperbolic relationship is pretty strong, although there are some faulty data around 50 μM peptide concentrations, overall the data has a hyperbolic shape.

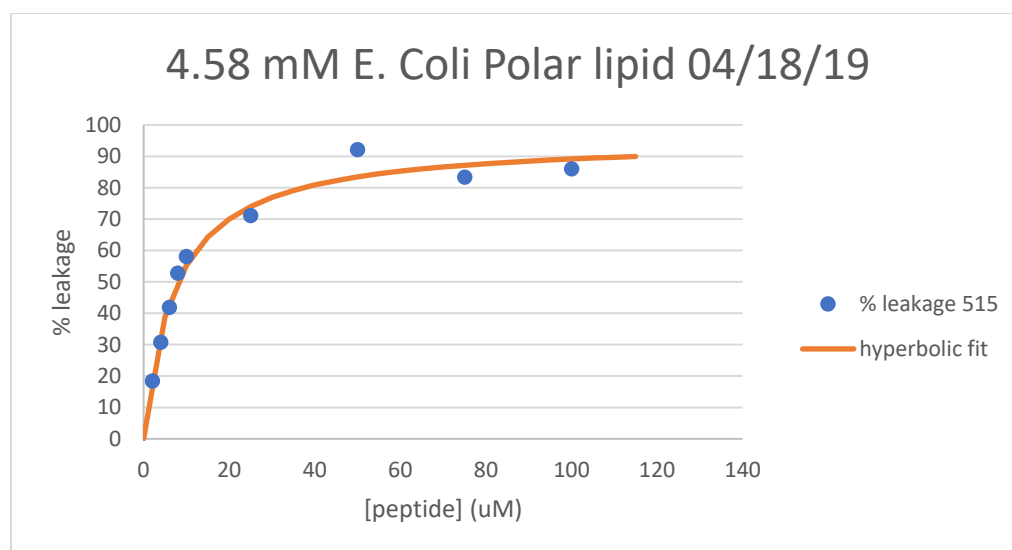


Figure 40- hyperbolic fit for first data fitting

Now ‘A’ is set to equal 100 as shown here:

$$\frac{100 * x}{B + x}$$

This should shift ‘B’ if ‘A’ wasn’t equal to 100 in the first data fitting.

The calculated parameters, errors, and R squared values that shows how well the data fits the linear fit are shown in Table 40 below.

A	100
B	8.256229
error	0.6757
R squared	0.968

Table 40- A and B values Using the second data fitting

Using the values of parameter ‘A’ and B in Table 40, a hyperbolic fit was overlaid on the data that was collected at 515 nm. This can be seen in Figure 41 below.

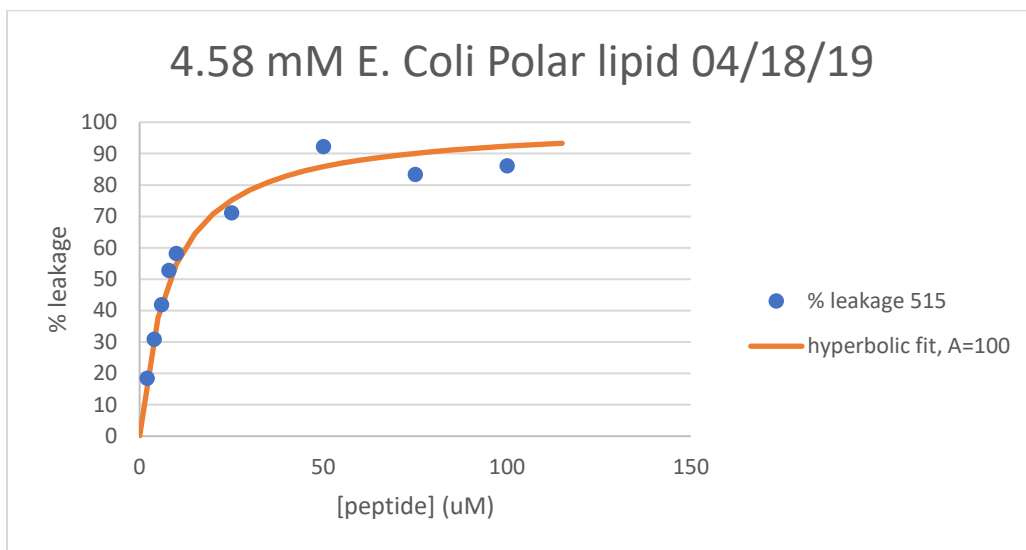


Figure 41- hyperbolic fit Using second data fitting

Lastly, an extra variable ‘n’ was added such that the new equation is:

$$\frac{100 * x^n}{B^n + x^n}$$

Using this equation, ‘A’ is set to 100. ‘B’ and ‘n’ parameters, their error values, and R squared value are calculated from the data and are shown in Table 41.

Using Table 41, the peptide concentration that would be required to achieve half of 100 percent leakage is 8.25 μM. Based on the small error rate and high R<sup>2</sup> value, we are confident in this data.

A	100
B	8.250601
error	0.7369
n	0.949171
error	0.09326
R squared	0.9691

Table 41- A and B values for the third data fitting

Using the values of A, B, and n in Table 41, a sigmoidal fit was overlaid on the data that was collected at 515 nm. This can be seen in Figure 42 below. Since the value of n in Table 41 is 1 that is equivalent to having the same equation in the second data fitting. This means that the fit isn't sigmoidal and is actually hyperbolic. This shows that there is no binding cooperation between the peptides. That means that initial binding and leakage does not facilitate further binding and leakage.

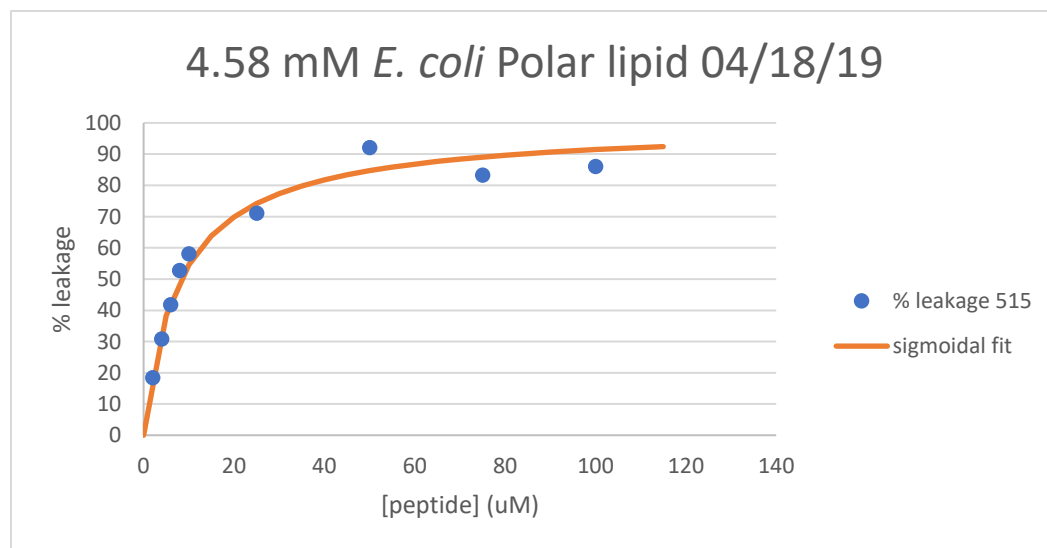


Figure 42- Sigmoidal fit Using third data fitting

## Conclusion

Antibiotics are used to treat bacterial infections. They are substances that prevent the normal metabolism of a disease-causing bacteria. There's an overwhelming amount of antibiotic

resistance occurring in bacteria right now. Trying to find a solution, this research focused on Maximin 3's behavior as a measure of how effective the AMP is against microbes under different conditions. The results generally showed an increase in leakage with increasing concentration of peptide. That means that initial binding and leakage does not facilitate further binding and leakage.

At all temperatures, the maximum leakage approaches 100% leakage. Based on the hyperbolic fit of the data, the B value was highest at 30 °C and lowest at 25 °C. This follows our hypothesis that since *Bombina Maxima* is a cold-blooded toad, the peptide would function best at lower temperatures. As of right now some AMPS are being used as topical creams against bacterial infections. We hope to continue studying AMPS and explore temperatures closer to body temperature. These measurements are preliminary and will be repeated

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