SELF-HEALING BIOLOGICAL MOLECULES FOR USE IN ENGINEERING MATERIALS

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Abstract

Phospholipid molecules are one of the fundamental building blocks of cell membranes in living organisms. These molecules are amphipathic with two hydrophobic fatty acid chains (tails) linked to a phosphate containing hydrophilic group (head) by a glycerol molecule. A bilayer lipid membrane (BLM), which is two phospholipids thick, is a 6-10 nm thick membrane and can be used to seal a porous material that has water on both sides. This paper presents how a specific phospholipid, 1-Stearoyl-2-Oleoyl-sn-Glycero-3-Phosphocholine (SOPC), is used to seal a single square aperture (25 x 25 μm) of a silicon substrate. The phospholipids are able to self-assemble into organized structures in the presence of water and will form a bilayer across the aperture when deposited on the aperture and substrate and inundated in water. Mechanical failure is induced using a pressure gradient across the substrate and upon failure the amphipathic nature of the phospholipids reconstructs the failed BLM and seals the aperture thus, introducing the concept of a self-healing biological molecule. This paper will also present testing done on a BLM formed using a method called the Droplet Interface Bilayer (DIB). This method uses water droplets placed in oil to form a monolayer of phospholipids around the droplets. Once the monolayer is formed around the droplet, a bilayer can be formed by connecting two droplets.

Introduction

Many biological molecules have a chemical composition that promotes the ability for the molecules to spontaneously self-assemble into organized structures. The chemical composition of phospholipids causes spontaneous self-assembly when the molecules are in an aqueous environment. One moiety of the phospholipids is polar causing it to be hydrophilic and the other moiety of the phospholipids is hydrophobic. The hydrophilic side of the phospholipids is called the “head” and the hydrophobic side is called the “tail.” Figure 1 shows an atomic schematic of a phospholipid molecule compared to the shorthand notation of the molecule. The molecule is phosphatidylcholine, specifically 1-Stearoyl-2-Oleoyl-sn-Glycero-3-Phosphocholine (SOPC), which has two hydrocarbon chains (fatty acids) that are the tail of the molecule with a chain length of 18 carbon atoms with a single cis double bond between the 9th and 10th carbon atom (counting from the methyl group) on one of the chains. The short hand notation of the molecule shows this double bond with a kink in one of the tails. These fatty acids are attached to a glycerol molecule that is also attached to the head. The head has two polar regions at the phosphate group and the nitrogen atom.

Figure 1. A short hand schematic of a phospholipid compared to a schematic of the atomic structure of SOPC consisting of carbon (light blue), hydrogen (white), oxygen (red), phosphorus (purple), and nitrogen (blue).

In an aqueous environment, phospholipids will self-assemble into organized structures that include micelles, liposomes, monolayers, planar bilayers, or a combination of these structures. The hydrophilic side of the molecule is attracted to water and the hydrophobic side is repelled from water. Figure 2 shows examples of these structures except for a monolayer.

Figure 2. Structures that phospholipids will form when placed in water.
Phospholipids are one of the components that formulate the structure of a cell membrane. They separate the cytoplasm of a cell from the surrounding environment by forming a planar bilayer. The bilayer formed by the phospholipids is impermeable to ion and fluid transport into and out of the cell.\(^1\) The transport of ions and fluid into and out of the cell is actively and passively performed by proteins embedded within the cell membrane. These embedded proteins are amphiphilic molecules as well that can span the entire membrane or half of the membrane. This amphiphilic nature of proteins allows them to interact with phospholipids and water and self-assemble into the organized structures formed by phospholipids. Figure 3 shows a schematic of part of a cell membrane surrounded by water with inserted proteins. The forces acting on the membrane allow the proteins and phospholipids to easily move in the plane of the bilayer, but they rarely move through the bilayer.

![Figure 3. A schematic of a cell membrane surrounded by water with two proteins embedded in the membrane.](image)

Phospholipids have been used to create membranes outside of the cell structure to study the molecules. A Bilayer Lipid Membrane (BLM) is a formed membrane of phospholipids. Haydon et al.\(^2\)-\(^11\) used optical and electrical means to quantify BLM properties (such as surface tension, thickness, and internal forces) formed over synthetic substrates with some aperture in the substrate. Needham et al.\(^11\)-\(^23\) continued the characterization work using droplets (or vesicles) in micropipettes and ultimately suggested using a method called the Droplet Interface Bilayer (DIB)\(^24\)-\(^25\) to study the properties of BLMs.

Droplet interface bilayers (DIBs) were suggested as a way to study bilayers without the need of a solid supporting structure. BLMs formed on supporting structures with apertures have several disadvantages that include the formation of lenses (bulk lipid solution that is greater than 2 molecules thick) in the bilayer (especially around the border), bilayer longevity, and knowing the substrate/lipid interaction. Sundaresan et al.\(^26\) showed that a supporting substrate can affect electrical measurements taken across a supporting substrate with a formed BLM. Bailey et al.\(^25\) discussed specific problems associated with the lens/bilayer/substrate interface and bilayer longevity. The DIB method of forming a bilayer uses water droplets submerged in oil. At the interface a monolayer of lipids is formed. The lipids can be suspended in the oil or as vesicles in the water. Once the water droplets are placed in the oil, a monolayer will form at the interface as shown in the schematic in Figure 4. When two of these droplets with a lipid monolayer are brought into contact, a bilayer can form between the two droplets without a supporting substrate and without the presence of lenses in the bilayer. Bilayers formed with the DIB method have a much longer longevity ranging from days to weeks where the longevity of bilayers formed over apertures are usually a few hours.

![Figure 4. Schematic of a water droplet with a lipid monolayer on the surface submerged in oil to show the orientation of the lipid molecules.](image)

Hopkinson and Leo\(^27\)-\(^28\) showed that planar BLMs formed from SOPC have the ability to seal a porous synthetic substrate inundated in an aqueous medium. A planar BLM spontaneously formed across open pores of a polycarbonate membrane (with one side being coated to form a hydrophilic surface and the other side being a hydrophobic surface) when SOPC in a solvent (n-decane) was added to the substrate. The BLM self-assembled and sealed the porous membrane from ion and fluid flow between two chambers filled with water. Impedance spectroscopy was used to verify the presence of the lipid molecules in the pores by comparing the measured impedance of the open pores to the measured impedance with the SOPC lipids added to the aperture. The increase in magnitude of the low frequency impedance indicated the presence of the SOPC lipids in the pores of the polycarbonate substrate.
Fluorescence spectroscopy was used to verify the presence of the lipids in the pores. The mechanical integrity of the BLM was tested with an increasing pressure gradient across the substrate until the BLM failed. The pressure where the BLM failed was found to follow the relationship

$$\Delta P \propto \frac{1}{r}$$  \hspace{1cm} (1)

where $P$ is pressure and $r$ is the radius of the pore. Figure 5 shows the test results of this relationship for a single aperture substrate and a porous substrate. The test showed that submicron porous substrates could hold over 100 kPa of pressure before bilayer failure.

![Figure 5. Failure pressure of BLM formed over porous polycarbonate substrate reported by Hopkinson and Leo.](image)

This paper will show that after a BLM mechanically fails, the self-assembly properties of the phospholipids will allow the BLM to reassemble or self-heal across the pores of the substrate. This paper will also present testing done on bilayers formed across a synthetic substrate with a single aperture formed using the DIB method. This method allows the formation of a BLM in an aperture without having the disadvantage of forming a BLM across an aperture as previously explained.

### Materials & Methodology
#### Substrate Preparation (self-healing)

The test fixture is composed of a stepper motor (model L1MGJ-M200XX060, EAD Motors, Dover, NH) used to drive a lead screw in a linear motion (see Figure 6). A stainless steel piston with an outside diameter of 12.2 mm is attached to the end of the lead screw, which has a thread pitch of 1.57 threads/mm. For each step the motor turns 0.9°, which translates into a linear piston motion of 1.59 μm. The piston is used to pressurize a lower fluid chamber inside the aluminum body of the test fixture, which has an internal volume of 5.0 mL. The lower fluid chamber is connected to a polycarbonate upper chamber via a 3.2 mm diameter hole. A track etched silicon chip acquired from Applied Nanostructures (Santa Clara, CA) is used as the substrate to support the BLM. The outer substrate dimensions of the chip are 6 x 6 x 0.3 mm. A single square aperture is etched into the center of the substrate. The aperture tapers through the thickness of the silicon substrate such that the aperture has dimensions of 450 x 450 μm on one side of the substrate and dimensions of 25 x 25 μm on the opposite side of the substrate. The chip has a thin silicon nitride layer covering the chip and is glued into the upper chamber using epoxy (model Scotch-Weld DP-105, 3M, St. Paul, MN) and the system is sealed using a rubber gasket. Two Ag/AgCl electrodes (2 mm diameter, World Precision) are placed one each in the upper and lower chambers. A pressure relief valve is connected to the lower fluid chamber and a fluid reservoir. Pressure is measured using a pressure transducer with a range of 0 to 41 kPa (model PX181B-006G5V, Omega Engineering, Inc., Stamford, CT), which has an accuracy of 0.3% of full scale. Pressure is recorded using a multifunction data acquisition system.
acquisition module (model USB-6009, National Instruments, Austin, TX) and Labview software (National Instruments, Austin, TX).

**Lipid Preparation and self healing test procedures**

SOPC is purchased in powder form from Avanti Polar Lipids (Alabaster, AL). SOPC powder is dissolved in n-decane (99% purity, Alfa Aesar, Ward Hill, MA) at a concentration of 40 mg/mL and mixed for 30 minutes using a sonicator (model 50, VWR, West Chester, PA). An aqueous salt solution is prepared with 0.1 M NaCl (100.0% purity, Mallinckrodt Baker, Inc., Paris, KY) and deionized water. The BLMs are reconstituted over the silicon substrate across the small side of the aperture, where its dimensions are 25 x 25 μm by using a pipette (model Calibra 822, Socorex, Switzerland) to deposit the phospholipids and solvent on the aperture.

For each trial the silicon substrate is coated with 1 μL of the lipid/n-decane mixture and then clamped between the lower and upper fluid chambers. Pressure is slowly applied by running the stepper motor at a fixed frequency of 1 Hz until BLM failure is observed. After BLM failure is reached, the stepper motor is immediately turned off to stop the application of pressure to the BLM and to allow the BLM to reform. After the reformation the stepper motor is turned on to begin a new pressurization cycle that is repeated for a total of three cycles. After these three pressurization cycles, the stepper motor is turned on for a duration of 50 seconds which causes the BLM to fail and the fluid to flow through the aperture. The BLM reformation the stepper motor is turned on again and the second pressurization cycle is repeated. A control trial is also performed by pressurizing the substrate with no BLM. The stepper motor is again run at a frequency of 1 Hz and the pressure is recorded.

**Electrical Impedance test procedure**

A well formed BLM acts as an RC circuit with a high resistance to current flow, with conductance values typically ranging between 10⁻⁹ and 10⁻⁵ S/cm and a capacitance value that is proportional to the area of the BLM. Therefore the DIB system can be modeled as an electrical circuit with the RC circuit of the BLM in series with a resistor (the resistance of the water droplets). An impedance analyzer (Autolab PGSTAT 12 with FRA 2 module, Eco Chemie B.V., The Netherlands) is used to apply a sinusoidal voltage potential across the Ag/AgCl electrodes and the analyzer simultaneously measures the current and calculates the electrical impedance at a specific frequency. A frequency range between 10 mHz to 100 kHz is used with a 5 mV voltage potential using Ag/AgCl electrodes.

For the DIB test, electrodes are made from 50 and 100 micron silver wire soldered to copper wire. The electrodes are then placed in chlorine bleach for 30 minutes and rinsed in de-ionized water. The electrodes are then dipped in a heated agarose solution (5 percent weight/volume 10 mM MOPS, 100 mM NaCl, near a pH of 7) three times to coat the electrode surface and render the surface hydrophilic for easier insertion in water droplets. For the test using BLMs formed for the self-healing across the porous substrate, the Ag/AgCl electrodes are not coated.

**Substrate Preparation (DIB)**

The fixture and the substrate used for testing the DIBs through an aperture are made from polycarbonate. A punch is used to make a circular substrate (Sterlitech PVP free polycarbonate filter membranes between 5 and 20 microns thick) 6.4 mm in diameter and an aperture is punch through the membrane (Harris micro-punch) at sizes of either 500 or 750 microns in diameter. The fixture is made from two pieces of polycarbonate that are 32 x 10 x 5.7 mm machined to clamp the polycarbonate membrane between a well. The well in each piece is machined to have a curved surface with a diameter of 4.8 mm and is 6.1 mm long. Two dimples are machined at the bottom of the curvature in the well in a line at the edge where the fixture holds the membrane. Figure 7 shows a picture of this fixture placed in a dish that catches any oil leaking through the seams of the fixture.

![Figure 7. A picture of the polycarbonate fixture with a polycarbonate membrane.](image_url)
Phospholipid Preparation (DIB)

1,2-Diphytanoyl-sn-Glycero-3-Phosphocholine (DPhPC) phospholipids are used for these tests because the there is no double bond in the tails and the transition temperatures of the molecule is higher. For this method, the phospholipids are stored in the water. When phospholipids are mixed with water, vesicles (either micelles or liposomes) will form. The preparation of the vesicles in water is similar to the preparation procedures used by Hwang et al. and is as follows. DPhPC is purchased in powder form from Avanti Polar Lipids (Alabaster, AL). A buffer solution of 10 mM MOPS, 100 mM NaCl, near a pH of 7 is added to the powdered lipids and vortexed for 10 seconds at a concentration of 2 mg/ml powder to buffer solution. The buffer/lipid solution is frozen and thawed for five cycles and then twice forced through a 0.1 μm polycarbonate membrane filter. The buffer/lipid solution is separated into several vials and refrozen for future use. The buffer/lipid solution is thawed at room temperature and used immediately for testing over the following month.

During the month of testing the buffer/lipid solution is stored in a refrigerator at approximately 4°C and is removed only to extract buffer/lipid solution for a test and is immediately returned to the refrigerator. A pipette is used to place two 0.75 μl of buffer/lipid solution droplets into an oil bath of hexadecane in the fixture well. The coated Ag/AgCl electrode tips are immediately positioned in the droplets. The electrodes are previously discharged in 10 mM NaCl and the potential difference between the electrodes is maintained at zero using the impedance analyzer. One electrode is held in place by a static holder and the other electrode is positioned by a digital micro-manipulator. Once the electrodes are positioned, the droplets are left for 15 minutes to form the monolayer at the interface of the water and oil (failure to allow the formation of a monolayer on the droplet surface will cause the droplets to coalesce into a single 1.5 μl droplet). The electrodes are used to position the droplets in contact through the aperture of the polycarbonate membrane. Once the monolayers are in proximity, the impedance analyzer is switched off and a charge builds between the electrodes. This charge helps extract excess oil from between the monolayers and discharges when the bilayer between the two droplets is formed.

Results

Self-Healing Tests

Impedance measurements across the aperture and the BLM are measured to verify the presence of a BLM. Control trials are performed in which the impedance of the aqueous solution and the silicon chip without a BLM is measured. BLMs are formed over the silicon substrate and the impedance is measured to insure the presence of the BLM across the aperture. Figure 8 shows several impedance measurements of the BLM formed across the silicon aperture compared to the baseline impedance of the silicon chip without a BLM. The figure shows that the impedance has increase by three orders of magnitude at the low frequency region. Figure 8 also shows that the phase deviates significantly from that of the baseline measurement. The capacitance of just the BLM is difficult to extract from this data because the silicon chip adds additional capacitance to the measurement.

![Figure 8](image_url)

Figure 8. (a) The magnitude of several impedance measurements of the BLM formed across the silicon chip aperture compared to the baseline without a BLM and (b) the phase of the comparisons.

A control trial for the pressurization test is performed in which the silicon substrate with the open aperture has fluid cyclically pumped through the aperture with no BLM formed over it. As shown in Figure 9 the pressure and current measured are plotted against time with an additional plot showing the pumping cycle of when the stepper motor is turned on and off. The current measurement shows that current starts at 990 nA and exponentially decays to 860 nA. This decay is assumed to be associated with the hydration of the electrodes. The pressure...
measurements show that the pressure does increase slightly when the stepper motor is on, indicating that the fluid flowing through the small aperture is causing a pressure gradient of 0.6 kPa.

![Image of graph showing current and pressure over time](image1)

Figure 9. Control trial in which a silicon substrate with a single aperture (25 μm x 25 μm) with a 70 mV DC potential across the aperture is pressurized cyclically for 600 seconds while the pressure and current are measured.

BLMs are then formed over the silicon substrate and then cyclically pressurized as previously explained. Figure 10 shows a single trial with a BLM formed over the silicon substrate. The current at the initiation of the trial is on the order of 100 pA and as the pressure is increased on one side of the BLM, the current rapidly increases. The reformation of the BLM happens more rapidly for the cases where the pressurization is discontinued immediately after BLM failure than for the cases where fluid is pushed through the aperture. Occasionally trials show interference from debris lodged in the aperture. In these instances the BLM forms around the object and the initial current measurement indicates that a BLM is formed across the aperture. The pressure then increases to values much higher than those found in Figure 10 indicating that the aperture is blocked by something other than just the BLM.

![Image of graph showing current, pressure, and pressurization over time](image2)

Figure 10. A trial in which a silicon substrate with a single aperture (25 μm x 25 μm) has a BLM formed over the aperture with a 70 mV DC potential across the BLM and is pressurized cyclically for 600 seconds while the pressure and current are measured.

After each pressurization trial, the impedance of the BLM is measured after reformation of the BLM and during BLM failure. The measurement of the failed BLM is made when the motor is pushing fluid through the aperture. Figure 11 shows the comparison between the impedance magnitudes of each of these measurements for a single trial. The impedance of the initially formed BLM and that of the “self-healed” BLM are almost identical, indicating that the BLM has reformed. The impedance for the failed case is similar to the baseline shown in Figure 8 without the BLM formed across the aperture.

![Image of graph showing magnitude and frequency](image3)

Figure 11. The measured impedance of the BLM after the initial formation, after the BLM has failed, and after the BLM has self-healed.

DIB Tests

DIBs are formed through the polycarbonate substrate as previously explained using DPhPC lipid vesicles in water. The bilayer can be modeled as an RC circuit and the water as a resistor in series with the
The mathematical equivalent impedance of an RC circuit in series with a resistor is used to approximate the resistance and capacitance of the bilayer and the resistance of the water from the measured data. The model of the impedance is

\[ Z(\omega) = \frac{R_1}{1 + j\omega R_1 C} + R_2 \]  

(2)

where \( R_1 \) is the resistance of the bilayer, \( R_2 \) is the resistance of the water, and \( C \) is the capacitance of the bilayer. With the estimated capacitance, the area and diameter (assuming the bilayer is circular) is calculated because the specific capacitance\(^{11} \) of the bilayer is about 0.6 \( \mu \text{F/cm}^2 \).

DIBs are formed in a polycarbonate fixture in water with the polycarbonate supporting membrane between the drops with either a 750 or 500 micron circular aperture punched out of the membrane. The purpose of the membrane with the aperture is to restrict the size of the bilayer. Figure 12 shows a picture taken of two droplets with inserted electrodes and one droplet protruding through the 750 micron aperture. The view is from under the fixture. The vertical blurred portion of the picture in the center is the membrane separating the two sides. The droplets are a little more than 1mm in diameter and the dimples are a little less than 1 mm in diameter.

Figure 12. Picture of two droplets in a polycarbonate fixture with a 5 micron thick membrane (with a 750 micron aperture) separated with inserted electrodes. The droplet on the left is in contact with the membrane and a portion of the droplet is bulging through the aperture in the membrane.

Figure 13 shows a plot of the measured impedance after two droplets are brought into contact through a 750 micron aperture. The bilayer resistance is 305 MΩ and the capacitance is 1.7 nF. The bilayer for this particular trial is approximately 0.0029 cm\(^2\) and 600 microns in diameter.

The model of the impedance is overlain on the data. Figure 13 shows a plot of the measured impedance of a bilayer formed through a 750 micron aperture with a plot of the impedance model overlain on the data.

Figure 14 shows a plot of the measured impedance after two droplets are brought into contact through a 500 micron aperture. The bilayer resistance is 5.4 GΩ and the capacitance is 720 pF as found using the same method to match the model to the data. The bilayer for this particular trial has an area of 0.0012 cm\(^2\) and is 390 microns in diameter.
The average failure pressure of the BLM formed over the silicon aperture, for the cases without debris, is 1.7 kPa with a standard deviation of 0.8 kPa. A previous study\textsuperscript{27,28} was performed to determine the failure pressure of a BLM formed over single and multiple pores with a polycarbonate membrane as the substrate. The study showed that the failure pressure was almost proportional to the inverse of the diameter of the pores in the substrate. The largest dimension of the aperture (the diameter of the pore for the previous study and the diagonal of the aperture in this study) is used to compare the results of this study with the results of the previous study. Extrapolating the single and multiple pore failure pressures of the previous study to the failure pressures of a substrate with a pore of 35 μm will result in failure pressures of 9.8 kPa and 0.57 kPa respectively. The failure pressure for this study falls within this range but closer to the multiple pore failure pressure. One would expect the failure pressure of the BLM on the silicon chip over a single aperture to be closer to the failure pressure of a BLM on a polycarbonate membrane with a single pore. Several things could be affecting the failure pressure of the BLM formed over the different substrates including but not limited to aperture geometries, substrate surface geometries, and the stiffness of the substrates. The apertures of the two substrates have different geometries (square vs circle). The surface properties of the substrates could have different hydrophilicities and therefore could affect the BLM. The polycarbonate membrane is very flexible and easily deforms while the silicon chip is rigid and deforms very little to pressurization before failure. In in-depth look at these differences could provide information that can be used in future studies to quantify the mechanical properties of the BLM apart from the properties caused by the substrates.

**DIB Test**

The data shows that the area of the formed bilayers does not completely cover the entire area of the apertures. For the best sets of data, which are the sets shown here in this paper, the bilayer diameters are 80 and 78 percent of the 750 and 500 micron apertures respectively. There are several reasons that could be causing the diameter of the bilayers to be smaller than the diameter of the apertures. First could be the machining tolerances of the fixture and the assembly of the fixture with the membrane. The fixture is machined by the author and the membrane is positioned by hand.
in the fixture. Therefore any variations in the dimensions of the components or positioning of the membrane could cause the droplets to not be perfectly aligned in the fixture to form a bilayer across the whole aperture.

A second reason for the diameter of the bilayers being smaller than the diameter of the apertures is due to the curvature of the droplets. The oil in the area under the edges of the aperture caught in the curvature of the droplets could impede the bilayer from forming across the entire aperture diameter. As the diameter of the aperture is decreased or the size of the droplet is increased, the area under the edge of the membrane may affect the total bilayer area.

Conclusion
SOPC BLMs were formed over a silicon substrate with a single aperture and pressurized until failure. After a short time period it was found that BLMs “self-healed” and reformed over the aperture. The failure pressure for the BLM formed over the square aperture was 1.7 kPa. Previous research has shown that the failure pressure of a BLM is inversely proportional to the largest dimension of the aperture. Therefore scaling the aperture down to sub-micron size openings will enable a BLM to hold much larger pressures before failure. Measurements of electrical current independently confirmed that BLMs which fail due to pressurization will reform.

DPhPC DIBs were formed through a circular aperture made in a polycarbonate membrane. The formed BLMs were limited in size by the size of the aperture as expected. The diameters of the formed BLMs were about 80 percent of the diameter of the apertures. This limitation on the size of the BLM was independent of using mechanical means to hold the droplets at a certain distance and only partially due to the size of the droplet as discussed. Forming BLMs using droplets on a synthetic substrate allows for the formation of BLMs on synthetic substrates without the incurrence of bulk lipid lenses in the bilayer, without the incurrence of a ring of lipids around the edge of the aperture and bilayer, and using a polycarbonate membrane as the substrate eliminates potential electrical contributions to bilayer impedance measurements that may be caused by other materials.

Reference


