INTRODUCTION

In nature, ion conduction plays a vital role in the regulation of metabolism, intra-cellular signaling, and active transport of species via enzymatic reactions within plant and animal cells. Specifically, proteins and ion channels create conductive pathways through an otherwise impermeable lipid membrane that separates regions of a cell. Recent interest in using biological molecules, namely proteins and ion channels, to create engineering devices and applications requires that these active elements be reconstituted into an artificial bilayer lipid membrane (BLM) that mimics their natural cellular environment. Together, the lipid membrane and the incorporated proteins and ion channels can be used to develop sensors, actuators, and power sources based on ion conduction. Research at Virginia Tech has found that bio-inspired materials, relying on ion conduction, can be used to develop fluidic pumps and power sources using biological molecules. In the past, these studies have involved the formation of a planar bilayer lipid membrane (BLM) formed across the pore(s) of a synthetic substrate. However, a new technique for BLM formation has been adopted that eliminates the need for a supporting substrate. Self-contained aqueous droplets are injected into a bath of organic solvent with dissolved lipid molecules and result in the formation of lipid monolayer-encased water droplets. When two droplets are brought into contact with each other, the hydrophobic tails of the lipid molecules zip together, creating a stable bilayer lipid membrane at the droplet interface. The incorporation of proteins into this lipid membrane interface makes it possible to tailor the permeability of the membrane and control transport of species from one “cell” to another. Preliminary results indicate the successful insertion of alpha-Hemolysin (αHL), a self-inserting protein that creates ion conductive pathways across the membrane. Controlling the transport of species from one compartment to another is demonstrated using feedback control of current flowing through the interface.

ABSTRACT

Ion conduction in materials enables active functions including actuation, sensing, and transport. Work at Virginia Tech has found that bio-inspired materials, relying on ion conduction, can be used to develop fluidic pumps and power sources using biological molecules. In the past, these studies have involved the formation of a planar bilayer lipid membrane (BLM) formed across the pore(s) of a synthetic substrate. However, a new technique for BLM formation has been adopted that eliminates the need for a supporting substrate. Self-contained aqueous droplets are injected into a bath of organic solvent with dissolved lipid molecules and result in the formation of lipid monolayer-encased water droplets. When two droplets are brought into contact with each other, the hydrophobic tails of the lipid molecules zip together, creating a stable bilayer lipid membrane at the droplet interface. The incorporation of proteins into this lipid membrane interface makes it possible to tailor the permeability of the membrane and control transport of species from one “cell” to another. Preliminary results indicate the successful insertion of alpha-Hemolysin (αHL), a self-inserting protein that creates ion conductive pathways across the membrane. Controlling the transport of species from one compartment to another is demonstrated using feedback control of current flowing through the interface.

ION CONDUCTION AND CONTROLLED TRANSPORT ACROSS LIPID MEMBRANES FORMED AT THE INTERFACE OF CELL-LIKE WATER DROPLETS

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Typically, suspended bilayer lipid membranes are formed on porous, synthetic substrates, including polycarbonate, Teflon, alumina, and silicon. The formation of BLMs on these substrates relies on the hydrophobic interactions between the amphiphilic (both hydrophilic and hydrophobic) phospholipid molecules and water. A phospholipid molecule is comprised of a hydrophilic, phosphate-based headgroup connected to either one or two hydrophobic, hydrocarbon tails. In the presence of water, the polar headgroups align toward the water, shielding the hydrophobic tails of the lipids. A bilayer lipid membrane consists of two lipid monolayers sandwiched together in which the hydrophobic tails interact in the interior of the membrane and the hydrophilic head groups comprise both of the outer surfaces. The geometry and surface chemistry of the supporting substrate, along with the method for BLM formation, determine how phospholipid molecules self-assemble into a bilayer structure on a synthetic substrate.

While the supporting substrate can be used to define the size of the BLM, it can also complicate the formation and characterization of BLMs. The droplet-interface bilayer (DIB) is a technique that eliminates the supporting substrate and provides long-lasting BLMs. A lipid monolayer self-assembles at the oil/water interface of an aqueous droplet injected into a nonpolar solution of phospholipids dissolved in organic solvent. Two lipid-encased droplets can then be brought together until they touch, upon which the hydrocarbon tails of the opposing monolayers “zip” together, forming a stable BLM at the droplet interface. Funakoshi, et al first attempted this technique as
intermediate validation that intersecting aqueous and lipid solution streams could be used to form BLMs via micro-fluidics\(^3\). Holden et al refined and expanded the approach to demonstrate that this technique produced BLMs that would last for days and that the droplets could be arranged and rearranged to form networks of nanoliter-volume water droplets interconnected by DIBs\(^4\).

In this paper, we report on the investigation of the droplet-interface bilayer formation technique for creating BLMs using both electrical impedance spectroscopy (EIS) and cyclic voltammetry (CV), neither of which has previously been employed on BMLs formed in this manner. Further, the conductance of the lipid membrane is increased upon incorporation of alpha-Hemolysin (\(\alpha\)HL) into the BLM. Lastly, controlled transport via integral feedback control demonstrates the ability to apply control techniques to bio-based systems.

**Experimental Methods**

The two phospholipids used in this study were 1,2-diphytanoyl-sn-glycero-3-phosphocholine (DPhPC) and 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine (POPE), purchased as lyophilized powders and used without further purification (Avanti, Inc.). A 10mg/ml lipid solution of DPhPC in hexadecane (Sigma) and a 10mg/ml solution of POPE in n-decane (Sigma) were prepared. A 100mM KCl (Sigma) solution in ultrapure water (18.2M\(\Omega\)-cm) was prepared and used as the electrolyte in the aqueous droplets. Alpha-Hemolysin (\(\alpha\)HL) from staphylococcus aureus (Sigma) was dissolved in 100mM KCl solution to a final concentration of 10µg/ml. Silver-silver chloride (Ag/AgCl) electrodes (~100µm Ø) were made from pure silver wire chlorided in household bleach (containing NaOCl) for 15-30 minutes.

**Droplet-Interface Bilayer (DIB) Formation**

The lipid solutions were simultaneously sonicated and heated to a temperature of 45°C, higher than the 41°C and 23°C glass transition temperatures for DPhPC and POPE lipids, respectively, prior to use. Heating the lipid solutions was performed in order to promote better monolayer formation (i.e. more compact lipid molecule packing around the droplet) while the lipids were in their fluid phase. Upon cooling, the well-packed monolayer is assumed to revert back to its less-fluid, more-rigid gel-state. The warm lipid solution was added to a shallow well milled into a piece of acrylic (PMMA). Quickly, two 400nl aqueous droplets were pipetted into the well such that the droplets were not touching each other. A 2x3 array of micro-machined divets (1mm-Ø, 0.5mm-deep, 1mm center-center spacing) in the bottom of the well helped to keep each droplet stationary and separated. After sitting apart in the lipid solution for 30-40 minutes to allow for sufficient monolayer formation around each droplet\(^5\), a Ag/AgCl electrode was inserted into each droplet. The droplets were then carefully brought into contact by hand using the inserted electrodes (Figure 1).

**Figure 1**: Egg-crate divets were machined into the acrylic fixture used for droplet-interface bilayer (DIB) formation (top left). An illustration of lipid-encased water droplets interfacing to form a BLM (top right). The procedural steps used to form DIBs (bottom).

**Electrical Measurements on the DIBs**

Electrical impedance spectroscopy (EIS) was performed using an Autolab PGSTAT12 Potentiostat/Galvanostat with FRA2 module controlled by FRA software (Eco Chemie). A 10mV (RMS) sinusoidal voltage was applied to the electrodes from 1MHz to 10 mHz during each measurement.

Cyclic voltammetry (CV) was performed using GPES software that controls the potentiostat functions of the Autolab system. The current through the BLM resulting from an increasing applied voltage is measured. Typically, the voltage was first held at a small negative potential and then increasing linearly at the rate of 5mV/s up to 500mV.

Current measurements on BLMs containing \(\alpha\)HL were taken with GPES software used to control the
Autolab PGSTAT12. Specifically, current measurements were measured at a sample rate of 1kHz for a constant applied potential.

**Feedback Current Control through a BLM/**

Current tracking was implemented using integral control through Simulink and ControlDesk software and dSpace hardware. The control voltage calculated in Simulink and originating in the dSpace hardware was supplied to the electrodes through the Autolab PGSTAT12. The resulting current was measured by Autolab, transformed into a proportional voltage signal, and directed back into dSpace/Simulink to complete the control loop. An Ithaco 4302 low pass filter (4-pole Butterworth filter) with a cutoff frequency at 50Hz was used to filter noise from the voltage signal proportional to the measured current.

**Results and Discussion**

**Electrical Impedance of DIBs**

Upon formation of bilayer lipid membranes (BLMs) at the interface of two aqueous droplets, the electrical impedance measurements showed the gradual extraction of organic solvent from between the two phospholipid monolayers that zip together (Figure 2). It is important to mention that measurements taken either with the droplets separated (as in Runs 1 and 2) or those taken with the electrodes placed directly into the lipid solution indicate that the surrounding lipid solution has some finite conductance that increases with increase lipid composition. In previous publications the authors neglected this component in their electrical measurements9, 10. However, at high enough lipid concentrations or small enough BLMs (with much larger resistance values), parasitic conduction through the lipid solution becomes significant.

The final, thinned BLM behaves as a parallel resistor-capacitor in series with the electrolyte solution resistance within the droplets. A resistance and capacitance of 1.5GΩ and 880pF, respectively, were measured for the DPhPC BLM shown in Figure 2. For a specific capacitance of 0.65μF/cm², the approximated area of interface is 0.14mm². Holden, et al measured capacitances of 350pF for 200nl droplets, indicating our interfacial areas are roughly 2-3 times the size they used10.

![Figure 2: Typical impedance measurements on a droplet-interface bilayer (DIB).](image)

Two different lipid solutions were used to form droplet-interface bilayers in this study: DPhPC/hexadecane and POPE/n-decane. Initially, POPE lipids were selected in order to evaluate DIB formation using a lipid with a lower glass transition temperature (Tg = 23°C). However, the difference in headgroup type and organic solvent used may have introduced additional parameters that affect lipid monolayer formation.

**Table 1: Resistance and capacitance values extracted from EIS measurements on multiple DIBs formed with both types of lipids**

<table>
<thead>
<tr>
<th>Trial</th>
<th>DPhPC/hexadecane</th>
<th>POPE/n-decane</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Rblm (MΩ)</td>
<td>Cblm (pF)</td>
</tr>
<tr>
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<td>20</td>
<td>670</td>
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<td>2</td>
<td>320</td>
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<td>3</td>
<td>450</td>
<td>940</td>
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<tr>
<td>4</td>
<td>1500</td>
<td>880</td>
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</table>

The approximated resistance and capacitance values from the impedance measurements on DIBs formed with the two different lipid solutions highlight the differences between the resulting BLMs. The lipid bilayers formed using DPhPC lipids typically exhibited much higher capacitance values. Needham and Haydons work offers an explanation for this behavior: smaller alkanes (such as n-decane) have a higher probability of residing in the surface of the bilayer, which increases the area per molecule of the lipid (reduces lipid packing) as well as the membrane thickness. Conversely, longer alkane chains (such as hexadecane) distribute more toward the center of the bilayer where a high degree of order in the hydrocarbon chains of the lipids constrains the organic solvent molecules to lay in parallel to the hydrocarbon chains. Thinner membranes have higher capacitances, as seen in the measurements made on DPhPC lipid membranes.
The impedance results also indicate a significant degree of variability in BLM resistance values. The extent to which the lipids form a tightly-packed monolayer before the droplets are brought together affects how resistive the resulting BLM is to ion conduction. Heating to above the glass transition temperature of the lipids was performed in order to make the lipids more mobile when the aqueous droplets were initially inject into the bathing solution. More time to allow for monolayer formation may be needed to achieve higher resistance values of the BLMs (a requirement for detecting single channel protein insertion events).

**BLM Failure due to Electric Fields**

Cyclic voltammetry measurements were preformed in order to identify failure due to an electrical potential. In these tests, the current flowing through the BLM was measured for a linearly increasing voltage. BLM failure was observed as both a sudden, irreversible increase in the current measured as well as visual inspection of the two droplets fusing to become one large droplet.

![Cyclic Voltammetry at 5mV/s](image)

**Figure 3:** Cyclic voltammetry measurements on DIBs signify BLM failure when the current suddenly spikes.

This analysis shows that the failure potential for droplet-interface bilayers may be inherently lower than those typically reported for supported DPhPC BLMs (390-410mV)\(^{12}\). Four separate trials on DPhPC BLMs indicate failure (which is also verified visually as the droplets fuse together to make one larger droplet upon BLM failure) between 100-200mV. The measurement on a POPE DIB, where n-decane was used as the organic solvent, shows that the failure potential is even lower—possibly due to both the more-fluid nature of the POPE lipids at room temperature and the higher tension state that this membrane sees due to n-decane absorption\(^{13}\).

**Protein Insertion Events**

In many areas of BLM research, the successful insertion of proteins into the bilayer verifies its bilayer structure. The incorporation of active elements such as proteins and ion channels into the BLM also provides ways to tailor the permeability of the lipid membrane for sensing and other active functions. Funakoshi, et al demonstrated insertion of gramicidin channels through brief current measurements of droplet-interface bilayers, while Holden, et al worked with a number of proteins, including alpha-Hemolysin\(^9\)\(^{10}\).

Alpha-Hemolysin (αHL) is a water-soluble transmembrane protein that can readily self-insert into a bilayer lipid membrane, creating ion conductive pathways across the membrane. For a constant applied voltage, increases in conductance upon protein insertion are measured as discrete steps in the measured current. Distinct increases of 100-200pS in conductance were seen (Figure 4) repeatedly on a DPhPC BLM in which one droplet contained the αHL proteins. These events were attributed to the successive insertion of the proteins into the BLM at the droplet interface.

![Cyclic Voltammetry at 5mV/s](image)

**Figure 4:** Evidence of α-Hemolysin insertion seen as discrete steps in the measured current through the BLM for an applied voltage of 70mV.

**Feedback Control Concepts on Bio-systems**

A controlled variable is continually compared with a desired value in order to compute a measure of error in a feedback control scheme. The defined controller uses the error measurement to compute a new control signal designed to drive the error to zero. In biological systems, one variable that could be controlled is the
transport of species, namely ions, across a BLM. The application of a voltage to an electrochemical system results in current flow in the form of ion movement. Therefore, in this example, we demonstrate the ability to use feedback control in order to specify a desired current flowing through the BLM. Integral control defines the dynamics of our controller, which takes measurements of error between the actual current and the desired current in order to compute a new control voltage. The plant, or system being controlled, is defined by the admittance—the inverse of the impedance—of a droplet-interface bilayer that does not contain proteins.

![Block-diagram of the feedback control scheme](image)

**Figure 5:** Block-diagram of the feedback control scheme used for current tracking.

The value of the integral gain (5x10^8) was selected based on the response of simulations of the closed-loop system. Specifically, simulations and experiments of feedback current control were performed on three different DIBs.

![Simulated closed-loop transfer](image)

**Figure 6:** Simulated closed-loop transfer for a BLM with a resistance of 320MΩ and a capacitance of 1nF. The top plot shows the magnitude versus frequency and the lower plot shows the phase versus frequency for the transfer function.

The predicted closed loop response indicates the ability to accurately track a current signal at frequencies less than about 10mHz. At higher frequencies, it is expected that the actual current through the BLM will have a reduced amplitude and exhibit phase lag of the desired signal. The simulated transfer function shown in Figure 6 used a BLM resistance of 320MΩ and capacitance of 1nF, which were measured by EIS. The same droplet-interface bilayer was tested to see if the current could be controlled experimentally (Figure 7 and Figure 8). The desired current signal used was a 100pA sinusoidal waveform tested at different frequencies.

![Desired current signal](image)

**Figure 7:** Demonstrated ability to track a 100pA sinusoidal, desired current signal at a frequency of 10mHz.

The measured current flowing through the bilayer lipid membrane tracked the desired signal very closely at a driving frequency of 10mHz. However at 100mHz, the actual current lagged the desired signal and also had a reduced amplitude.

![Actual vs. Desired Current](image)

**Figure 8:** Demonstrated ability to track a 100pA sinusoidal, desired current signal at a frequency of 10mHz.

The application of integral feedback control has shown that the current flowing through a bilayer lipid membrane can be controlled. Relating this current to the transport of ionic species could enable precise regulation of the contents within each cell-like water...
droplet and move toward controlled interactions between interfacing droplets.

**Conclusion**

Droplet interface bilayers (DIBs) form in nonpolar lipid solutions when two lipid monolayer-encased aqueous droplets are brought into contact. This technique provides a simple, reliable method for the formation of bilayer lipid membranes (BLMs) and eliminates the need for a synthetic, supporting substrate. In this work, electrical impedance spectroscopy (EIS) and cyclic voltammetry (CV) were used to characterize the resulting BLMs. These techniques provide information about the electrical properties of the lipid membranes as well as determine when they will fail due to applied electrical potentials. Current measurements indicating successful insertion of αHL proteins into the interfacial BLM demonstrate that the interface is indeed a bilayer and that the permeability and selectivity of the interface can be tailored using proteins and/or ion channels. Lastly, integral feedback current control was demonstrated on DIBs. The incorporation of the lipid membranes into feedback loops shows for the first time that bio-based systems can be integrated into external control schemes in which their dynamics can be augmented for specific purposes.

**References**


