Development and Characterization of Multiple Nanopore Systems for Molecular Sensing

By
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A PROJECT

submitted to

Oregon State University

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degree of

Honors Baccalaureate of Science in Bioengineering
(Honors Scholar)

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Abstract approved:

\[ \text{Signature} \]

Jacob Schmidt

Artificial lipid bilayer technologies are effective models for studying cell membrane properties. Droplet bilayers rely on microfluidics and provide advantages over other bilayer methods due to their compatibility with automated development systems that increase yield while minimizing cost. This paper describes the implementation of bilayer technologies using membrane protein channels (\(\alpha\)-hemolysin) combined with NeutrAvidin to detect DNA in multiple nanopore systems. The NeutrAvidin recognizes and binds a biotinylated DNA with high specificity, blocking the protein channel. This method has been used previously but here we substantially enhance reproducibility and detection capabilities by incorporating multiple nanopore systems with parallel arrays. Correlations between applied voltage, concentration, and number of ion channels were analyzed and predicted using a mathematical model in order to optimize the capabilities of these systems. The resulting relationship between the quantity of channels available and the time it takes to block has been shown to be critical when introducing an analyte into the system, as it provides a reference for analysis of various blocking events in multi-nanopore studies.

Key Words: lipid bilayers, NeutrAvidin, droplet bilayers, nanopores, ion channels

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

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Development and Characterization of Multiple Nanopore Systems for Molecular Sensing

Application of Membrane Arrays to Detect DNA Through Simultaneous, Reversible Blocking Studies

Tyler T. Schmeckpeper
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Oregon State University Honors Thesis
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Introduction

Artificial lipid bilayer technologies (ALBs) have received significant attention over the last 50 years due to their potential medical and scientific applications such as drug delivery and cancer therapy [1-2]. A variety of different bilayer technologies exist including painted, substrate, tethered, and droplet bilayers [3-5]. Low cost and highly controllable conditions have made ALBs ideal for studying ion channels and pore proteins as well as implementing studies to use these pores for novel applications. These applications range single molecule sensing [6-8] to nanopore DNA sequencing [9-12]. Recently developed artificial lipid bilayer formation techniques have enabled parallelization and automation of analysis, significantly increasing the measurement throughput of these artificial bilayer platforms. Use of a multi-channel amplifier allows for the simultaneous and reproducible measurement of up to 32 bilayers [13].

The integration of membrane proteins into ALBs allows for extensive study of ion channels using electrophysiological measurements of current. Channel current recordings of these proteins have shown to be very fast and accurate in the identification of analytes such as water-soluble polymers, organic molecules, peptides [14-15], and DNA [16] using the transmembrane toxin, α-hemolysin (αHL), as a sensing agent. αHL is a protein most commonly known for lysing red blood cells by introducing heptameric protein pores that disrupt the integrity of the cell membrane. The αHL nanopores are excellent sensing agents in ALBs because each pore can be detected through electrical techniques. This detection comes from the pore integration decreasing the overall electrical resistance of the bilayer. An amplifier can also detect
modification of the size or blockage of the pore, and an engineered system can take advantage of this property for many applications. Since measurement is purely electrical, detection does not require fluorescent or radioisotopic labeling. Furthermore, the data from these electrical measurements is easy to acquire and analyze computationally, making all-in-one devices for untrained operators possible.

A unique experiment utilizing ALBs successfully studied cocaine molecules. Concentrations of 300 ng/mL were detected using α-hemolysin integrated into ALB nanopores on a chip with a cocaine-binding aptamer. Aptamers are nucleic acid sequences that have been engineered to specifically bind to target molecules upon which the conformation of the aptamer is changed. In the cocaine detection study, a negatively charged aptamer was used as an electrophoretically mobile element. When an electrical gradient was applied across the ALB, the individual aptamers and cocaine molecules could move freely across the bilayer because they are too small to block an α-hemolysin pore. However, when the aptamer bound a cocaine molecule, the conjugate’s conformation and size were large enough to block the pore. A visible reduction in current occurs across the ALB when the channel is blocked, and the timing of this event correlates with the concentration of the analyte [17].

Similar binding techniques that utilize a droplet bilayer formation method can be used to detect small molecular analytes while also taking advantage of large-scale bilayer development techniques. Various small molecules, and even proteins, fall within the physical constraints of detection using these nanopores, which have the capacity for unprecedented analytical use through simple electrical measurement and cost effective procedures. However, ALBs are very fragile and regularly fuse or fail
to produce significant results. To combat this, we are using parallel arrays for production and analysis, which will drastically increase the amount of bilayers that can be tested. This will ultimately increase the detection capabilities of ALBs while also potentially creating a realistic, affordable analytical technique to replace current lab methods for molecular analysis. Furthermore, the parallelization of the bilayer systems opens up many new applications that were either unapproachable or not effective when measuring single bilayers individually.

Current methods of detection are both expensive and time consuming, often requiring molecular labeling [18-20]. While nanopores provide a cheap and reproducible means for detecting molecules at low concentrations, current applications utilize single nanopore systems or solid-state nanopores [21]. We have developed a platform based on artificial bilayer arrays and multiple, simultaneous, nanopore sensing that will ultimately increase the detection capabilities of ALBs while maintaining low costs. By incorporating various changes to current sensing methods, we were able to determine and quantify the relationships of molecular blocking with applied voltage, concentration, and nanopore volume in the system in order to optimize and develop a model for future testing. DNA was chosen because of its stability and structure in addition to its negative charge that makes it ideal for electrophoretic movement. By incorporating a universal molecule such as DNA, future studies will be able to apply the obtained correlations for reference in various other molecular studies.
**Existing Theory**

Stochastic (random) single molecule sensing has been shown to correlate directly with the mathematical probability of the nanopore being free or blocked. This probability is dependent on the molecular concentration, time, and association constant ($K_{on}$) and follows an exponential decay mathematical model. By incorporating first-order kinetic theory, the average time for each individual block can be calculated with $C$ (molecule concentration) and $K_{on}$.

[1] $P_{o} + DNA \rightleftharpoons P_{o} \cdot DNA$

[2a] $P_{free}(t) = e^{-K_{on}Ct}$  

[2b] $P_{not\ free}(t) = 1 - e^{-K_{on}Ct}$

[3] $P_{free}(t) - P_{free}(t - dt) = \frac{-dP_{free}}{dt} dt \Rightarrow T_{block} = \int_{0}^{\infty} tP(t) dt \Rightarrow T_{block} = \frac{1}{CK_{on}}$

**Proposed Theory**

We hypothesize that the number of available, blockable channels will alter this mathematical model. Because the proposed detection method relies on multiple nanopores for faster sensing, the previously mentioned model must be changed to accommodate another variable ($N$). In addition, the probability for each blocking event must be taken into consideration, which should directly affect future blocking by decreasing the overall probability, as shown in equations 4-6 for a two nanopore situation.

[4] $P_{both\ free}(t) = P_{free1} \cdot P_{free2} = e^{-K_{on}Ct} e^{-K_{on}Ct} = e^{-2K_{on}Ct}$

[5] $P_{1\ bound, 2\ free}(t) = K_{on1}C e^{-K_{on1}Ct} dt \cdot e^{-K_{on2}Ct}$

[6] $P_{2\ bound, 1\ free}(t) = K_{on2}C e^{-K_{on2}Ct} dt \cdot e^{-K_{on1}Ct}$
Because we are using the same molecule (DNA+N) and ion channel (αHL) for each binding event, we can safely assume that $K_{on1} = K_{on2}$, in order to calculate the probability that either pore is blocked shown in equation 7.

\[ P_{1\text{ or }2\text{ bound}}(t) = (K_{on1} + K_{on2})Ce^{-(K_{on1} + K_{on2})Ct} dt = 2K_{on}Ce^{-2K_{on}Ct} \]

\[ T_{\text{block}} = \frac{1}{2K_{on}C} \Rightarrow \text{for all } N, \quad T_{\text{block}} = \frac{1}{NK_{on}C} \]

Equation 8 shows the final, derived equation that will allow us to predict the time to binding event for each nanopore present in an ALB system.

**Materials & Methods**

**Chip Assembly**

Bilayers were formed on chips designed by Lu and coworkers [13] (Figure 1-A). A 75 µm Delrin (McMaster Carr-8573K281) partition makes up the middle of the chip, containing an aperture 200-1000 um in diameter and two outer circular openings in line with the aperture, which is enclosed by a 0.0625” thick acrylic sheet (McMaster Carr-8560K171) below and a 0.25” thick acrylic sheet above. The top acrylic sheet contains three circular openings that are collinear with the Delrin partition. The bottom piece has a rectangular cutout that aligns with and connects the circular openings of the above parts. Below the bottom piece is an additional piece of acrylic that encloses the chamber. This design has been repeated eight times on a single piece of acrylic and Delrin to form chips with eight individual bilayer-forming chambers. The openings and apertures were laser cut (VersaLaser) and each piece
was assembled using VHB foam tape (McMaster Carr-7170A33). These chips allow for the formation of lipid bilayers using a microfluidic droplet technique [13].

**Bilayer Formation**

450uL of the measurement buffer solution (MB) (1M KCL, 10mM HEPES, pH 7.8) that contains 250ug/mL of 1,2-diphytanoil-sn-glycerol-3-phosphocholine (DPhPC) (Avanti Polar Lipids) is dispensed into the bottom well (Figure 1-B). 25 uL of Decane with 10 mg/mL of DPhPC lipids is then dispensed into the top well, forming a lipid monolayer (Figure 1-C). After 15 minutes, 50 uL of the MB with 100 ng/mL of αHL (List Bio-120) is added to the top well, which forms a second lipid monolayer upon contact (Figure 1-D). Shortly after, 50uL of additional buffer is added to the bottom well which brings the monolayers together to form an artificial bilayer (Figure 1-E).

**α-Hemolysin Incubation and Insertion**

The αHL (initially stored at 100 µg/mL in MB) was added in a 3:1 ratio with cholesterol (Sigma-C8667) and concentrated in MB to 90 µg/mL. The mixture was stored at 20 °C overnight and diluted to desired concentration prior to use. New incubated αHL was made weekly to avoid degradation of heptamers. After stable bilayers were formed, the incubated αHL (100 ng/mL) was added to the system, where the heptamers would self assemble into the ALB.

**Electrical Measurement**

Electrodes are placed in both the top and bottom wells and a voltage is applied (70-100mV) to detect a corresponding current (initially zero) based on the expected resistance of the bilayer. Measurements were taken using a Digitada 1440A digitizer
and a Molecular Devices 200B amplifier (Axon Instruments) for single channel experiments, and a multichannel amplifier (Tecella Inc) for parallel measurement. Custom, printed circuit boards (PCBs) have been developed by the Lu group to allow simultaneous measurement of all eight bilayers in a singular chip (Figure 1-G). Eight bleached silver wire electrodes (CC Silver and Gold-SFW22a) are connected to the PCB (Figure 1-H), which is connected to the amplifier. Up to 4 of these chips and PCBs were used simultaneously to measure 32 artificial bilayers at a time on a multi-channel amplifier.

**DNA+N Assay**

The αHL present in the buffer solution heptamerizes and forms ion channels whose integration into the bilayer can be identified by an increase in current (~70pA) [6]. Following the formation of these channels, biotinylated DNA (Biosynthesis-sequence can be found in public domain) is introduced into the system with NeutrAvidin (Life Technologies-A2666). To block nanopores, the measurement protocol applied a voltage (100-150mV) for ninety seconds to induce blocking of the nanopore and an opposite voltage (0-100mV) for 10 seconds to unblock the pore.

**Results**

**Ion Channel Integration**

α-Hemolysin (αHL) channels were integrated into the artificial lipid bilayers (ALBs) at various concentrations (50-500 ng/mL). The ion channel insertion was measured through an increase in current of approximately 90pA while holding 100mV (or 60pA while holding 70mV) across the bilayer (Figure 2), which agrees
with the conductance (1 nS ~ 1 M KCl) reported [6]. Typical bilayers had appropriate resistances ranging from $10^6 - 10^8$ ohm cm$^2$ [22], leading to sub-picoamp currents when applying 100mV. For all experiments, channel insertion was facilitated using a voltage protocol that alternated between 150mV and -150mV. At this voltage, channel insertion occurred typically within 5 minutes. At lower voltages, the time for channel insertion events to occur was longer. Furthermore, incubation of monomeric αHL in liposomes composed of DPhPC and cholesterol before addition to the bilayer significantly increased the chances of heptameric protein insertion as compared to alternate protocols where monomeric αHL was added directly to the bilayer system (Table 1). In the latter case, channel formation would take much longer and incomplete channels were more common, with channels opening transiently as well.

**Electrophoretic Movement**

Analytical use of ALBs require effective blocking of the αHL channels as this directly translates into a measureable change in current. Biotinylated DNA bound to NeutrAvidin (DNA+N) was added to the system at varying concentrations after bilayer formation and channel insertion to characterize blocking rates and dependence of blocking to analyte concentration. The negatively charged DNA+N conjugate was electrophoretically propelled towards and across the bilayer by an electric field. To confirm that the blocks were occurring due to DNA+N occupying the pore, we added DNA+N to both sides of the bilayer. When the conjugate was added to the top well, a negative voltage was applied and blocked pores resulted in increased current (Figure 3A and 3C). When the conjugate was added to the bottom well, a positive voltage
was applied and resulted in decreased current (Figure 3B). Since the direction of the electric field that induces this effect is dependent on which side of the bilayer the DNA+N conjugate is located (top/bottom), blocks should only occur while applying the appropriately directed voltage. In all experiments performed with DNA+N, blocks were observed at the appropriate sign voltage (negative on top, positive on bottom).

**Channel Blocking**

Experimental bilayers with as many as 24 ion channels were used to quantify blocking characteristics at different voltages (100-150mV) and DNA+N concentrations (50 nM-2.5 μM). Each blocking event was signified by a sudden, permanent decrease in current dependent on the applied voltage. At 100mV, 80-90pA current decreases were observed, while 100-110pA decreases were observed at 125mV. Multiple blocks also occurred simultaneously and consecutively (Figure 3) with 20pA variation from incomplete blocking by the conjugate. The number of channels present was determined to be dependent on the concentration of αHL used to initially form the nanopores. However, it was observed that all current passing through the bilayer was not necessarily guaranteed to be blocked. Blocking percentages were then recorded and found to be dependent on voltage, concentration and number of pores. 81% of the current in the ALBs were blocked at 100mV, 250 nM when an average of 12.2 channels were present throughout the tests (n=12.2), and 75% was blocked at 100mV, 1 μM when an average of 19 channels were present (n=19). However, this percentage decreases significantly to 52% at 125mV, 250 nM when an average of 2.2 channels existed (n=2.2), suggesting that more channels (larger n) allow for greater chance of complete blocking of the bilayer. It is also
important to note that residual currents were present in these systems, caused by partial opening and blocking of channels. This led us to conclude that the remaining currents may not consist of complete channels at all.

Effects on blocking time of both the concentration of DNA+N and the voltage applied were also explored. The average time to block at 100mV and 250 nM DNA+N was 5.3 seconds (n=12.2), which decreased to 3.9 seconds when the concentration was raised to 1 µM (n=19) without changing the voltage (Table 2). Additionally, when the voltage was increased to 125mV but concentration held at 250 nM DNA+N, the average time to block increased substantially to 27.4 seconds (n=2.2). This result disagrees with the expected results of higher voltages causing quicker blocks. As a result, we explored the possibility that the number of available channels for blocking was also a contributing factor to the total time to block. This data suggests that the applied voltage, as well as DNA+N concentration, is inversely related to the time to block a single channel, however blocking time decreases significantly as the number of αHL pores (n) increases. We also suspect that the average time to block was related to how many available channels exist at the time of block. The average time for each individual block was recorded, with corresponding data suggesting a nonlinear relationship that varies with voltage and concentration. This led us to believe that the nanopores are independent of one another.

**DNA Removal**

Ion channel gating often occurred due to molecular instability or contamination (theorized) and can lead to false positive data since channel gating results in a similar current decrease profile as a channel block by DNA+N conjugates.
To eliminate this uncertainty, a ramping voltage was applied to the bilayer after multiple blocks occurred. The ramp (-50mV to 0mV) slowly decreased the electrostatic force keeping the DNA+N lodged into the channel, allowing the conjugate to leave the pore when Brownian motion becomes stronger than the electrostatic force (Figure 4). As seen in the figure, the observed current decreases slowly in correspondence to the decreasing applied voltage. Each ‘bump’ in the graph signifies a DNA+N conjugate unblocking a nanopore, thus allowing the current to increase negatively. These unbinding event measurements elucidate whether reductions in current while previously applying a blocking voltage (-125mV) were due to a channel gating (transient channel closure) or due to a block with the DNA+N conjugate.

**Correlations**

In order to accurately associate correlations between the different experimental parameters and check the validity of the proposed theory, the average blocking time multiplied with the corresponding concentration was recorded and graphed against the number of channels present in the bilayer (Figure 5). This data showed exponential regression fitting when plotted, with an $R^2$ value of 0.921 and exponential equation that matches very closely to what was predicted using the mathematical model described earlier in the paper, $y = 9832.6x^{-1.179}$. This data concludes that the number of channels has an inverse, nonlinear effect on the average time it takes to block each nanopore.
Discussion

The incorporation of multiple αHL channels provides a significant advantage over single nanopore sensing. By increasing the amount of protein pores present, the number of statistically relevant events per experiment increases, effectively reducing the time required to produce conclusive data. The time to block was calculated as the time from the previous block to the blocking event, as we suspect that a blocking event immediately changes probability of another block occurring and that each nanopore behaves independently of each other. As demonstrated, the likelihood of blocking increases dramatically with each additional pore, which increases the speed of nanopore-based sensing devices.

Based on experimental results, we have concluded that significant correlations exist between the time for a molecular sensing event and applied voltages, molecule concentration, and the number of channels present in the ALB. We showed that the average time to block directly relates to how many available channels exist at the time of block (Figure 5). This relationship between the quantity of channels available to block and the time it takes to block is critical when introducing an analyte, into the system, as it will provide a reference for analysis of various blocking events in multi-nanopore studies. Using this mathematical model, a reference curve can be constructed, ultimately allowing for rapid determination of the molecular concentration present in a system, if the number of nanopores is known, or vice versa. This data specifically relates to the αHL nanopores and can thus be translated to detection for various other molecules, as long as the association constant, $K_{on}$, is determined.
It is also worth noting that incorporating monomeric αHL into liposomes consisting of DPhPC and cholesterol (3:1) significantly improved the chances of incorporation of full heptameric channels in our bilayers. The liposomes effectively mimic the use of red blood cells or surfactant micelles used in other studies [23-24], providing a membrane for oligomerization of αHL monomers. Furthermore, observations suggest that αHL continues to oligomerize up to 1 week after initial incubation, but begins to degrade within that time period as well. Complete analysis of this incubation process will increase effectiveness of commercially available αHL and allow oligomerization of αHL in a cell-free environment with minimal purification.

Further analysis would be particularly appropriate for studies involving aptamer binding to target neutral analytes as was reported earlier [17]. Because the aptamer is a nucleic acid sequence and the analyte is uncharged, the system would behave similarly to the experimental design used here. Increasing the number of nanopores in aptamer binding studies would reduce the time required to acquire statistically relevant information about the analyte.

Various other approaches to nanopore detection utilize single αHL channels in ALBs or multiple solid-state nanopores [21]. However the advantages of multiple nanopore measurement can apply to many different technologies for protein detection, molecular tagging, and various other single molecule applications. The production capacity and sensing capabilities of ALBS increases substantially by integrating multi-pore sensing with the corresponding parallel arrays mentioned in this paper. Further considerations that should be taken into account for ALB use
include microfluidic requirements, smaller bilayer development, and contamination prevention. The addition of these two enhancements will hopefully lead to further applications of single molecule detection while maintaining a relatively low and realistic cost.
<table>
<thead>
<tr>
<th>Number of Trials</th>
<th>Average Time to Channel Formation (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>αHL Monomers</td>
<td>4</td>
</tr>
<tr>
<td>αHL Heptamers</td>
<td>8</td>
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</tbody>
</table>

**Table 1.** Comparing the effect that incubation has on the rate of nanopore formation. The αHL monomers self assemble into heptamers in the presence of cholesterol and liposomes, which can later insert more efficiently into the ALBs.

<table>
<thead>
<tr>
<th>Concentration (nM)</th>
<th>Average Channel Number</th>
<th>% Of Current Blocked</th>
<th>Average Time to Each Block</th>
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</thead>
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<tr>
<td>200</td>
<td>1.7</td>
<td>100</td>
<td>11.3</td>
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<tr>
<td>250</td>
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<td>81</td>
<td>5.3</td>
</tr>
<tr>
<td>600</td>
<td>2</td>
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<td>1500</td>
<td>1.1</td>
<td>100</td>
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</tr>
</tbody>
</table>

**Table 2.** The data shows the effect that concentration (nM) and the number of channels have on the time for each ion channel block to occur at 100mV only. While concentration can be seen to increase the rate of block, the average number of channels is the most important variable in determining both the percentage of current blocked and the average time to each individual block.
**Figure 1.** Parallel bilayer array and development schematic  
(A) Exploded assembly of the chip. The small hole in the partition is in line with the middle well of the top sheet. (B) First step of bilayer formation. Buffer is added to bottom channel. (C) Decane then added through top, middle well to form self-assembled monolayer. (D) Buffer then added to top well to form another monolayer. (E) Bilayer forms from two monolayers after more solution is added to bottom well. (F) Diagram of 8-well chip array. (G-H) Printed circuit board with bleached silver wire electrodes connected to base. (I) Schematic for 32-bilayer testing apparatus, where each chip and circuit board is help in place using acrylic plates. Figure taken from Lu et al.
Figure 2. A single αHL channel is shown inserting into the artificial lipid bilayer by an applied voltage of 100mV. The increase in current is caused by a decrease in the resistance of the bilayer, which increases the current based on ohm’s law, \( V = IR \).
Figure 3. Electrophysiological measurements of channel blocking 
(A) Two channels being blocked by DNA+N at 125mV, 250 nM. DNA+N located in the top well. (B) Five channels being blocked at 150mV, 100 nM αHL concentration. Located in the bottom well, which explains the positive current. (C) Sixteen channels being blocked at 100mV, 1 μM αHL concentration. DNA+N located in the top well.
Figure 4. Ramping the applied voltage from -50mV to 0mV to release DNA+N from αHL nanopore. The slow decrease in voltage allows us to observe the exact moment that the conjugate unblocks the channel. Two channels are unblocked in this figure.
Figure 5. Graphing the effect that the number of channels present in the ALB (N) has on the time it takes to block an individual nanopore. A and C show the 1\textsuperscript{st} order exponential decay that is exhibited by the system, while B shows an inverse x-axis which forms a linear trend due to the power trend in A and B. Error bars calculated using 95% confidence intervals for exponential regression. Corresponding equations signify high similarities to an exponential regression fit. $R^2$ values verify the quality of the fit of the data points to the trend line.

(A) 250 nM and 1 \textmu M DNA+\text{N} concentrations at 100mV multiplied by the time to block vs. the number of blockable channels (N); (B) Concentration multiplied by time to block vs. inverse number of blockable channels (1/N). (C) Graph showing the weighted averages of figure 5A (250 nM and 1 \textmu M) vs. the number of blockable channels.
References


