# Investigations on Transmembrane Ion Channels Suspended Over Porous Silicon Membranes

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## ABSTRACT

This work presents a method to characterize the electrochemical properties of transmembrane ion channels and lipid bilayer membranes. The system is composed of a 3 µm thick porous silicon membrane with the epithelial sodium channel (ENaC) proteins fused into a lipid bilayer membrane (LBM) supported on the porous silicon layer. The LBM was composed of two synthetic phospholipids:1,2-diphytanoyl-*sn*-glycero-3-phosphoserine and 1,2-diphytanoyl-*sn*-glycero-3-phosphoethanolamine. Electrical Impedance spectroscopy was performed from 0.1 Hz to 100 KHz. The electrochemically-fabricated porous silicon template had pore diameters in the range 0.2~ 2 µm. The LBM was formed by means of the Langmuir-Blodgett and Langmuir- Schaefer techniques, at a bilayer surface tension of 40 mN/m in room temperature. The electrolyte-PSi system showed on average a capacitance of about 9.76 µF/cm$^2$. The lipid bilayer membrane showed a capacitance of about 0.63 µF/cm$^2$. The ENaC channels resulted in a capacitance of about 0.57 µF/cm$^2$.

*Keywords:* Porous silicon; Lipid Bilayer Membranes; Transmembrane proteins; Electrical impedance

## I. INTRODUCTION

Artificial black lipid membranes have been studied significantly in literature for their remarkable ability to mimic biological membranes [1] allowing biologists to perform studies on membrane proteins [2] [3]. Studying ion channels, in particular, requires investigating their functionalities in relation to their structures, which is accompanied by tremendous difficulties challenging when studying them in their native tissues [4]. Nonetheless, the lack of strength in black lipid membranes, and their high vulnerability to rupture possess a great challenge to biologists. Hence, efforts to understand the structure and function of intercellular membrane proteins fused into lipid bilayers have become reliant on micro and nanofabricated template technologies such as sol gel, alumina, mica-based microarrays, and porous silicon templates for protein analysis [5-12]. However, one drawback of these technologies is the difficulty associated with studying transmembrane proteins, due to the contact of the substrate to the lower membrane side of the proteins, leading to protein denaturation and conformational changes [6]. To overcome this difficulty, technologies based on porous materials such as porous silicon have been investigated to support lipid bilayer membranes for studying transmembrane proteins and biosensing applications [7-11]. However, none of these have demonstrated ability to fully address transmembrane proteins from underneath the porous support frame.

We present a device composed of artificial lipid bilayer membranes (BLM) from synthetic phospholipids deposited on electrochemically-fabricated porous silicon membranes to be used for incorporating transmembrane proteins into the lipid bilayers and investigated them using electrical impedance spectroscopy. The LBMs are formed using the Langmuir-Blodgett [13] and Langmuir-Schaeffer techniques [14,15]. The lipids used for LBM formation in this work have been previously demonstrated to be used for incorporating the Epithelial Sodium Channel (ENaC) proteins by Berdiev and Benos [2] and Ismailov et. al [3], in which negatively charged phosphatidylserine and the neutral lipid phosphatidylethanolamine are used to form the supporting LBM. The porous silicon template depicted in figure 1, acts as a mechanical support allowing ion flux to pass through bilayer and porous silicon an electrolyte solution on the backside of the substrate.

## II. POROUS SILICON TEMPLATE

Porous silicon was prepared electrochemically [16] on a n-type silicon substrate with a low resistivity and a <100> crystallographic direction. A process was developed to fabricate a porous silicon structure that has a thickness of about 3 µm and pore diameters of about 0.2~2 µm. In this process a volumetric concentration of 49% HF: 95% Ethanol: Deionized Water equal to 30:15:55 was used. The electrochemical cell was light-illuminated at room temperature. A current density of about 10-15 mA/cm$^2$ was applied for over duration of 20-30 minutes. The sample was then rinsed in DI water for 10 minutes, then immediately immersed in Ethanol to reduce the water capillary effect when
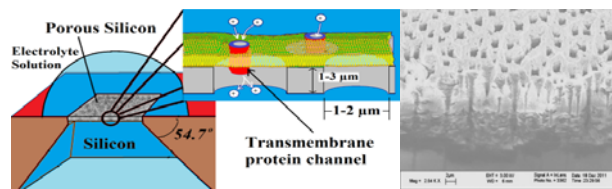


Figure 1. Illustration of the artificial biological membrane with SEM image of a porous silicon support with 0.5 µm wide pores etched through a 3 um thick silicon membrane.

drying. The resulting porous silicon membrane had an area of about $3.6 \times 10^{-4}$ cm². A scanning electron micrograph of the porous membrane is presented in figure 1.

## III. LIPID BILAYER DEPOSITION

Many techniques have been developed for depositing lipid bilayer membranes such as lipid vesicle spreading [17], the Montel-Mueller method [18], spin coating [19,20], and the Langmuir- Blodgett and Langmuir- Schaefer techniques molecules. A Lamgmuir-Blodgett (LB) trough was used [13-15], which offer the advantage of tight packing of the lipid to deposit a lipid bilayer membrane on the structure using the Langmuir-Blodgett and Langmuir-Schaeffer techniques as illustrated in figure 2. Prior to deposition, the porous silicon membrane is wetted with water from both sides, and then the back opening is tightly sealed with a copper foil over an electric insulator.

Two phosphoglyceride lipids: 1,2-diphytanoyl-*sn*-glycero-3-phosphoserine ($C_{46}H_{89}NO_{10}PNa$) and 1,2-diphytanoyl-*sn*-glycero-3-phosphoethanolamine ($C_{45}H_{90}NO_8P$), with the molecular weights 870.163 and 804.172 g/mol respectively and supplied by the Avanti Polar Lipids, Inc were mixed in a one-to-two parts ratio. Phosphatidylserine and phosphatidylethanolamine are two of the most common phospholipids in biological membranes, comprising 25% of the plasma membrane of red blood cells and more than 70% of that of the Escherichia coli (E. Coli) bacteria.

The Langmuir-Blodgett technique offers the advantage of tightly packing the lipid molecules, hence resulting in a bilayer membrane that is as strong and tight as possible. Tight packing of lipid bilayers is a feature biologically achieved by the presence of cholesterol in mammalian cell membranes. Figure 2 below illustrates the effect of surface pressure on membrane formation. The goal is to pack the lipids tight enough to form a contiguous layer that provides molecular ordering without supplying so much pressure that the lipids begin to fold over on themselves. Thus, a bilayer should be packed as close to the solid-state For this experiment, a total of 9 μL of the phospholipid mixture was added to the surface of deionized water and were compressed at a speed of 1.5 cm/min and at a surface pressure of 40 mN/m using a LB trough by Nima Technology LTD.
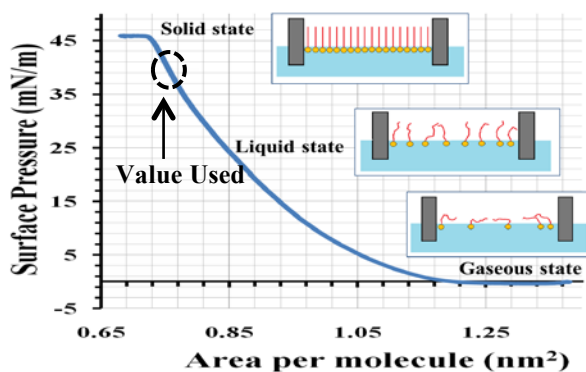


Figure 2. Surface pressure vs. Area isotherm of LB/LS deposited membranes.

The first monolayer was deposited by retracting the porous silicon samples upwards at a speed less than 2 mm/min. The decrease in the area of monolayer on the water surface to maintain the surface pressure was greater than the area coated on the porous silicon substrate. This indicated a deposition transfer ratio greater than or equal to unity, as defined by Langmuir and Schaefer [15]. The second monolayer was deposited using the Langmuir-Schaefer method. Proteoliposomes containing Epithelial Sodium Channel (ENaC) Proteins were spread over the lipid bilayers immediately after deposition.

## IV. PROTEIN FUSION

The method to prepare the amiloride-sensitive Na⁺ channel proteins of the ENaC/degenerin family was detailed by Berdiev and Benos in [2]. In brief, the method starts by linearization of plasmids containing the ENaC cDNA, the plasmid then undergoes an *in-vitro* transcription process into messenger RNA using T7 RNA Polymerase proteins. The translation process of the messenger RNA to ENaC proteins is done using the TNT transcription-translation kit.

Following a procedure to elute and concentrate the ENaC proteins, the proteins are reconstituted into proteoliposomes by incubating the proteins with the lipids: phosphatidylethanolamine, phosphatidylserine, and phosphatidylcholine at a 50:30:20 ratio consecutively. Alternatively, vesicles containing the ENaC proteins may also be prepared by injecting the cRNA molecules of each of the three subunits of the ENaC proteins into the oocytes of an adult female Xenopus [2].

Lipid bilayer membranes were deposited on one side of the porous silicon layer. Immediately after LBM deposition, vesicles containing ENaC channel proteins were spread on the lipid bilayer membrane. The electrolyte solution was a 99% purity sodium chloride (NaCl) dissolved in deionized water at a concentration of 0.1M.

Atomic force microscopy of protein fused lipid membranes on the surface of the porous silicon bilayer are presented in figure 3. The image presented shows both natured proteins with a 30 nm elongated shape and larger areas (100 nm²) where membrane fusion exceeded the solid state surface pressure transition causing the lipid bilayer to fold over onto itself and denature some of the fused proteins. Reducing surface pressure in the LB trough can eliminate folding of the lipid bilayer and providing accurate control of the amount of the protein incorporated lipid micelles fused onto the surface.
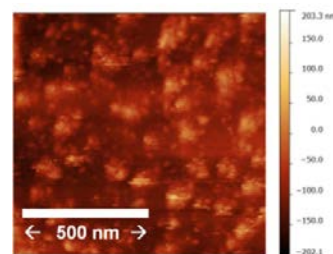


Figure 3. Tapping mode AFM after ENaC protein fusion.

# I. IMPEDANCE ANALYSIS

Characterization of the Electrochemical Impedance was performed at room temperature using a Princeton Applied Research VersaSTAT3 potentiostat. A three-electrode system was used as shown in the setup in figure 4. In which a platinum wire was used as the working electrode, the counter electrode was a copper foil that tightly covers the 0.9 mm × 0.9 mm back hole of the structure supporting the porous silicon layer. The reference electrode was a standard 4M potassium chloride (KCl) saturated with silver chloride electrode by Accumet, with a thin Luggin-Haber capillary that was built in the lab to fit in the micro electrochemical system. The tip of the reference electrode is set at a close proximity to the working electrode in one compartment, and the counter electrode in the bottom compartment. The height and location of the reference and working electrodes from the porous silicon surface were adjustable by a precision translation stage.
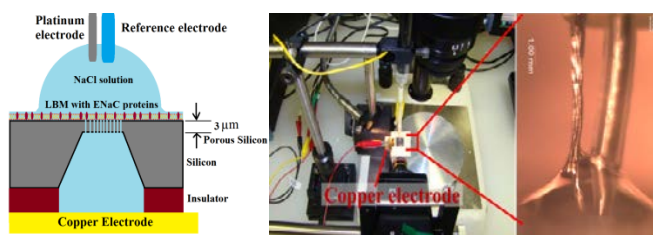


Figure 4. Schematic of the experiment (left). Experimental setup (middle). Platinum and the reference electrodes penetrating the electrolyte above the Psi surface (right).

In their work, Naumowicz et. Al. [21] performed EIS measurements on lipid bilayer membranes formed from 3-sn-phosphatidylcholine and 1,2-dimyristoyl-sn-glycero-phosphoethanolamine covering an area of about .04-.08 cm² using sinusoidal signals with a peak amplitude of 4 mV. On the other hand, it was reported in [22] that the breakdown potential measured for phosphatidylcholine is over 200 mV in the pH range 4-8. In this study, a 100 mV sinusoidal signal was applied in the range of frequencies 0.1-100,000 Hz. The peak amplitude of the applied signal is comfortably below the breakdown potential reported for phosphatidylcholine in [26]. It is also high enough to take into account the porous silicon layer impedance with accurate measurements.

The lipid bilayer membrane is electrically modeled as impedance composed of a resistance in parallel with a capacitor [12], addition of the ENaC channels is modeled by adding another impedance parallel to the LBM impedance. In series with them is the impedance of the porous silicon membrane, and the electrolyte resistivity. The simplest equivalent circuit of an electrochemical system takes into account the resistance of the electrolyte solution, which is modeled as a resistance $R_{electrolyte}$ connected in series in the equivalent circuit of the system, since the entire current must pass through the electrolyte [37]. The impedance of the system includes the series combination of the impedance of the system and the electrolyte resistance.

The real and imaginary components of the impedance, $Z_{S3}$, of the completed membrane with ENaC vesicles are spread on the lipid bilayer membrane to incorporate the transmembrane ion channels. The equivalent impedance of this Electrolyte-PSi-LBM-ENaC system is shown in the figure 5. The real and imaginary impedances are given by:

$$Z_{S3_{Re}} = R_{electrolyte} + \frac{R_{PSi}}{1+R_{PSi}^2 C_{PSi}^2 \omega^2} + \frac{R_t}{1+R_t^2 C_t^2 \omega^2}, \quad (1)$$

$$Z_{S3_{Im}} = \frac{R_{PSi}^2 C_{PSi}\omega}{1+R_{PSi}^2 C_{PSi}^2 \omega^2} + \frac{R_t^2 C_t \omega}{1+R_t^2 C_t^2 \omega^2}, \quad (2)$$

where,

$$R_t = \frac{R_{LBM}R_{ENaC}}{R_{LBM}+R_{ENaC}}, \quad (3)$$
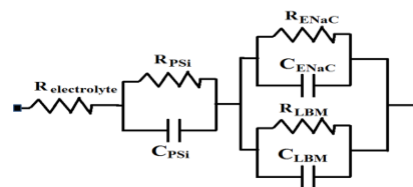
$$C_t = C_{LBM} + C_{ENaC}. \quad (4)$$



Figure 5. Equivalent impedance of the system.

To determine the impedance of the lipid bilayer and the transmembrane proteins, the impedance spectra over the range 0.1 Hz to 100 KHz were measured for the porous silicon membrane alone as a control experiment, then with the addition of the lipid bilayer membrane, and finally with the spreading of the ENaC vesivcles. The impedance spectra of each of the three regimes are shown in figure 6.
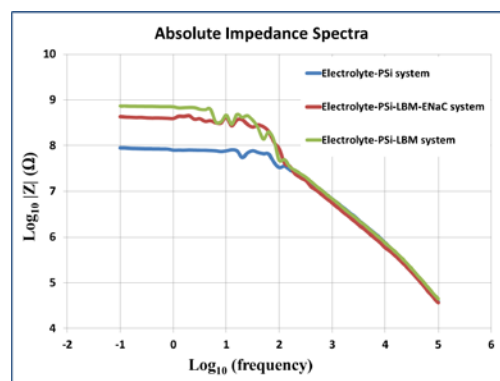


Figure 6. Electrochemical Impedance Spectra.

The data shows that impedance decreases with increasing frequency for all regimes, and that no significant difference is observed at high frequencies. This is attributed to the decrease of the reactance known to be inversely proportional to the frequency. The system composed of a porous silicon with a lipid bilayer membrane has a higher impedance than the system with incorporated protein channels. The high impedance in the porous silicon-LBM system is expected and is attributed to the low permeability of the LBM to ions; resulting in an increase in the impedance. Following the incorporation of the ion channel proteins, the impedance

decreases due to the presence of the sodium channels in the lipid bilayer. This difference in the impedance is more prominent at low frequencies, due to decreases in the impedance difference with increasing frequencies.

The Nyquist plots associated with each of the three regimes are shown in figure 7. A second order polynomial regression of the imaginary versus real impedance of each of the three systems was used over the entire range of frequencies to generate the nyquist cuvrve. The resonance due to the silicon substrate that occurs between 70-80 Hz (seen in figure 6) and results in data scattering near that range of frequencies. The electrolyte-PSi system showed on average a capacitance of about 9.76 $\mu F/cm^2$. The lipid bilayer membrane showed a capacitance of about 0.63 $\mu F/cm^2$, this capacitance is in agreement with that obtained for black lipid membranes of the same phosopholipid structure (0.67-0.95 $\mu F/cm^2$) [2]. The ENaC channels resulted in a capacitance of about 0.57 $\mu F/cm^2$. It is also slightly larger than, but still in good agreement with the EIS data taken on ENaC on living frog cells which recorded a capacitance of 0.33 $\mu F/cm^2$ [24].
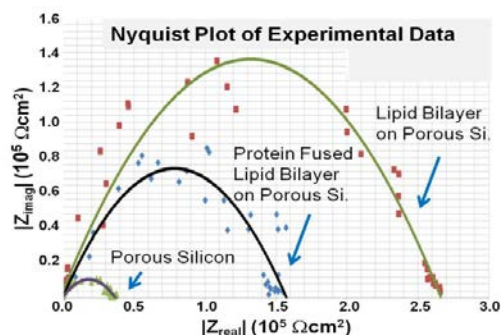


Figure 7. Nyquist plots. The Experimental data fitted by solid lines for each of the three experimental conditions.

## II.   CONCLUDING REMARKS

This article demonstrates a protein functionalized porous silicon bio-membrane tested using electrochemical impedance spectroscopy. The resulting capacitance of the ENaC protein fused within the lipid bilayer is comparable to published BLM and in-vivo path clamp experiments. However, the result shows promise for the use of thin porous silicon membranes as a templating technique for the creating and testing or artificial biological membranes.

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