Integrated Techniques for Transmembrane Protein Sensing

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ABSTRACT

Devices with 150μm diameter apertures were microfabricated in a silicon substrate using well-known cleanroom technologies for the measurement of ion channel proteins. The capacitance of the device was reduced with a 75μm SU-8 layer and the surface was made hydrophobic with chemically vapor deposited polytetrafluoroethylene. A novel approach using phase sensitive detection was then used to measure single channels of the bacterial transmembrane porin protein OmpF inserted into phospholipid bilayers formed across the aperture of the fabricated device.

Keywords: microfabrication, ion channel, sensor, lipid bilayers

1 INTRODUCTION

Ion channel proteins are of considerable interest because they produce the excitation of nerves and muscles. They have been routinely measured in many laboratories with both patch clamp and ion channel reconstitution techniques. Patch clamping refers to the technique where a cell membrane with embedded ion channels is sucked into the opening of a glass pipette forming a high resistance giga-ohm seal and is held at a known potential [1]. Discrete switching events between open and closed states of the ion channels in the membrane result in current fluctuations which are measurable using conventional low noise current amplifiers[2]. Ion channel reconstitution replaces the pipette tip with a small opening in a suitable substrate so that a lipid membrane can be formed across the aperture with Montal-Mueller techniques [3]. Ion channels are then inserted into this membrane so that they can be studied in a known environment [4].

Efforts have been made to replace the patch-clamp with microfabricated devices that are suitable to perform the same ion channel measurements. Researchers have focused on fabricating small apertures in glass [5], Si/SiO2 [6-10] and silicone elastomers [11]. Different issues such as ease of fabrication, noise properties of the final device and the ability to form a high resistance giga-ohm seal, suitable for single channel measurement, have been addressed.

There have also been efforts to genetically engineer transmembrane proteins for stochastic sensing of organic analytes. It has been shown that by genetically engineering and modifying the inner pore regions of α-hemolysin channel proteins, specific analytes can be detected and identified[12, 13]. Using engineered proteins and an integrated device, a sensor capable of detecting single molecule analytes can be envisioned. Previously we have demonstrated a working microfabricated silicon device that is capable of such measurements [6-8, 14]. Here, we demonstrate a phase sensitive detection technique used to record single channels of transmembrane proteins inserted into bilayer membranes formed on the device. This technique rejects background ambient noise and enables measurements in surroundings where common voltage clamp, current recording methods fail.

2 EXPERIMENTAL

Samples were prepared using 4”, double-sided polished Si (100) wafers having a thickness of 440μm. The substrates were patterned using photolithography and standard AZ4330 resist and then etched in a deep silicon reactive ion etcher (STS Advanced Silicon Etcher) using the Bosch process. The aperture was designed to have a 150μm diameter centrally located in a 1 mm diameter region thinned to a final thickness of 150μm. A thermal oxidization of 200nm followed to produce an electrically insulating layer on the surface. In order to reduce the capacitance of the device, 75μm of SU-8 100 resist was patterned around the central aperture with conventional photolithography. A polytetrafluoroethylene (PTFE, Teflon) layer was then chemically vapor deposited using the deep reactive ion etcher with C4F8 as the gas source. The hydrophobic termination lowers the surface energy and increases the contact with the lipid hydrocarbon chains, thereby allowing formation of a high resistance giga-seal[8]. All fabrication was performed at Arizona State University in the Center for Solid State Electronics Research cleanroom.

Lipid bilayer experiments were performed using a Teflon bilayer chamber with a 5 mm diameter opening between two baths of electrolyte solution. Both baths were filled with 3 ml KCl solution (1.0M for the bilayer measurements and 0.75M for the OmpF porin

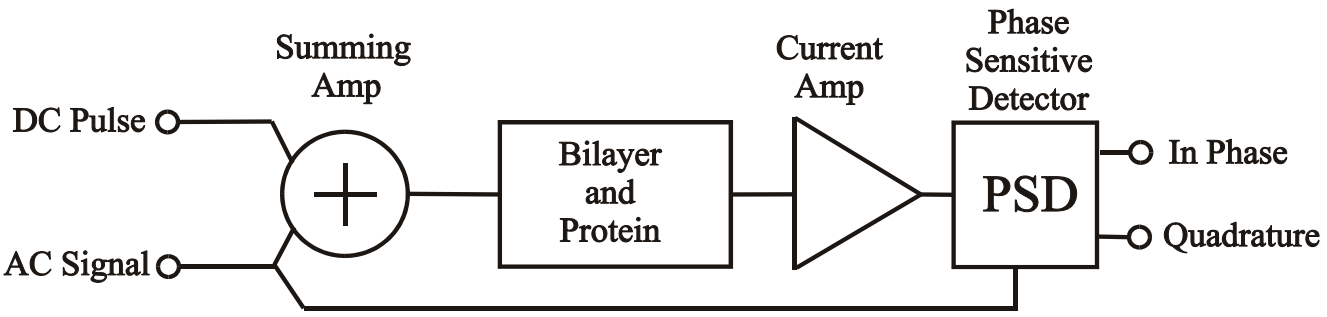

Figure 1: Block diagram of the final instrumentation set up used for phase sensitive detection of the conductances of ion channels inserted into suspended lipid bilayers. A DC holding potential and an AC reference signal are added together using a summing circuit and the resulting voltage waveform is applied to the lipid bilayer system. A low noise current amplifier is used to magnify the current response of the system to the applied potential waveform. The AC current response is compared to the reference signal of the lock-in amplifier using phase sensitive detection and the final in-phase and quadrature components are output to a text file using LabVIEW software.

measurements), buffered with 20 mM N-(2-Hydroxyethyl) piperazine-N'- (2-ethanesulfonic acid) (HEPES) at pH 7.4. The device was sandwiched between the baths with the aperture in the center of the opening. Each bath was connected to external electronics with Teflon coated silver wires, each with one end chloridized in 5% NaOCl. Lipids (1,2-Dioleoyl-sn-Glycero-3-Phosphoethanolamine and 1,2-Dioleoyl-sn-Glycero-3-Phosphocholine) (DOPE: DOPC, 4:1) were dissolved in n-Decane (10mg/ml) and used to form a bilayer with the techniques of Montal and Mueller [3]. Current response and channel conductance were measured using an Axon Instruments Axopatch amplifier, a Keithley 236 source measure unit, a Stanford Research Systems SR 830 lock-in amplifier, a Stanford Research Systems SR570 current preamplifier and a National Instruments DAQ PCI card programmed with LabVIEW software. The lock-in reference signal used for phase sensitive measurements was a 20mV RMS, 15Hz sinusoidal waveform.

3 DISCUSSION AND RESULTS

A silicon aperture was previously designed and fabricated which allowed for long term stable formations of lipid bilayers using Montal-Mueller techniques [3]. Common silicon processing techniques were used for precise control of device parameters on the micron level. A 1 mm diameter, circular area of the wafer was thinned to ~150μm final thickness in the area where the aperture was formed. Once the wafer was thinned, a 150μm aperture was etched though the center of the thinned region using backside alignment lithography and the Bosch deep silicon etch process. SU-8 was then patterned into the thinned region and onto the surface of the device in order to reduce the capacitance and decrease the noise of the device [6, 7]. It was found that PTFE chemically vapor deposited on the surface helps to ensure a high resistance seal with a lipid bilayer [8]. Previously, these devices were used to measure characteristic current recordings of OmpF porin channels using conventional voltage clamp electronics [6-8].

One main problem in the recording of ion channel proteins is the inherent noise of the total measurement setup. Voltage clamp measurements record the current through the lipid bilayer and ion channels as well as currents arising from noise factors including shot noise, 1/f noise, the input voltage noise of the amplifier headstage and dielectric noise due to thermal fluctuations in lossy dielectric materials [15-18]. Noise levels can become large enough to drown out the desired current response of the ion channels. One way to overcome such noise issues in measurements is to use phase sensitive techniques to increase the signal to noise ratio.

The main goal of using the lock-in amplifier was to demonstrate that it could be used to measure single ion channels of transmembrane proteins. OmpF porin protein was used as the model protein for the phase sensitive measurements because it was previously measured on the silicon device using the Axopatch 200B current amplifier [2]. Here, a Stanford Research Systems SR830 digital lock-in amplifier was used for the phase sensitive detection. The digital lock-in amplifier provides an advantage over conventional analog lock-in amplifiers because problems such as harmonic rejection, output offsets, limited dynamic reserve and gain error are significantly reduced by digital signal processing techniques [19]. A Stanford Research SR570 low-noise current amplifier was used to amplify the signal from the lipid bilayer system.

Using the lock-in amplifier, a low valued (<25mV RMS) sinusoidal signal was applied to the bilayer system. The closing of OmpF porin channels is not statistically favorable unless the applied voltage is greater then 100mV DC [20-22]. The AC signal from the lock-in amplifier could be directly applied to the bilayer system so that as channels insert the magnitude of the measured conductance increases with characteristic steps. With just the low magnitude AC signal of the lock-in amplifier the closing of the channels was not statistically favorable. Therefore, an adder circuit was designed and built with an operational

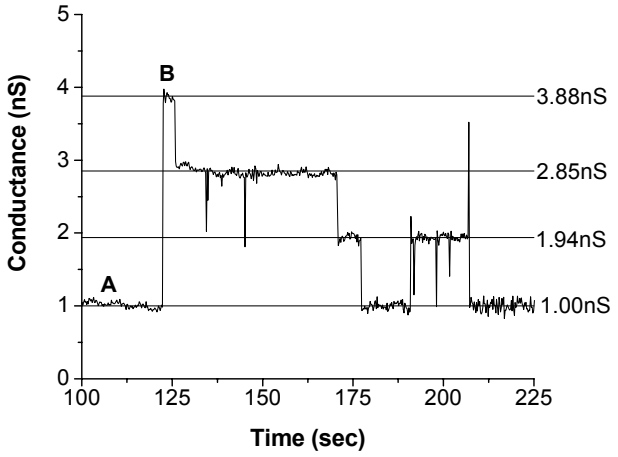

Figure 2: Conductance measurement using a lock-in amplifier of OmpF porin protein inserting into a lipid bilayer suspended on the silicon device. The bath solutions were 0.75M KCl solution with a 150mV DC holding potential added to a 25mV RMS lock-in reference signal. The conductance as a function of time shows discrete steps due to the opening and closing of single channels of the OmpF porin.

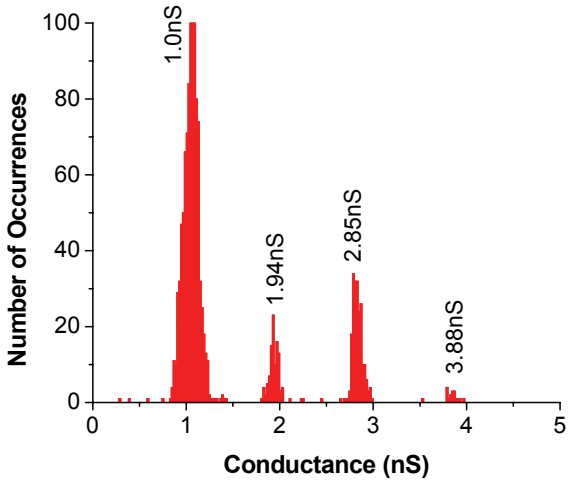

Figure 3: The bilayer and protein system conductance from Fig. 2 compiled into a histogram showing peaks corresponding to the conductance of the single channels. The average of the differences between each adjacent peak can be used to determine the average single channel conductance of the protein, in this case 0.96nS.

amplifier (OPA27) to apply the summation of the AC signal from the lock-in amplifier and a DC signal from another external voltage source to the lipid bilayer system. The additional voltage source was a Keithley 236 source meter that was used to apply a DC potential high enough to make channel closing statistically favorable. A block diagram of the final instrumentation setup is shown in Fig. 1.

Before measuring lipid bilayers using the lock-in amplifier, the system (Fig. 1) was calibrated using resistor and capacitor elements with values similar to those of experimental ion channels measured with conventional low noise current amplifiers. The most important part of the lock-in measurement is the relationship between the phase of the reference voltage signal output with regards to the measured current signal input. Low-pass filtering of the signal in the current amplifier as well as measurement cable capacitance will result in a phase shift which must be zeroed out. A capacitor circuit element was measured with the instrument setup shown in Fig. 1 and used to set the phase of the lock-in to 90° similar to methods presented in [23]. This ensures that the in-phase output from the lock-in accurately reflects the real component of the measured current signal.

Once the system was calibrated, measurements were performed to determine the conductance of OmpF porin protein. First, lipid bilayers were formed in 0.75M KCl solution using the Montal-Mueller painting technique on the silicon aperture and measured with the lock-in amplifier as a function of time. The sampling rate of individual points was limited to 5Hz because of the serial port and software interface between the lock-in amplifier and the data recording computer. OmpF porin was added to the grounded bath and a bias of 150mV was applied across the membrane. OmpF is a trimer with three conducting channels per protein so that when it is measured in the system, there are three distinct conductance levels per protein. Fig. 2 shows the initial high resistance bilayer (point A), followed by the insertion of OmpF porin (point B) and then by the closing of individual channels due to an applied bias of 150mV. The insertion and subsequent closing of three channels in Fig. 2 is most probably due to a single OmpF protein.

The data from the conductance plot as a function of time can be plotted as a histogram where each peak corresponds to the conductance of a different number of OmpF porin channels [24]. An amplitude histogram was plotted from the recording presented in Fig. 2 and is shown in Fig.3. The first peak at ~1nS corresponds to a 1GΩ membrane formed across the aperture. Successive peaks correspond to integer multiples of a fundamental conductance, associated with that of single channels of the OmpF porin protein. The average of the differences between each adjacent peak can be used to determine the average single channel conductance of the protein, in this case 0.96nS. For 0.75M KCl, a conductance of about 1nS is commonly reported in the literature [25, 26].

4 CONCLUSION

A silicon based device was fabricated with suitable properties for lipid bilayer formation and single ion channel measurements. The Bosch process was used to etch an

aperture of 150μm diameter in the silicon substrate and a 75μm thick, capacitance reducing, SU-8 layer was patterned around the aperture. PTFE was then chemically vapor deposited on the surface to help ensure a high resistance seal with a lipid bilayer. A lock-in amplifier was used to measure conductance changes of single channels of OmpF porin protein inserted into bilayers formed on the silicon device demonstrating the ability to use such techniques for measurement of ion channel proteins. Optimization of the phase sensitive detection still requires an increase in the sampling rate of the measured data in order to record channel fluctuations that occur on millisecond time scales. The microfabricated device, in conjunction with phase sensitive techniques, demonstrates a sensor capable of measuring stochastic conductance fluctuations of engineered proteins resulting from the detection of specific analyte molecules.

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