

Planar lipid bilayer reconstitution with a micro-fluidic system

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A planar lipid bilayer which is widely used for the electrophysiological study of membrane proteins in laboratories is reconstituted using a micro-fluidic system, in a manner that is suitable for automated processing. We fabricated micro-channels on both sides of the substrate, which are connected through a 100–200 μm aperture, and showed that the bilayer can be formed at the aperture by flowing the lipid solution and buffer, alternately. Parylene coating is found to be suitable for both bilayer formation and electric noise reduction. Future applications include a high-sensitivity ion sensor chip and a high-throughput drug screening device.

Introduction

Understanding the function and interaction of proteins is a significant and formidable task in post-genome studies.¹ After the success of the DNA array chip, the idea of a “protein chip” had emerged in which proteins are patterned to a substrate in an array, so that their different interactions can be monitored in parallel.^{2,3} Although applications of protein array chips diverge widely, their primary targets are mostly water-soluble proteins. On the other hand, handling of membrane proteins (*e.g.* ion channel, pump, receptor and transporter) has been tremendously challenging, since they are bound in a plasma membrane structure.¹ They have to be kept in the lipid bilayer structure throughout the analytical process, otherwise they lose their original activities.

There are two major methods for the functional study of membrane proteins, *i.e.* patch clamping⁴ and the planar bilayer method.^{5,6} In the patch clamping method, a cell that contains a target membrane protein is held tightly against a glass pipet by suction, and the potential or channel current between the inside and the outside of the cell is recorded. The planar bilayer method (*e.g.* the Langmuir–Blodgett method) utilizes an artificial planar lipid bilayer reconstituted at a tiny aperture opened typically on a Teflon sheet. Electrophysiological study of the membrane protein is performed after the incorporation of a target membrane protein into the bilayer. In this method, the composition of lipid and surrounding electrolyte can be arbitrarily determined, and inner-cellular membrane proteins can also be analyzed.

However, both methods are laborious processes, and required experience and skill. Recently, several groups have been developing on-chip patch clamping systems.^{7,8} In those systems, a cell is aspirated to a tiny aperture on a chip instead of a glass pipet and the standard patch clamping process follows. The on-chip integration of a planar lipid bilayer has been less explored; possibly because the formation of the bilayer is rather unstable and relies on many parameters, such as the amount and distribution of lipid and the pressure difference across a hole.

In this paper, we present novel micro-devices and methods to reconstitute the planar lipid bilayer membrane on a chip, which is suited to the automated multi-channel study of membrane proteins. The bilayer membrane is formed at the etched aperture on a silicon substrate using either a micro-fluidic system or a liquid droplet injection system.

Preliminary test

To start with, we examined how a lipid bilayer forms on a tapered aperture made on a silicon substrate by flowing lipid solution and buffer (Fig. 1). Firstly, a silicon chip with an anisotropically etched hole (150 μm) is placed over a buffer droplet (0.1 M KCl + 0.1 M glucose) on a glass substrate. At this moment, the buffer surface stays at the front of the aperture. Then, 1 μL of lipid solution (20 mg asolectin per mL *n*-decane) is placed next to the aperture and spread over it by tilting the substrate. Lastly, the buffer droplet is placed on the aperture. The formation of the bilayer is observed using an inverted microscope (Olympus IX-71) with a $\times 20$ to $\times 40$ objective lens through transmitted light. When the lipid bilayer is formed, its circular edge is clearly observed as shown in Fig. 2(a), which is in accordance with the findings of Ide *et al.*^{9,10} The area outside the bilayer is a thick layer of lipid solution (annulus) and the small dots should be micelles of lipid molecules. In this report, only one combination of lipid (asolectin taken from soy bean), solvent (*n*-decane) and buffer has been tested. However, other combinations found in the literature (*e.g.* phosphatidylcholines and phosphatidylserines for lipids, and hexa-decane and squalene for solvent⁵) should work as well.

The thickness of the initial layer of lipid solution on the aperture is found to be crucial for the bilayer formation. A bilayer is formed spontaneously when it is thin enough. However, when the amount of lipid solution is too much, it merely stays as a thick layer. Thus, the substrate should be wettable to the solvent so that excess of lipid solution spreads

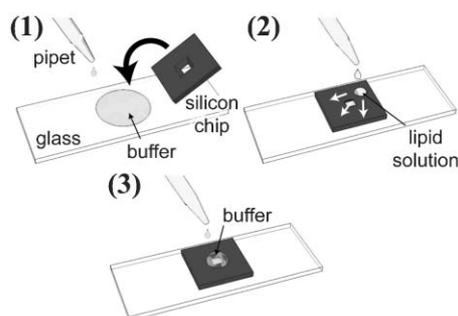


Fig. 1 Preliminary test of bilayer reconstitution at an anisotropically etched aperture on silicon.

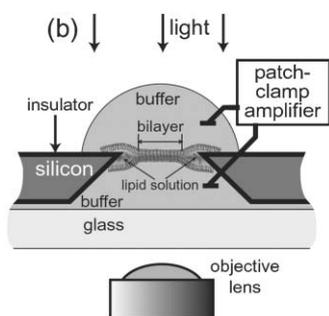
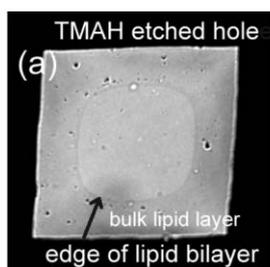


Fig. 2 (a) Lipid bilayer formed at a 150 μm TMAH etched hole. (b) Cross sectional diagram of experimental setup.

Table 1 Success rate of the bilayer formation method in Fig. 1 and contact angle of water and lipid solution on different surfaces

Surface	Success rate	Contact angle/degrees (water)	Contact angle/degrees (<i>n</i> -decane)
SiO ₂ (0.5 μm)	1/10	25	4
Parylene (5 μm)	2/10	85	7
Cytop [®]	0/10	100	30

out easily. At the same time, it has to be electrically insulated to diminish the noise for the electrophysiological recording. We examined three different surface coatings, SiO₂ (thermally oxidized), Parylene and perfluoro polymer (Cytop[®]) as listed in Table 1. The solvent for the lipid (*n*-decane) has a smaller surface tension than water, so it basically spreads well (due to its small contact angle) on any surface. The bilayer was successfully formed with the SiO₂ and Parylene surfaces. However, it did not form on the Cytop surface, which still has an *n*-decane contact angle of 30 degrees.

The transitional trans-membrane current is measured to confirm if it is a bilayer. Conventional Ag/AgCl electrodes are inserted into the top and bottom of the membrane and the current is measured with a patch clamp amplifier (Nihon Kohden, CEZ-2400). The whole setup is placed in a shielded box and grounded to minimize the ambient electric noise. Since

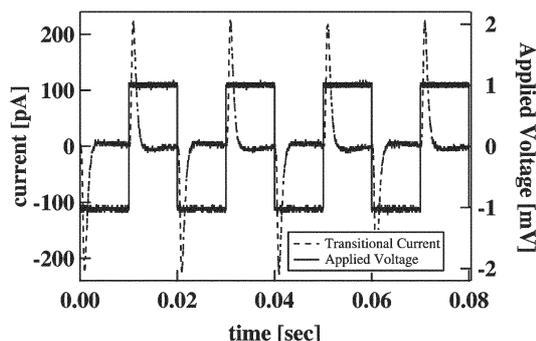


Fig. 3 Transitional current across the bilayer when a ± 1 mV square signal is applied.

silicon is a conductive material, the thin dielectric coating on the surface works as a capacitor, which introduces an electric noise large enough to affect the channel current recording. With the 0.5 μm thermally oxidized chip, the noise was unacceptably large (~ 50 pA). With a 5 μm Parylene coating, it is reduced to less than 10 pA. Thus, we chose the Parylene coating for further experiments. The capacitance of the bilayer membrane is measured by integrating the transient current when a ± 1 mV p-p square wave signal is applied (Fig. 3). It is calculated to be $0.5 \mu\text{F cm}^{-2}$, which is in accordance with values found in the literature.^{5,9,10}

Chip design and fabrication

Fig. 4 illustrates the conceptual diagram of the membrane protein chip. It consists of two fluidic channels on both sides of the silicon substrate and they are connected with apertures. A lipid bilayer is formed at the aperture by flowing lipid solution and buffer alternately (details are described later). After the bilayers are formed, membrane proteins will be incorporated (by fusing liposomes prepared with proteins, for example) and their activity is recorded with the integrated electrodes.

The fabrication process is shown in Fig. 5. It starts with the oxidation of a 280 μm thick silicon wafer. A tiny hole (50 μm in diam. and 200 μm in depth) is etched by ICP-RIE (Fig. 5(ii)), and the back side channel is defined and etched by TMAH (Figs. 5(i) and (iii)). Then, the front channel is defined and etched again by ICP-RIE until an aperture of the desired size appears (Fig.(iv)). Parylene is deposited on the entire surface for electric insulation (Fig. 5(v)). Finally, channels on both sides are sealed by bonding glass plates that already have electrodes patterned (Fig. 5(vi)).

The photographs of the fabricated double-layered channel device are shown in Fig. 6. The channel width and depth are 500 μm and 100 μm , respectively. The aperture size on the front is 150 μm .

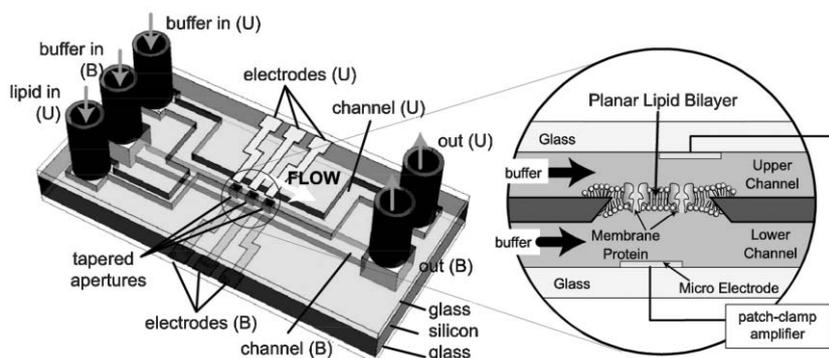


Fig. 4 Conceptual diagram of a membrane protein chip.

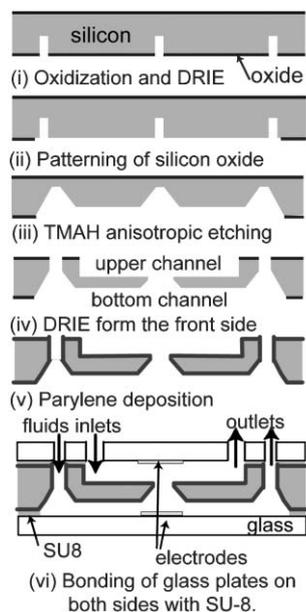


Fig. 5 Fabrication process.

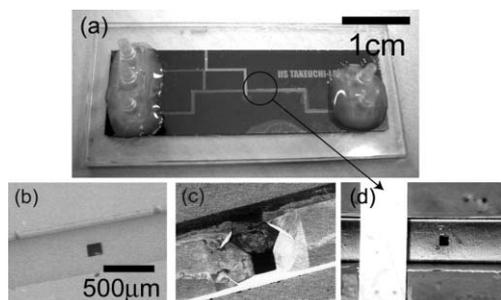


Fig. 6 Images of the double-layered channel device. (a) Overview. SEM pictures of (b) upper and (c) bottom channels. (d) After bonding of electrode patterned glass.

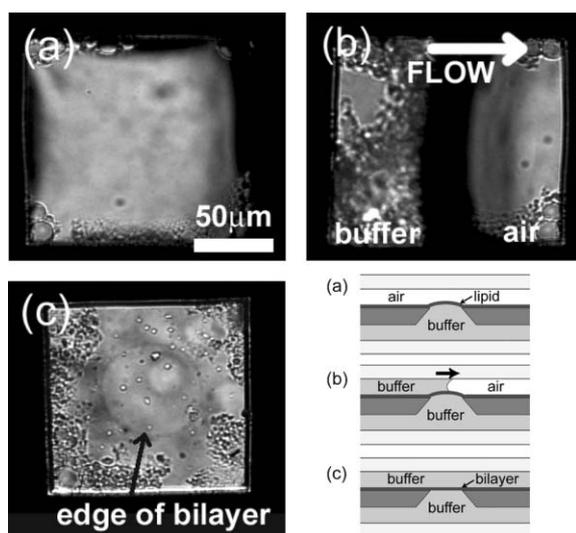


Fig. 7 Sequence of the bilayer formation process for the channel device. (a) Interface of the buffer and air. (b) Interface of the buffer in the upper channel when passing the aperture. (c) Lipid bilayer reconstituted.

Results

Procedure of the bilayer formation is illustrated in Fig. 7. All the flows are driven by syringe pumps (Kd Scientific, KDS

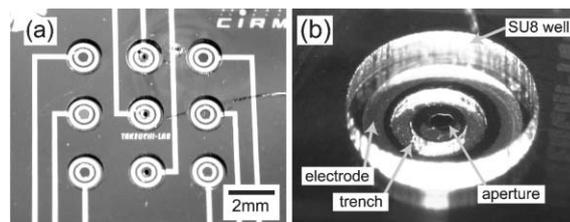


Fig. 8 Images of the well array device. (a) 3×3 array of apertures. (b) Close-up view of single well.

200). Firstly, the bottom channel is filled with buffer. The surface of the buffer stays at the front of the aperture because of the surface tension. Then, the lipid solution is introduced to the upper channel and flushed with air. At this stage, a thin layer of lipid solution remains over the buffer surface at the aperture (Fig. 7(a)). Finally, buffer is slowly introduced into the upper channel at a flow rate of $\sim 0.1 \mu\text{L min}^{-1}$ ($\sim 40 \mu\text{m s}^{-1}$ velocity). The interface of buffer passing the aperture is monitored (Fig. 7(b)). The bilayer is formed spontaneously after the interface of buffer passes as shown in Fig. 7(c).

With this approach, it could be possible to reconstitute many bilayers simultaneously if there are many apertures in the channel. However, for the multi-channel electrophysiological recording, it is not suitable, because all the detection sites (bilayers) are electrically connected by a buffer.

To solve this problem, we fabricated another device, in which the upper channel is replaced by wells made of SU-8 (well array device, shown in Fig. 8). In Fig. 8(b), the sizes of the aperture and well are $200 \mu\text{m}$ and 1.5 mm , respectively. The bilayer formation process is illustrated in Figs. 9(a)–(c). Again, the bottom channel is filled by the buffer and lipid solution (approximately 10 nL) is injected by a glass capillary connected to a syringe pump (WPI, miro4). Since the lipid solution spreads well both on the glass capillary and the substrate, the excess amount of lipid is dragged out of the capillary during injection. Thus, it was difficult to control the exact amount of lipid injected. To solve this problem, the trench surrounding an aperture is fabricated so that the excess amount of lipid solution flows into it and keeps the lipid layer on the buffer interface thin. Finally, a droplet of buffer ($\sim 1 \mu\text{L}$) is placed and a bilayer is spontaneously formed as shown in Fig. 9(d).

Discussion

Both devices presented in this paper consist of two fluidic compartments (*i.e.* channels and wells) on the top and bottom

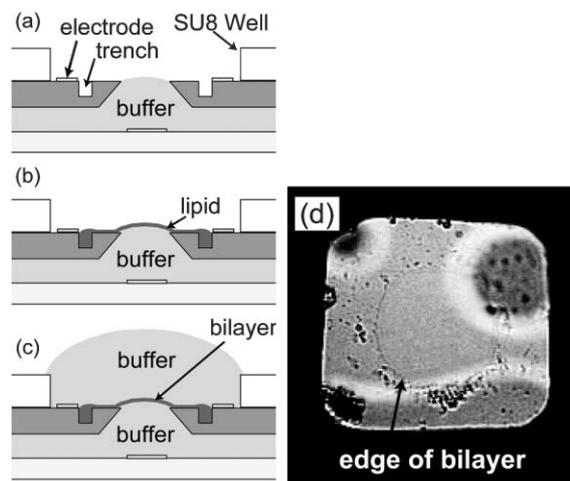


Fig. 9 (a)–(c) Sequence of the bilayer reconstitution process for the well array device. (d) A bilayer reconstituted.

of the substrate and a bilayer is formed spontaneously at the narrow side of the tapered aperture when a thin layer of lipid solution is sandwiched by the buffer. The double-layered channel device is a completely closed system and the buffer inside does not evaporate. Thus, it is suitable for applications that take a long time. Furthermore, it should be possible to make more than one bilayer if many apertures are fabricated along the channel. In this case, the electrophysiological recording in an individual bilayer cannot be performed, since they are electrically connected through the buffer. It can be used for the interaction analysis of membrane proteins based on fluorescence. In the well array device, all the detection sites are electrically separated, so that individual recordings in parallel becomes possible. However, it requires a manipulation system or x - y stage to deliver liquid injection to an individual site.

Concluding remarks

We showed that the planar bilayer membrane is reconstituted at an aperture using a silicon based micro-fluidic system. It is achieved by flowing or injecting lipid solution and buffer, alternately, into the fluidic compartments. Thick Parylene coating is found to be suitable for the bilayer formation and electric noise reduction. In the present method, the laborious manual technique required in the conventional methods is eliminated, which is suitable for automated parallel processing.

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