Technical Note

Lipid Bilayer Microarray for Parallel Recording of Transmembrane Ion Currents

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Lipid Bilayer Microarray for Parallel Recording of Transmembrane Ion Currents

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This paper describes a multiwell biochip for simultaneous parallel recording of ion current through transmembrane pores reconstituted in planar lipid bilayer arrays. Use of a thin poly(*p*-xylylene) (parylene) film having micrometer-sized apertures ($\phi = 15-50\ \mu m$, $t = 20\ \mu m$) led to formation of highly stable bilayer lipid membranes (BLMs) for incorporation of transmembrane pores; thus, a large number of BLMs could be arrayed without any skillful technique. We optically confirmed the simultaneous formation of BLMs in a 5×5 matrix, and in our durability test, the BLM lasted more than 15 h. Simultaneous parallel recording of alamethicin and gramicidin transmembrane pores in multiple contiguous recording sites demonstrated the feasibility of high-throughput screening of transmembrane ion currents in artificial lipid bilayers.

In parallel to the huge effort of biologists turning toward proteomics, new generations of microarrays for parallel screening, such as protein chips, have appeared.[1−3] These microchips may considerably accelerate the exhaustive study of protein structures and functions. Parallel chips for ion channel recording are thus of prime importance, especially for drug discovery, because this class of membrane proteins is involved in a wide variety of diseases.[4] Ion channel activity is most often monitored electrically using the patch-clamp technique on the plasma membrane of cells.[5] Planar patch-clamping systems, using microfabricated apertures, have the advantage of automatically trapping the cell on a micrometer-sized aperture by microfluidic pressure control. This technique can even be performed in parallel,[6,7] although it sometimes suffers from unstable electrical sealing at the recording site.

An alternative way to monitor ion channel activity is the artificial bilayer lipid membrane (BLM) method, in which many types of ion channels have been reconstituted and proven to function.[8,9] An excellent electrical sealing provides extremely sensitive monitoring (down to the single-molecule level), while the compositions of membrane and incorporated proteins can be precisely controlled. In addition, only a small fraction of membrane extract from cells expressing target ion channels is necessary. Thus, high-throughput screening (HTS) systems based on artificial BLMs will become a low-cost and highly sensitive alternative to the patch-clamp based systems. However, the major obstacle against developing such an array chip has been the low reproducibility and stability of BLMs.[8] Use of microfabricated apertures has been expected to be beneficial in improving the reproducibility and stability of BLMs, and many studies have been devoted to developing reliable and useful systems.[10−18] Nonetheless, a parallelized and addressable BLM chip for simultaneous recording has not yet been developed. We suppose it is because BLM formation still relies on the conventional techniques, i.e., either the painting method or Langmuir−Blodgett method. In the former technique, the solvent containing phospholipids is manually painted over the aperture, resulting in poor reproducibility. The latter technique is difficult to realize in microfluidic structures.

We have proposed the use of microfluidic channels to deliver lipid solution and buffer solution[19] into micromachined apertures

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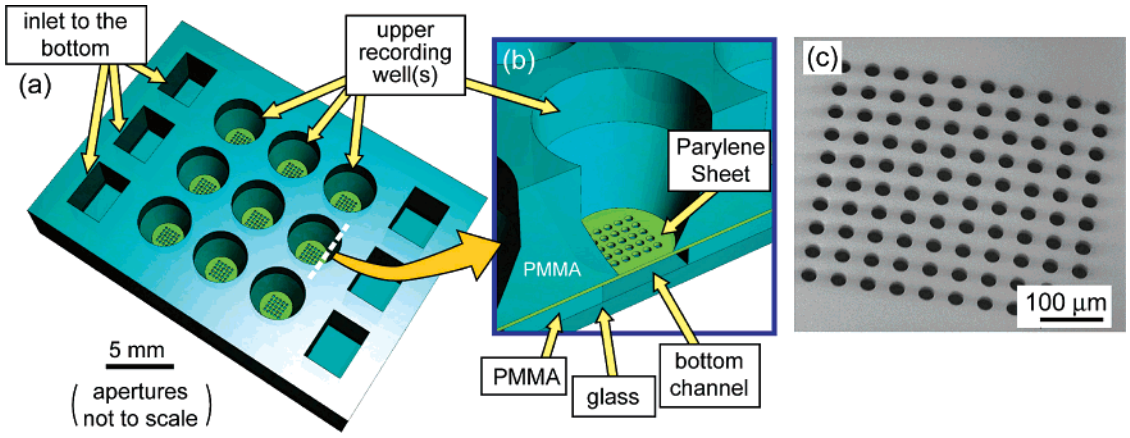

Figure 1. Schematic of the lipid bilayer array chip. (a) Overview and (b) close-up of sectional view at the recording well. A thin sheet of parylene with microfabricated apertures were sandwiched between two PMMA plates that serve as upper recording wells and bottom channels. Planar lipid bilayers were formed across the apertures by first filling the upper wells with buffer, then introducing lipid solution and buffer into the bottom channel. (c) SEM image of 100 apertures opened in a parylene film.

to achieve uniform distribution. We demonstrated the simultaneous formation of four BLMs in a PMMA plastic device.[20] However, due to the poor spatial resolution of machining on plastic, the initial thickness of the lipid layers distributed over the apertures was thick (~50 μm). Thus, external pressure was required to thin the lipid membrane down to the bilayer, which added another complexity in the system. In this paper, we present a microfluidic chip that consists of micromachined apertures formed in a poly(*p*-xylylene) (parylene) sheet. Apertures of 15–50 μm in diameter and 20 μm in thickness significantly improved the reproducibility and stability of BLMs in our microfluidic platform. We demonstrated the first simultaneous recording of transmembrane current in several recording sites of an addressable BLM chip.

EXPERIMENTAL SECTION

Design and Fabrication. A schematic of the general design is depicted in Figure 1. As shown in Figure 1a, the chip consists of multiple recording wells on the front substrate and fluidic channels on the bottom substrate. As shown in Figure 1b, a parylene sheet that has micrometer-sized apertures is sandwiched between those two substrates. A scanning electron microscopy (SEM) image of the apertures is shown in Figure 1c. The fabrication process of the parylene sheet is illustrated in Figure 2a.[21,22] First, a 20 μm thick parylene film was vapor deposited on a single-crystalline silicon substrate. Then, aluminum was deposited on parylene and patterned by the standard photolithographic process. Using aluminum as a mask, parylene was etched by an oxygen plasma. After removing the aluminum, the parylene sheet with microapertures was peeled off from the silicon substrate using tweezers. The packaging structure is illustrated in Figure 2b. PMMA plastic substrates ($W = 40$ mm, $D = 25$ mm) on which recording wells ($\phi = 4$ mm, $H = 5$ mm) and fluidic channels ($W = 2$ mm, $H = 0.5$ mm, $L = 24$ mm) were machined were glued

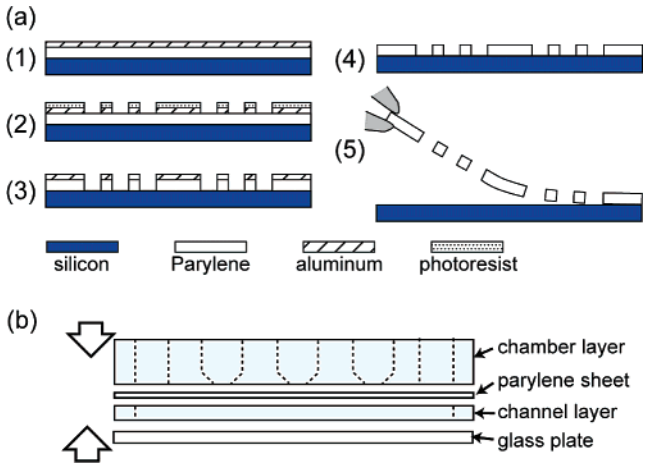

Figure 2. (a) Fabrication process of the parylene sheet: (1) deposition of parylene and aluminum; (2) patterning of aluminum; (3) parylene etching; (4) aluminum removal; (5) peeling off the patterned parylene sheet from the substrate. (b) Gluing chamber and channel substrates made of PMMA plastic for packaging.

by an adhesive (Super X 8008, Cemedine, Japan) to sandwich the parylene sheet. A cover glass plate was finally glued to close the bottom channels. Several prototypes with a varying number of recording wells and apertures were fabricated. We typically used apertures with diameters ranging between 10 and 50 μm in arrays of 9 (3 × 3), 25 (5 × 5), or 100 (10 × 10) in one recording well. This arrayed design was used because the mechanical stability and sealing resistance of BLM increases as the membrane size decreases,[13] while the statistical probability that ion channels added in a buffer solution incorporate into the BLM is proportional to the surface area. We thus split a large total surface of BLM into many parallelized subsurfaces.

Reagents. For the lipid solution, 10 mg of 1,2-diphytanoyl-*sn*-glycero-3-phosphocholine (DPhPC) (Avanti Polar Lipids, U.S.A.) dissolved in 1 mL of *n*-decane was used. For the buffer solution, 300 mM KCl + 10 mM MOPS at pH = 7.0 was used. Stock solutions of gramicidin from *Bacillus brevis* (Sigma, U.S.A.) and α-hemolysin from *Staphylococcal aureus* (Sigma, U.S.A.) were prepared at 1 mg/mL and 0.5 mg/mL concentrations in methanol

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and buffer solution, respectively. Gramicidin forms a pore upon dimerization, permitting a flow of monovalent cations at conductance of 10−20 pS per pore.[23] α-Hemolysin forms a heptameric pore in a lipid bilayer membrane.[24] Liposomes containing alamethicin from *Trichoderma viride* (Sigma, U.S.A.) were prepared using a freeze and thaw method after hydrating the dried mixture of 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC, Avanti Polar Lipids, U.S.A.), 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE, Avanti Polar Lipids, U.S.A.), and alamethicin at a molar ratio of 0.32 mM/0.34 mM/0.25 μM with buffer solution. Alamethicin exhibits a voltage-dependent conductance, in which the number of monomers associated determines the multiple conductance levels.[25]

Lipid Bilayer Formation. All the fluidic manipulation was performed by simple manual pipetting, without any tubing and pumping setup. First, 40 μL of buffer solution was put in the upper well, and 8 μL of lipid solution followed by 80 μL of buffer solution was flowed into the bottom channel. The layer of lipid solution was formed over apertures after the lipid solution passed through.[19,20] For the channel reconstitution, 3 μL of the stock solution of transmembrane pore monomers was added into the upper recording well.

Optical and Electrical Monitoring. The above-mentioned procedure was performed on an inverted optical microscopy stage (IX-71, Olympus, Japan) covered by a Faraday cage to protect it from the ambient noise. Formation of the BLM was optically monitored from the bottom of the chip with a transmitted light. The membrane current was measured using a patch-clamp amplifier (CEZ-2400, Nihon Kohden, Japan) connected to a couple of Ag/AgCl electrodes; one was inserted in the recording well (recording electrode), and the other was inserted in the bottom channel (common electrode). Electric current was recorded using a digital data acquisition system (Digidata 1322A and pCLAMP ver. 9, Molecular Devices, U.S.A.). For the multiple well recording, up to three amplifiers were used for one chip device, as illustrated in Figure 3. Upper recording wells are electrically isolated, so the channel current in each well can be monitored.

RESULTS AND DISCUSSION

Optical and Electrical Monitoring in a Single Recording Well. Arrays of BLMs are formed in a well, over the micrometer-sized apertures micromachined in the parylene sheet. After the fluidic procedure, the circular border separating a thin lipid bilayer and a thick supporting annulus (Plateau−Gibbs border[8,26]) appeared spontaneously within 10 min in all apertures of 5 × 5 arrays (apertures $\phi = 47$ μm), as shown in Figure 4a. An increase in the lipid bilayer area was observed when voltage step (50−100 mV) was applied across the membrane, which is a proof of lipid bilayer formation.[27,28] We should note that this Plateau−Gibbs border was no longer visible in apertures smaller than 40 μm, probably due to the optical diffraction.

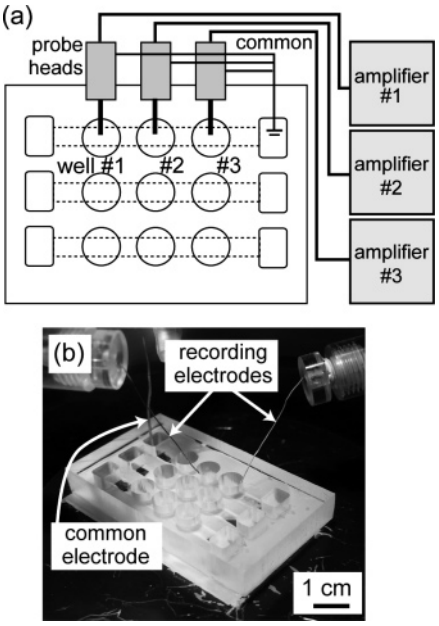

Figure 3. (a) Electrical connection scheme. (b) Patch-clamp amplifier heads are plunged into recording wells in which different transmembrane pores are reconstituted into BLMs. Grounds of the amplifier heads are connected together to the common bottom channel.

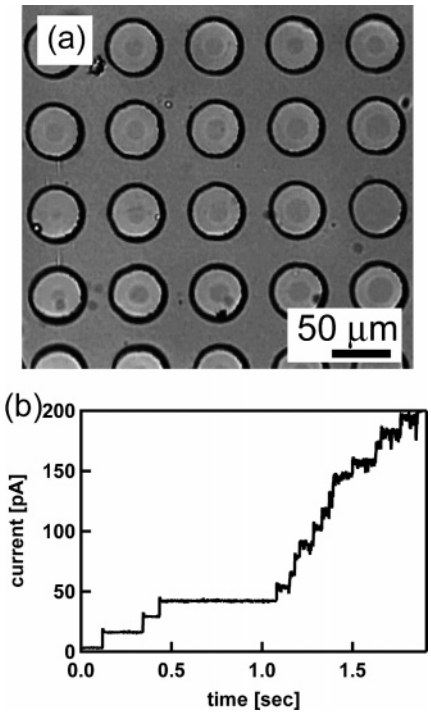

Figure 4. (a) Microscopic image of 25 planar bilayers formed in an array of 47 μm diameter apertures. The circular zone inside each aperture delimits the lipid bilayer. (b) Current through α-hemolysin membrane pore protein reconstituted in a single well device with 100 apertures having 40 μm diameter. Stepwise increase of channel current was observed at 60 mV voltage clamp condition, indicating the insertion of channel proteins.

BLM formation was monitored electrically with a device that has 100 apertures of $\phi = 15$ μm. Electric capacitance was measured by applying square signals across the membrane using

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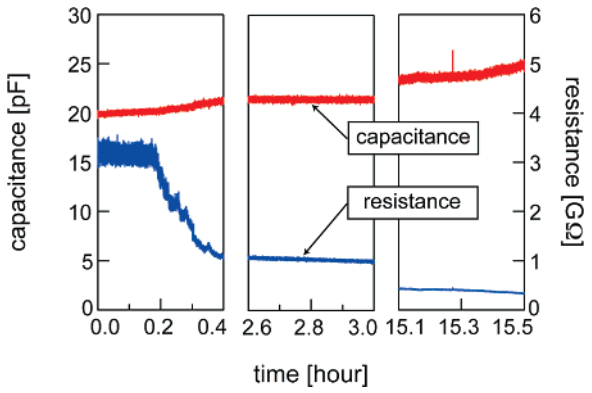

Figure 5. Capacitance and sealing resistance measurement performed on one of the recording site of the 3 × 3 chip. Recording was done in three time slots. The high level of the capacitance confirms the existence of bilayers, while the decreasing of the resistance, starting at 0.2 h, is due to the reconstitution of gramicidin ion channel into bilayers.

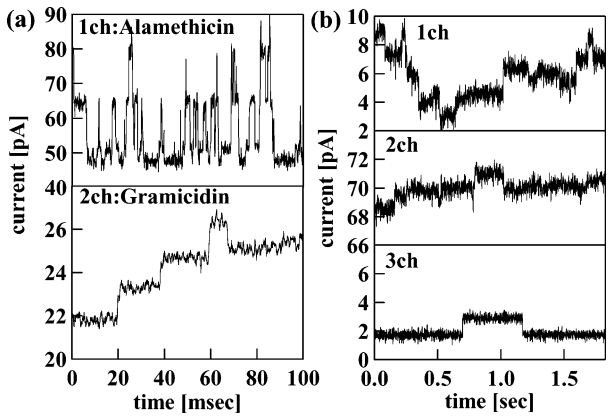

Figure 6. Simultaneous recording of transmembrane ion currents. (a) Recording of alamethicin and gramicidin transmembrane pores in two adjacent wells. Clamping voltage in each well was 80 mV. (b) Recording of gramicidin current in three adjacent wells at 100 mV clamping voltage.

a built-in function of pCLAMP software. A rapid increase in electric capacitance was observed upon the BLM formation, and the specific capacitance (C_s, capacitance over the total area of apertures) reached 0.17 μF/cm^2. This value should be comparable to C_s of BLM formed with *n*-decane reported in the literature (~0.4 μF/cm^2),[26] considering that the actual total membrane area should be smaller than the aperture area. This BLM was also confirmed to be biofunctional by recording a ion channel signals from α-hemolysin reconstituted in the membrane. With a device that has 100 apertures of $\phi = 40$ μm, a steplike increase in membrane current that represents formation of single pores was observed at a 60 mV voltage clamp condition within 10 min after addition into the recording well (Figure 4b).

If we sequentially changed the position of the recording electrode among the wells, the signal recording was successful typically in three to five wells out of nine (a 3 × 3 wells chips was used for this experiment). During this scanning process, the lipid membrane was sometimes broken, probably due to the mechanical shock caused by electrode insertion to the wells. In order to avoid such breakage of the membrane and to improve the successful ratio of the recording signals, we need to prepare nine sets of electrodes and amplifiers, which was not possible in this study due to the limited number of amplifiers.

In addition, the chip could be washed and reused many times, maintaining the same successful rate of recording. The chip is washed three successive times with an ultrasonic bath, and dried with an air spray gun for the next use.

Bilayer Duration Test. One of the recording sites in the 3 × 3 array chip was used for this experiment. After the BLM formation, the capacitance and sealing resistance were measured intermittently. The upper well, inlet, and outlet of the bottom channel filled with buffer solution, were covered with a mineral oil to avoid drying. Measurement was performed during 24 min time slots, starting, respectively, at 0, 2.6, and 15.1 h after the bilayer formation. The capacitance remained at a high value (~20 pF), indicating the existence of the thin BLMs for at least 15 h, as shown in Figure 5. Meanwhile, electrical resistance decreased from 3 GΩ initially to 0.2 GΩ after 15 h. That is because of gramicidin incorporation into the BLM.

Simultaneous Electrical Recording in Multiple Wells. To demonstrate the feasibility for HTS applications, we conducted the parallel recording of transmembrane pore activities using a device equipped with nine recording wells and multiple recording electrodes. First, we performed the simultaneous recording of two types of antibiotic channels: gramicidin and alamethicin. After the membrane formation, the buffer solution of each channel was introduced into each adjacent well. Both alamethicin and gramicidin channel current, which have different timescales of stochastic opening, were successfully recorded at a 10 kHz sampling frequency, as shown in Figure 6a. Next, we recorded the gramicidin channel current in three adjacent wells at a 1 kHz sampling frequency. Current steps due to the opening and closing of the gramicidin channel were clearly observed in all three recording wells simultaneously (Figure 6b). This result is, to our knowledge, the first successful parallel recording of different transmembrane pores inserted in an addressable array of artificial lipid bilayers in a single chip.

CONCLUSION

We have developed a method for the reconstitution of stable BLMs in arrays using a microfluidic chip. On this chip we succeeded in simultaneous and parallel recording of ion channel current through transmembrane pores. Due to its simple procedures, we expect that the chip could be readily utilized in regular biological laboratories.

The key feature that enabled the stable bilayer formation is the use of a thin parylene sheet in which small apertures are micromachined. We presume that the thickness of the aperture restricts the initial thickness of the lipid layer, so thinning readily occurs spontaneously to make a bilayer without any external driving force. High durability of the bilayers was achieved because a thin membrane becomes more robust with a smaller surface area at the given thickness. Recording in a larger number of wells should be achievable by increasing the number of wells, electrodes, and amplifiers. We believe that our method will provide a route to a new generation of highly parallelized ion channel analysis.

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