Highly Reproducible Method of Planar Lipid Bilayer Reconstitution in Polymethyl Methacrylate Microfluidic Chip

Hiroaki Suzuki, Kazuhito V. Tabata, Hiroyuki Noji, and Shoji Takeuchi*

Institute of Industrial Science, The University of Tokyo 4-6-1 Komaba, Meguro-ku, Tokyo 153-8505, Japan

Received September 16, 2005. In Final Form: November 15, 2005

We developed a highly reproducible method for planar lipid bilayer reconstitution using a microfluidic system made of a polymethyl methacrylate (PMMA) plastic substrate. Planar lipid bilayers are formed at apertures, 100 μm in diameter, by flowing lipid solution and buffer alternately into an integrated microfluidic channel. Since the amount and distribution of the lipid solution at the aperture determines the state of the lipid bilayer, controlling them precisely is crucial. We designed the geometry of the fluidic system so that a constant amount of lipid solution is distributed at the aperture. Then, the layer of lipid solution was thinned by applying an external pressure and finally became a bilayer when a pressure of 200−400 Pa was applied. The formation process can be simultaneously monitored with optical and electrical recordings. The maximum yield for bilayer formation was 90%. Using this technique, four lipid bilayers are formed simultaneously in a single chip. Finally, a channel current through gramicidin peptide ion channels was recorded to prove the compatibility of the chip with single molecule electrophysiology.

Introduction

Reconstituted planar lipid bilayers, or black lipid membranes (BLMs), suspended across apertures are used for electrophysiological analysis of membrane proteins,[1] including functional analysis ion channels[1,2] and DNA/polymer identification using nanopores.[3,4] Unlike supported lipid bilayers, or tethered membranes, which are stable and can be micropatterned on a substrate,[5−7] BLMs have excellent electric sealing properties that are capable of recording signals through single channel proteins. However, the reproducibility of BLM reconstitution in conventional techniques is very low, and experience and skill are required. Thus, reconstituting BLMs has been a limiting factor toward high throughput processing of such analyses.

Conventionally, the painting method and Langumuir−Blodgette (LB) method are used to produce lipid bilayers suspended at a small aperture opened in a polymer sheet or septum.[1] Then, target membrane proteins are incorporated into the bilayer by a diffusive process or vesicle fusion for analysis. To form a BLM in the painting method, lipids dissolved in a nonpolar solvent are spread over the aperture submerged in aqueous solution. The layer of the lipid solution gradually thins down and finally becomes a bilayer. However, the process is unsteady and must usually be repeated many times to finally create a bilayer. This is because distributing an appropriate amount of lipid solution repeatedly is extremely difficult, and reconstituted BLMs are fragile. The repeatability of the LB method, in which two lipid monolayers are raised to form a bilayer, should be high. However, it requires a bulky buffer bath setup and is not suitable for integration into microfluidic systems.

Attempts have been made to reconstitute BLMs in microfabricated apertures for the purpose of high-throughput processing of electrophysiological analyses. A variety of methods and materials are used to form well-defined microapertures (e.g., lithography of single-crystal silicon[8−12] and epoxy photoresist,[13,14] ion beam etching of glass,[15,16] and molding of Teflon[17]). They used either the painting[10,15,16,18] or the LB method[8,11,13,14,17] for the reconstitution procedure of lipid bilayers, which should encounter the aforementioned problems.

Ide et al.[19,20] previously developed a system that allows simultaneous optical and electrical observation of single ion channels in horizontally reconstituted BLMs. In this system, a BLM is formed at the aperture opened in a horizontally placed polymer sheet, so that the incorporated proteins as well as a bilayer can be optically observed via a biological microscope with high magnification.

We aim to establish a system that works as a platform for the multichannel electrophysiological recordings of membrane proteins, or a "membrane protein chip". To fabricate multiple BLMs in a single chip, the reproducibility of the bilayer reconstitution must be improved dramatically. Our approach is

* Corresponding author. Tel.: +81-3-5452-6650; fax: +81-3-5452-6649; e-mail: takeuchi@iis.u-tokyo.ac.jp.

(1) Miller, C. *Ion Channel Reconstitution*; Plenum Press: New York, 1986.
(2) LaTorre, R.; Alvarez, O. *Phys. Rev.* **1981**, *61*, 77−150.
(3) Wang, H.; Branton, D. *Nat. Biotechnol.* **2001**, *19*, 622−623.
(4) Sutherland, T. C.; Long, Y.-T.; Stefureac, R.-I.; Bediako-Amoa, I.; Kraatz, H.-B.; Lee, J. S. *Nano Lett.* **2004**, *4*, 1273−1277.
(5) Groves, J. T.; Ulman, N.; Boxer, S. G. *Science* **1997**, *275*, 651−653.
(6) Kung, L. A.; Kam, L.; Hovis, J. S.; Boxer, S. G. *Langmuir* **2000**, *16*, 6773−6776.
(7) Sackmann, E.; Tanaka, M. *Trends Biotechnol.* **2000**, *18*, 58−64.
(8) Osborn, T. D.; Yager, P. *Langmuir* **1995**, *11*, 8−12.
(9) Schmidt, C.; Mayer, M.; Vogel, H. *Angew. Chem., Int. Ed.* **2000**, *39*, 3137−3140.
(10) Pantoja, R.; Sigg, D.; Blunck, R.; Bezanilla, F.; Heath, J. R. *Biophys. J.* **2001**, *81*, 2389−2394.
(11) Peterman, M. C.; Ziebarth, J. M.; Braha, O.; Bayley, H.; Fishman, H. A.; Bloom, D. M. *Biomed. Microdevices* **2002**, *4*, 231−236.
(12) Suzuki, H.; Tabata, K.; Kato-Yamada, Y.; Noji, H.; Takeuchi, S. *Lab Chip* **2004**, *4*, 502−5.
(13) Cheng, Y. L.; Bushby, R. J.; Evans, S. D.; Knowles, P. F.; Miles, R. E.; Ogier, S. D. *Langmuir* **2001**, *17*, 1240−1242.
(14) Ogier, S. D.; Bushby, R. J.; Cheng, Y. L.; Evans, S. D.; Evans, S. W.; Jenkins, A. T. A.; Knowles, P. F.; Miles, R. E. *Langmuir* **2000**, *16*, 5696−5701.
(15) Fertig, N.; Meyer, C.; Blick, R. H.; Trautmann, C.; Behrends, J. C. *Phys. Rev. E: Stat. Phys., Plasmas, Fluids, Relat. Interdiscip. Top.* **2001**, *64*, 040901.
(16) Fertig, N.; Blick, R. H.; Behrends, J. C. *Biophys. J.* **2002**, *82*, 3056−62.
(17) Mayer, M.; Kriebel, J. K.; Tosteson, M. T.; Whitesides, G. M. *Biophys. J.* **2003**, *85*, 2684−2695.
(18) Fertig, N.; George, M.; Klau, M.; Meyer, C.; Tilke, A.; Sobotta, C.; Blick, R. H.; Behrends, J. C. *Receptors Channels* **2003**, *9*, 29−40.
(19) Ide, T.; Yanagida, T. *Biochem. Biophys., Res. Commun.* **1999**, *265*, 595−9.
(20) Ide, T.; Takeuchi, Y.; Aoki, T.; Yanagida, T. *Jpn. J. Physiol.* **2002**, *52*, 429−34.

10.1021/la052534p CCC: $33.50
Published on Web 01/14/2006

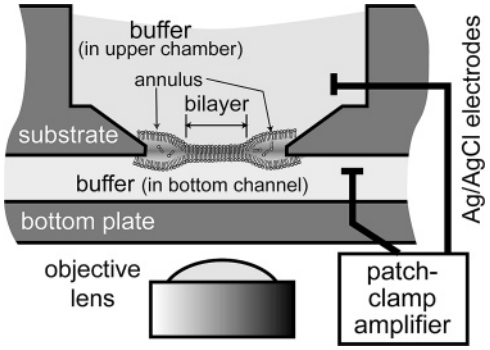

Figure 1. Conceptual diagram of the membrane protein chip.

to use a microfluidic system for the bilayer reconstitution procedure. Since our method should allow precise control of fluids, the reproducibility should be improved. A conceptual diagram of the membrane protein chip we developed is shown in Figure 1. The bilayer reconstitution method used is a modified version of the method in Ide et al.[19,20] It has two fluidic compartments on the front and bottom of the substrate, a chamber and a channel, respectively. They are connected through a tapered hole, where the lipid bilayer is to be formed at the smaller opening. Since the bilayer is formed horizontally, its formation process can be optically observed under a microscope. For the electric recording, Ag/AgCl electrodes are inserted into both fluidic compartments.

In this study, we fabricated this device with PMMA plastic that is dielectric and easy to process. The bilayer reconstitution procedure and geometry of the aperture were optimized by examining the bilayer formation process in detail. To improve the reproducibility, the amount of lipid solution applied over the aperture must be controlled precisely. We designed the shape of the aperture so that a constant amount of lipid solution remains at the aperture. Then, the layer of lipid solution is thinned by applying a static pressure to form a bilayer. Since the distribution of the lipid solution is constant in each operation, the bilayer formation was repeatable. With the optimal design, four lipid bilayers are reconstituted simultaneously in a single chip. A gramicidin peptide channel, a nanopore that passes monovalent cations, is incorporated into the bilayer.

Experimental Procedures

Materials and Fabrication. Microfluidic devices are fabricated by machining a 1.5 mm thick PMMA plate (Mitsubishi rayon, Acrylite L, Japan), as shown in Figure 2. A chamber, a channel, and a tapered hole are machined using CAD/CAM 3-D modeling machine (Modia Systems, MM-100, Japan) with a positioning accuracy of 6 μm, as shown in Figure 2a. The tapered hole is machined with a 100 μm end-mill, as in Figure 2b, and a through hole, or aperture, is opened with a 100 μm drill bit at its bottom, as in Figure 2c. The diameter of the opening is fixed at 100 μm, and the width and the height of the bottom channel are 2 and 0.5 mm, respectively. The height of the sidewall of the opening is defined as *H*, as shown Figure 2c. A cover glass plate is glued on the bottom side to close the channel. The bottom channel can be used for the exchange or addition of chemicals or proteins for further analyses. We chose PMMA because, being a nonconductive and low dielectric material ($\epsilon = 3-4$), reducing the electric noise to a level small enough to allow the detection of single channel current was easier. Also, PMMA is transparent and easy to machine fine structures. In addition, its hydrophobic property is suitable for bilayer formation. Finally, it does not absorb organic solvents, unlike poly(dimethylsiloxane), which is often used for microfluidic chips. For the formation of multiple bilayers, a chip with four apertures was fabricated, as shown in Figure 2d,e.

Bilayer Reconstitution Procedure. The bilayer formation method is illustrated in Figure 3. Prior to the experiment, a plastic cylinder

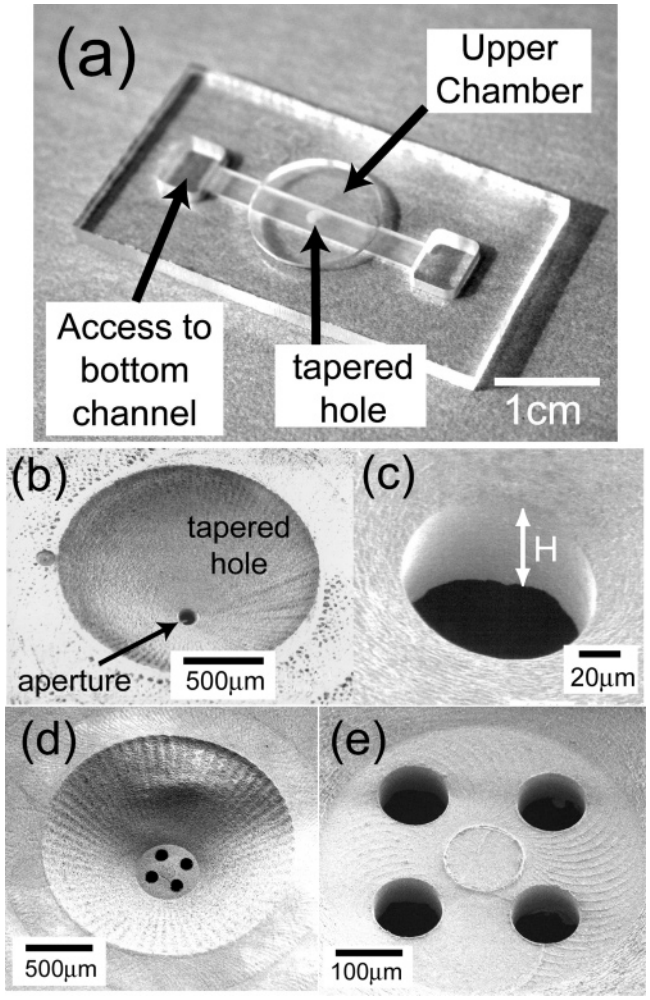

Figure 2. (a) Overview of the membrane protein chip made of PMMA. SEM images of (b) a tapered hole and (c) a close-up of a 100 μm aperture opened at the bottom. (d and e) Device with four apertures.

8 mm in diameter is glued to the upper chamber, and the 1.5 mm silicone tube is connected for pressure adjustment. For the lipid solution, 20 mg of asolectin (Sigma) in 1 mL of *n*-decane (Kanto Chemical, Japan) is prepared. For fluorescent observation, 0.02 mg of Rhodamine B labeled phosphatidylethanolamine in chloroform (Avanti Polar lipids, Inc.) is added to 1 mL of lipid solution. For the buffer, 10 mM KCl water is used. First, 50 μL of buffer (0.5 mm in height) was added to the upper chamber, as shown in Figure 3, panel 1. The interface of the buffer stops at the small opening due to surface tension. Next, the lipid solution is placed into the bottom channel, as shown in Figure 3, panel 2, and is sucked out from the channel inlet by being absorbed in cotton, as shown in Figure 3, panel 3. At this point, a layer of the lipid solution remains on the buffer surface at the aperture. Next, the buffer is placed into the bottom channel, so that the layer of the lipid solution is sandwiched between the buffer layers, as shown in Figure 3, panel 4. To cancel out the pressure difference across the lipid layer, the horizontal water levels in upper and lower compartments are equalized. Finally, a lid is placed on the upper chamber using vacuum grease. Thus, adjusting the pressure in the upper chamber is possible using an external pressure source, as shown in Figure 3, panel 5. As the pressure is applied, the layer of lipid solution thins down to become a bilayer. The pressure is stopped when a bilayer is formed. Injection of a protein-containing solution and buffer exchange can be performed from the inlet of the bottom channel.

Experimental Setup. All experiments were performed on an inverted microscope (Olympus, IX-71, Japan) to monitor the lipid membrane under either bright field or fluorescent observation. To acquire the three-dimensional distribution of the lipid solution,

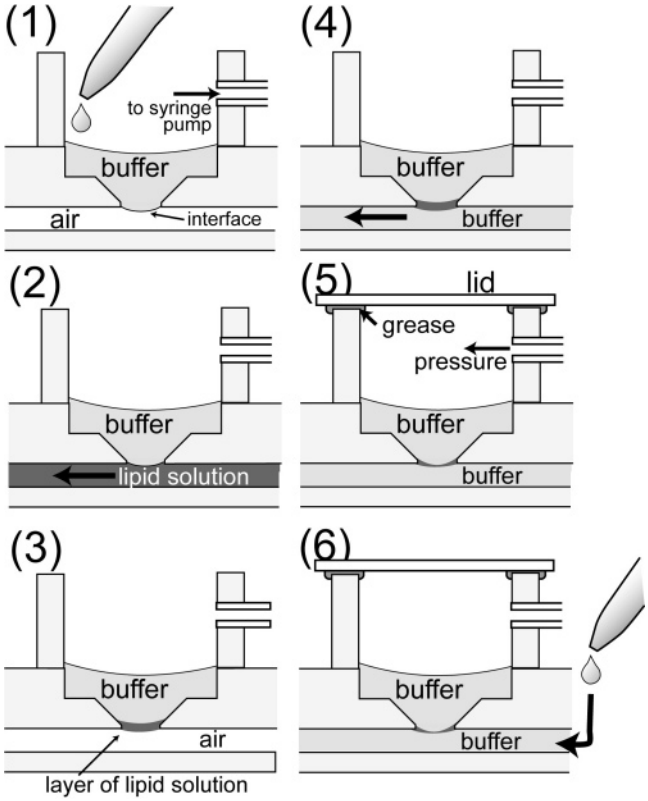

Figure 3. Sequence of the bilayer formation procedure.

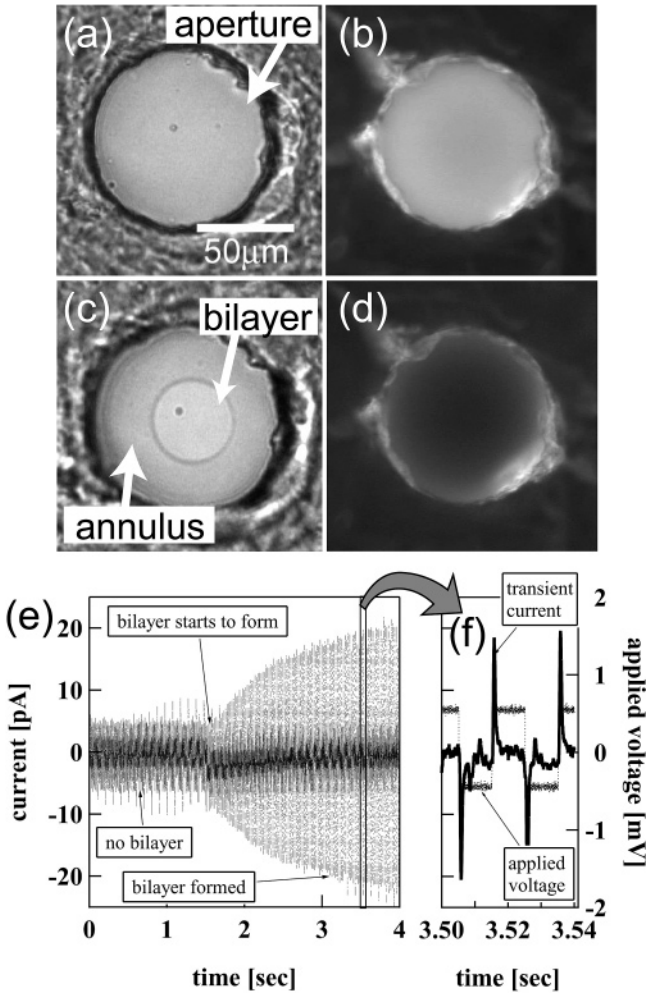

Figure 4. Images of the aperture (a and b) before and (c and d) after the planar lipid bilayer is formed. Images on the left column (a and c) are observed with bright field illumination, and images on the right column (b and d) are fluorescent images. (e) Transient current across a bilayer when 0.5 mVp−p square signal is applied at 50 Hz. (f) Larger magnification plot of the current signal at $3.5 < t < 3.54$.

fluorescent confocal microscopy (Yokogawa Elect. Co., CSU-22, Japan) was used. We scanned in the *z* direction with a PZT positioning system for an objective lens (PI, E-662, Germany). For electric monitoring, Ag/AgCl electrodes from the device were connected to a patch clamp amplifier (Nihon Kohden, CEZ-2400, Japan), and the output signal was recorded with a 200 MHz digital oscilloscope (Yokogawa Elect., DL1640, Japan). For the membrane capacitance measurement, a square wave signal generated by a function generator (Agilent Technologies, 33220A) was sent to the amplifier. The whole system was carefully grounded and enclosed in a Faraday cage. The air pressure in the upper chamber was adjusted by the displacement of a syringe pump (WPI, UMP-2) and was monitored with a pressure sensor (Copal Electronics, PA-100, Japan).

Results

Optical and Electrical Monitoring of Bilayer Formation. Images of the aperture during the bilayer formation process are shown in Figure 4a−d. A bright field image before applying the pressure is shown in Figure 4a, which corresponds to step 4 in Figure 3. At this stage, a thick layer of lipid solution sandwiched by buffer covered the aperture. With fluorescent observation, as shown in Figure 4b, emission from the fluorescent lipid can be seen uniformly inside the aperture, showing that a thick layer of lipid solution remains only inside the aperture. As the pressure in the upper chamber increased, the fluorescent emission gradually became dimmer because the layer of the lipid solution was thinning. When the film thickness approached a few hundred angstroms, lipid molecules aligned on both water/solvent interfaces attracted each other by van der Waals forces,[1] and a bilayer formed spontaneously by self-assembly. Under an optical microscope, an indistinct circular edge, or Plateau−Gibbs border, appears from the center, as shown in Figure 4c.[12,19−21] The area outside the bilayer is the annulus or bulk region, in which the thicker layer of lipid solution remained to support the bilayer. The application of an electric voltage across the membrane added a compressive force and caused the shape of the annulus to change in such a way that the diameter of the bilayer increased.[1,19] By the time the bilayer formed, fluorescent emission from the center area can barely be observed as compared to the emission from the rim, as shown in Figure 4d. The bilayers typically lasted for a few to tens of minutes.

Bilayer formation was also monitored electrically. A bilayer membrane works as a thin dielectric separating two conductive layers (buffers) and exhibits capacitance of around 0.4 (μF/cm^2).[1,22] The transient current across the bilayer membrane when a 0.5 mVp-p square signal is applied is shown in Figure 4e. Before a bilayer is formed, the peaks of the capacitive transient current are less than ±5 pA. At $t = 1.5$ s, the peak value began to increase along with the bilayer formation. The peak amplitude of the output signal increased as the area of the bilayer expanded and was constant when the area was stable. A larger magnification plot of the signal at $t = 3.5$ s is shown in Figure 4f. In our measurement system, the peak amplitude was proportional to

(21) Suzuki, H.; Tabata, K.; Kato-Yamada, Y.; Noji, H.; Takeuchi, S. *μTAS*; Malmo: Sweden, 2004; pp 246−248.

(22) Fujiwara, H.; Fujihara, M.; Ishiwata, T. *J. Chem. Phys.* **2003**, *119*, 6768−6775.

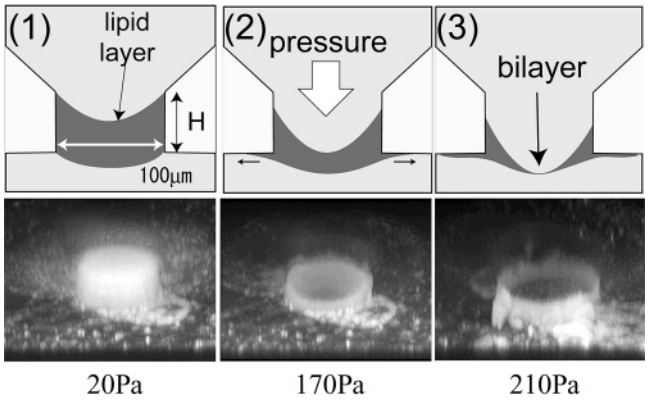

Figure 5. Process of bilayer formation. Illustrations of sectional view (upper column) and three-dimensional distribution of fluorescent lipids measured with a scanning confocal microscope (lower column).

the capacitance at <120 pF. The capacitance of the bilayer shown in Figure 5c was 8 pF, with its area of 1.66×10^{-5} cm^2 (a circle 46 μm in diameter). The specific capacitance was 0.48 μF/cm^2, which is in accordance with the literature.[1,22]

Reproducibility Assessment. From the preliminary tests, we found that bilayers are likely to form if the initial thickness of the lipid solution layer is less than a few micrometers. However, such layers are easy to break by the shear force due to the buffer introduction into the bottom channel. This problem can be solved by designing the geometry of the aperture so that a thick layer of lipid solution, in tens of micrometers, remains at the aperture. Such a layer rarely breaks even with a mechanical shock (e.g., a gentle touch with a finger). If the initial amount of lipid distributed is always the same, the layer becomes a bilayer repeatedly at a constant pressure.

However, to measure out a small amount of lipid solution, on the order of picoliters, was not a trivial task since nonpolar solvents have a very low surface tension. We found that a simple approach produced the best reproducibility. When the edge of the tapered hole is made to have the vertical side-wall (*H* in Figures 2 and 5), the lipid solution is stuck in the cylindrical volume defined by the aperture diameter and the sidewall after the buffer introduction in Figure 3, panel 4.

A schematic of the thinning sequence and corresponding 3-D images observed with fluorescent confocal microscopy are shown in Figure 5. At first, the lipid solution is typically 30−50 μm thick, as shown in Figure 5, panel 1. As the pressure gradually increased, by 1−2 Pa/s, the lipid solution was pushed down, and it became thin in the center area, as shown in Figure 5, panel 2. The displaced lipid solution spread along the bottom surface around the aperture. Finally, when a bilayer was formed, a large volume of lipid solution only existed at the rim of the aperture, as shown in Figure 5, panel 3.

We tested five devices with different values of *H* (0, 21, 47, 52, and 82 μm) to seek the optimum height, while the diameter of the aperture was kept at 100 μm. For each device, the procedure in Figure 3 was repeated 9−12 times to examine the reproducibility. In each trial, the device was washed with hexane in an ultrasonic bath and dried to remove residual lipids and solvent that could lower repeatability. The success rate of the formation of a thick lipid layer at step 4 in Figure 3 is plotted as shaded bars in Figure 6.The initial layer of lipid solution was repeatedly formed with $20 < H < 60$ μm devices (50−200 pL in volume). When *H* was smaller than 20 μm, the layer of lipid solution did not fully form, breaking when the buffer was placed into the bottom channel. When $H = 82$ μm, air bubbles were sometimes

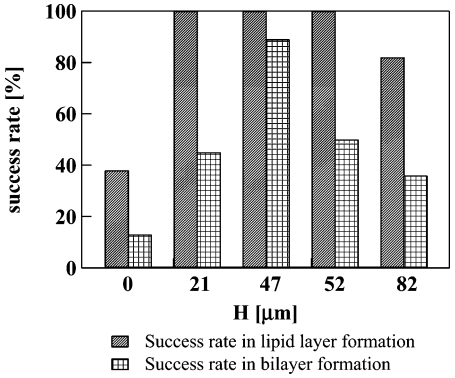

Figure 6. Success rate in lipid layer and bilayer formation with different *H*.

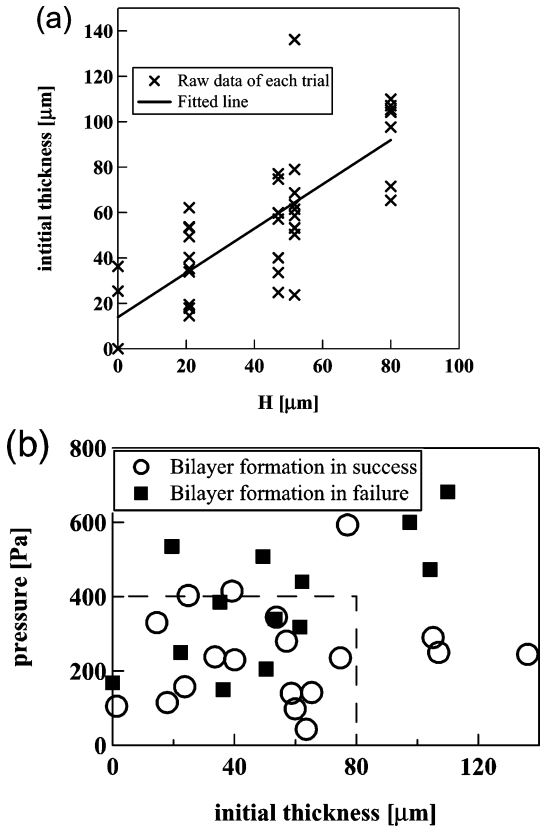

Figure 7. (a) Initial thickness of lipid solution layer at the aperture with different *H*. (b) Applied pressure for bilayer formation (circles) and membrane breakdown (squares) with different initial thicknesses.

trapped at the aperture. The checked bars show the success rate of bilayer formation, after pressure application, for layers that lasted longer than 30 s. The success rate was 90% with the $H = 47$ μm chip.

The initial thickness of the lipid solution layer is shown in Figure 7a, as measured by adjusting the focal plane of the objective lens at the upper and lower surfaces of the lipid layer with a PZT controller. The initial thickness was almost proportional to *H* with fluctuations of ±20 μm. Then, pressure was applied in the upper chamber to form a bilayer. In cases when the bilayer did not form, it broke during the final thinning process similarly to a soap bubble breaking. The applied pressure when either the lipid layer became a bilayer (circles) or broke down (squares) is shown in Figure 7b. In the figure, bilayers were formed when the initial thickness was less than 80 μm and the applied pressure was smaller than 400 Pa. At pressures greater than 400 Pa, the

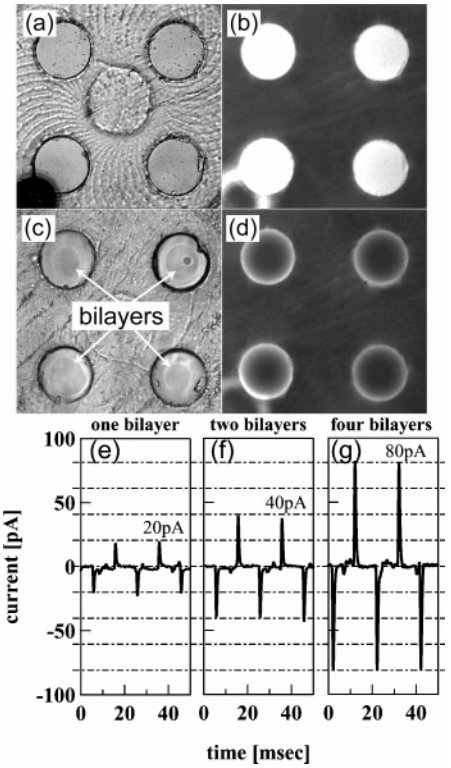

Figure 8. Images of four apertures during the bilayer formation process. (a) Initial state and (b) its fluorescent image. (c) Final state when four bilayers are formed and (d) its fluorescent image. (e−g) Transient current across the multiple bilayers when 0.5 mVp−p square signal is applied at 50 Hz.

chance of breaking increased because the curvature of the lipid layer became larger.

From the results shown in Figures 6 and 7, the optimum H existed at around 50 μm. When $H < 20$ μm, the lipid layer broke because of buffer introduction or small disturbances. When $H > 60$ μm, a larger pressure was required for thinning, increasing the chance of breaking at the final thinning phase. Among the successfully formed bilayers, ones that were formed at smaller pressures tended to last more than 10 min, while those formed at larger pressures tended to break in a few minutes.

Simultaneous Reconstitution of Multiple Bilayers. Simultaneous formation of multiple bilayers was done with the four aperture chip shown in Figure 2d,e. The procedure was exactly the same as that for the single aperture chip. Microscopic images of a four-aperture device with $H = 43$ μm are shown in Figure 8a−d. The initial state is shown in Figure 8a,b. From the fluorescent image in Figure 8b, thick and uniform layers of the lipid solution remained at all apertures. By increasing the pressure, they thinned uniformly, and four bilayers were successfully formed, as shown in Figure 8c,d. Note that each of the bilayers had an almost uniform size. The average size and standard deviation of the diameter of bilayers calculated from 12 samples were 56 and 13.7 μm (24% of the average), respectively. In addition, all four bilayers formed in approximately 50% of the trials. The transient current across the bilayer membranes is shown in Figure 8e−g. In Figure 8e, where only one bilayer membrane formed, the peak value of the transient current was approximately 20 pA. In Figure 8f,g, two and four bilayers formed, respectively. The peak values were approximately 40 and 80 pA, respectively, which is proportional to the number of bilayers formed. Thus, in the present device, the formation of bilayers optically observed under the microscope can be confirmed with electric measurement simultaneously.

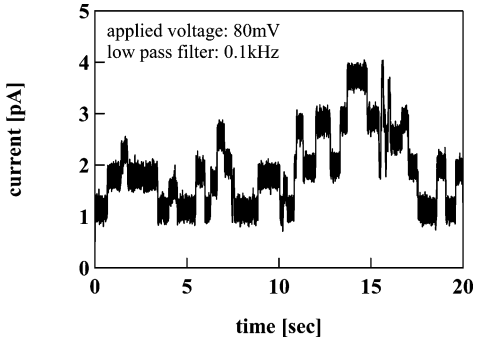

Figure 9. Single channel current recording through gramicidin incorporated in the lipid bilayer.

Recording of Single Ion Channel Signal. The gating event of the gramicidin peptide ion channel was recorded to confirm the capability of the PMMA chip for electrophysiology at the single molecular level. This experiment was performed with a single aperture chip. Gramicidin is a small peptide ion channel that opens and passes monovalent ions along with the dimerization.[23] After the bilayer formation, 10^{-9} M gramicidin (Sigma-Aldrich Co.) dissolved in methanol was added from the inlet to the bottom channel. They diffused and were incorporated in the bilayer membrane spontaneously. The time series of the transmembrane current with an 80 mV voltage-clamp is shown in Figure 9. The standard deviation of the background noise in the setup was 0.16 pA, which mostly came from the microscope and the syringe pump due to imperfect shielding. Before the gramicidin containing methanol was added to the channel, there was no signal except for the constant drift corresponding to the sealing resistance of 80 GΩ. As time passed, the steplike signals in Figure 9 began to appear due to the gating events of the gramicidin channels. The current of approximately 1 pA corresponded to the flow of K^+ ions through a single gramicidin dimer. This confirms that the bilayer was reconstituted successfully since gramicidins formed ion channels only in the bilayer structure.[19] Also, it proves that our device can conduct electrophysiology at the single molecular level.

Conclusion

Planar lipid bilayers were formed with high reproducibility in a PMMA microfluidic chip. Distributing a constant amount of lipid solution at apertures was possible by flowing solutions into the channel, which contributed to the repeatable bilayer formation. The formation process of bilayers was monitored simultaneously with optical and electrical means, allowing multiple options for analytical methods. The yield of the bilayer formation reached 90% when the height of the sidewall of the aperture edge was around 50 μm. Using this method, four bilayers were formed simultaneously in a single device. We showed that the PMMA device is compatible with the recording of ion channel currents at the single molecular level.

The planar lipid bilayer method provides the most accurate functional analysis of ion channel proteins. However, lipid bilayers are not readily reproducible. With our device and method, even multiple lipid bilayers are repeatedly reconstituted in a single chip with one-time operation. In the four-aperture device presented here, recordings at individual sites were not possible since all bilayers were electrically connected by the buffer. We purposely

(23) Borisenko, V.; Lougheed, T.; Hesse, J.; Fureder-Kitzmuller, E.; Fertig, N.; Behrends, J. C.; Woolley, G. A.; Schutz, G. J. *Biophys. J.* **2003**, *84*, 612−622.

designed it so that the apertures come close enough to observe the bilayer formation in a single photographic frame. However, designing the channel system so that the multiple apertures are electrically independent to allow multichannel recordings is straightforward. Fabricating additional channels into the system to deliver different reagents or samples to detection sites is also possible. We believe that this technique will help to develop high-throughput screening systems for membrane proteins.

Acknowledgment. The authors are grateful to Prof. T. Ide in JST for his guidance on the bilayer reconstitution method presented in his literatures.[19,20] This project is supported by the Program for the Promotion of Basic Research Activities for Innovative Biosciences (PROBRAIN) under the supervision of the Ministry of Agriculture and Fisheries in Japan.

LA052534P