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Stable lipid bilayers based on micro- and nano-fabrication

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In this review, we will discuss our recent approaches for improving the mechanical stability of free-standing bilayer lipid membranes (BLMs) by combining with BLM formation and micro- and nano-fabrication techniques. BLMs were prepared across a microaperture fabricated in silicon (Si) chips or nanoporous alumina films, and their mechanical stability and electric properties were investigated. BLMs spanned over the porous alumina showed background noise currents small enough for recording activities of low-conductance channels, though further stability enhancement of porous alumina films was necessary. BLMs suspended in a thin Si_3N_4 septum showed a dramatic improvement of BLM stability. The BLMs were resistant to a voltage of ± 1 V and the membrane lifetime was 15–43 h with and without incorporated channels. The membrane containing gramicidin channel exhibited tolerance to repetitive solution exchanges, though the electric properties of the BLMs are necessary to be improved. The realisation of BLMs having both mechanical stability and proper electric properties will open up a variety of applications including highly sensitive biosensors and high-throughput drug screenings for ion channels.

Keywords: bilayer lipid membrane; ion channel; porous alumina; Si microfabrication

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Introduction

Ion channel proteins are of great interest as subjects of basic physiological studies and targets of high-throughput screening for drug discovery. Reconstitution of ion channel proteins in free-standing bilayer lipid membranes (BLMs) provides an excellent system for drug screening under chemically controlled conditions (*1*). In addition, ion channel proteins embedded in free-standing BLMs are useful for designing highly sensitive biosensors, because channel proteins have signal amplification ability as well as specific recognition ability (*2*, *3*). Conventional BLMs are prepared by classical methods, e.g. painting (black membrane) (*4*), monolayer folding (*5*, *6*) and tip–dip methods (*7*). These membranes have been used for functional analysis of channel proteins and designing highly sensitive biosensors (*3*). However, the fragility of BLMs hinders their wide range of applications and confines the bilayer method to laboratory use.

Extensive studies have been made to improve the stability of free-standing BLMs by preparing BLMs in microfabricated devices (*3*, *8–13*) or supporting BLMs with hydrogels (*14–17*). These efforts led to prolonged membrane lifetime of several tens of hours (*18*, *19*). However, mechanical stability of the BLMs has not been improved to the desired extent. Considering that patch-clamped membranes with the lifetime of several hours are still more widely used partly due to the compatibility with solution exchanges which enables easy drug application and removal, one of the most desirable goals of membrane stability for free-standing BLMs is tolerance to solution exchanges while allowing channel-current recordings rather than just the improvement of membrane durability (*3*).

In this review, we will discuss our recent approaches for preparation of stable BLMs through the combination of micro- and nano-fabrication techniques and BLM formation. The BLMs were prepared across a micro-aperture fabricated in silicon (Si) chips (*20*) or nanoporous alumina films (Figure 1). The stability of the BLMs was characterised in terms of tolerance to mechanical and electrical shocks, membrane lifetime, breakdown voltage and tolerance to solution exchanges. Electric properties of the BLMs as a platform for channel-current recordings are also discussed. Successful coupling of BLMs and such microfabrication techniques will realise widespread applications of artificial lipid bilayers, including high-throughput drug screening and sophisticated biosensors.

Micro- and nano-fabrication and BLMs formation

Traditional free-standing BLMs are formed across apertures in plastic septa. Micrometre-sized (from $\sim\mu$m to $\sim$ several 100 μm) apertures are usually formed

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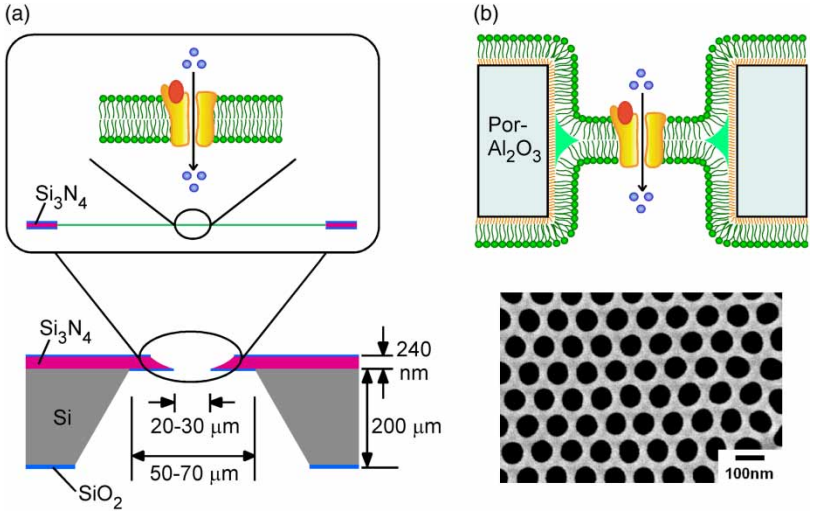

Figure 1. Schematic of a BLM formed across (a) a microfabricated Si chip and (b) a nanoporous alumina film. A SEM image of an anodic porous alumina film is also shown. Anodisation was conducted in 0.3 M oxalic acid at room temperature and an applied potential of 40 V.

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in micrometre-thick septa. Such scale discrepancy between the micrometre-thick septa and nanometre-thick BLMs may result in instability of the BLMs. When free-standing BLMs are spanned over nanopores (*21*, *22*) or suspended in nanometre-thick substrates (*20*), the membrane stability will presumably be enhanced. We have examined BLM formation across anodic porous alumina films or microfabricated Si chips to improve the BLM stability (Figure 1). Anodic porous alumina is a self-ordered nanopore insulator whose pore sizes are tunable by anodisation of aluminium (Al) in an appropriate acid solution. Increased membrane stability by reduction in aperture size is expected for porous alumina, whereas total BLM area is still large to facilitate incorporation of proteins, which is favourable for designing highly sensitive biosensors. On the other hand, Si microfabrication by the lithography techniques is currently well established. Our Si-based approach to improve the BLM stability is microfabrication of apertures in a nanometre-thick Si_3N_4 film deposited on a Si substrate (*20*). The use of nanometre-thick septa will reduce the stress on the lipid bilayer structure at the contact with the septa. We have fabricated anodic porous alumina films and Si chips having microapertures and investigated the BLM formation using both substrates.

Fabrication of anodic porous alumina films

Figure 2(a) schematically shows the fabrication procedure of porous alumina films. An Al sheet (thickness 0.5 mm, purity 99.999%) was first electropolished in a mixture solution of perchloric acid and ethanol (1:4, v/v). Anodisation of the electropolished Al sheet was conducted using either aqueous oxalic (0.3 M) or phosphoric acid (0.1 M) as electrolyte. In the case of phosphoric acid, porous alumina was obtained in a one-step anodisation process. Al was anodised for 7 min at -4°C and an applied voltage of 160 V. In the case of oxalic acid, porous alumina was obtained by a two-step anodisation process. First, Al was anodised for 30 min in 0.3 M oxalic acid at room temperature and an applied voltage of 40 V. Then the oxide layer was removed by wet etching in a 10 wt% phosphoric acid solution containing 4 wt% chromium (VI) oxide at 60°C for 20 min. The remaining pattern on the Al served as a mask for the second anodisation process using the same parameters as the first step except for anodisation time of 10 min. After anodisation, a layer of SU-8 3010 photoresist (Microchem Co., Newton, MA, USA) was spun on the porous alumina side and patterned by the standard photolithography to form apertures with a diameter of $\sim$250 μm. The remaining Al was removed by etching with 36 wt% hydrochloric acid containing saturated copper(II) sulphate, yielding a porous alumina membrane with closed pores at the bottom (barrier layer). Finally, the barrier layer was removed by dipping in 5 wt% phosphoric acid at 40°C. This procedure also worked to widen the diameter of nanopores. The porous alumina films thus fabricated were silanised by treating with 2% (v/v) 1H,1H,2H,2H-perfluorooctyl-trichlorosilane in *n*-hexadecane for 2 h.

Fabrication of microapertures in Si chips

Apertures with a diameter of 20–30 μm (Figure 1(a)) were fabricated in a FZ Si (100) wafer ($>$9000 Ω cm, 200 μm in thickness), one side of which was coated with a 240 nm thick Si_3N_4 layer (Semitec, Chiba, Japan). Figure 2(b) shows the procedure for the fabrication

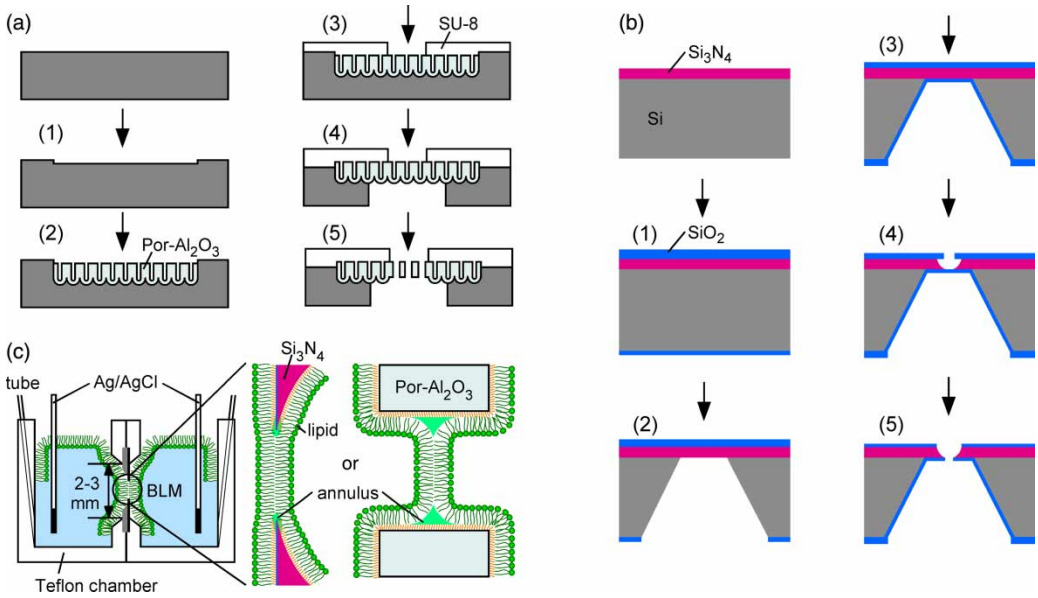

Figure 2. Schematic diagram of the process for the fabrication of (a) a nanoporous alumina film and (b) a microaperture in a Si chip. (a) (1) Electropolishing; (2) anodisation; (3) patterning; (4) removal of Al and (5) removal of barrier layer. (b) (1) Thermal oxidation and sputtering of SiO_2; (2) pattering and anisotropic etching of Si; (3) sputtering of SiO_2; (4) isotropic etching of Si_3N_4 and (5) removal of SiO_2. (c) Schematic of a BLM formed by the monolayer folding method.

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of the apertures. The wafer was first thermally oxidised and then the Si_3N_4 side was coated with a SiO_2 layer using the sputtering method. The former oxide layer was patterned by the standard photolithographically, followed by anisotropic etching in 25% tetramethylammonium hydroxide at 90°C. Then, a SiO_2 layer was deposited on the bare Si_3N_4 surfaces formed by the anisotropic etching. After photolithographically patterned, circular holes were fabricated in the Si_3N_4 layer by isotropic etching in 85% phosphoric acid at 150°C. Finally, the SiO_2 layer beneath the holes was removed by 5% hydrofluoric acid to form apertures. Before BLM formation, the Si chip thus fabricated was silanised by treating with 2% (v/v) 3-cyanopropyldimethylchlorosilane in acetonitrile for 24 h.

BLM formation

BLMs were prepared by folding up two lipid monolayers across the anodic porous alumina films or microfabricated Si chips (Figure 2(c)). First, the substrate thus fabricated was set in the middle of a Teflon chamber, and then precoated with a thin layer of *n*-hexadecane by dropping chloroform containing *n*-hexadecane. The substrate separated two 1.5 ml compartments in the chamber. A 1400 μl portion of 2.0 M KCl solution containing 10 mM HEPES/KOH (pH 7.4, abbreviated as KCl buffer) or 2.0 M CsCl solution containing 10 mM HEPES/CsOH (pH 7.4, abbreviated as CsCl buffer), filtered just before use through a cellulose acetate filter (pore size 0.20 μm), was added to each side of the chamber. The water level in both compartments was set below the aperture. Then, a small amount (10 μl) of a lipid solution dissolved in chloroform/*n*-hexane (1:1, v/v) was spread on the aqueous solutions. The composition of the lipid solution used was 10 mg/mg L-α-phosphatidylcholine (PC): L-α-phosphatidylethanolamine (PE): cholesterol (Chol) = 6:2:2 (w/w) for porous alumina films and 2 mg/ml PC:PE:Chol = 7:1:2 (w/w) for Si chips. PC and PE were those extracted from egg (Avanti, Alabaster, AL, USA) and composed of various acyl chain lengths. As the total surface area of the porous alumina films was much larger than that of the Si chips, higher lipid concentration was required for BLM formation. After solvent evaporation, BLMs were formed by gradually raising the water level until it surpassed the aperture. The incorporation of gramicidin into the BLMs was performed by adding gramicidin solutions to the KCl buffer in both the compartments under constant stirring. Incorporation rate was higher for the porous alumina films (56%, $n = 9$) than for the Si chips (37%, $n = 35$) when gramicidin was added to give a final concentration of 0.14–28 ng/ml, which was probably due to much larger total membrane area for the porous alumina substrate. Current recordings were performed with an Axopatch 200B patch-clamp amplifier (Molecular Devices, Sunnyvale, CA, USA). The signal was filtered at 1.0 kHz low-pass filter, digitised at 10 kHz and stored on-line using a digital data acquisition system (Digidata 1440 and pCLAMP software ver. 10.2, Molecular Devices). The data were analysed with a pCLAMP ver. 10.2

using a 500 Hz low-pass filter. The total capacitance including BLMs and the substrates was determined from the measured current when a voltage ramp was applied.

BLMs spanned over anodic porous alumina

Alumina films with a pore diameter of 50–80 nm and a thickness of 1–12 μm (aspect ratio:10–150) were obtained by anodisation in oxalic acid (Figure 1(b)), whereas those with a pore diameter of 200–350 nm and a thickness of 0.2–6 μm (aspect ratio:1–30) were obtained by anodisation in phosphoric acid (Figure 3). BLM formation was examined with these alumina films. When the aspect ratio was high, BLMs were not formed due to air bubbles trapped inside the nanopores. On the other hand, BLMs were successfully formed across the alumina films with the low aspect ratio of 1–3 and pore diameter of 200–350 nm. BLMs having a resistance of 5–100 GΩ were formed with a probability of ~80% ($n = 20$) after precoating the porous alumina with a thin layer of *n*-hexadecane. The peak-to-peak current noise was 1–2 pA after filtered at 500 Hz, which is suitable for recording low-conductance channels, such as gramicidin channels. Gramicidin is a natural ion channel-forming pentadecapeptide. When gramicidin monomers form a membrane-spanning dimer, they form a channel that is permeable to monovalent cations. Figure 4 shows examples of gramicidin single-channel currents. Stepwise currents corresponding to transitions between open and closed states were clearly observed, showing a sufficiently low noise level for single-channel recordings. The current showed no transient currents during opening and closing steps within the available time resolution (<2 ms), as shown in Figure 4(c). This electric property is favourable for recording channel currents with fast open ↔ close kinetics. Further addition of gramicidin led to increased number of channels in the BLM, showing multiple openings in the current trace (Figure 4(a)). The observed conductance level (21 pS in 2.0 M KCl, Figure 4(b)) is in the range (20–25 pS in 2.0 M KCl) of conductance reported by others (*23*, *24*). These results suggest that functional free-standing BLMs are successfully formed across the porous alumina films. However, no dramatic improvement of the BLM stability was observed probably due to the fragility of the porous alumina itself. The membranes were often ruptured by mechanical shocks, for example, stirring of the aqueous solutions. Further improvement is necessary to enhance the stability of the porous alumina films by optimising the size and edge shape of the holes in the photosensitive polymers supporting the porous alumina films.

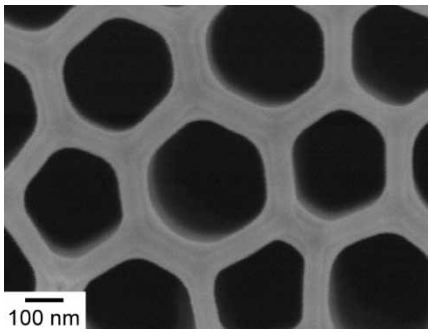

Figure 3. A SEM image of an anodic porous alumina film having low aspect ratio. Anodisation was conducted in 0.1 M phosphoric acid at −4°C and an applied potential of 160 V.

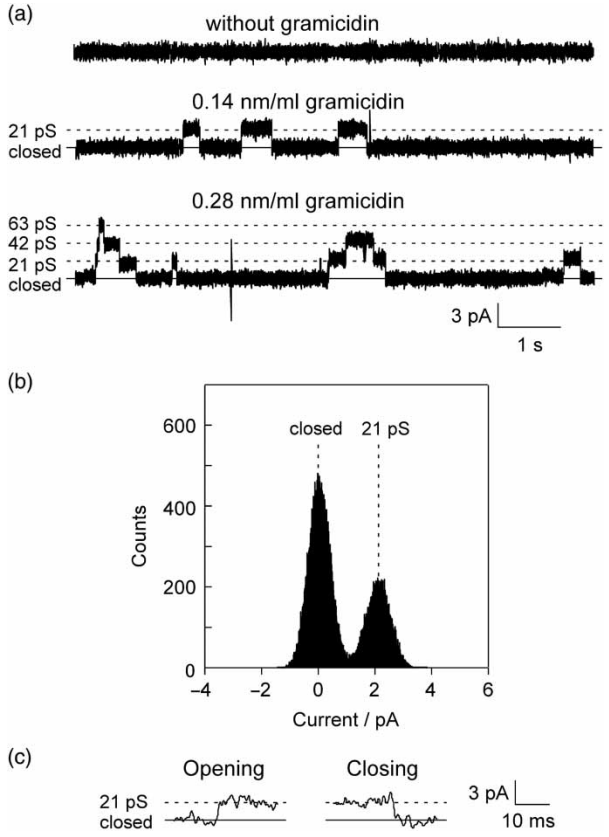

Figure 4. (a) Examples of gramicidin single-channel currents recorded at an applied potential of +100 mV. (b) Current-amplitude histogram of gramicidin channel traces recorded under conditions given in (a). (c) A time-expanded current trace for gramicidin channel transitions corresponding to opening and closing. Applied potential was +100 mV.

BLMs suspended in Si chips

Figure 5(a) shows a scanning electron microscopy (SEM) image of the edge of an aperture fabricated in a Si_3N_4 septum. The aperture was prepared in a 240 nm thick Si_3N_4 septum by isotropic etching. The aperture edge was smoothly thinned with the edge angle of about 9–22° owing to the isotropic etching. As theoretical calculation

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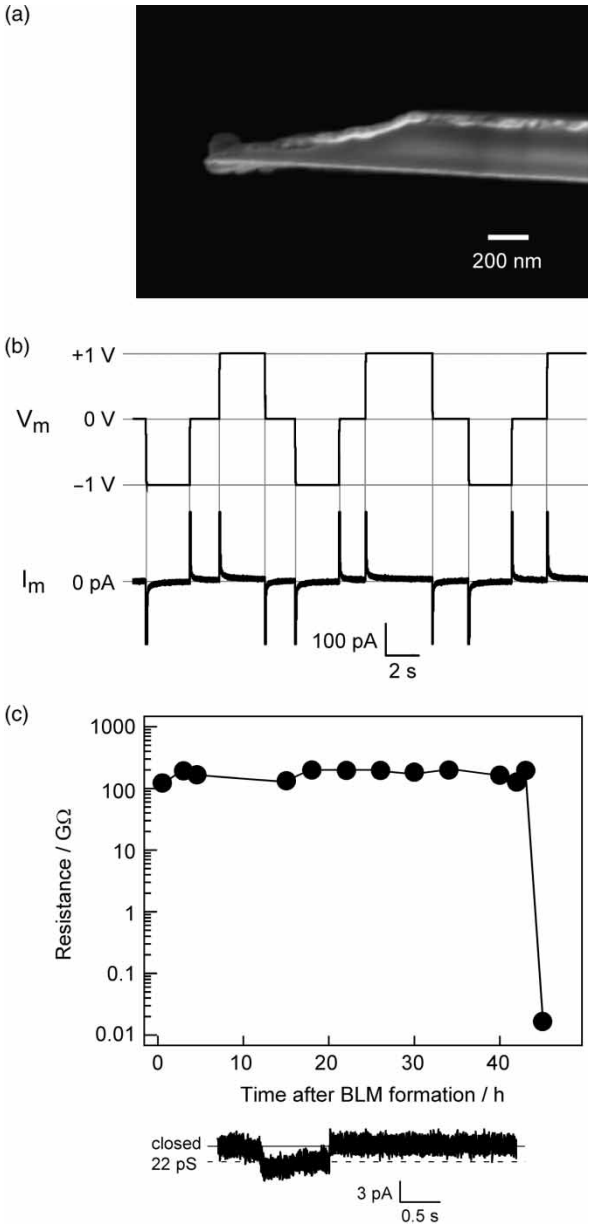

Figure 5. (a) A cross-sectional SEM image of an aperture microfabricated in a Si_3N_4 septum by the isotropic etching. (b) An example of membrane current (I_m) when the applied potential (V_m) was switched stepwise $+1\,V \rightarrow 0\,V \rightarrow -1\,V$ and vice versa. (c) (top) A time course of the resistance obtained from a BLM which exhibited the lifetime of 43 h. Membrane resistance higher than 200 GΩ was plotted as 200 GΩ. (bottom) An example of gramicidin single-channel current recorded 43 h after the BLM formation. Applied potential was −100 mV.

suggests that the annulus–septum contact angle should be small for a stable arrangement of BLMs (*19*, *25*, *26*), the tapered aperture edge, as well as nanometre thickness of the septum, is favourable to improve the BLM stability.

Formation of free-standing BLMs across the aperture was investigated by the folding method. BLMs having a resistance of 3–>100 GΩ were successfully formed in the apertures with high probability (92%, 49 membranes out of 53 trials) when the Si_3N_4 septum around the aperture was precoated with a thin layer (10 nl) of *n*-hexadecane. Solvent-free BLMs were also formed without the precoating; however, the success probability decreased to ~30%. Therefore, BLMs formed after the precoating were investigated for the following study. The present BLMs exhibited high stability as well as high-sealing property. The membranes were not broken by electrical shocks, such as plugging-off and re-connecting the Ag/AgCl electrodes inserted in the aqueous solutions surrounding the BLMs. Membranes survived when a constant voltage of ± 1 V was applied (100%, $n = 10$). Even when the applied potential was switched stepwise $+1\,V \rightarrow 0\,V \rightarrow -1\,V$ and vice versa, still 90% of the membranes (9/10) survived these treatments (Figure 5(b)). Considering that the breakdown voltage for conventional BLMs has been reported *ca.* 300–500 mV (*27*, *28*), the present membranes showed much higher stability to electrical stimulation.

Membrane durability was then examined with five BLMs. Membrane lifetime, defined as the duration for which BLMs retained membrane resistance higher than 1 GΩ, was 15–48 h ($n = 2$) without incorporated channels. When gramicidin ion channels were incorporated into BLMs, these membranes also showed a similar lifetime of 15–43 h ($n = 3$). No significant differences in membrane properties (resistance and capacitance) were observed among these membranes with various lifetimes. In the case of the longest lifetime, the BLM exhibited resistance higher than 100 GΩ up to 43 h and then the resistance decreased below 1 GΩ after 45 h (Figure 5(c)). Gramicidin channel activity was observed 43 h after the BLM formation, suggesting the functionality of the present BLM even over 40 h after the membrane formation.

Another stability required for widespread application of BLMs is tolerance to mechanical shocks induced by solution exchanges. Figure 6(a) shows examples of single-channel currents from a BLM containing gramicidin when aqueous solutions were exchanged in series KCl → CsCl → KCl. In KCl buffer, conductance level of 20 pS was observed. Then, the aqueous solutions surrounding the BLM were exchanged by sucking up KCl buffer with tubes and subsequently adding CsCl buffer with a micropipette. The water level was kept higher than the aperture to rule out the possibility of membrane re-folding. After repeating this process 5–15 times for a thorough solution exchange, single-channel currents were still observed with a higher conductance level of 53 pS. Changing the aqueous solutions back to KCl buffer led to the channel currents with the conductance of 22 pS. The observed conductance levels in both solutions were very close to reported values (25 pS in 2 M K^+ and 55 pS in 2 M Cs^+) (*23*). These results demonstrate that the present BLM containing gramicidin survived repetitive solution exchanges without any

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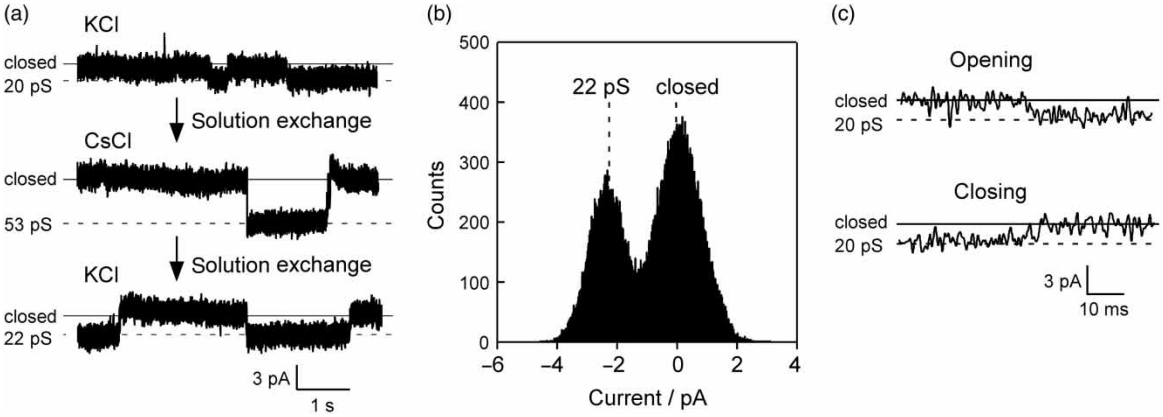

Figure 6. (a) Examples of single-channel currents recorded from a BLM containing gramicidin channel at an applied potential of − 100 mV when the aqueous solutions surrounding the BLM were exchanged from KCl to CsCl and then back to KCl. The water level was kept higher than the aperture in which the BLM was formed. (b) Current-amplitude histogram of gramicidin channel traces recorded at − 100 mV in KCl buffer. (c) A time-expanded current trace for gramicidin channel transitions corresponding to opening and closing. Applied potential was − 100 mV.

perturbation of single-channel conductance. When the solution exchange experiments were examined with different BLMs including a solvent-free membrane, all the BLMs containing gramicidin showed tolerance to solution exchanges ($n = 7$), confirming the high mechanical stability of the present BLM system.

The background current noise was 2–3 pA (peak-to-peak) after filtered at 500 Hz. This noise level is higher than those of BLMs prepared by the folding method using insulator septa, such as porous alumina (Figure 4) and conventional Teflon films. The amplitude histogram of gramicidin channel was much broader for the Si chip (Figure 6(b)) than the porous alumina film (Figure 4(b)) due to the higher background noise. The higher noise level for the Si chip is probably due to large capacitance (mean ± SEM was 252 ± 19 pF) of the present BLM chip, which stemmed from semiconductor Si. Similar large capacitance of several hundred pF has also been reported for BLMs prepared in a Si chip (*18*). As Si has high dielectric constant of 11–12, the use of Si resulted in a large capacitance of the total chip containing the BLMs, leading to relatively large noise currents.

The open time of the gramicidin channel in the present BLMs was distributed in the range from 59 ms to 6.0 s with a mean open time of 0.35 ± 0.04 s ($n = 191$). The range of open time and mean open time was similar to that reported for gramicidin incorporated into BLMs prepared by the conventional method (*29–31*). Transient current changes were observed for the open ↔ close transitions: 12 ± 2 ms for openings and 13 ± 2 ms for closings ($n = 191$) (Figure 6(c)). The transients were slower than those obtained with conventional black films (~4 ms) (*23*) and BLMs prepared in porous alumina (<2 ms) (Figure 4(c)). The slower transient currents are again probably due to the large capacitance from Si. Further improvement is necessary for recording activities of fast-gating channels, for example, the application of capacitance-reducing layer to the Si chips, which can also work for reducing background noise currents.

Conclusion

Mechanical instability has been an obstacle for the application of BLM systems. Although a large number of fabrication techniques have been proposed, mechanical stability of BLMs has not been dramatically improved. In addition, most of these studies are based on the painting method which needs a large amount of unvolatile organic solvents to form BLMs. As a large amount of organic solvents may denature vulnerable channels, we showed a different approach to form stable BLMs, where the amount of solvents was minimised. The key feature that stabilised BLMs is the nanometre-scale design of the septum: reduction in membrane size by the use of porous alumina and reduction in membrane stress at the contact with the septum by the use of smoothly tapered apertures in a nanometre-thick Si_3N_4 septum. Although further enhancement of membrane stability is necessary, the electric properties of the porous alumina as substrate-suspending BLMs are suitable for recording both low-conductance and fast-gating channels. On the other hand, BLMs suspended in a thin Si_3N_4 septum showed a dramatic improvement of BLM stability. The membrane containing gramicidin channel exhibited tolerance to repetitive solution exchanges, though the electric properties of the BLMs are necessary to be improved. The next step is realisation of stable BLMs with proper electric properties for recording activities of biological channels. Establishment of such BLMs will lead to realisation of various applications of BLMs including a smart miniature

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biosensor and a high-throughput analysis of ion-channel proteins.

Acknowledgements

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