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Free-Standing Lipid Bilayers Based on Nanoporous Alumina Films

Ayumi Hirano-Iwata[1,2], Tasuku Taira[3], Azusa Oshima[1], Yasuo Kimura[3] and Michio Niwano[1,3]

[1] Graduate School of Biomedical Engineering, Tohoku University
6-6 Aoba, Aramaki, Aoba-ku, Sendai, Miyagi 980-8579, Japan
[2]PRESTO, Japan Science and Technology Agency (JST)
4-1-8 Honcho Kawaguchi, Saitama, 332-0012, Japan
[3]Laboratory for Nanoelectronics and Spintronics, Research Institute of Electrical Communication
Tohoku University, 2-1-1 Katahira, Aoba-ku, Sendai, Miyagi 980-8577, Japan

1. Introduction

Ion-channel proteins are of great interest as subjects of basic physiological studies and main targets of drug discovery. Reconstitution of channel proteins in free-standing bilayer lipid membranes (BLMs) provides an excellent system for drug screening under chemically-controlled conditions. However, the fragility of BLMs hinders their applications and confines the bilayer method to laboratory use. Traditional free-standing BLMs are formed across micrometer-sized apertures in plastic septa [1], which seems too large to stably suspend BLMs with the thickness of 4-5 nm. In this study, we propose a method for improving the BLM stability by preparing BLMs across nanoporous alumina films. The use of porous alumina reduced individual membrane size to improve the BLM stability, while total BLM area is still large to facilitate protein incorporation, which is favourable for designing highly sensitive biosensors. The stability of the BLMs was investigated in terms of membrane lifetime and breakdown voltage. Electric properties of the BLMs as a platform for channel-current recordings are also discussed.

2. Experimental Section

Fig. 1 shows the fabrication procedure. Porous alumina films with aspect ratio of 1-3 were prepared, since unsuccessful BLM formation was observed for those with higher aspect ratios. A cleaned Al sheet (thickness 0.5 mm, purity 99.999%) was electropolished in a mixture of perchloric acid and ethanol (1:4, v/v). Then the Al sheet was anodized in 0.1 M phosphoric acid at an applied voltage of 160 V. A layer of CRC-8810 photosensitive resin was spun on the porous alumina side and patterned by the standard photolithography to form an aperture with a diameter of 100 μm. The remaining Al was removed by etching in hydrochloric acid containing saturated copper(II) sulfate. Finally, the bottom part of the oxide layer was removed by dipping in 5 wt% phosphoric acid, which also worked to widen the pore diameter. The porous alumina films thus fabricated were silanized by treating with 2% (v/v) 1H,1H,2H,2H-perfluorooctyltrichloro silane in n-hexadecane for 2 h.

BLMs were prepared by the monolayer folding method after precoating the porous alumina film with n-hexadecane. The composition of the lipid solution was 10 mg/ml L-α-phosphatidylcholine: L-α-phosphatidylethanolamine: cholesterol = 6:2:2 (w/w) in chloroform/*n*-hexane (1:1, v/v). Buffer solution used for gramicidin was 2.0 M KCl containing 10 mM HEPES/KOH (pH 7.4) and that for alamethicin was 0.5 M KCl containing 10 mM HEPES/KOH (pH 7.4). The incorporation of peptide channels (gramicidin or alamethicin) into the BLMs was made by adding the peptide solutions to the buffer solutions. Current recordings were performed with an Axopatch 200B patch-clamp amplifier (Molecular Devices). The signal was low-pass filtered at 1-5 kHz, digitized at 10 kHz, and stored on-line using a data acquisition system (Digidata 1440 and pCLAMP 10.2, Molecular Devices). The data were analyzed using pCLAMP 10.2 software.

3. Results & Discussion

Anodic porous alumina films with a pore diameter of 200-350 nm and a thickness of 200-500 nm were prepared on a CRC resin. BLMs having the resistance of 1 − >100 GΩ were formed with the probability of ~80% (n=101). The observed membrane capacitance was 17±1 pF (mean±SEM, n=67) after subtracting the capacitance of the CRC resin and porous alumina. The total exposed BLM area of 4.4 x 10^{-5} cm^2 was calculated based on a surface porosity of 56%, which was obtained by pixel analysis from SEM images. This area is equal to that of a BLM with a diameter of ~75 μm. The mean specific capacitance was 0.39±0.3 μF/cm^2 (n=67), which agrees well with those reported for artificial BLMs [1]. Possibility of the BLM formation across the CRC resin was excluded either by the specific capacitance and tapered edge shape of the resin.

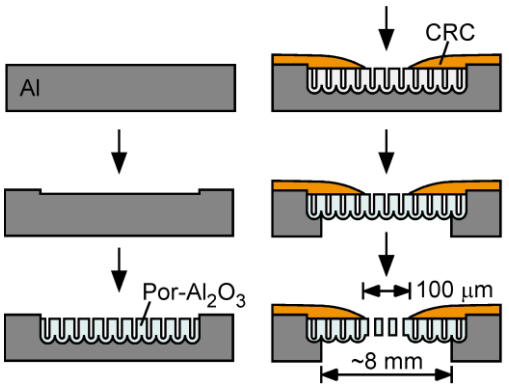

Fig. 1. Fabrication process of an anodic porous alumina film.

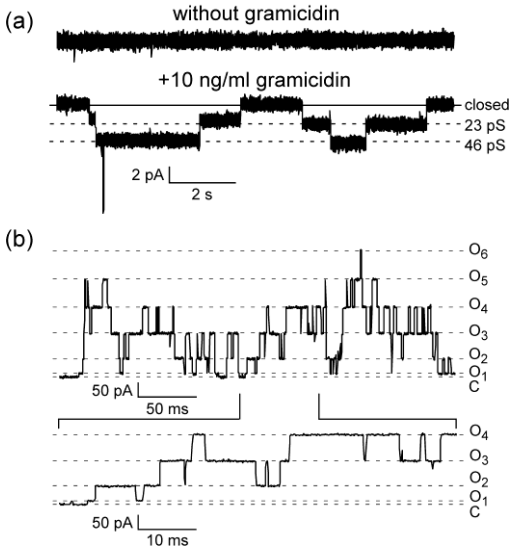

Fig. 2. Representative gramicidin and alamethicin single-channel currents.

Functionality and electric properties of the BLMs were then examined by recording single-channel activities of gramicidin and alamethicin. The noise level of the BLM was examined by recording single-channel activities of gramicidin whose conductance is relatively low [2]. Alamethicin is known to show fast transitions from one conductance state to the next one [3] and used for examining the transient responses of the BLMs. Fig. 2a shows examples of gramicidin single-channel currents. Stepwise currents were clearly observed with the conductance of 23 pS. This level is similar to reported values [2,4], suggesting the functionality of the present BLMs. The peak-to-peak noise current filtered at 500 Hz was about 1-1.5 pA. This noise level of the gramicidin-containing BLMs is smaller than that (~3 pA) observed with Si/SiN substrates having apertures of 20-30 μm [4] and is comparable to that (~1-1.5 pA) observed for a polymer substrate where BLMs with similar actual size (~75 μm) were formed [5]. These results suggest that porous alumina can be used as a low-noise platform for suspending BLMs. Fig. 2b shows examples of alamethicin single-channel currents. The conductance levels were very close to reported values [6], again confirming the functionality of the present BLMs. The expanded trace showed that our recording system clearly resolved the fast transitions of alamethicin channels. The trace showed no transients during opening and closing steps (< 0.5 ms). This electric property is favorable for recording activities of biological channels with fast open↔close kinetics.

The stability of BLMs spanned over the porous alumina films was investigated in terms of membrane lifetime and tolerance to applied potential. Membranes withstood an applied potential of ±1 V (100%, n=14). Even when the applied potential was switched stepwise +1 V ↔ 0 V ↔ -1 V, still 71% of the membranes (n=14) survived these treatments. Thus the present BLMs withstand much higher voltage than the breakdown voltage (~220-330 mV) reported for BLMs in apertures of 50-100 μm [3,5,7]. Considering that the present BLMs have the total membrane size of ~75 μm, much higher BLM stability was achieved by supporting large membrane area with nano-mesh structure of the porous alumina films. Membrane lifetime, defined as the duration for which BLMs retained resistance higher than 1 GΩ, was 16-30 h (n=2) with and without gramicidin channels. This lifetime was longer than that (several hours) of BLMs formed in conventional μm-scale apertures [5,7,8]. Gramicidin channel activities were observed 16 h after membrane formation, demonstrating the long-lasting functionality and stability of the BLMs. When we compare the membrane stability with that of our previously reported BLMs which were formed across a microaperture (20-30 μm) with a smoothly tapered edge fabricated in a Si/SiN chip [4], similar tolerance to applied potential and longer lifetime (>40 h) was observed for the BLMs in a Si/SiN chip. This comparison suggests that tapered edge shape of the aperture is also important to improve the membrane stability. Meanwhile much better electric properties were obtained for the BLMs spanned over porous alumina films. Thus the combination of the nanoporous alumina and tapered edge shape will realize the BLMs with further improved membrane stability and electric properties suitable for current recordings of biological channels with fast open↔close transitions.

3. Conclusions

We have succeeded in preparation of stable BLMs by using anodic nanoporous alumina films as a substrate suspending BLMs. Porous structure reduced the individual membrane size, leading to increased BLM stability. Since the large total area is favorable for incorporating large number of proteins, porous alumina-based BLMs are useful for designing highly sensitive biosensors.

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