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Short communication

Rapid fabrication of Teflon micropores for artificial lipid bilayer formation

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ABSTRACT

A number of recent studies have dealt with the development of biosensors using single-channel recording with an artificial lipid bilayer. However, the fragility of these bilayers and current noise present serious problems in their application towards biosensor development. To address this problem, many experimental investigations employing micropores in the formation of lipid bilayers have been reported. In this work, we present a method for the fabrication of micropores using commercially available low-melting Teflon film and a heated tip. This method allowed for the rapid (in a few seconds) and reproducible fabrication of micropores 2–3 μm in diameter. We employed a single-channel recording using a gramicidin channel and confirmed that the bilayer membrane can form on micropores by the painting method. The bilayer formed is stable under high voltage (+1000 mV). Fabricated micropores possess a conical shape with sharp edges, features which facilitated the formation of artificial lipid bilayers which could be utilized for low-noise and high voltage recording due to decreased access resistance and increased bilayer stability. These advantages promise to improve the performance of artificial lipid bilayers when employed in the development of flexible biosensors.

1. Introduction

The use of patch-clamping cells has proven to be a powerful approach in the elucidation of fundamental ion channel protein biophysics since its invention by Neher and Sakmann (1976; Hamill et al., 1981; Sakmann and Neher, 1995). This technique allows for the probing of ion channels under native conditions and for the investigation of activity following changes in the physical and chemical surroundings.

In 1962, Mueller et al. reported on a method for preparing synthetic artificial lipid bilayers, or bilayer lipid membranes (BLMs), using a plastic partition with a small aperture painted with lipid extracts of cow brain (Mueller et al., 1962). These artificial lipid bilayers have been used for single-channel recording and applied to various types of channel proteins with a view to investigating physiological and pharmacological properties. Furthermore, these techniques allow for the measurement of the properties of intracellular membranes, a task not possible with patch clamp pipettes. It is also possible to examine the effects of changes in the channel environment since in artificial lipid bilayer experiments, experimental conditions such as ion concentration in aqueous solution and lipid content in the membranes can be controlled.

Recently, a number of studies have been reported concerning the generation of biosensors using artificial lipid bilayer techniques. Examples include ultrasensitive single-molecule detection (Bayley and Cremer, 2001; Bezrukov et al., 1994; Braha et al., 2000; Gu et al., 1999; Movileanu et al., 2000), potential single-DNA sequencing (Kasianowicz et al., 1996; Meller et al., 2000; Vercoutere et al., 2001; Mathe et al., 2004; Sanchez-Quesada et al., 2004; Howorka et al., 2001), the modulation of ion selectivity (Gu et al., 2000), nanoelectroosmotic transportation (Gu et al., 2003), and studies in the area of nanochemistry (Luchian et al., 2003; Gu et al., 2001).

However, the artificial lipid bilayer technique has several limitations. Lipid bilayers typically form across an aperture with diameter in the range of 10–100 μm. These materials are very fragile, easily disrupted by electrical or mechanical stimulation, and are unsuitable for low-noise recordings due to the high capacitance across the large lipid bilayer area.

To address these issues, there has been increasing interest towards the development of micropores for use as lipid bilayer supports and subsequent investigation. Recently, micropores have often been produced with apertures in silicon (Liu et al., 2009; Maurer et al., 2007; Osborn and Yager, 1995; Pantoja et al., 2004), glass (Fertig et al., 2001), PDMS (Klemic et al., 2002) and silicon nitride (Morgan et al., 2004; Peterman et al., 2002). Although numerous reproducible and well-defined apertures can be pro-

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duced in silicon-based materials using standard micro fabrication techniques, silicon does have some major disadvantages. Native silicon has very low resistance compared with lipid bilayers (which is in the GΩ range), so that large background currents can occur. In the case of insulated silicon supports, there is often a large shunt capacitance across the lipid bilayers due to the use of thin dielectric films, leading to increased electrical noise and restricted bandwidth, whilst silicon nitride membranes themselves are extremely fragile. Furthermore, the fabrication route is time-consuming (Sandison and Morgan, 2005).

On the other hand, Teflon is a well-suited polymer for use in the planar lipid bilayer technique due to its chemical stability, good electrical properties (high resistivity, low dielectric constant, and low dielectric loss (Penner, 1995)), and ability to support planar lipid bilayers that are stable for several hours (Schlue and Hanke, 1993; Wonderlin et al., 1990). Furthermore, Mayer et al. reported a fabrication method of Teflon micropores using AF-Teflon, and showed low-noise channel recording (Mayer et al., 2003). However, this method requires several steps over a period of hours for the fabrication of micropores due to the unwieldiness of AF-Teflon.

To resolve this, here we present new simple methods for the rapid fabrication of Teflon micropores. Micropores were fabricated on commercially available low-melting Teflon film (ETFE heatproof temperature 150 °C) using a heated tip. Using these methods, pores 2–3 μm in diameter were quickly fabricated (in a few seconds) with high reproducibility. We demonstrate the use of micropores in ETFE for planar lipid bilayer experiments, confirm formation of BLMs in micropores using single-channel recordings of gramicidin ion channels, and demonstrate stability of the BLMs by applying a high membrane voltage.

2. Materials and methods

2.1. Fabrication of micropores in Teflon film and designing a cell for channel recording

Fig. 1 summarizes the fabrication of micropores in ETFE film. A hole (diameter 0.4 mm) was made in the center of a Ag/AgCl plate (thickness 0.5 mm) and pasted to an ETFE film (thickness 12 μm, purchased from Nilaco) using epoxy adhesive (Alardaite Rapid). The tip was created by electrochemically polishing (10% KOH, 20 V_{rms} 60 Hz) Tungsten wire (Ø 0.5 mm), heating using a nichrome wire (Ø 0.2 mm), positioning to the center of the ETFE film on a cover glass using an optical microscope (IX-50, Olympus), and movement of the tip to the ETFE film using the focusing system. Following contact of the cover glass with the heated tip, a hole is generated in the ETFE film at the point of contact between the tip and the cover glass.

Fig. 1(C) shows a cross section of the completed cell. The chamber was made using a silicon O-ring (inner diameter 11.0 mm fineness 2.6 mm) separating the Ag/AgCl plate on the *Cis* and *Trans* side. The cell was then clamped using an M2 screw and nat.

2.2. Formation of artificial lipid bilayer

Diphytanoylphosphatidylcholine (DphPC) was purchased from Avanti Polar Lipids, Inc. Bilayers were formed using the painting method (Mueller et al., 1962). Lipid solution (20 mg/ml DphPC in *n*-decan) was painted by generating air bubbles at the tip of a pipette (a small amount of lipid solution (20 μL) was attached to the tip of a micropipette and excess solvent was blown away in pure water) in the base solution (2 M KCl, 10 mM Hepes, pH 7.3) and passing these bubbles through to the ETFE pore. When too much lipid solution was painted to the pore, air bubbles were sprayed with a new pipette to remove excess lipid solution in order to effect thinning.

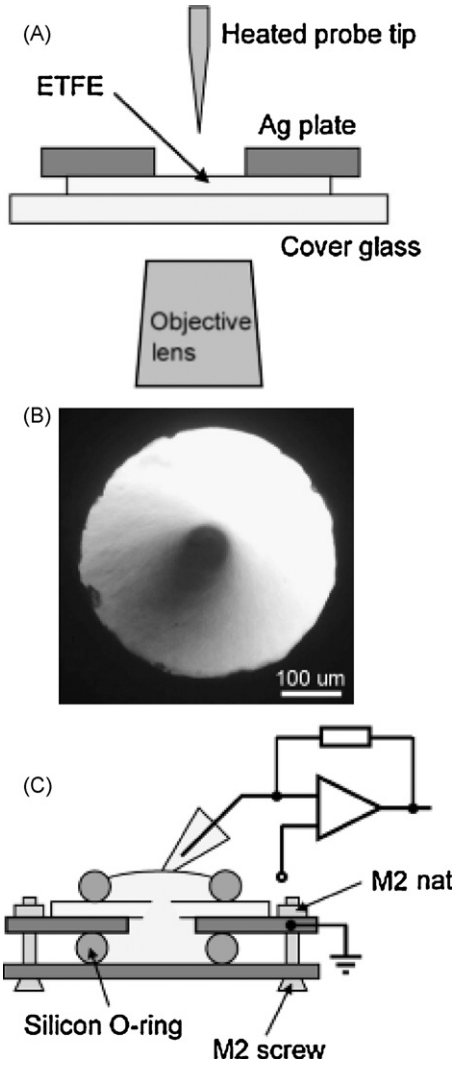

Fig. 1. Fabrication process of ETFE micropores. (A) Schematic view of fabrication process. A hole is made in the center of the Ag/AgCl plate (25 mm × 25 mm, thickness 0.5 mm) using a micro drill (Ø 0.4 mm), and to this is attached an ETFE film (thickness 12 μm) using epoxy adhesive and the material is then placed on a cover glass. (B) Positioning of the heated tip in the center of the Ag hole using an optical microscope (IX-50 Olympus) and controlling approach to the ETFE film using focusing systems. (C) Cross-section of the designed cell.

Current recordings were performed using a patch clamp amplifier (CEZ-2400, Nihon Kohden, Japan), and the data were non-filtered. The sampling rate of the data was 1 kHz and analyzed using commercial software (pClamp10.2, Molecular Devices).

3. Results and discussion

3.1. Micropores in Teflon film

Fig. 2 shows SEM images of fabricated ETFE pores. Fig. 2(A)–(C) shows images of ETFE pores fabricated using various tip temperatures. When fabricated using a non-heated tip, pores did not form (Fig. 2(A)). On the other hand, when the tip temperature was too high, deformations of ETFE occurred (Fig. 2(C)). When the tip was heated to suitable temperatures, ETFE pores were fabricated with diameters of ca. 3 μm and with a conical shape that complemented the taper of the tips (Fig. 2(I)). ETFE has a low-melting point and the pores formed complemented the shape of the tip at moderate temperatures (Fig. 2(B)). The heating conditions of a tip depend on the current applied to the Nichrome wire. Therefore, by examining

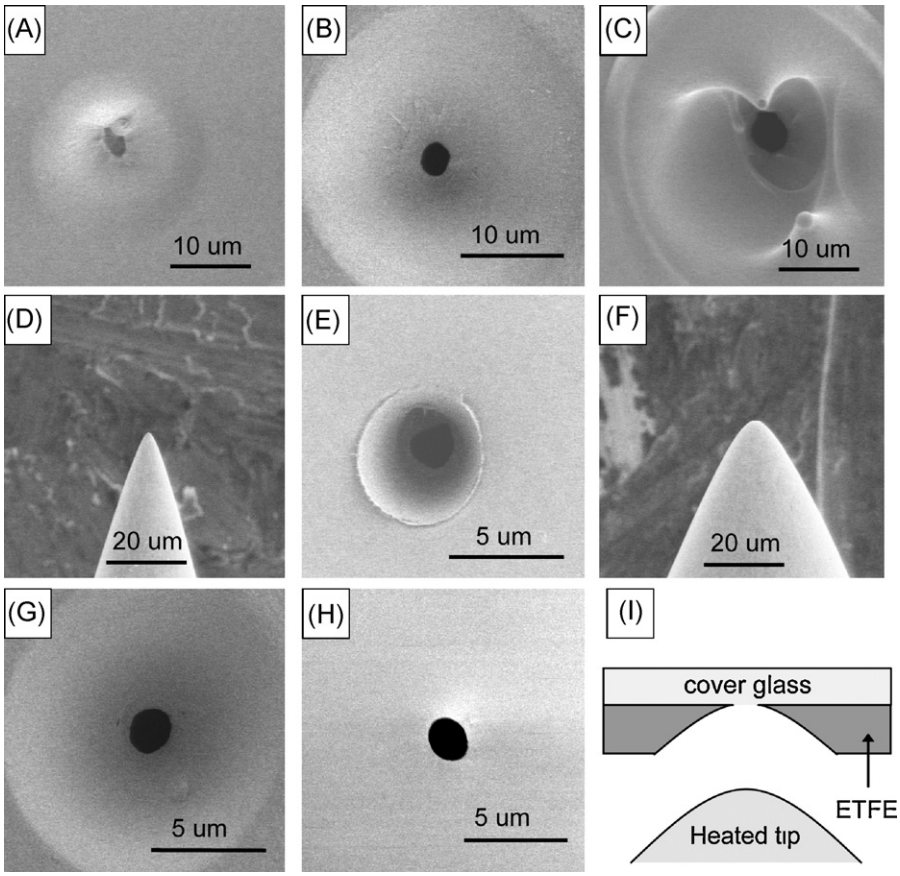

Fig. 2. SEM images of fabricated micropores. (A) Approach of a non-heated tip to ETFE film. (B) Pore fabricated using a tip of moderate temperature. (C) Pore fabricated using an excessively heated tip. (D) Electrochemically polished Tungsten wire under 20 V_{rms}. (E) Pore fabricated by tip (D). (F) Electrochemically polished Tungsten wire under 15 V_{rms}. (H) Pore fabricated by tip (F). (H) Pore shape on the cover glass side. (I) A cross-section of the pore fabricated.

the most suitable temperature beforehand, we are able to fabricate conical ETFE pores in a reproducible manner.

Fig. 2(D)–(G) shows the dependency of pore shape on tip sharpness. Fig. 2(D) shows a sharp tip (conical angle is ca. 30°) and Fig. 2(E) shows an ETFE pore fabricated using a sharp tip. Fig. 2(G) shows a dull tip (conical angle is ca. 60°) and Fig. 2(F) shows an ETFE pore fabricated using a dull tip (Fig. 2(G)). The pore shown in Fig. 2(E) has a smaller aperture angle compared with pore shown in Fig. 2(F). It is apparent that the aperture angle of the conical pore is dependent on the sharpness of the tip. From this result, it was thought that using a dull tip at the most suitable temperature would allow for the fabrication of conical pores that possess large aperture angles. These types of conical pores are well-suited for lipid bilayer experiments since large aperture angles obviate the disadvantage of high electrical access resistance of micro apertures (Wonderlin et al., 1990).

Fig. 2(H) shows the cover glass side of the ETFE pore (Fig. 2(G)). On the opposite side to the tip side, the shape of the pore is not conical but flat since the ETFE film was in contact with the flat cover glass. This shows that the pore has a conical shape on the tip side and a flat shape on the cover glass side. The edge of the aperture is very sharp, similar to the pores fabricated by the shaving method, and this aspect is suitable for the painting method which may require subsequent thinning of the lipid solution (Wonderlin et al., 1990).

3.2. Bilayer experiments on micropores

Lipid bilayer experiments on micropores usually employ the Montal–Mueller method (Mayer et al., 2003; Montal and Mueller, 1972; Sandison and Morgan, 2005; O'Shaughnessy et al., 2007) since it is difficult to generate membranes that spontaneously form a thin bilayer in small apertures (Hirano-Iwata et al., 2008). However, we performed bilayer experiments on micropores (diameter 3 μm) using the painting method. After painting lipid solution over the aperture, air bubbles were sprayed onto the aperture to remove excess lipid solution and the material was allowed to rest until the membrane spontaneously formed a thin bilayer at 100 mV. In a few minutes, the membrane current attained was 0.3–0.5 pA, indicating bilayer lipid membrane resistance (over 200 GΩ). To confirm the formation of a bilayer lipid membrane directly, the membrane was examined using a gramicidin channel (Fig. 3(A)). Gramicidins are produced by the soil bacterium *Bacillus brevis* and form well-defined cation-selective ion channels in bilayer lipid membranes (Kelkar and Chattopadhyay, 2007). One can see 3 pA conductance steps in the current–time trace, which is a typical characteristic of gramicidin ion-channels in bilayer lipid membranes. These data confirm that a lipid bilayer was successfully formed and that a single-channel recording can be obtained on a micropore generated using the painting method. The removal of excess lipid solution using air bubbles, in addition to the shape of the pore, facilitate successful thinning of the lipid solution.

The stability of lipid bilayers was evaluated by applying a membrane voltage. Bilayers become more stable with decreasing aperture diameter (Mayer et al., 2003), but are disrupted irreversibly at around 500 mV (Kramar et al., 2007; Mayer et al., 2003). Fig. 3(B) shows the results of applying a high voltage to a lipid bilayer on a micropore. When applying a voltage of 1000 mV for a few seconds the transmembrane current reaches saturation, but after returning the voltage to 100 mV the initial current

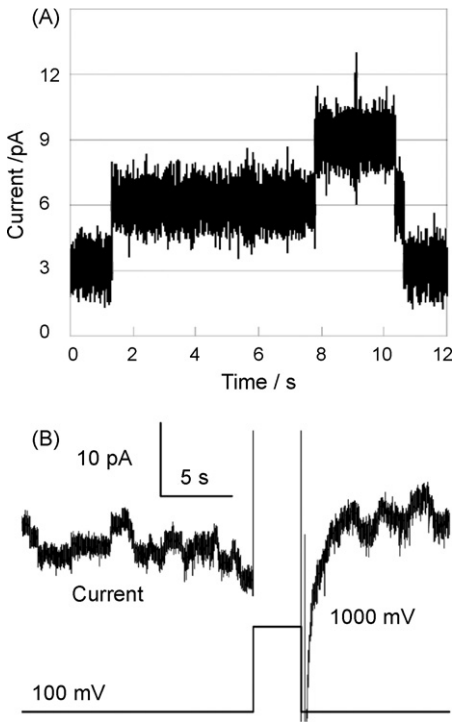

Fig. 3. Bilayer experiments on micropores. (A) Channel recording of gramicidin. Base solution comprised 2 M KCl, 10 mM Hepes (pH 7.4) and applied voltage of +100 mV with 0.1 nM gramicidin. 3 pA conductance steps were consistently observed. (B) Stability of bilayer membrane under high voltage. Conductance steps of gramicidin were observed at +100 mV and +1000 mV. At first, the transmembrane current was saturated to the value of the amplifier, but the current was restored at +100 mV.

was restored. If the lipid bilayer is disrupted irreversibly, the aforementioned current behavior should not be observed. This result suggests that the lipid bilayer could exist intact under high voltage. In the painting method, thinning of the lipid droplet occurs spontaneously and the resulting structure comprises a lipid bilayer surrounded by an annulus of the bulk solution. This affords a thin bilayer area small enough to withstand high voltages.

This type of stability of lipid bilayers against large applied voltages is clearly beneficial for studying ion channels with small single-channel conductance (Peterman et al., 2002) and for the generation of devices incorporating artificial lipid bilayers (Denyer et al., 1998; Fertig et al., 2002; Olapinski et al., 2008; Schmidt et al., 2000; Xu et al., 2001).

4. Conclusion

In this study, we have developed a method for the rapid and reproducible fabrication of Teflon micropores (diameter of 2–3 μm) using a heated tip and commercial low-melting Teflon film. When using a dull tip (conical angle ca. 60°) with moderate temperature, the generated pore has a conical shape with large aperture angle and sharp edges. These features are suitable for low-noise recording due to reduced access resistance and membrane capacitance. Furthermore, we confirmed the formation of lipid bilayers using the painting method. The bilayers formed on the micropores were very stable under high membrane voltage (1000 mV for at least for 2–3 s). This shows the potential use of our fabricated micropores as flexible biosensors with low-noise and high voltage channel recording.

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