Anal. Chem. **2008**, *80,* 7792–7795

Lipid Bilayers at the Gel Interface for Single Ion Channel Recordings

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Single-channel recording using artificial lipid bilayers is along with the patch-clamp technique a very powerful tool to physiologically and pharmacologically study ion channels. It is particularly advantageous in studying channels that are technically difficult to access with a patch pipet. However, the fragility of the bilayers and the difficulty to incorporate ion channels into them significantly compromises measurement efficiency. We have developed a novel method for forming artificial lipid bilayers on a hydrogel surface that significantly improves the measurement efficiency. Bilayers formed almost instantly (<1 s) and were able to incorporate various types of ion channel proteins within a short time (<30 s) enabling multichannel measurements. These results indicate that this method can potentially be applied to developing high-throughput screening devices for drug design.

Single-channel recording using artificial lipid bilayers is a popular technique to physiologically and pharmacologically characterize ion channels.[1] This technique has also been applied to develop single-molecule sensor devices, although it is limited to laboratory use. There are two major problems that significantly reduce single-channel measurement efficiency and limiting sensor applicability: the membrane fragility and low incorporation rate of ion channels into artificial bilayers. Lu et al. showed that stable bilayers were supported by an agarose gel though they were not suited for single-channel recording.[2] We reported the agarose-supported bilayers were more stable than conventional self-standing bilayers and that electrical and optical single-channel recordings were possible with these types of bilayers.[3−5] Several attempts have been made to form durable bilayers by supporting or encapsulating bilayers with a hydrogel.[6−9] However, the bilayer durability has not been improved to the desired extent by these technologies. Thus, the bilayer method is still limited to laboratory use. It is quite difficult to achieve bilayers available for use outside the laboratory by reinforcing conventional painted or folded bilayers. An alternative way to overcome these difficulties and produce practical devices is to develop methods to form bilayers much more simply and promptly than conventional techniques. If nonspecialists can form bilayers rapidly on-site and be able to insert ion channels into them, bilayers with high stability or long lifetimes are not required because several minutes should be enough for analyte detection.

Rather than focusing on durable bilayers, we developed several methods for artificial bilayers that are not especially durable but can be formed easily, rapidly, and repeatedly.[3] In this paper, we report a novel technique for making bilayers at a gel surface that solves both membrane fragility and the low incorporation rate of ion channels. This method makes it possible to significantly increase measurement efficiency such that bilayers can be used as practical single-molecule sensors. Furthermore, it is notable that this technique enables us to make multichannel single-channel recording systems because of its simplicity.

EXPERIMENTAL SECTION

Preparation of Vesicles. Membrane vesicles from bovine trachea,[10] porcine uterine sarcolemma,[11] skeletal muscle sarcoplasmic reticulum,[12] and *Torpedo* electric organ[4] were prepared as previously reported. Vesicles were suspended in 150 mM KCl, 300 mM sucrose, and 10 mM Hepes (pH 7.4) and stored at −80 °C.

Bilayer Formation at the Gel/Water Interface. Glass pipets for bilayers at the gel/water interface were made from glass capillary tubing with outer and inner diameters of 1.5 and 0.9 mm, respectively, using a pipet puller (P-97, Sutter Instrument). The pulled capillary tubes were fabricated with a microforge apparatus (MF-900, Narishige, Japan) to make tips with a wide aperture. The diameter of the aperture at the tip of the pipet was 30−50 μm. Hydrogel (0.5−2% agarose VII or Sepharose 4B in 150 mM

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10.1021/ac801224a CCC: $40.75 © 2008 American Chemical Society
Published on Web 09/19/2008

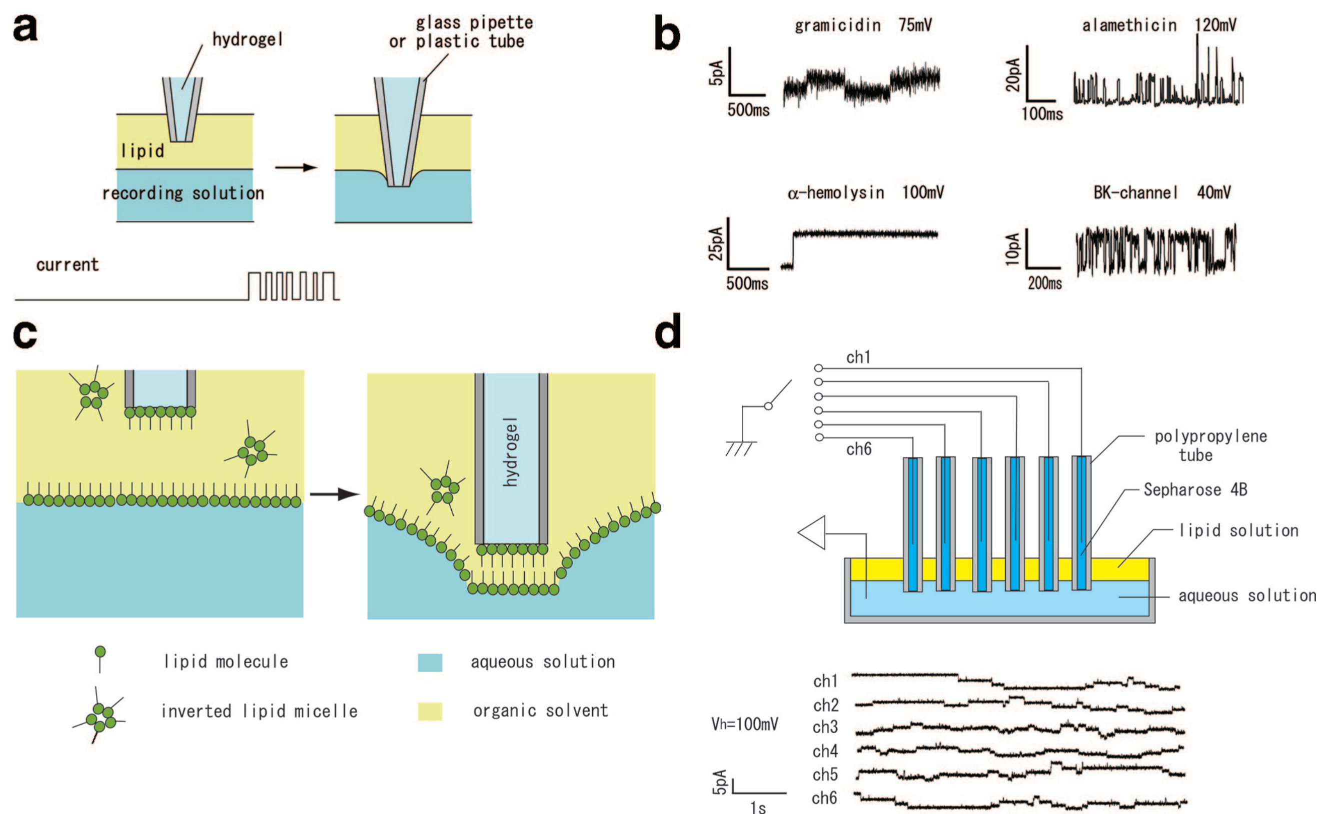

Figure 1. Artificial lipid bilayers formed at the hydrogel/water interface. (a) Procedure to produce bilayers at the gel/water interface. A fine tube filled with hydrogel (tip aperture of 30−200 μm) plunged into a recording solution through a lipid solution layer. The bottom is a hypothetical current record. (b) Current records for a various types of channels. Indicated channels were added to the bath solution before the recordings (0.3 μg/mL alamethicin, 0.02 μg/mL gramicidin D, 10 μg/mL hemolysin, and 10 μg/mL bovine tracheal vesicles, respectively). The recording solution contained 150 mM KCl and 10 mM Hepes (pH 7.4). The membrane potential was held at the indicated voltage. (c) A bilayer was produced from the thick membrane. Organic solvent and inverted lipid micelles were removed between two lipid monolayers resulting in a lipid bilayer membrane. (d) Multichannel single-channel recording of gramicidin. Six bilayers were formed at a time with polypropylene tubes as shown in (a). The tip diameter was ~0.1 mm. The tubes were fixed with each other and moved together. The bath chamber was a 35-mm culture dish. The bath solution contained 5×10^{-9} M gramicidin D, 1 M KCl, and 10 mM Hepes (pH 7.4).

KCl, 10 mM Hepes, pH 7.4) was dissolved by heating, aspirated into the pipet from the tip, and then left at room temperature to cool. Plastic tubes with a larger aperture (100−200 μm) were filled with hydrogel the same way. A 96-well polystyrene culture plate was used as the bath chamber to measure channel currents. An aqueous solution including channel-forming peptides or membrane vesicles was poured into the well. A lipid solution (20−50 mg/mL phospholipids in *n*-decane) was then layered over the recording solution layer. For bilayer formation, it is only required that the recording solution is covered with the lipid solution. It is not needed to precisely control either the amount of or the surface area of the solutions. Bilayers were made by moving a hydrogel-filled glass pipet or a plastic tube through this lipid solution layer into the aqueous solution. The depth of the tip dipped into the solution was usually 10 μm−1 mm. However, it was not necessary to be precise as using a coarse manipulator for dipping was sufficient. Channels were incorporated spontaneously into the bilayer at the gel/water interface.

Bilayer Formation at the Gel/Gel Interface. Bilayers were formed at the hydrogel/hydrogel interface as previously described.[5] Heated and dissolved hydrogel was poured into the well of a 96-well culture plate and cooled at room temperature. A lipid solution (20−50 mg/mL phospholipids in *n*-decane) was layered over the cooled hydrogel. A small amount (<2 μL) of vesicle suspension containing 1−10 mg of proteins/mL was added to the lipid solution layer. A hydrogel bead made at the tip of a fine tube (see Figure S-2 in Supporting Information, SI) was moved downward until contacting the bottom hydrogel layer resulting in a bilayer. Channel proteins in a vesicle membrane were incorporated into the bilayer by vesicle fusion.

RESULTS AND DISCUSSION

Bilayers at the Gel/Water Interface. Figure 1a shows how to make bilayers. A fine tube such as a glass pipet or a polypropylene tube is filled with hydrogel and plunged into an aqueous solution through a phospholipid solution layer. The depth of the tip is not exact, ranging from several tens of micrometers to 1 mm. Bilayers are formed repeatedly by plunging the tubes into the solution through lipids (see Figure S-1 in SI) allowing channel recordings. We could form bilayers with this method much more easily and promptly (<1 s) than previous methods. Figure 1b represents channel current records taken with bilayers formed by this method. Channel-forming peptides such as gramicidin and alamethicin or certain proteins like hemolysin were incorporated into the bilayers by adding them to the aqueous solution. Channel proteins such as BK-channels were reconstituted into the bilayers by vesicle fusion. To accomplish this, we also took advantage of conditions such as hyperosmolarity in the

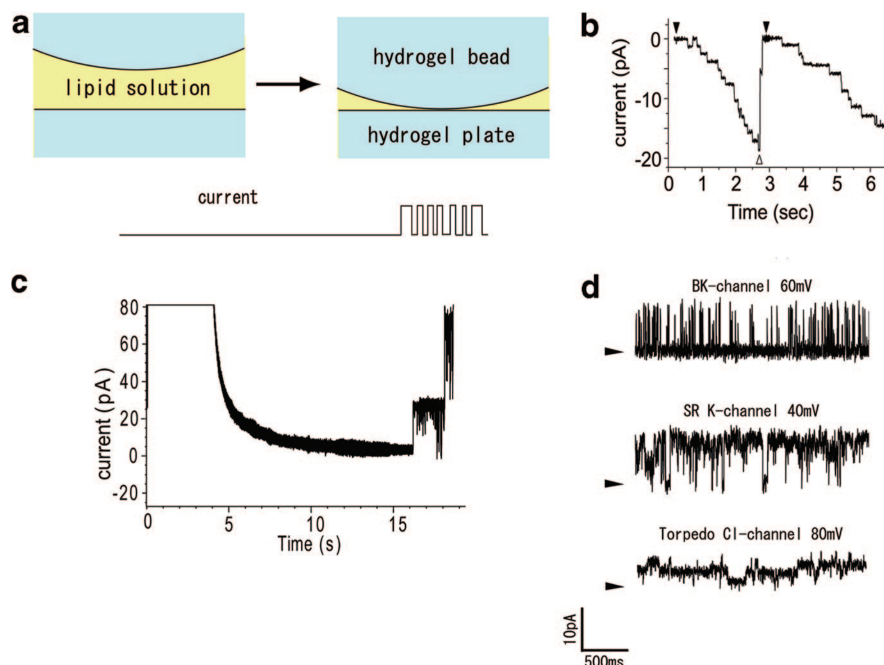

Figure 2. Artificial lipid bilayers formed at the hydrogel/hydrogel interface. (a) Procedure to produce bilayers at the hydrogel/hydrogel interface. A hydrogel bead made at the tip of a fine tube was moved downward and brought into contact with a hydrogel plate. Excess lipids and solvent were forced out of the space between the two hydrogels resulting in a lipid bilayer membrane. (b) A gramicidin channel current recorded from a bilayer formed at the hydrogel/hydrogel interface. Arrow heads indicate where the bead was lifted causing detachment from the hydrogel plate (▽) and lowered causing contact with the hydrogel plate (▼) in the lipid solution. (c) Incorporation of a BK-channel into the bilayer. A membrane vesicle suspension prepared from bovine trachea was added onto the hydrogel plate. A bilayer was then made as in (a). The figure shows a portion of the repeated incorporation. The gel bead had been lifted resulting in the disappearance of the bilayer. At time zero, the gel bead was brought into contact with the gel plate reproducing the bilayer. The BK-channel was incorporated into the bilayer 16 s after bilayer reproduction. The large transient current beginning at time zero is a capacitive current arising from the rapid increase in membrane capacitance. The gel bead was 0.2 mm in diameter. (d) Single-channel records from bilayers at the hydrogel/hydrogel interface. Indicated channels were reconstituted into the bilayer.

vesicle lumen that are known to promote vesicle fusion with self-standing layers. As we reported in previous papers,[3−5] the agarose support did not significantly affect the basic properties of the channels inserted into the bilayer as they resembled those from conventional bilayers (see, for example, Figure S-3 in SI).

There are two popular methods to make artificial bilayers for channel recordings. The tip-dip method[13] folds two lipid monolayers at an air/water interface to make bilayers. The painting method[14] has bilayers made from a thick lipid solution layer by removing excess lipids and solvent resulting in two monolayers on the surface of the lipid solution coming into contact. Our method is much more simple and prompt than either of these two as bilayers are formed at the gel/water interface almost instantly. The tip-dip method has the advantage that one can form solvent-free bilayers. However, it is difficult to make a large (>10 μm in diameter) bilayer, and even for small bilayers, it is necessary to manipulate the glass pipet quite carefully. On the other hand, the painting method makes large bilayers quite easily. However, initially there is a thick solvent layer between the two monolayers and it takes rather long to remove excess lipid and solvent. This method is quite inconvenient when applied to multichannel measurements because the waiting time for the thinning process differs from one bilayer to the next. With the method described in this paper, bilayers are made promptly by rather roughly manipulating hydrogel-filled tubes, meaning it is possible to make a number of bilayers easily and simultaneously (Figure 1c). Thus, it is most suited for simultaneous recordings from multiple bilayers. In fact, we successfully measured channel currents from six bilayers using a very simple apparatus (Figure 1d). It is only required that the recording solution is covered with the lipid solution to efficiently form bilayers. The formation rate was significantly dependent on neither the shape nor the size of the bath chamber. This should be advantageous when making a multichannel device and when combining our method with other technologies such as microfluidics.

Bilayers at the Gel/Gel Interface. As explained above, bilayers were made rapidly at the gel/water interface. Furthermore, amphiphilic channel-forming molecules such as gramicidin, alamethicin, and hemolysin were easily incorporated into these bilayers. However, unlike these channels, channel proteins from biological membranes could not be incorporated into bilayers just by adding them to the solution. Several techniques including vesicle fusion have been used to reconstitute channel proteins into artificial bilayers. However, incorporating proteins by these techniques in which channel proteins haphazardly incorporate into bilayers are probabilistic and time-consuming. This incorporation problem has significantly reduced the efficiency of current measurements. To address this problem, we developed a novel method for making bilayers in which bilayers were made at the hydrogel/hydrogel interface.[5] Bilayers were formed by contacting two hydrogel layers, one a hydrogel bead and the other a hydrogel

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plate, in a lipid solution as shown in Figure 2a. This process of bilayer formation, in principle, is the same as that shown in Figure 1a because there is a water layer on the surface of the hydrogels that stimulates bilayer formation since the excess lipids and solvent are forced out of the space between the two hydrogels due to the polar environment.

Figure 2b is a gramicidin current recorded with a bilayer made by the method described in Figure 2a. This current trace is a segment of consecutive experiments in which the upper hydrogel bead was repeatedly moved up and down. Lifting the hydrogel bead (▽) caused the current level to reach zero, coinciding with the disappearance of the bilayer. Lowering (▼) the bead resulted in current step increases indicating the formation of a new bilayer and incorporation of the channels. As shown in this figure, a bilayer was formed almost instantly after bringing two gels into contact with each other. The greatest advantage of this method is that we can incorporate channel proteins into bilayers much more rapidly than conventional methods. Figure 2c indicates a single-channel current of a BK-channel protein recorded from a bilayer made with this method. The vesicle suspension had been added onto the lipid solution layer before plunging the hydrogel bead. This current record is like that in Figure 2b but for a BK-channel. The gel bead was moved up and down to contact the gel plate at time zero. Incorporation of the BK-channels could be repeated a number of times, normally occurring within 30 s (see Figure S-4 in SI).

This incorporation method was applicable to other types of channel proteins such as rabbit skeletal sarcoplasmic reticulum channels and *Torpedo* anion channels as shown in Figure 2d. All channel types were incorporated into the bilayers within 30 s of bringing the two hydrogels into contact with each other, although the time required for incorporation depended on the vesicle type. Judging from the voltage dependency of the bilayer channels incorporated, channels were reconstituted into bilayers by vesicle fusion from the underside of the bilayers. It is likely that reducing the space between the gels resulted in concentrating the vesicles, which promoted fusion with the bilayers. It is also possible that the mechanical pressure exerted on the vesicles by pressing the gel bead onto the gel plate induced fusion with the bilayers. The channel properties measured with bilayers at the gel/gel interface were like those measured with conventional bilayers (see, for example, Figure S-5 in SI).

As shown in the introduction, the purpose of this study was to develop methods to form bilayers and insert ion channels simply and rapidly. Recently, several papers have described the formation of durable bilayers by encapsulating them with hydrogels.[6−9] Although these bilayers are similar in appearance to ours, the comparison between these and ours is limited because of the divergent purpose and formation method.

CONCLUSION

In this study, we have made novel methods to produce lipid bilayers for single-channel recordings that allow us to overcome two conventional barriers that reduce measurement efficiency. We conclude that our method is significantly more efficient for high-throughput devices for single-channel recordings than any current alternatives. In future work, we will report on the progress of this artificial bilayer technique including our goal to automate the procedure by applying laboratory chip technology that will allow for a more rapid, higher volume method.[15,16]

ACKNOWLEDGMENT

The authors thank Dr. Peter Karagiannis for carefully revising the manuscript. This work was supported by grants from the Ministry of Education, Culture, Sports, Science and Technology of Japan (Molecular and System Life Science, and Innovative nanoscience of supermolecular motor proteins working in biomembranes).

SUPPORTING INFORMATION AVAILABLE

Experimental details and channel recording data. This material is available free of charge via the Internet at http://pubs.acs.org.

Received for review June 16, 2008. Accepted August 18, 2008.

AC801224A

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