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Rapid Report

Horizontal 'solvent-free' lipid bimolecular membranes with two-sided access can be formed and facilitate ion channel reconstitution

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Abstract

We present here an easily used method and apparatus for formation of horizontal 'solvent-free' lipid bilayer membranes affording two-sided access. These horizontal bilayers allow direct delivery of submicroliter volumes of samples onto the membrane upper surface increasing the efficacy of reconstitution by several orders of magnitude, as demonstrated using *Staphylococcus aureus* α-toxin. Also, they permit creation of locally high and transient transbilayer osmotic gradients to initiate fusion of ion-channel containing liposomes with planar membrane, which, following fusion, leaves the membrane and channel in essentially symmetric bathing solutions. This method is especially advantageous for cases where thickness of the membrane, absence of hydrocarbon solvent, or presence of differing lipid compositions in the two monolayers is critical.

From the early 1960s until now several methods for formation of planar lipid bilayer membranes have been developed, such as hydrocarbon solvent-containing 'painted' membranes [1], and 'solvent free' membranes folded from lipid monolayers [2,3], including dip-stick (tip-dip) bilayers [4,5] (see, for review, Ref. [6]). These methods each have distinct advantages and have been used for modeling the ion and nonelectrolyte transport of cell membranes, often by the reconstitution and study of protein channels or other membrane-active substances.

Two advantages of folded membranes are that it is possible to form asymmetric bilayers, and to minimize possible channel denaturing effects of hydrocarbon solvent. In some cases 'solvent-free' bilayers can be 'painted' by using a special solvent that does not dissolve in the bilayer matrix, such as squalene [7], or by using hexadecane which can be 'frozen-out' of the membrane [8]. These methods allow the formation of bilayers with properties close to those of solvent-free membranes, but also can produce leaky bilayers and other conditions not practical for some channel reconstitution studies.

One reliable method of ion-channel reconstitution into planar bilayers is the method of fusing liposomes with planar membranes, which takes place more readily to 'painted' than to 'solvent-free' planar membranes [9,10]. The fusion is promoted by establishing a temporary transbilayer osmotic gradient, followed by a technically challenging perfusion of osmoticant and residual liposomes from membrane bathing solution to prevent multiple fusion events so that single channels can be studied in symmetric bilayer bathing solutions.

Reconstitution may be facilitated by forming planar bilayer membranes in a horizontal orientation. This allows the directed delivery of samples onto the membrane. In one step this achieves an acceleration toward the membrane and a locally high but transient concentration at the membrane of the added sample. Among the previous membrane formation methods, two-sided access horizontal bilayers have been formed only by the painting technique, and are either solvent-containing or formed from hexadecane solutions [11,12]. However, the 'painted' membranes, since they contain hydrocarbon solvent, have a thicker hydrocarbon region which can affect the functioning of reconstituted proteins such as Ca^{2+}-ATPase [13], and may denature protein. The tip-dip method forms horizontal solvent-free bilayers, but has the disadvantage of one-sided access and does not permit 'layering' of samples onto the membrane.

In this work we present a new device and method for

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forming horizontal folded 'solvent-free' planar lipid bilayer membranes, which have two-sided access and permit directed delivery of samples to the membrane. Experiments using *S. aureus* α-toxin show the amplification of channel forming efficiency and improved liposome fusion that is possible in this system. A preliminary report of part of this work has been published in abstract form [14].

Materials and methods

Membranes were formed at room temperature (22–24° C) by folding together two lipid monolayers across a round aperture of 0.1–0.5 mm diameter by the apparatus and method described below. For membrane formation egg phosphatidylcholine (PC) and bovine brain phosphatidylserine (PS) (Avanti Polar Lipids, Pelham, AL) and hexadecane (Aldrich, Milwaukee, WI) dissolved in *n*-pentane (Fluka, Buchs, Switzerland) were used.

Powdered *S. aureus* α-toxin with 32.5 u/μg hemolytic activity (Calbiochem, La Jolla, CA) was dissolved to 306 μg/ml concentration in 80 mM octyl glucoside and 10 mM NaCl stock and frozen in 10-μl aliquots. Before use, the toxin was thawed on melting ice and diluted 10-fold in membrane bathing solution, or in 'heavy buffer' containing 459 mM urea, 10 mM KCl, 10 mM Hepes, 0.1 mM Na-EDTA (pH 7.4) and 0.02% sodium azide.

α-Toxin proteoliposomes were formed from PC/PS

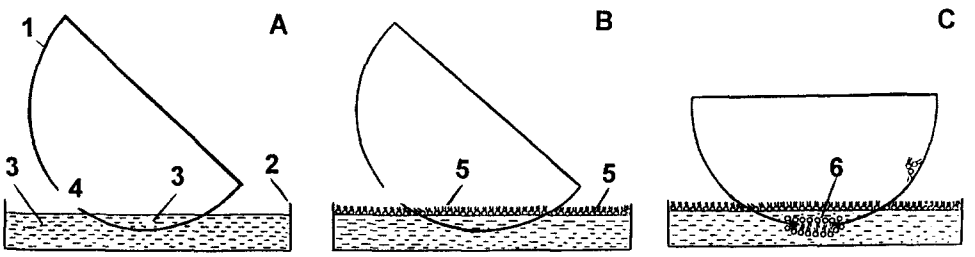

Fig. 1. Scheme of the three basic stages of formation of horizontal 'solvent-free' lipid bimolecular membranes (arbitrary scale and sizes). (A) The upper chamber (1) containing a bilayer aperture (4) is rotated above the lower chamber (2) so that buffers (3) in each chamber do not contact the aperture. (B) Lipid monolayers are spread on the buffer surfaces of each chamber. (C) The upper chamber is rotated to dip the aperture through the monolayers into the aqueous phase, forming a bilayer and bringing it to the final horizontal orientation.

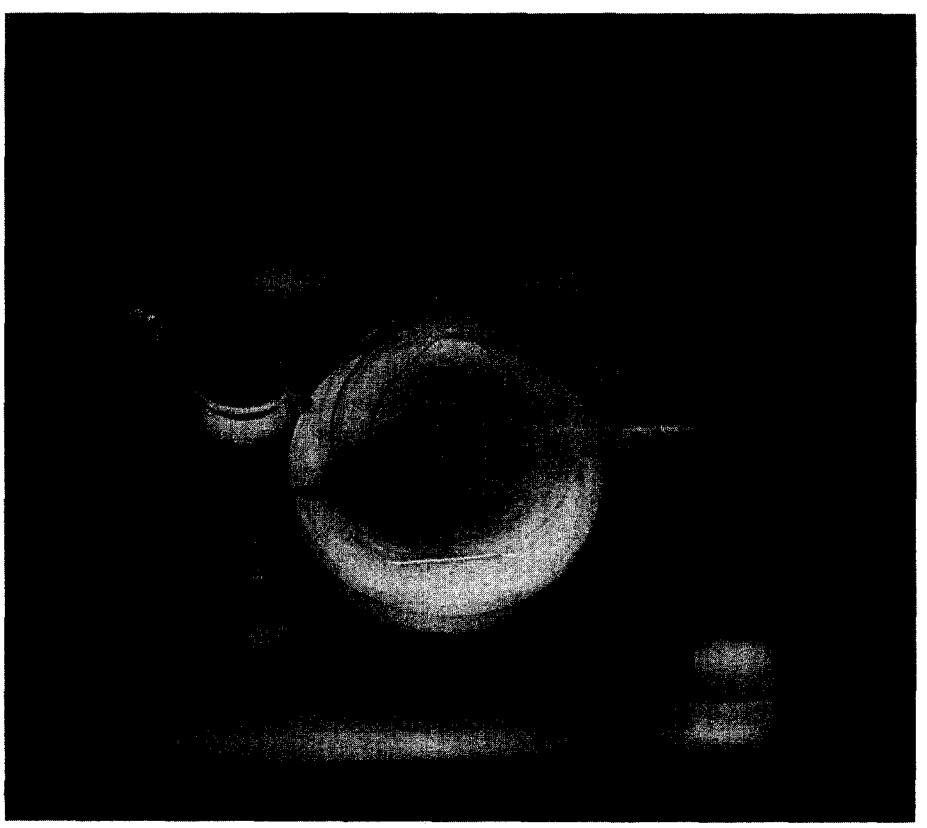

Fig. 2. Photograph of the working prototype with hemispheric upper chamber and suspended electrodes used in these experiments (see Materials and methods).

(2:1, w/w) in 'heavy buffer' essentially following a 'fast dilution technique' [3]. The final liposome suspension contained 0.5 μg/ml lipid, 2.14 μg/ml α-toxin and 0.56 mM octyl glucoside.

For electrical measurements a pair of Ag-AgCl-KCl-agar-agar-bridges were fabricated within standard pipette tips [15]. The voltage clamp circuit was constructed using an OPA 128LM (Burr Brown, Tucson, AZ) amplifier with output signal to a 132A Dual Beam Oscilloscope and 7004A *X–Y* recorder (Hewlett Packard, Santa Clara, CA).

Results and discussion

(1) Apparatus and method of formation of horizontal solvent-free bilayers. Fig. 1 shows the three basic stages of membrane formation: the bilayer aperture on a hemispheric upper chamber is positioned above the buffer/air interface (Fig. 1A), lipid monolayers are spread on buffer surfaces (from a 1% solution in pentane), the aperture is treated with hexadecane (0.5% in pentane; not shown) and the pentane allowed to evaporate (Fig. 1B), and the hemisphere is rotated to dip the aperture through the lipid monolayers and into the aqueous phase to form the bilayer and bring it to a final horizontal orientation (Fig. 1C). The rotational symmetry of the thin-walled upper chamber provides essentially equal changes in hydrostatic pressure on both sides of the bilayer during its formation, allowing the bilayer to form and remain stable in the aperture.

The working prototype used to explore the technique is shown in Fig. 2. The upper chamber was fabricated from the hemispheric bottom of a thin-walled (0.6 mm) polyethylene centrifuge tube (40 mm diameter; Beckman, Palo Alto, CA). A 5–6 mm diameter hole was made on the pole of the hemisphere, across which a thin (≈ 18 μm) Teflon film (Chemical Fabrics, Merrimack, NH) containing the membrane aperture is affixed by silicone grease (as in some vertical bilayer systems [2,16]). For better electrical isolation and smoother transition of the leading edge of the upper lipid monolayer onto the Teflon partition, the pole region of the hemisphere is thinned by sanding of its convex surface (to which the Teflon partition is attached). The hemisphere is attached along the equator to a flat, acrylic ring (≈ 10 mm wide) and a rigid rod that can be axially rotated by a rotation stage (RSP-1; Newport, Irvine, CA). An acrylic base, to which the rotation stage is fixed, includes a recess to hold the lower chamber (polystyrene 60 × 15 mm Petri dish), the holder for the 'lower' electrode, and a port for an optic fiber for illumination. The 'upper' electrode is attached to the upper chamber (Fig. 2) and remains in contact with the buffer while the chamber rotates during bilayer formation. If a larger electrode is used, it can be mounted on a separate holder, or can be in electrical contact with the upper buffer through a flexible salt-agar-agar bridge in plastic tubing.

Bilayers formed by this method are generally stable for hours and have the expected electrical characteristics (low leakage current, low noise and high specific capacitance). Membranes were easily formed by this technique across apertures up to 0.5 mm diameter (larger membranes were not attempted).

The working volumes and surface areas of the upper and lower chambers in the illustrated prototype are ≈ 10 ml and ≈ 0.4 ml, and ≈ 24 cm^2 and 4.4 cm^2, respectively (Fig. 2). Monolayers were formed from 5 μl and 10 μl of the 1% lipid solution added to the surfaces of upper and lower chambers, respectively. However, to conserve lipids and other reagents, the surface areas (and volumes) can be easily changed almost to any needed values by constructing chambers of different sizes, by changing the suspension height of the upper chamber relative to lower chamber, or by constructing a lower chamber that has a curvature matching that of the upper chamber (not shown).

Perhaps the best upper chambers would be those made entirely of thin-walled Teflon that smoothly thins down to the membrane aperture, or those having a double-walled construction in which the Teflon partition is mechanically clamped between two walls. However, these designs would be more complex to fabricate. We have explored another, more easily made variant of the upper chamber, one having hemicylinder contours made of thin-walled acrylic. This design has the additional advantage of formation of flat seals with the Teflon partition.

All varieties of this chamber permit formation of horizontal membranes by other means, such as from a lipid monolayer of bipolar lipids [17], or from monolayers spontaneously (re)organized from liposomal membranes as earlier described by Schindler [3] (which we have employed routinely).

Because the membrane aperture (and the bilayer itself) moves during membrane formation, it is convenient to observe the chamber from above with a stereomicroscope that can move in three dimensions. However, if only low magnification is used to observe the chamber, the microscope can remain stationary.

(2) Ion-channel reconstitution showing the advantages of the method. To explore the advantages of the apparatus and the method of formation of horizontal 'solvent-free' lipid bilayers, reconstitution studies using *S. aureus* α-toxin were carried out. Reconstitution of channels was carried out in two ways: by addition of α-toxin to the membrane bathing solution, and through fusion of α-toxin-containing liposomes with the horizontal bilayer. For each of these methods of reconstitution, the horizontal bilayer permits two modes by which samples can be added: by stirring into the bulk bathing solution (mode 1), and by layering in a dense solution onto the bilayer upper surface from above (mode 2).

Fig. 3 shows a current recording of α-toxin channels following addition by the stirring mode (mode 1). In 0.1 M NaCl, 10 mM Hepes (pH 7.3) the channel conductance is ≈ 60 pS at 80 mV applied potential (positive on toxin addition side). The channels have asymmetric conductance (about 1.3 times greater for one polarity of voltage than for

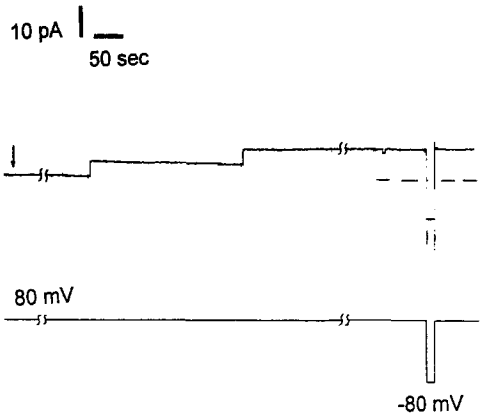

Fig. 3. Channels reconstituted by stirring of α-toxin into the bulk bilayer buffer. Upper trace is a current record of a horizontal bilayer containing two α-toxin channels. Lower trace is a diagram of applied potential (sign of potential indicated is for toxin addition side). Bilayer was formed on a ≈ 140 μm aperture from PC/PS (2:1, w/w) in symmetric 0.1 M NaCl, 10 mM Hepes (pH 7.3). The upper chamber solution (0.4 ml) was then brought to 4 mM octyl glucoside (a concentration that had no effect on membrane conductance but that facilitates α-toxin channel-forming activity). At the arrow, a total 0.153 μg α-toxin was stirred into the bulk phase of the upper buffer. The dashed line shows the current level of the unmodified membrane. Note the asymmetric currents with voltage polarity reversal characteristic of α-toxin channels.

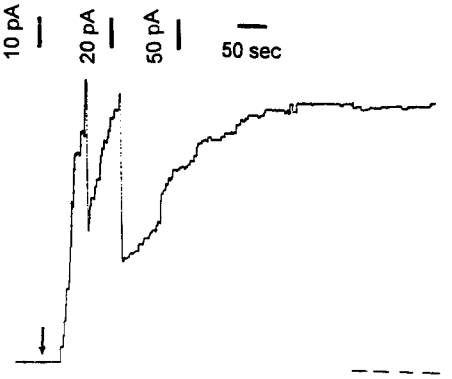

Fig. 4. The rapid kinetics of membrane current induced by α-toxin when layered from above onto the horizontal bilayer. At the arrow, 0.2 μl of 15.3 μg/ml α-toxin sample diluted in 'heavy' buffer (see Materials and methods) also containing 4 mM octyl glucoside was layered onto the upper surface of the membrane, without stirring of the bulk buffer. Membrane potential was 80 mV (positive on toxin addition side) and the dashed line shows the current level of unmodified membrane. Lipid and buffer composition as in Fig. 3.; the current scale changes 5-fold during the trace. Note that the total amount of α-toxin added was 53 times less than that in Fig. 3, but the number of channels is about 45 times greater. Therefore the efficiency of reconstitution in this experiment is enhanced by a factor of ≈ 2600 (see explanation in the text).

the other in these conditions), characteristic of α-toxin, as described by others (see, for example, Ref. [18]).

Our experiments showed that detergent was required for α-toxin channel formation. No channel activity was seen when α-toxin was added to the membrane bath without detergent. Under the latter conditions, when the concentration of α-toxin became as high as a few μg/ml, no channels were observed, but there was membrane breakdown.

To control for detergent artifacts in estimation of α-toxin channel forming efficiency we showed that as much as 4 mM octyl glucoside final concentration did not cause detectable changes in bare membrane and α-toxin channel conductance. This concentration was present in the upper buffer before α-toxin addition (Fig. 3). In the described conditions, addition of 0.2–0.4 μg/ml final concentration of α-toxin typically induced 1–3 channels within 15 min of addition.

Fig. 4 shows a current recording following local layering of α-toxin onto the upper surface of the horizontal membrane (mode 2). A submicroliter volume (0.2 μl) of α-toxin (15.3 μg/ml toxin) in 'heavy buffer' (see Materials and methods) containing 4 mM octyl glucoside was layered on the membrane (arrow). Shortly after addition, the bilayer conductance increased dramatically, and reached a steady state at ≈ 5 min.

In Fig. 4, at steady state the conductance of the membrane corresponds to ≈ 100 α-toxin channels (calculated from single channel data) arising from addition of only ≈ 0.003 μg α-toxin. A total of 0.153 μg α-toxin added in the stirring mode (in Fig. 3) gave rise to only two channels. The data shown in Figs. 3 and 4 were typical for each method of addition, for bilayers of the same area. Therefore, the horizontal orientation of the membrane allows creation of a locally high and transient concentration of sample at the membrane surface enabling a greater than 2600-fold increase in α-toxin channel forming activity. Such amplification of efficiency is likely to occur for other membrane-active substances as well. The real amplification of efficiency produced by layering actually is much greater since some of the sample is lost because the lumen of the micropipette used for layering was about three times larger than the bilayer aperture, and because of diffusive effects.

Therefore, the use of horizontal membranes is promising for evaluating channel forming activity and efficacy of preparations when availability of substance (e.g., channels, surfactants, etc.) is limited, as for in vitro translated protein. Our development of 'solvent-free' horizontal membranes has particular advantages in cases where membrane hydrocarbon solvent content or membrane thickness, as well as differing lipid compositions in the two monolayers are critical factors for reconstitution [19,20].

Because in the layering mode all of the sample reaches the membrane essentially simultaneously, channel formation begins and reaches steady state rapidly (Fig. 4). This mode of addition affords great control over number of channel insertions. By simply stirring the upper buffer after a channel is inserted, additional insertions can be prevented. Layering of sample by hand-held pipette can limit the reproducibility of delivery of sample to the membrane. However, the use of a pipette mounted on a micromanipulator would essentially eliminate this source of variation.

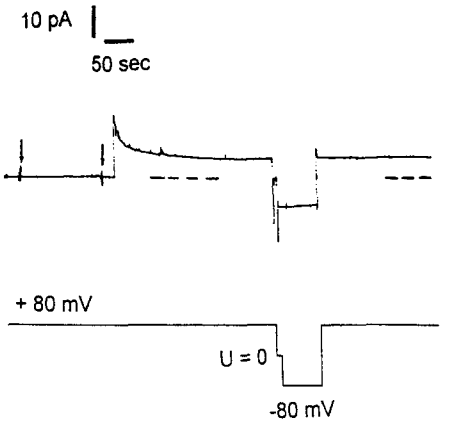

Fig. 5. Current record of horizontal bilayer showing reconstitution of an α-toxin channel by the method of liposome fusion (upper trace). The dashed line shows the current level of unmodified membrane, and lower trace is a diagram of applied potential. At the first arrow, 0.4 μl of liposomes reconstituted with α-toxin were layered on the upper surface of the bilayer. At the second arrow, 2 μl of 3 M NaCl was layered onto the same side of the bilayer to induce a trans-bilayer osmotic gradient to initiate liposome fusion [9,10]. A current jump corresponding to fusion of a liposome containing an α-toxin channel. The current relaxation is due to diffusion of the concentrated NaCl away from the bilayer, after which the reconstituted α-toxin channel remains in the membrane in essentially symmetric buffers. Note the asymmetric currents characteristic of α-toxin, after the relaxation (see also Fig. 3). Lipid and buffer composition as in Fig. 3 except that octyl glucoside was omitted.

An advantage of the use of horizontal bilayer for ion-channel reconstitution following liposome fusion is shown in Fig. 5. The solvent-free horizontal membrane was formed in symmetric solutions of 0.1 M NaCl, 10 mM Hepes (pH 7.3). At the first arrow, a 0.4 μl aliquot of reconstituted α-toxin liposomes formed in 'heavy' buffer was layered on the upper surface of the membrane. At the second arrow a 2 μl aliquot of 3 M NaCl was layered onto the membrane to initiate fusion. After a single fusion event (current jump) and approx. 3 min for depletion of the local high NaCl concentration (without stirring), the reconstituted α-toxin channel remains in the bilayer in essentially symmetric solutions. As before (Fig. 4), for reconstitution by liposome fusion the number of channels in the membrane can be well controlled by simply stirring the upper buffer after the needed number of channels are observed.

The 'heavy' buffer in which the liposomes were formed and added contained approx. 459 mM urea. In some of these experiments, it was noted that addition of the 3 M NaCl was not required for fusion (but it did enhance fusion probability). These cases may occur because of the osmotic gradient that develops across the liposome membrane upon the depletion of external urea from the local environment of the liposome to the bulk membrane buffer. Optimization of this method of inducing fusion (e.g., by appropriate choice of bilayer and liposome lipid compositions, osmoticant and channel, and sample delivery method) may eliminate the need for any additional osmotic manipulations [9,10].

We note three additional advantages (i–iii) and two minor disadvantages (iv–v) of this technique:

(i) Because the same area of the Teflon partition is submerged in buffer in every experiment, the contribution of the partition's capacitance to the total capacitance is highly reproducible. In vertical solvent-free bilayer systems, this contribution depends not only on the surface tension of the buffers and wettability of the chamber's working surfaces, but also on the volumes of buffer used in a given experiment. This feature is particularly advantageous for control of formation (monitored by capacitance changes) of small solvent-free membranes used for high-resolution recording, well as permitting more precise measurement of bilayer capacitance. (ii) The possibility of formation of salt bridges between the two bilayer chamber compartments is almost totally eliminated. Because of this, exhaustive drying of the chamber between experiments is not necessary. (iii) The problems of electrical noise, bubbles and carryover of chemicals between experiments caused by use of syringes to control volumes in vertical bilayer chambers are also eliminated. (iv) For the layering mode of addition, (mode 2), because the sample reaches the membrane at high concentration, artifacts caused by contaminants may be more obvious, and for both the layering and mixing modes of additions, contaminants that are more dense than the upper buffer may collect at the membrane. (v) Mechanical stirring of the upper buffer requires a different stirring configuration than can be used for the lower chamber.

The first 'solvent-free' planar bilayers were formed using an apparatus with a vertically movable septum containing the membrane aperture [2]. The septum was moved downward through a water/air interface covered by lipid monolayer(s), inducing the spontaneous formation of a vertical lipid bilayer membrane. However, this apparatus has not been widely used because of its complexity and problems of electrical isolation. Our apparatus is based on the same principle, but avoids these problems.

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