Video Article

Lipid Bilayer Experiments with Contact Bubble Bilayers for Patch-Clampers

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Abstract

Lipid bilayers provide a unique experimental platform for functional studies of ion channels, allowing the examination of channel-membrane interactions under various membrane lipid compositions. Among them, the droplet interface bilayer has gained popularity; however, the large membrane size hinders the recording of low electrical background noise. We have established a contact bubble bilayer (CBB) method that combines the benefits of planar lipid bilayer and patch-clamp methods, such as the ability to vary the lipid composition and to manipulate the bilayer mechanics, respectively. Using the setup for conventional patch-clamp experiments, CBB-based experiments can be readily performed. In brief, an electrolyte solution in a glass pipette is blown into an organic solvent phase (hexadecane), and the pipette pressure is maintained to obtain a stable bubble size. The bubble is spontaneously lined with a lipid monolayer (pure lipids or mixed lipids), which is provided from liposomes in the bubbles. Next, the two monolayer-lined bubbles (~50 μm in diameter) at the tip of the glass pipettes are docked for bilayer formation. Introduction of channel-reconstituted liposomes into the bubble leads to the incorporation of channels in the bilayer, allowing for single-channel current recording with a signal-to-noise ratio comparable to that of patch-clamp recordings. CBBs with an asymmetric lipid composition are readily formed. The CBB is renewed repeatedly by blowing out the previous bubbles and forming new ones. Various chemical and physical perturbations (*e.g.*, membrane perfusion and bilayer tension) can be imposed on the CBBs. Herein, we present the basic procedure for CBB formation.

Video Link

The video component of this article can be found at https://www.jove.com/video/58840/

Introduction

For ion channels, the cell membrane is not simply a supporting material but a partner for generating the ion flux. Functionally, the membrane is an electrical insulator in which ion channels are embedded, and all cell membranes are imparted with a resting membrane potential. In previous studies, an arbitrary membrane potential was imposed from an external circuit by which electrical current through the channels was measured. This quantitative evaluation of the ion flux at different membrane potentials revealed the molecular properties of these channels, such as their ion-selective permeation and gating functions[1,2]. The membrane platform for functional studies of ion channels is either the cell membrane or the lipid bilayer membrane. Historically, single-channel electrical current recordings were first performed in lipid bilayers[3,4], and the relevant techniques were developed for cell membranes, such as the patch-clamp method (**Figure 1A**)[5,6]. Since then, these two techniques have evolved separately for different purposes (**Figure 1**)[7,8].

Membrane lipids and bilayer membranes are currently the focus of research for their roles in supporting the structure and function of channel proteins. Therefore, the ready availability of methods to vary the lipid composition in bilayers is in high demand. Lipid bilayer formation methods such as the planar lipid bilayer (PLB)[8,9,10,11], water-in-oil droplet bilayer[12], and droplet interface bilayer (DIB)[13,14,15,16,17,18,19] techniques (**Figure 1**) are common choices, providing an opportunity for examining the channel function under varying lipid compositions[20]. Although the DIB is technically much easier to produce than the conventional PLB, the large size of the DIB has created a disincentive for patch-clampers to apply it for studying single-channel current recordings with usual-sized conductance (<100 pS).

To circumvent the background noise, the bilayer area must be minimized. This issue recalls the repetitions of history in developing electrophysiological techniques for lipid bilayers (**Figure 1**). In the early days, a small-sized bilayer (1-30 μm in diameter) was formed at the tip of a pipette (tip-dip method; **Figure 1C**)[21,22,23], rather than using a free-standing bilayer (~100 μm in diameter) on a hydrophobic septum in a chamber (**Figure 1B**). The tip-dip method allowed for electrical measurements with much lower background noise[24]. Our experiences with the PLB[25,26], tip-dip[22,23,27], and patch-clamp[28,29,30,31] methods led us to a novel idea of forming lipid bilayers by using the principles of the water-in-oil bilayer. We have referred to this as the contact bubble bilayer (CBB) method[20,32]. In this method, rather than hanging the water droplets in an oil phase (**Figure 1D**), a water bubble is blown from a glass pipette (with tip diameter of approximately 30 μm) into the oil phase (**Figure 1E and 2**), where the bubble is maintained by applying a steady pressure. A monolayer forms spontaneously at the water-oil interface at the surface of the bubble. Then, two bubbles are docked through the manipulation of two glass pipettes, and the bilayer is formed as the two monolayers approach each other, yielding an equilibrium bilayer area. The size of the bubble is controlled by the intra-bubble pressure (holding pressure), and likewise

the bilayer size. An average diameter of 50 μm is frequently used. Although the volume of the bubble is small (<100 pL), it is connected to the larger volume of the pipette solution that is in the microliter range, constituting the bulk electrolyte phase.

There are many benefits to use the CBB method (**Table 1**). As a lipid bilayer formation technique, membranes of various lipid compositions can be produced, and asymmetric membranes are more readily formed[32] than are those by the conventional folding method[33]. The bilayer can be mechanically manipulated, unlike the conventional PLB that can only be bent with a hydrostatic pressure difference[34,35]. By changing the holding pressure, the bubbles either expand or shrink, leading to increased or decreased membrane tension[32]. The bilayer is mechanically detachable into monolayers, similar to the freeze-fracture technique[36,37] of membranes in morphological studies, but with the CBB, a maneuver allows for repeated detach and attach cycles[32]. The small volume of the electrolyte solution within the bubble allows efficient fusion of channel-reconstituted liposomes into the bilayer, and the probability of getting channel recordings is much higher than with the conventional PLB technique. The small bubble volume also allows rapid perfusion (within ~20 ms) once another injection pipette is inserted into either of the bubbles. Unlike the patch-clamp method, a broken CBB-based membrane is re-formed immediately and repeatedly, and pipettes can be used several times a day. By integrating benefits of the patch-clamp and PLB methods, the CBB provides a versatile platform to vary the physicochemical conditions of the membrane, allowing for unprecedented studies of channel-membrane interactions.

Before presenting a detailed protocol of the CBB formation process, the physicochemical background of the bilayer formation is presented first,which will be helpful for patch-clampers to resolve experimental difficulties relating to the membrane formation that are encountered.

CBB experiments impart lessons of surface chemistry science[38]. The CBB is similar to a soap bubble blown from a straw into the air, where likewise, a water bubble is blown into an organic solvent. One will notice that a water bubble is hardly inflated when membrane lipids are not included in either the water bubble or the organic solvent. In the absence of amphipathic lipids, the surface tension at a water-oil interface is high, and the intra-bubble pressure for blowing a bubble will be high. This is a realization of the Laplace equation ($\Delta P = 2\gamma/R$, where ΔP is the intra-bubble pressure, γ is the surface tension, and R is the bubble radius). When the concentration of lipids in either the organic phase or the electrolyte solution is high, the density of lipids in the monolayer increases, as dictated by the Gibbs adsorption isotherm ($-d\gamma = \Gamma_i \, d\mu_i$, where Γ_i is the surface excess of compound i, and μ_i is the chemical potential of component i)[39], leading to a lower surface tension and ease of bubble formation. In the CBB, the bilayer can be observed from a tangential angle (**Figure 2**), and the contact angle between the monolayer and bilayer is measurable. This angle represents an equilibrium between the surface tensions of the monolayer and bilayer (Young equation: $\gamma_{bi} = \gamma_{mo} \cos(\theta)$, where γ_{bi} is the bilayer tension, γ_{mo} is the monolayer tension, and θ is the contact angle). The changes in the contact angle indicate changes in the bilayer tension, given that the monolayer tension is evaluated from changes in the contact angle as a function of the membrane potential (Young-Lippmann equation: $\gamma_{mo} = C_m V^2/4 \, (\cos(\theta_0) - \cos(\theta_v))$, where C_m is the membrane capacitance, V is the membrane potential, and θ_0 and θ_v are the contact angles at 0 and V mV, respectively)[40,41,42]. When two bubbles are close enough, they approach each other spontaneously. This is due to the van der Waals force, and we can visually observe this dynamic process in CBB formation.

A CBB system consists of distinct phases: namely, a bulk oil phase, water bubbles coated with a monolayer, and a contacting bilayer (**Figure 3**). These are reminiscent of the multiple phases observed in a PLB, such as a solvent-containing torus around the bilayer phase and a thin organic phase sandwiched by two monolayers[43,44]. In the CBB, the monolayer phase is continuous with the bilayer leaflet, and lipid molecules readily diffuse between the monolayer and the leaflet. The monolayer phase covers most of the bubble surface, constituting the major phase that serves as a lipid reservoir. Because the hydrophobic tail of lipids in the monolayer extends outward to the bulk oil phase, the bilayer interior or the hydrophobic core opens to the bulk oil phase. Thus, a hydrophobic substance injected into the oil phase close to the bilayer is able to readily access the bilayer interior. This is the membrane perfusion technique we had developed recently[45], by which the lipid composition in the bilayer is changed rapidly (within a second) during single-channel current recordings. We found that the cholesterol content in the bilayer could be reversibly controlled by switching the cholesterol perfusion on and off[45]. In the event that the concentration of the relevant substance in the monolayer and bilayer differs, the concentration gradient of the relevant substance is immediately dissolved through diffusion, which is known as the Marangoni effect[46,47]. On the other hand, flip-flops across the monolayers are slow[48,49,50].

Using the CBB method, the bilayer is formed under versatile physicochemical conditions, such as an electrolyte pH as low as 1 [51], a salt (K^+, Na^+, *etc.*) concentration up to 3 M, a membrane potential as high as ±400 mV, and a system temperature of up to 60 °C.

There are several options for formation of the CBB and incorporation of channel molecules therein. For formation of the monolayer at the water-oil interface, lipids are added either in an organic solvent (lipid-out method; **Figure 4A, 4C**) or in a bubble as liposomes (lipid-in method; **Figure 4B, 4D**). Notably, the lipid-in method allows for the formation of asymmetric membranes[15,32]. Channel molecules soluble in aqueous solution (*e.g.*, channel-forming peptides) are directly added into the bubble (**Figure 4A, B**)[52,53], whereas channel proteins are reconstituted into liposomes, which are then added into the bubble (**Figure 4C, D**). Herein, the formation of CBBs by the lipid-in method for either a channel peptide (polytheonamide B (pTB); **Figure 4A**) or a protein (KcsA potassium channel, **Figure 4C**) is shown.

Protocol

1. Prepare Liposomes

1. Disperse phospholipids (*e.g.*, 10 mg in powder) in chloroform at a desired concentration (*e.g.*, 10 mg/mL).
2. Evaporate chloroform.
    1. Place the phospholipid solution in a round-bottom flask and set it on a rotary evaporator (see **Table of Materials**) connected to a N_2 gas cylinder. Rotate the flask under N_2 flow at room temperature until a thin phospholipid film appears (after ~30 min).
    2. Place the open flask into a desiccator that is connected to a vacuum pump. Using the vacuum pump, aspirate the inside of the desiccator for several hours to remove the chloroform thoroughly.
3. Add an appropriate volume of an electrolyte solution to the flask and suspend the phospholipid to obtain a 2 mg/mL phospholipid suspension.
4. Sonicate the suspension for several tens of seconds using a bath sonicator (see **Table of Materials**) to obtain a multilayered vesicle (MLV) suspension.

5. For preparation of proteoliposomes that contain ion channel proteins, add a protein solution (with the proteins solubilized using appropriate detergents; 2% volume is the maximum) to the MLV suspension and sonicate for several seconds using the bath sonicator.

2. Prepare Large-Bore Glass Pipettes

1. Set a glass capillary to a pipette puller and fabricate micropipettes with a fine tapered tip through two-step pulling.
2. Set the micropipette on a microforge and contact the tip of the micropipette to a platinum filament at the tapered portion with a diameter of 30 to 50 μm.
3. Heat the filament briefly (5 s) and immediately turn it off.
   NOTE: This manipulation forms a crack at the heating point, whereby the tip of the micropipette is cut off, leaving a wide bore with a diameter of 30 to 50 μm.

3. Treat the Surface of Glass Slide with a Shallow Concave Well (Siliconization for a Water-Repellent Finish)

1. Clean the surface of the glass slide with a shallow well with distilled water and ethanol.
2. Apply an appropriate volume (*e.g.*, 100 μL) of a siliconizing reagent (water-repellent) onto the hole slide glass.
3. Dry the reagent completely in the air.
4. Place the glass slide on the stage of an inverted microscope.

4. Form the CBB and Perform Electrophysiological Measurement

1. Add 100 μL of hexadecane into the shallow well of the siliconized hole slide glass.
   NOTE: For the lipid-out method, phospholipids are dispersed in hexadecane (20 mg/mL) beforehand.
2. Fill the electrolyte solution up to half the length of the micropipette, using a tuberculin syringe.
3. Set the micropipette onto the micropipette holder with a pressure port, allowing the Ag/AgCl wire electrode to soak into the pipette electrolyte solution.
4. Connect one of the micropipette holders to the head stage of a patch-clamp amplifier and the other one to the electrical ground.
5. Connect a microinjector to the pressure port of the micropipette holder.
6. Set the micropipette to an appropriate position above the stage of an inverted microscope by manipulating the micromanipulator.
7. Adjust the electrode offset potential.
   1. Place 1 μL of the same electrolyte solution used to fill the micropipette on the flat surface around the shallow well of the hole slide glass, creating an electrolyte dome.
   2. Soak the tip of both micropipettes into the electrolyte dome by manipulating the micromanipulator.
   3. Adjust the electrode offset potential of the patch-clamp amplifier.
   4. Confirm the correct offset at the end of experiments by breaking the CBB via application of a high membrane potential (electrical breakdown; using Zap on the amplifier), causing the two bubbles to be fused into one (bubble fusion).
   5. Correct the liquid junction potential[54] in the cases where asymmetric electrolyte solutions are used, such that the calculated value is added to the applied membrane potential for the true membrane potential.
      NOTE: The liquid junction potential is calculated using the program JPCalc[55].
8. Draw the liposome solution from the tip.
   NOTE: When water-soluble channels are examined using the lipid-out method, suctioning of the liposome solution is not necessary.
   1. Place 1 μL of liposome solution on the flat surface around the shallow well of the hole slide glass (liposome-containing dome).
   2. Manipulate the micromanipulator and insert the tip of the micropipette into the liposome-containing dome.
   3. Aspirate the liposome-containing solution by lowering the pressure inside the micropipette holder using the microinjector.
   4. Repeat the procedure for the other pipette.
9. Manipulate the micromanipulator and dip the tip of the micropipette into the hexadecane in the shallow well.
10. Blow a water bubble slowly by increasing the pressure until the bubble reaches the desired size (*e.g.*, 50 μm in diameter) and maintain the same pressure thereafter.
11. Discard the bubbles by passing the tip through the oil-air interface if it is hard to keep the size of the bubbles stable.
12. Repeat steps 4.8 to 4.9 until stable bubbles are formed.
13. Manipulate the bubbles to allow contact between them (**Figure 5**).
    NOTE: Sometimes, the bubbles approach each other spontaneously to form the CBB. In other cases, the bubbles are close but do not contact each other. In this case, push the bubbles each other mechanically.
14. Fine-tune the pressure to maintain the bubble size, because the size may gradually change even at the constant intra-bubble pressure.
15. Set the membrane potential to the appropriate value using the patch-clamp amplifier and wait for the channel current to emerge (**Figure 6**).

5. Measure Bilayer Capacitance

1. Measure the bilayer electrical capacitance (C_{el}) by applying a ramp potential.
   NOTE: When the rate of the voltage change in the ramp command is 10 mV/10 ms (or 1 V/s) followed by -10 mV/10 ms, the amplitude of the current jump upon changes in the slope corresponds to the read of the membrane'scapacitance value (*e.g.*, 100 pA → 100 pF).
2. Evaluate the bilayer area of two bubbles stacked one on the other and focus the microscope at the bilayer level to view the edge of the bilayer (**Figure 6**).
   NOTE: The bilayer shape is mostly circular, and the area is calculated from the radius.

3. Calculate the specific membrane capacitance (C_{sp}) by dividing the electrical capacitance by the bilayer area ($C_{sp} = C_{el}/A$).
4. Calculate the bilayer thickness (thickness of the hydrophobic core) using $D_c = (\varepsilon_r\varepsilon_0)/C_{sp}$ (where ε_r and ε_0 represent the permittivity of the hydrophobic region of the bilayer and the permittivity of a vacuum, respectively).

Representative Results

A typical CBB had a diameter of 50 µm (**Figure 5, 6**) and the specific membrane capacitance in hexadecane was 0.65 µF/cm^2. The bubble size was arbitrarily controlled by the intra-bubble pressure. When small bubbles are necessary for low-noise recordings, the tip diameter should be correspondingly small. For example, for a bubble size of 50 µm in diameter, the tip diameter should be 30 µm.

Once the CBB had formed, the channel molecules either in aqueous solution or in the liposome were spontaneously inserted into the bilayer within a span of a few to dozens of minutes. Insertion of the channels was confirmed by the stepwise increase of current amplitude (**Figure 7**) under the applied membrane potential. The smaller membrane area (<1,000 µm^2) relative to that in the conventional PLB and DIB systems substantially improved the electrical signal-to-noise ratio.

Current recordings could be continued until the membrane was disrupted and the two bubbles merged. New bubbles were blown and the CBB formed immediately and repeatedly. The pipette could be used repeatedly within a day.

Duty cycle of attach and detach. A bilayer formed by the CBB method can be disintegrated into two monolayers. Detach-attach of the CBB can be repeated by manipulating the two bubbles (duty cycle of detach-attach). This process was monitored by the appearance of the channel current of pTB, a peptide channel from a marine sponge, as it was readily inserted into the lipid bilayer to form a monovalent cation-selective pore[52,56]. The pTB channel current emerged immediately after attaching the two monolayers, and the current became larger as the area of contact increased (**Figure 8**). The amplitude of the current was synchronized with the detach-attach manipulation of the CBB.

Single-channel measurements of the KcsA channel. The KcsA potassium channel is pH sensitive, being activated by acidic intracellular pH[57]. Thus, the electrolyte solution was set to be asymmetric[58,59,60]. In step 4.2 of CBB formation, the pipette solution of the left side was set at pH 4, whereas that of the right side was set at pH 7.5[25]. In step 4.7.1, a proteoliposome suspension, rather than liposome suspension, was placed onto the slide glass for aspiration into the left pipette. Accordingly, the KcsA channel was oriented in the membrane with its cytoplasmic domain facing the left bubble. Liposomes with the protein:lipid weight ratio of 1:2000 are suitable for single-channel current recording, and those with a 1:10 ratio are for macroscopic current recording.

Asymmetric membrane. An asymmetric lipid bilayer can be formed using different liposome suspensions for each bubble (lipid-in)[15,32]. The orientation of the KcsA channel in the membrane was regulated by setting an asymmetric solution pH (pH 4_{in}/pH 7_{out}), leading to its cytoplasmic side being toward the left side. Accordingly, the left side was assigned as "in," whereas the right side was assigned as "out." To examine lipid dependence on the gating of the KcsA channel, four types of CBB (including an asymmetric CBB) were used; namely, PG_{in}/PG_{out}, PG_{in}/PC_{out}, PC_{in}/PG_{out}, and PC_{in}/PC_{out} (PG: phosphatidylglycerol) (**Figure 9**). The KcsA channel exhibited high open probability (>90%) only in the PG_{in}/PG_{out} and PG_{in}/PC_{out} membranes. The KcsA channel requires the existence of anionic phospholipids in the inner leaflet of the membrane for a high open probability[26].

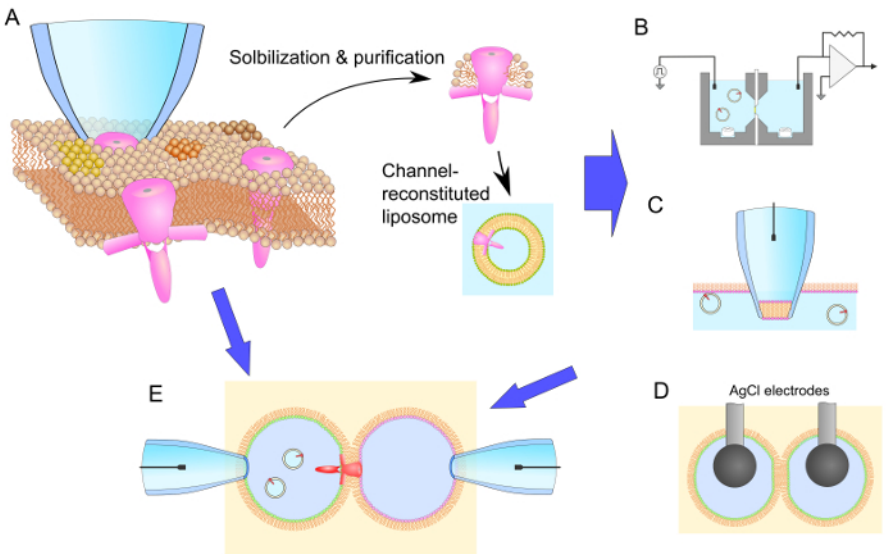

Figure 1: **Lipid bilayer and patch-clamp methods.** Various methods have been developed for forming the lipid bilayer. (A) Patch-clamp method. (B) Conventional planar lipid bilayer method, which provides a free-standing, mostly vertical, bilayer on a small hole. (C) Tip-dip method. A monolayer at the air-water interface is positioned on the tip of a glass electrode. Various modifications have been developed. (D) Droplet interface bilayer method. (E) Contact bubble bilayer method. Please click here to view a larger version of this figure.

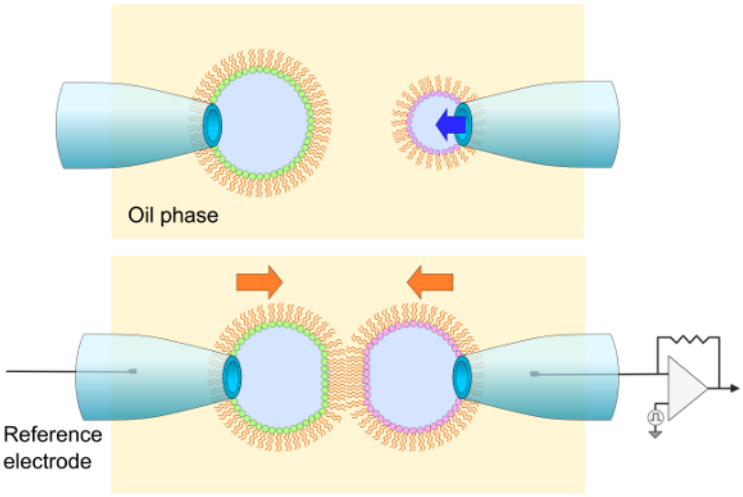

Figure 2: **Contact bubble bilayer formation.** As a bubble is blown into an oil phase, lipid molecules transfer spontaneously to the water-oil interface. Different types of lipids as liposomes are included in each bubble (lipid-in), and monolayers are formed exclusively by the relevant lipids. Docking two monolayers generates an asymmetric bilayer membrane. Please click here to view a larger version of this figure.

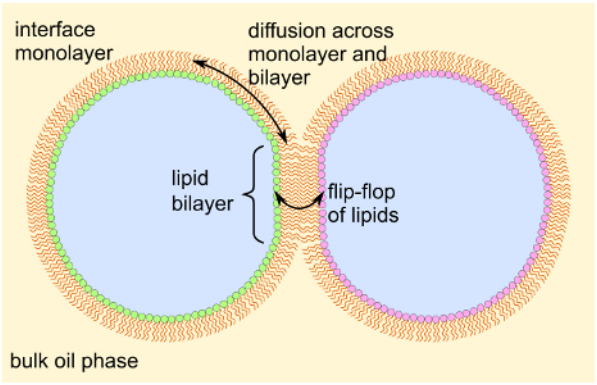

Figure 3: **Distinct phases in the contact bubble bilayer, and the dynamics of lipids therein**. Lipids lining the bubble constitute a phase where they belong to either a monolayer or a bilayer. Flip-flop of lipids across the bilayer is infrequent, and the asymmetric membrane is retained for a long time. Please click here to view a larger version of this figure.

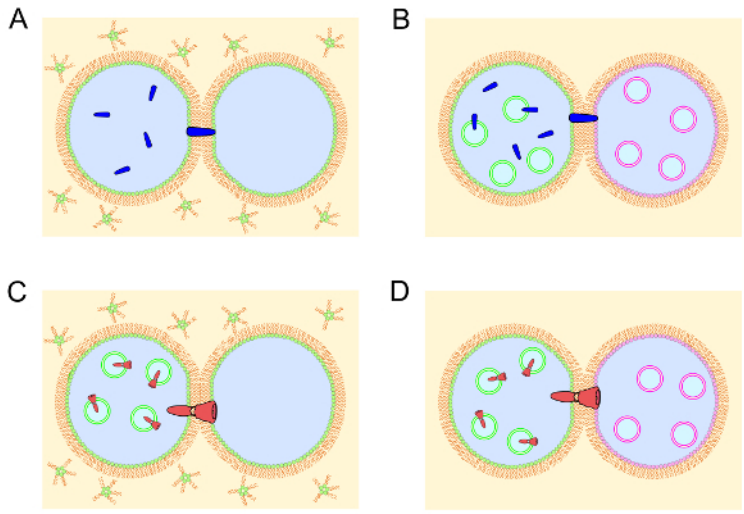

Figure 4: **Delivering lipids with the lipid-out or lipid-in method.** Lipids are added either in an organic solvent (lipid-out; A and C) or in aqueous solution as liposomes (lipid-in; B and D). In the lipid-in method, an asymmetric membrane is formed. Channel-forming substances that are soluble in aqueous solution (blue), such as peptides, are added into one of the bubbles and spontaneously inserted into the bilayer. In the lipid-in method, a part of the channels is inserted into liposomes, which are then fused to the bilayer. Channel proteins (red) are reconstituted into liposomes in either the lipid-out or lipid-in method, which are then added into one of the bubbles and spontaneously fused to the bilayer. Please click here to view a larger version of this figure.

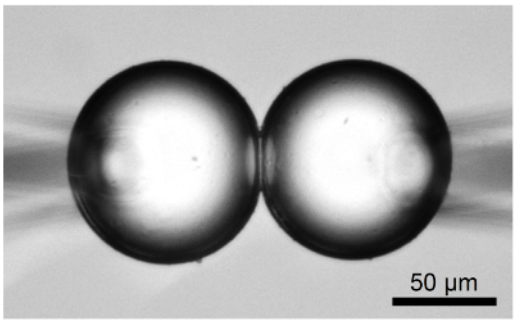

Figure 5: **Formation and microscopic image of the contact bubble bilayer.** The bilayer is observed from a tangential direction. Please click here to view a larger version of this figure.

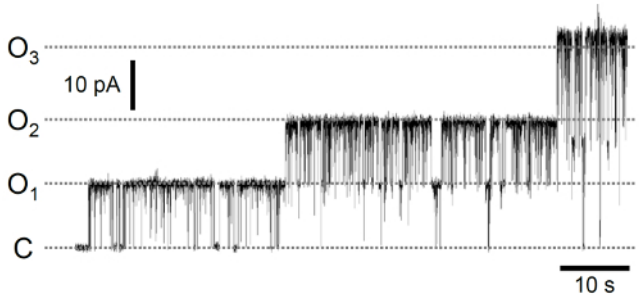

Figure 6: **Observation of the bilayer for measuring the bilayer area.** Two bubbles are stacked one on the other through pipette manipulation, and the microscope is focused at the level of the contact bubble bilayer. Please click here to view a larger version of this figure.

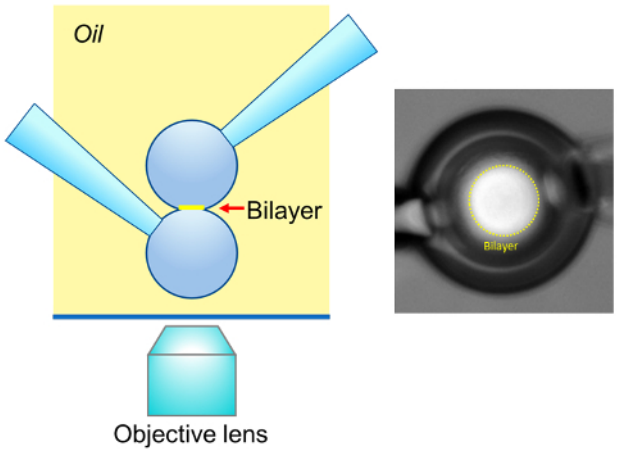

Figure 7: **Stepwise insertion of the KcsA channel into the contact bubble bilayer membrane.** The E71A mutant of the KcsA channel was used to show the immediate detection of the inserted channels. Please click here to view a larger version of this figure.

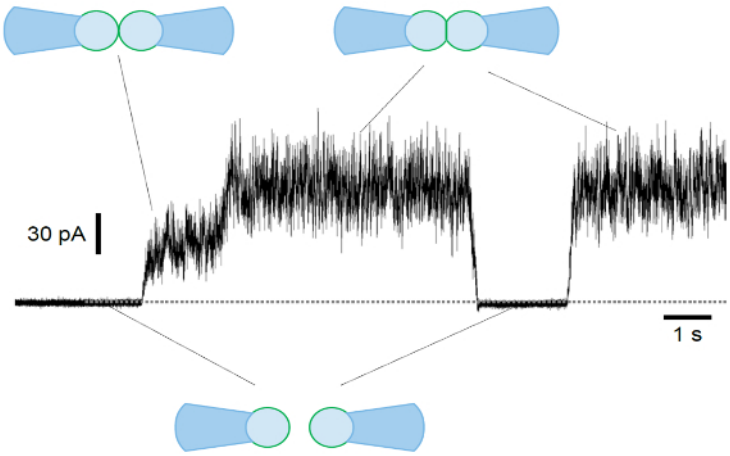

Figure 8: **Detach-attach of monolayers and responses of channels.** A peptide channel, polytheonamide B, was added into one of the bubbles, which was then spontaneously incorporated into the membrane. When detached, channels in the membrane withdrew but were retained at the membrane leaflet of the channel-added side. Upon attachment, the channels were inserted into the membrane. Please click here to view a larger version of this figure.

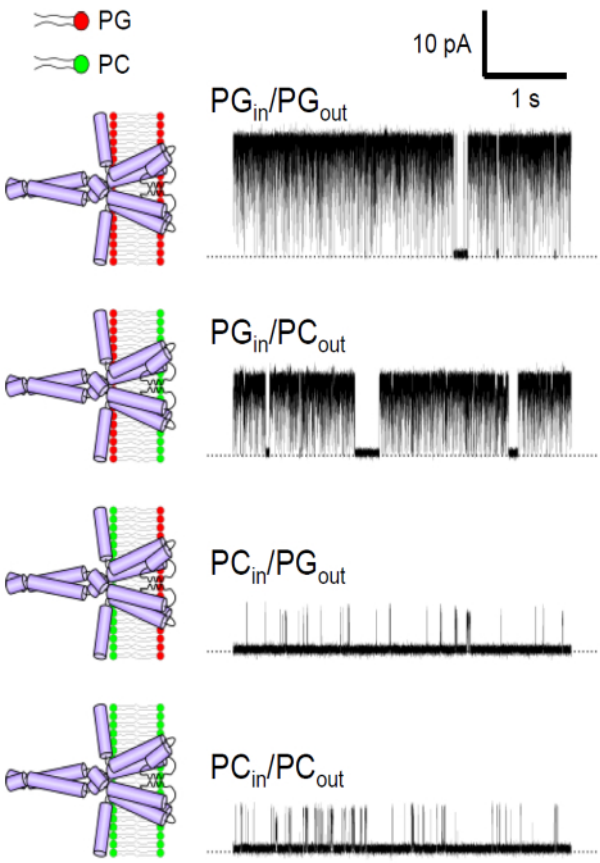

Figure 9: **Formation of an asymmetric membrane and channel activity dependent on the composition of the leaflet.** The KcsA channel is regulated by acidic lipids, such as PG, in the inner leaflet of the membrane. Please click here to view a larger version of this figure.

	Conventional PLB	Liposome patch	DIB	CBB
Success rate[1]	high	low	very high	very high
Immediate reformability[2]	yes	no	intermediate	yes
Asymmetric membrane	yes	no	yes	yes
Solution exchange	slow	very fast	slow	fast
S/N ratio[3]	low	high	low	high
Membrane deformation	no	no	no	yes[4]
Mechanical manipulation	no	yes[5]	yes	yes
Membrane perfusion	no	no	yes	yes
Lipid compositions	diverse	limited[6]	diverse	diverse
[1]The success rate of forming stable lipid bilayer per trial.				
[2]Formation of a new membrane quickly after break down of a membrane. Also, membrane is formed repeatedly.				
[3]The signal-to-noise (S/N) ratio represents the electrical background noise primary generated from the membrane capacitance.				
[4]Concave or convex membrane can be formed by applying different inner pressure between two bubbles.				
[5]Only increasing the membrane tension.				
[6]Phospholipids that can form giant unilamellar vesicles are applicable, but the giga-seal may not be attained.				

Table 1: Characteristics of various lipid bilayer membranes.

Discussion

The CBB method of lipid bilayer formation is based on the principle of a water-in-oil droplet lined by a monolayer[20]. Technically, the procedures for forming CBBs are easy, especially for patch-clamp researchers, who are proficient in manipulating glass micropipettes. The electrophysiological setup for the patch clamp is readily used in the CBB when two pipette manipulators with microinjectors are available. On the other hand, because the CBB is a successor of the conventional PLB, for which a large amount of physicochemical knowledge has been accumulated[8,61], this background as well as knowledge of surface chemistry[38] is helpful for operating and manipulating CBBs. The CBB serves a versatile platform for studying channel-membrane interplays. By using the CBB method, the bilayer can be modulated in terms of chemical composition and physical manipulation. In the CBB, lipids lining the bubble can diffuse freely across the border of the monolayer phase and leaflet of the bilayer, which enables various experimental procedures. Various physical and chemical modifications to the bilayer are now made possible, and we have developed other techniques such as the bilayer attach-detach and the membrane perfusion methods[45]. We have extended CBBs for *in vitro* channel protein synthesis, where channel synthesis was performed in the bubble, and newly synthesized channel proteins were subjected to spontaneous transfer to the bilayer under application of a membrane potential where nascent channel functions of the KcsA channel was traced from the time of initiating the transcription/translation of KcsA DNA[62].

Among the steps of the protocol, maintaining the bubble size is critical. The bubbles may slowly swell or shrink spontaneously because lipid transfer to the interface is slow and the monolayer tension is prone to change. In particular, the first bubble inflated just after suction of the liposome solution (steps 4.8.3 and 4.10) is hard to maintain because the monolayer tension is lowered by the liposomes accumulated at the tip of the pipette. Discarding the initial bubbles and subsequent formation of the bubbles provide the stable CBB. Visual tracing of the bubble size and fine tuning of the pressure are necessary.

There are experimental limitations to the CBB method. Although the CBB is designed for electrophysiological measurements, the electrical resistance of the bilayer for certain lipid compositions may not be high enough (*e.g.*, 100 GΩ) for single-channel recordings. For example, dioleoyl phosphatidylcholine is frequently used for liposomes, but it forms electrically leaky CBBs at 25 °C[63]. CBBs cannot even be formed with lipid species whose phase transition temperature is above the recording temperature. Indeed, CBB formation was difficult with dipalmitoyl phosphatidylcholine at room temperature, but raising the temperature above T_m circumvented the problem[64]. In these experiments, the temperature was controlled by using a transparent heat plate (see **Table of Materials**) beneath the slide glass[62]. The phase transition temperature of the frequently used lipid diphytanoyl phosphatidylcholine is below 0 °C [65], and CBBs are readily formed[32,45,51] at a broad temperature range.

Phospholipids of the water-oil interface are provided from either the lipid-in or lipid-out method, where the transfer rate is relatively slower in the lipid-out than in the lipid-in method[63]. In addition, for reasons unknown, the electrical resistance of the bilayer membrane is relatively smaller with the lipid-out method than with the lipid-in method.

In the protocol (step 4.8), a solution of liposomes and channel-reconstituted liposomes (1 µL) is loaded from the tip of the pipette (steps 4.9 and 4.10), and the rest of the pipette contains the previously filled electrolyte solution. This is merely for conserving materials, such as lipids and channel molecules. Consequently, the liposomes diffuse gradually toward the upper pipette solution, and after a while, the lipid concentration at the tip of the pipette becomes insufficient to form lipid monolayers. In this case, fresh liposome solution should be aspirated from the tip (step 4.8). This temporal concentration change can be circumvented when the whole pipette is filled with the solution containing lipids and channel molecules, whereas liposomes gradually settle down into the bubbles. In this case, bubbles are renewed.

Electrophysiologically, the CBB size is larger than that of the patch clamp, thus yielding a larger membrane capacitance. However, the series resistance (the electrical resistance in a series of membrane resistance) is much lower (~100 kΩ) than that for the patch clamp (pipette resistance > 1 MΩ), which accelerates the speed of the voltage clamp and attenuates the background noise and series resistance errors in the voltage-clamped membrane potentials[66,67,68]. The background noise is a function of the membrane capacitance and the series resistance, where a low series resistance complements a high membrane capacitance, resulting in low background noise[9].

As a rule of lipid bilayer experiments, detergents should not be used to wash glassware. Even a trace amount of detergent perturbs the integrity of the bilayer. Organic solvents such as chloroform/methanol and ethanol should be used for such cleaning purposes instead.

Overall, the CBB integrates the benefits of both the patch clamp (*e.g.*, the mechanical manipulation of the membrane) and the PLB (*e.g.*, the ability to modify the lipid composition of the membrane). Various types of channel-forming substances and channel proteins have been studied[52,69,70,71]. The development of this method is opportune since increasingly more researchers are focusing on channel-membrane interplay, and the CBB provides a versatile platform for various experiments. Further experimental developments in the CBB method are expected.

Disclosures

The authors have no conflict of interest to disclose.

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