17 Sellers Street
Cambridge, MA 02139
tel. +1 617 945 9051
www.JoVE.com

~~Standard Manuscript Template~~

Lipid Bilayer Experiments with Contact Bubble Bilayers for Patch-~~Clamper~~Clampers

AUTHORS: Masayuki Iwamoto and Shigetoshi Oiki
Department of Molecular Physiology and Biophysics
University of Fukui Faculty of Medical Sciences
Yoshida-gun, Fukui, Japan

Masayuki Iwamoto: iwamoto@u-fukui.ac.jp

CORRESPONDING AUTHOR: Shigetoshi Oiki; oiki-fki@umin.ac.jp

KEYWORDS: Lipid bilayer, patch- clamp, water-in-oil droplet, monolayer, ion channel, electrophysiology, surface chemistry.

SHORT ABSTRACT: ~~*(10 words minimum, 50 words maximum)50*~~
~~Contact~~Herein, we present a protocol for the formation of lipid bilayers using a contact bubble bilayer ~~is a water-in-oil droplet bilayer, which can be mechanically manipulated via glass pipettes. From a patch pipette (tip diameter = 30 μm), a~~method. A water bubble ~~was~~is blown from a patch pipette into an organic solvent, whereby a monolayer ~~was~~is formed at the water–oil interface~~, subjected~~. Two pipettes are manipulated to ~~be docked~~ dock the bubbles to form a bilayer.

LONG ABSTRACT: ~~*(150 words minimum, 300 words maximum) 250,*~~
Lipid ~~bilayer experiments~~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, ~~water-in-oil droplet bilayers, such as the~~ the droplet interface bilayer ~~(DIB), have~~has gained popularity; ~~but a~~however, the large membrane size hinders the ~~recordings~~recording of low electrical background noise ~~that the patch clampers anticipate.~~. We have established ~~the~~a contact bubble bilayer (CBB) method~~, which~~ that combines the benefits of planar lipid bilayer and patch-clamp methods, such as the ability to vary the lipid ~~compositions~~composition and to manipulate the bilayer ~~mechanical manipulability~~mechanics, respectively. ~~With an experimental~~ Using the setup for conventional patch-~~clamping~~clamp experiments, CBB-based experiments can be readily performed. ~~Electrolyte~~In brief, an electrolyte solution in a glass pipette ~~was~~is blown into an organic solvent phase (hexadecane), and ~~a bubble was~~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~~) through the transfer of lipids from either the electrolyte solution or the organic solvent.~~), which is provided from liposomes in the bubbles. Next, two monolayer-lined bubbles (~50 μm in diameter) at the tip of the glass pipettes ~~were docked for bilayer formation. Thereafter, pipette pressure was maintained~~ are docked for ~~a stable bubble size. By applying a high membrane potential (~1000 mV), a membrane was broken and the two bubbles were fused, whereby the electrode offset potential is readily adjusted. The CBB is renewed by blowing out a~~

書式変更: フォント : Times New Roman, 12 pt, 太字 (なし) , フォントの色 : 自動
書式変更: 左揃え, インデント : 最初の行 : 0 mm
書式変更: フォント : Calibri, 12 pt
書式変更: 左揃え
書式変更: 上 : 25.4 mm, 端からフッターまでの距離 : 10.7 mm, 段落番号 : 現在の位置から開始
書式変更: フォント : Calibri, 12 pt
書式変更: フォント : Calibri
書式変更: 左揃え, 改ページ時 1 行残して段落を区切らない
書式変更: フォント : Calibri
書式変更: フォント : Calibri
書式変更: 左揃え, 改ページ時 1 行残して段落を区切らない
書式変更: フォント : Calibri
書式変更: フォント : Calibri
書式変更: 左揃え
書式変更: 左揃え, 改ページ時 1 行残して段落を区切らない
書式変更: フォント : Calibri

~~fused bubble and forming new bubbles.~~bilayer formation. Introduction of channel-reconstituted liposomes ~~in a~~into the bubble ~~led~~leads to the incorporation of ~~the~~ channels in the bilayer, allowing for single-channel current ~~recordings~~recording with a signal-to-noise ratio comparable to ~~patch-clamp recordings.~~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 ~~can be applied~~ (e.g., membrane perfusion and bilayer tension) can be imposed on ~~CBB and here~~the CBBs. Herein, we present the basic procedure for CBB formation.

INTRODUCTION: ~~*(150 words minimum, 1500 words maximum)*~~

~~Ion channels carryout their function in the membrane, without which the channel function that is required for generating ion flux is never attained. Functionally, the membrane is an electric insulator in which ion channels are embedded, and all the cell membranes are imparted with the resting membrane potential. In experiments, an arbitrary membrane potential is imposed from an external circuit by which electric current through channels is measured. Measurements of ion flux at different membrane potentials constitute quantitative studies of channel molecular properties, such as ion permeation and gating (1, 2). The membrane platform is served by either the cell membrane or lipid bilayer membrane. Historically, single channel electric current recordings were first performed in lipid bilayers (3, 4), and the relevant techniques were developed for cell membrane, such as the patch-clamp (Fig. 1E)(5, 6). Since then these two techniques have been evolved separately for different purposes (Fig. 1)(7, 8).~~

~~Membrane lipids and bilayer membranes are currently in focus for their roles in supporting structure and function of channel proteins, and the readily availability of methods to vary the lipid compositions in bilayers is in demand. The lipid bilayer methods such as planar lipid bilayer (PLB)(8–11) and a water-in-oil droplet bilayer (12), including droplet interface bilayer (DIB)(13–19) (Fig. 1), are methods of choice, providing an opportunity for examining channel function under varying lipid compositions (20). Even though DIB is technically much easier than the conventional planar lipid bilayer, the large size of the bilayer has created a disincentive for patch-clampers to apply it for studying single channel current recordings having usual sized conductance (< 100 pS).~~

~~To circumvent the background noise, the bilayer area must be minimized. This issue recalls to the repetitions of history for developing electrophysiological techniques (Fig. 1). In early days, a small size bilayer (1 – 30 μm in diameter) was formed at the tip of a pipette (tip-dip method; Fig. 1C)(21–23), rather than using a free-standing bilayer (~100 μm in diameter) on a hydrophobic septum in a chamber (Fig. 1B). The tip-dip method allowed electric measurements with much lower background noise (24). Our experiences with the planar lipid bilayer (25, 26), tip-dip (22, 23, 27) and patch-clamp (28–31) methods led us to an idea of a novel method for forming a lipid bilayer using the principles of water-in-oil bilayer. This is referred to as the contact bubble bilayer (CBB) method (20, 32). Rather than hanging the water droplets in an oil phase (Fig. 1D), a water bubble was blown from a glass pipette (the tip diameter of approximately 30 μm) into the oil phase (Fig. 1F and 2), where the bubble was maintained by applying steady pressure. A monolayer was formed at the water-oil interface at the surface of the bubble. Two bubbles were~~

書式変更: フッター

書式変更: フォント：Times New Roman, 12 pt

~~docked through manipulation with two glass pipettes, and the bilayer was spontaneously formed as the two monolayers approached each other, yielding an equilibrium bilayer area. The size of the bubble is controlled by the intra-bubble pressure (holding pressure), and so is the bilayer size. The average size of 50 µm in diameter is frequently used. The volume of the bubble is small (< 100 pL), but it is connected to the pipette solution, whose volume is in µL range.~~

~~There are many benefits to usin the CBB (Table 1). As a lipid bilayer method, membranes of various lipid compositions can be formed and asymmetric membranes are more readily formed (32) than by the conventional folding method (33). The bilayer is able to be mechanically manipulated, which cannot be performed in the conventional planar lipid bilayer, except for bending the bilayer 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. The bilayer is mechanically detachable into monolayers, similar to the freeze-fracture technique (36, 37) of membranes in morphological studies, but this unprecedented maneuver allows for repeated detach and attach cycles (32). A small volume of the electrolyte solution within the bubble allows efficient fusion of channel-reconstituted liposomes to the bilayer, and the probability of getting channel recordings is much higher than with the conventional planar lipid bilayer 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, a broken membrane is reformed immediately and repeatedly, and pipettes can be repeatedly used several times a day.~~

~~Before presenting a detailed protocol for the formation of the CBB~~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 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)[8–11], water-in-oil droplet bilayer[12], and droplet interface bilayer (DIB)[13–19] techniques (Figure 1) are common choices, providing an opportunity for examining 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

書式変更: フッター

書式変更: フォント : Times New Roman, 12 pt

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)[21–23], 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[28–31] 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 ~~in CBB~~ is presented first, which ~~is~~will be helpful for patch-clampers to ~~solve issues when~~resolve experimental difficulties relating to membrane formation that are encountered.

The Sense of surface chemistry in the CBB: 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

書式変更: フッター

書式変更: フォント : Times New Roman, 12 pt

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)[40–42]. When two bubbles are close enough, they approach each other spontaneously. This is due to 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[48–50].

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,C) or in a bubble as liposomes (lipid-in method; Figure 4B,D). 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) ~~**The Sense of surface chemistry in the CBB:** CBB experiments impat lessons of surface~~

書式変更: フォント : Calibri

書式変更: フォント : Calibri

書式変更: フッター

書式変更: フォント : Times New Roman, 12 pt

chemistry science (38). CBB is similar to a soap bubble blown from a straw into the air. A water bubble is blown into an organic solvent, and one will notice that a water bubble is hardly formed when membrane lipids are not included in either the water bubble or the organic solvent. In the absence of amphipathic lipids, 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 ($\Delta P = 2\gamma/R$; ΔP: intra-bubble pressure, γ: surface tension, R: 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 becomes higher as dictated by the Gibbs absorption isotherm ($d\gamma = \Gamma_i\, d\mu_i$; Γ_i: the surface excess of compound i, μ_i; the chemical potential of component i) (39), leading to lower surface tension and ease of bubble formation. In CBB, bilayer is observed from a tangential angle, and the contact angle between monolayer and bilayer is measurable. This angle represents equilibrium of surface tension of monolayer and bilayer (Young equation; $\gamma_{bi} = \gamma_{mo} \cos(\theta)$; γ_{bi}: the bilayer tension, γ_{mo}: the monolayer tension, θ: the contact angle), and the changes in the contact angle indicate changes in surface tension (40–42). When two bubbles are close enough, they are approach each other spontaneously. This is due to van der Waals force, and we are visually observing this dynamic process in CBB formation.

CBB provides unique phases (Fig. 3), which is reminiscent of multiple phases observed in a planar lipid bilayer, such as a torus around the bilayer phase (43, 44). In CBB, a monolayer phase is continuous with a leaflet of the bilayer, and lipid molecules readily diffuse between the monolayer and the leaflet. A monolayer phase covers most of a bubble surface, constituting a major phase serving as a lipid reservoir. When a concentration gradient of a lipid constituents exists between a monolayer and a leaflet, the relevant molecule diffuses rapidly down the gradient, which is known as the Marangoni effect (45, 46). On the other hand, flip-flops across the monolayers are slow (47–49).

For CBB composed of certain lipid species, the electrical resistance of CBB is low and cannot be used for single-channel current recordings. Even the bilayer cannot be formed. This is mostly because of the physical feature of constituent lipids, whose phase transition temperature is above the recording temperature. For example, dioleoylphosphatidylcholine (DOPC) is frequently used for liposomes, but the electrical resistance is low at 25 °C (50). CBB formation was difficult with dipalmitoylphospatidylcholine (DPPC) at room temperature but was possible above the phase transition temperature (51). The frequently used lipid diphytanoylphospatidylcholine (DϕPC) has its phase transition temperature below 0 °C (52), and CBB is readily formed (32, 53, 54) at a broad temperature range.

As the hydrophobic tail of lipids extends outwardsare 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.

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 we recently developed (53), by which the lipid composition in the bilayer is changed rapidly, within a second, during single-channel

書式変更: フォント : Calibri

書式変更: フッター

書式変更: フォント : Times New Roman, 12 pt

~~current recordings. We found that cholesterol content in the bilayer can be reversibly controlled by switch on and off of the cholesterol perfusion.~~

PROTOCOL:

1 ~~For formation of the monolayer at the water-oil interface, lipids are added either in an organic solvent (lipid-out) or in a bubble as liposomes (lipid-in) (Fig. 4). In either case, lipids transfer to the water-oil interface, while the transfer rate is relatively slow in lipid-out that in the lipid-in method (50). In additon, the electrical resistance of the bilayer membrane is relatively smaller in lipid-out than that with the lipid-in method for reasons unknown. Notably, the lipid-in method yields asymmetric membranes (15, 32).~~Prepare liposomes

~~Channel molecules soluble in aqueous solution (e.g., channel-forming peptides) can be directly added in a bubble (55, 56), and channel proteins are reconstituted into liposomes, which are then added in a bubble.~~

~~1 Preparation of liposomes~~

~~1)~~1.1 ~~Phospholipids in chloroform:~~ Disperse phospholipids (e.g., 10 mg in powder) in chloroform at ~~an appropriate~~ a desired concentration (e.g., 10 mg/~~ml~~mL).

~~2)~~1.2 ~~Chloroform evaporation:~~ Evaporate chloroform

~~i)~~1.2.1 Place the phospholipid solution in a round-bottom flask and set it on a rotary evaporator (see table of materials) connected ~~with~~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).

~~ii)~~1.2.2 Place the open flask ~~in~~into a desiccator~~, which~~ that is connected to a vacuum pump. ~~Aspirate~~Using the vacuum pump, aspirate the inside of the desiccator ~~using the vacuum pump~~ for several hours to remove the chloroform thoroughly.

~~3)~~1.3 ~~Phospholipid suspension~~Suspend the phospholipid: Add an appropriate volume of an electrolyte solution to the flask and suspend the phospholipid to obtain a 2 mg/~~ml~~mL phospholipid suspension.

~~4)~~1.4 Sonicate the suspension for several tens of seconds using a bath sonicator (see table of materials) to obtain a multilayered vesicle (MLV ~~liposome~~) suspension.

~~5)~~1.5 For preparation of proteoliposomes that contain ion channel proteins, add ~~less than 2% volume of~~ a protein solution (with the proteins ~~are~~ solubilized using appropriate detergents; 2% volume is the maximum) to the MLV ~~liposome~~ suspension and sonicate for several seconds using the bath sonicator.

~~2.~~2 ~~Preparation of~~ Prepare large-bore glass pipettes

~~1)~~2.1 Set a glass capillary (see table of materials) to a pipette puller (see table of materials) and fabricate ~~two~~ micropipettes with a fine tapered tip through two-step pulling.

~~2)~~2.2 Set the micropipette on a microforge (see table of materials) and contact the tip of the micropipette to a platinum filament at the tapered portion with a diameter of 30 to 50 µm.

書式変更: フォント : Calibri

書式変更: フッター

書式変更: フォント : Times New Roman, 12 pt

~~3)~~2.3 Heat the filament ~~for a moment~~briefly (5 s) and immediately turn it off. This manipulation forms a crack at the heating point ~~and~~, whereby the tip of the micropipette is cut off, leaving a wide bore with a diameter of 30 to 50 µm.

~~3.~~3 ~~Surface treatment~~Treat the surface of hole slide glass (slide glass with a shallow concave well; see table of materials; siliconization for a water-repellent finish)

~~1)~~3.1 Clean the surface of ~~a~~the hole slide glass with distilled water and ~~EtOH~~ethanol.

~~2)~~3.2 Apply an appropriate volume (e.g., 100 µL) of a siliconizing reagent (water-repellent~~, e.g., Sigmacote) on to~~; see table of materials) onto the hole slide glass.

~~3.3 Use after~~Dry the reagent ~~dries~~ completely~~.~~

~~3.4 The hole slide glass is placed on the stage of the inverted microscope.~~

~~4 Formation of the CBB and electrophysiological measurement~~

~~3.3 Equipment: micropipette holders (x 2); The micropipette holder should be equipped with a pressure port. An Ag/AgCl wire electrode was used.~~ in the air.

3.4 Place the ~~siliconized~~ hole slide glass on the stage of an inverted microscope (see tables of materials).

4 Form the CBB and ~~add~~perform electrophysiological measurement

~~1)~~4.1 Add 100 µ~~l~~L of hexadecane into the ~~hole~~shallow well of the siliconized hole slide glass.

Note: For the lipid-out method, phospholipids are dispersed in hexadecane (20 mg/mL) beforehand.

~~2)~~4.2 Fill the electrolyte solution up to half the length of the ~~glass pipette~~micropipette, using a tuberculin syringe.

~~3)~~4.3 Set the micropipette ~~to~~onto the micropipette holder~~, which is mounted on~~ with a ~~micro manipulator, and~~ pressure port (see table of materials), allowing the Ag/AgCl wire electrode (see table of materials) to soak ~~the Ag/AgCl wire electrode~~ into the pipette electrolyte solution.

~~4)~~4.4 Connect one of the micropipette holders to the head stage of a patch-clamp amplifier ~~and another~~(see table of materials) and the other one to the electrical ground.

~~5)~~4.5 Connect a ~~micro injector~~microinjector (see table of materials) to the pressure port of the micropipette holder.

~~6)~~4.6 Set the micropipette to an appropriate position above the stage of an inverted microscope by manipulating the ~~micro manipulator.~~micromanipulator (see tables of materials).

4.7 ~~Suction~~Adjust the electrode offset potential.

4.7.1 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.

4.7.2 Soak the tip of both micropipettes into the electrolyte dome by manipulating the micromanipulator.

4.7.3 Adjust the electrode offset potential of the patch-clamp amplifier.

4.7.4 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).

書式変更: フッター

書式変更: フォント : Times New Roman, 12 pt

4.7.5 Correct the liquid junction potential[54] in the case 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].

~~7)~~4.8 Draw liposome solution from the tip

~~Place 1 µl droplet of~~Note: When water-soluble channels are examined using the lipid-out method, suctioning of the liposome solution is not necessary.

~~i)~~4.8.1 Place 1 µL of liposome solution on the flat surface around the ~~hole~~shallow well of the hole slide glass (liposome-containing dome).

~~ii)~~4.8.2 Manipulate the ~~micro manipulator~~micromanipulator and insert the tip of the micropipette into the ~~droplet~~liposome-containing dome.

~~iii)~~4.8.3 Aspirate the ~~droplet~~liposome-containing solution by lowering the pressure inside the micropipette holder using the ~~micro injector~~microinjector.

~~iv)~~4.8.4 Repeat the procedure for the other pipette.

~~8)~~4.9 Manipulate the ~~micro manipulator~~micromanipulator and dip the tip of the micropipette into the hexadecane ~~on~~in the ~~hole~~shallow well.

~~9)~~4.10 ~~A~~Blow a water bubble ~~was~~ slowly ~~blown~~ by increasing the pressure until the bubble ~~reached a~~reaches the desired size (e.g., 50 µm in diameter~~),~~) and maintain the same pressure ~~was maintained~~thereafter (maintaining pressure).

~~10)~~4.11 ~~If it is hard to keep the size of the bubbles stable, discard~~Discard the bubbles by passing the tip through the oil~~–~~–air interface if it is hard to keep the size of the bubbles stable.

~~11)~~4.12 Repeat steps 4.8 to 4.9 until stable bubbles are formed.

4.13 ~~Manipulators are used~~Manipulate the bubbles to allow contact between ~~bubbles.~~them (Figure 5).

~~12)~~Note: Sometimes, the bubbles approach each other spontaneously ~~approach~~ 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.

~~13)~~4.14 ~~The bubble size should be maintained by fine-tuning~~Fine-tune the pressure to maintain the bubble size, because the size may gradually change even at the constant intra-bubble pressure.

~~14)~~4.15 Set the membrane potential to the appropriate value using the patch-clamp amplifier and wait for the channel current to emerge~~.~~ (Figure 6).

~~In our study, a solution of liposomes and channel-reconstituted liposomes was drawn from the tip into the pipette electrolyte solution (step 9 and 10). This is merely for conserving materials such as lipids and channel molecules. Consequently, the sample and lipid concentration gradually decrease because of diffusion towards the upper pipette solution, and after a while, monolayers cannot be formed. In this case, fresh lipid solution should be aspirated from the tip (step 7). This temporal concentration change can be circumvented when the whole pipette is filled with the solution containing lipids and channel molecules, whereas liposomes gradually settled down into the bubbles.~~Measure~~In this case, bubbles are renewed. When the phase transition temperature (T_m) is high, CBB must be performed at above the T_m, and the temperature is controlled using a transparent heat plate beneath the slide glass (57)~~

書式変更: フォント : Calibri

書式変更: フッター

書式変更: フォント : Times New Roman, 12 pt

~~5 KcsA potassium channel specific protocol (58–60)~~

~~1) Asymmetric electrolyte solution: The channel is activated only when the cytoplasmic side is acidic (61). Accordingly, in step 2 in the CBB formation, 'functional' orientation of the KcsA channel in the membrane was controlled by setting the pH of the two pipette solutions asymmetric (i.e., pH4 and 7) (25).~~

~~2) In step 7 for the CBB formation, proteoliposome suspension rather than liposome suspension is placed on the glass vessel for aspiration into pipettes.~~

~~3) Concentration of KcsA: In the case of the KcsA potassium channel, use of liposomes with the protein/lipid weight ratio of 1/2000 is suitable for single-channel current recording, and that of 1/10, for macroscopic current recording.~~

~~6 Adjustment of the electrode offset potential~~

~~Setting the zero offset potential is prerequisite for the electrophysiological measurements.~~

~~1) Upon aspirating the liposome solution, two electrodes are soaked in a same liposome dome. Now, two AgCl electrodes are connected via a electrolyte solution of the dome, and the electrode offset potential can be adjusted.~~

~~2) At the end of experiments, CBB is broken by applying high membrane potential (electrical breakdown; using Zap in the Axopatch amplifier) and the two bubbles are fused into one (bubble fusion) to confirm the correct offset.~~

~~3) In case of asymmetric solutions, the liquid junction potential (62) should be corrected using the program JPCalc (63).~~

~~7.~~5 ~~Measurement of~~ bilayer capacitance

~~1)~~5.1 ~~The~~Measure the bilayer ~~electric~~electrical capacitance ~~is measured~~(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/~~sec),~~s) followed by –10 mV/10 ms, the ~~measured current~~ amplitude of the current jump upon changes in the slope corresponds to the ~~jump in current upon the application of ramp potential.~~ read of the membrane's capacitance value (e.g., 100 pA → 100 pF).

~~2)~~5.2 ~~To evaluate~~Evaluate the bilayer area~~,~~ of two bubbles ~~are~~ stacked ~~next to each~~one on the other, and focus the microscope ~~is focused~~ at the bilayer level~~.~~ ~~The~~ to view the edge of the bilayer ~~is visually discernible.~~ (Figure 6).

~~Specific membrane capacitance can be measured from the electric~~Note: The bilayer shape is mostly circular, and the area is calculated from the radius.

~~3)~~5.3 Calculate the specific membrane capacitance ~~and the bilayer area as~~ (C_{sp} ~~= Cel/A. The specific membrane~~) by dividing the electrical capacitance ~~in hexadecane is 0.65 μF/cm^2.~~ by the bilayer area ($C_{sp} = C_{el}/A$).

~~4)~~5.4 ~~Bilayer~~ Calculate the bilayer thickness (thickness of the hydrophobic core) ~~is evaluated as~~ 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~~.~~).

~~8 Asymmetric membrane~~

書式変更: フッター

書式変更: フォント : Times New Roman, 12 pt

~~An asymmetric membrane can be formed, when different types of lipids are added in each bubble, in the form of liposomes (lipid-in) (15, 64).~~

REPRESENTATIVE RESULTS:

A typical CBB ~~has~~had a diameter of 50 µm (~~Fig.~~Figure 5,6) and the specific membrane capacitance in hexadecane was 0.65 µF/cm^2. The bubble size ~~is~~was arbitrarily controlled by the intra-bubble pressure. ~~The size of the CBB is governed by the bubble size because the bilayer is spontaneously formed as two bubbles approach each other.~~ 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 ~~is~~had formed, the channel molecules either in aqueous solution or in the liposome ~~are~~were spontaneously inserted into the ~~CBB~~bilayer within a span of a few ~~minutes~~ to dozens of minutes. Insertion of the channels ~~can be~~was confirmed by ~~a~~the stepwise increase of current amplitude (~~Fig. 6~~Figure 7) under the applied membrane potential. The smaller membrane area (<~~-~~1000 µm^2) relative to that in the conventional PLB and DIB ~~system~~systems substantially ~~improves~~improved the electrical signal-to-noise ratio.

Current recordings ~~can~~could be continued until the membrane ~~is~~was disrupted and the two bubbles ~~are~~ merged. New bubbles ~~can be~~were blown and the CBB ~~is~~ formed immediately and repeatedly. The pipette ~~can~~could be used repeatedly ~~used, and the new bubbles readily form another CBB~~within a day.

~~**The duty cycle of attach and detach:** Similar to freeze fracture procedure for morphological studies usingf the electron microscope, 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). Such a process is also monitored by the appearance of the channel current of polytheonamide B (pTB), a peptide channel from a marine sponge, as it is readily inserted into the lipid bilayer to form a monovalent cation-selective pore (55, 65). The pTB channel current emerged immediately after attaching the two monolayers and current became larger as the area of contact increased (Fig. 7). The amplitude of the current was synchronized with the detach-attach manipulation of the CBB.~~

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:

書式変更: フッター

書式変更: フォント : Times New Roman, 12 pt

The KcsA potassium channel is pH sensitive, being activated by acidic intracellular pH[57]. Thus, the electrolyte solution was set to be asymmetric[58–60]. 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: ~~Asymmetric~~
An asymmetric lipid bilayer can be formed using different liposome ~~suspension~~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~~, i.e.,~~; namely, PG_{in}/PG_{out}, PG_{in}/PC_{out}, PC_{in}/PG_{out}, and PC_{in}/PC_{out} (~~Fig. 8). Here, the pH of the two bubbles was set asymmetrically as $pH4_{in}/pH7_{out}$.~~PG: phosphatidylglycerol) (Figure 9). The KcsA channel exhibited high open probability (>~~-~~90%) only in the PG_{in}/PG_{out} and PG_{in}/PC_{out} ~~membrane~~membranes. The KcsA channel requires the existence of anionic phospholipids in the inner leaflet of the membrane for a high open probability ~~(26).~~[26].

Tables and Figures:

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.

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.

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.

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

書式変更: フッター
書式変更: フォント：Times New Roman, 12 pt

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.

Figure 5: Formation and microscopic image of the contact bubble bilayer. The bilayer is observed from a tangential direction.

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.

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.

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.

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.

DISCUSSION: ~~*(3–6 paragraphs)*~~

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

書式変更: フッター

書式変更: フォント：Times New Roman, 12 pt

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, dioleoylphosphatidylcholine 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 dipalmitoylphosphatidylcholine 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 diphytanoylphosphatidylcholine 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 our 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.

~~The CBB is a method to form a lipid bilayer based on the principle of water-in-oil bilayer (20). Technically, procedures for forming CBB is easy, especially for patch-clampers. Electrophysiological setup for the patch-clamp is readily used in the CBB when two pipette manipulators with microinjectors are available. On the other hand, CBB is a successor of the conventional planar lipid bilayer, for which a large amount of physicochemical knowledge has been accumulated, and this background, as well as knowledge of surface chemistry, are a prerequisite for operating and manipulating CBB. The CBB serves a versatile and unprecedented~~

書式変更: インデント：最初の行： 1.77 字

書式変更: フォント：Calibri

書式変更: フッター

書式変更: フォント：Times New Roman, 12 pt

~~platform for studying channel-membrane interplays. By using the CBB method, bilayer can be modulated in terms of chemical composition and physical manipulation. In CBB, lipids lining a bubble are freely diffusible across the border of the monolayer phase and leaflet of the bilayer, which enables various experimental procedures. Various applications are now made possible and we have developed unprecedented techniques such as the attach-detach and the membrane perfusion. We extended CBB for *in vitro* channel protein synthesis (*in bulla* synthesis), in which channel synthesis was performed in the bubble, and newly synthesized channel proteins are subjected to spontaneous transfer to the bilayer where nascent channel functional of the KcsA channel was traced from the time of initiating the transcription/translation of KcsA DNA (57).~~

~~There are experimental limitations in the CBB method. CBB is designed for electrophysiological measurements, but the bilayer resistance should be as high as 100 GΩ. This high resistance membrane cannot be attained for lipid molecules having T_m above experimental temperature. Raising temperature above the T_m circumvents the problem. Electrophysiologically, the bilayer size is larger than that of the patch clamp, yielding larger membrane capacitance in the CBB method. However, the series resistance (the electric resistance in series of the membrane resistance) is much lower (~100 kΩ) than that for the patch-clamp (the pipette resistance > 1 MΩ), which accelerates the speed of voltage clamp and attenuates the background noise and the series resistance errors in the voltage-clamped membrane potentials (66–68). The background noise is a function of the membrane capacitance and the series resistance, and a low series resistance complements high membrane capacitance, resulting in low background noise (9).~~

~~Overall, CBB integrates the benefits of patch-clamp, such as the mechanical manipulability of the membrane, and those of planar lipid bilayer, such as the ability to modify lipid composition of the membrane. Various types of channel-forming substances and channel proteins have been studied (55, 56, 69, 70). Development of this method is opportune since more and more researchers are focusing on channel-membrane interplay, and the CBB provides a versatile platform for various experiments. Further experimental developments are expected in CBB.~~

~~**Trouble shooting**~~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[66–68]. 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].

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, 69–71]. 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.

書式変更: フッター

書式変更: フォント : Times New Roman, 12 pt

Troubleshooting: As a general rule of lipid bilayer experiments, detergents should not be used ~~in the washing of glass wears~~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.

ACKNOWLEDGEMENTS:

The authors would like to thank Mariko Yamatake and Masako Takashima for technical assistance. This work was supported in part by KAKENHI grant numbers 16H00759~~,~~ and 17H04017 (SO).

DISCLOSURES:

The authors have no conflict of interest to disclose.

REFERENCES

1. Hille, B ~~(2001).~~ *Ion channels of excitable membranes* ~~(~~. Sinauer Associates Inc~~,~~. Sunderland~~). 3rd Ed.~~. (2001).
2. Oiki, S ~~(2015).~~ Channel function reconstitution and re-animation: a single-channel strategy in the postcrystal age. ~~*J Physiol*~~*The Journal of Physiology*. **593**~~:~~, 2553–2573~~.~~, doi: 10.1113/JP270025 (2015).
3. Mueller, P~~, et al. (1962).~~, Rudin, D.O., Tien, H.T., Wescott, W.C. Reconstitution of cell membrane structure in vitro and its transformation into an excitable system. *Nature*. **194** (4832~~):~~), 979–980~~.~~, doi: 10.1038/194979a0 (1962).
4. Hladky ~~SB~~, S.B., Haydon ~~DA (1970)~~, D.A. Discreteness of conductance change in bimolecular lipid membranes in the presence of certain antibiotics. *Nature*. **225**~~:~~, 451–453~~.~~ (1970).
5. Neher, E~~.~~, Sakmann, B ~~(1976).~~ Single-channel currents recorded from membrane of denervated frog muscle fibres. *Nature*. **260**: 799-802~~.~~ (1976).
6. Hamill ~~OP~~, O.P., Marty, A~~.~~, Neher, E~~.~~, Sakmann, B~~.~~, Sigworth ~~FJ (1981)~~, F.J. Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. *Pflugers Arch*. **391** (2~~):~~), 85–100~~.~~ (1981).
7. Sakmann, B~~.~~, Neher, E ~~(2009).~~ *Single-Channel Recording* ~~(~~. Springer~~.~~ New York~~). 2nd Ed.~~. (2009).
8. ~~Miller C ed. (1986)~~ *Ion Channel Reconstitution* ~~(~~. Springer~~.~~ New York. (1986).
9. Wonderlin ~~WF~~, W.F., Finkel, A~~.~~, French ~~RJ (1990)~~, R.J. Optimizing planar lipid bilayer single-channel recordings for high resolution with rapid voltage steps. ~~*Biophys J* 58(2):289–97.~~*Biophysical journal*. **58** (2), 289–297, doi: 10.1016/S0006-3495(90)82376-6 (1990).
10. Oiki, S ~~(2012).~~ Planar Lipid Bilayer Method for Studying Channel Molecules. *Patch Clamp Techniques*~~, ed Okada Y (Springer-Verlag), pp.~~ 229–275~~.~~ (2012).

書式変更: フッター

書式変更: フォント : Times New Roman, 12 pt

11.- Kapoor, R., H. Kim, J., Ingolfson, H., Sparre Andersen, O (2008). Preparation of Artificial Bilayers for Electrophysiology Experiments. *J Vis Exp*Journal of Visualized Experiments. (20), doi: 10.3791/1033. (2008).

12.- Funakoshi, K., Suzuki, H., Takeuchi, S (2006). Lipid bilayer formation by contacting monolayers in a microfluidic device for membrane protein analysis. *Anal Chem*Analytical Chemistry. **78** (24), 8169–8174., doi: 10.1021/ac0613479 (2006).

13.- Bayley, H. *et al.* (2008) Droplet interface bilayers. *Mol Biosyst*Molecular BioSystems. **4** (12), 1191–1208., doi: 10.1039/b808893d (2008).

14.- Watanabe, R., Soga, N., Hara, M., Noji, H (2016). Arrayed water-in-oil droplet bilayers for membrane transport analysis. *Lab on a Chip*. **16** (16), 3043–3048., doi: 10.1039/c6lc00155f (2016).

15.- Hwang WL, W.L., Chen, M., Cronin, B., Holden MA, M.A., Bayley, H (2008). Asymmetric droplet interface bilayers. *J Am Chem Soc*Journal of the American Chemical Society. **130** (18), 5878–5879., doi: 10.1021/ja802089s (2008).

16.- Tonooka, T., Sato, K., Osaki, T., Kawano, R., Takeuchi, S (2014). Lipid bilayers on a picoliter microdroplet array for rapid fluorescence detection of membrane transport. *Small (Weinheim an der Bergstrasse, Germany)*. **10** (16), 3275–82., doi: 10.1002/smll.201303332 (2014).

17.- Dixit SS, S.S., Kim, H., Vasilyev, A., Eid, A., Faris GW (2010), G.W. Light-driven formation and rupture of droplet bilayers. *Langmuir*. **26** (9), 6193–6200., doi: 10.1021/la1010067 (2010).

18.- Malmstadt, N., Nash, M. a, Purnell RF, R.F., Schmidt JJ (2006), J.J. Automated formation of lipid-bilayer membranes in a microfluidic device. *Nano Lett*letters. **6** (9), 1961–5.1965, doi: 10.1021/nl0611034 (2006).

19.- Najem JS, J.S. *et al.* (2015) Multifunctional, Micropipette-based Method for Incorporation And Stimulation of Bacterial Mechanosensitive Ion Channels in Droplet Interface Bilayers. *J Vis Exp* (105):1–10.*Journal of Visualized Experiments*. (105), doi: 10.3791/53362 (2015).

20.- Oiki, S., Iwamoto, M (2018). Channel-Membrane Interplay in Lipid Bilayer Membranes Manipulated through Monolayer Technologies. *Biol Pharm Bull*Biological & Pharmaceutical Bulletin. **41**:, 303–311. (2018).

21.- Andersen OS (1983), O.S. Ion movement through gramicidin A channels. Single-channel measurements at very high potentials. *Biophys J* 41(2):119–33.*Biophysical journal*. **41** (2), 119–133, doi: 10.1016/S0006-3495(83)84414-2 (1983).

22.- Oiki, S., Danho, W., Madison, V., Montal, M (1988) M2delta. M2 delta, a candidate for the structure lining the ionic channel of the nicotinic cholinergic receptor. *Proc Natl Acad Sci U S A* 85(22):8703–7.*Proceedings of the National Academy of Sciences of the United States of America*. **85** (22), 8703–8707 (1988).

23.- Oiki, S., Koeppe RE, R.E., Andersen OS (1995), O.S. Voltage-dependent gating of an asymmetric gramicidin channel. *Proc Natl Acad Sci U S A*Proceedings of the National Academy of Sciences of the United States of America. **92**(March): (6), 2121–2125., doi: 10.1073/pnas.92.6.2121 (1995).

書式変更: フッター

書式変更: フォント : Times New Roman, 12 pt

24.- Sigworth FJ, F.J., Urry DW, D.W., Prasad KU (1987), K.U. Open channel noise. III. High-resolution recordings show rapid current fluctuations in gramicidin A and four chemical analogues. *Biophys J* 52(6):1055–64.*Biophysical journal*. **52** (6), 1055–1064, doi: S0006-3495(87)83299-X [pii] 10.1016/S0006-3495(87)83299-X (1987).
25.- Iwamoto, M, et al. (2006)., Shimizu, H., Inoue, F., Konno, T., Sasaki, Y.C., Oiki, S. Surface structure and its dynamic rearrangements of the KcsA potassium channel upon gating and tetrabutylammonium blocking. *J Biol Chem*The Journal of Biological Chemistry. **281** (38):), 28379–28386., doi: M602018200 [pii] 10.1074/jbc.M602018200 (2006).
26.- Iwamoto, M,., Oiki, S (2013). Amphipathic antenna of an inward rectifier K+ channel responds to changes in the inner membrane leaflet. *Proc Natl Acad Sci U S A* 110(2):749–54.*Proceedings of the National Academy of Sciences of the United States of America*. **110** (2), 749–754, doi: 10.1073/pnas.1217323110 (2013).
27.- Oiki, S,., Koeppe RE, R.E., Andersen OS (1994), O.S. Asymmetric gramicidin channels: heterodimeric channels with a single F6Val1 residue. *Biophys J*Biophysical Journal. **66** (6):), 1823–32.1832 (1994).
28.- Ando, H,., Kuno, M,., Shimizu, H,., Muramatsu, I,., Oiki, S (2005). Coupled K+-water flux through the HERG potassium channel measured by an osmotic pulse method. *J Gen Physiol*The Journal of general physiology. **126** (5):), 529–38.538, doi: 10.1085/jgp.200509377 (2005).
29.- Kuno, M,. *et al.* (2009) Temperature dependence of proton permeation through a voltage-gated proton channel. *J Gen Physiol*The Journal of general physiology. **134** (3):), 191–205., doi: 10.1085/jgp.200910213 (2009).
30.- Iwamoto, M,., Oiki, S (2011). Counting Ion and Water Molecules in a Streaming File through the Open-Filter Structure of the K Channel. *J Neurosci* 31(34):12180–8.*The Journal of neuroscience*. **31** (34), 12180–12188, doi: 10.1523/JNEUROSCI.1377-11.2011 (2011).
31.- Chang HK, H.K., Iwamoto, M,., Oiki, S,., Shieh RC (2015), R.C. Mechanism for attenuated outward conductance induced by mutations in the cytoplasmic pore of Kir2.1 channels. *Sci Rep*Scientific Reports. **5**., doi: 10.1038/srep18404. (2015).
32.- Iwamoto, M,., Oiki, S (2015). Contact Bubble Bilayers with Flush Drainage. *Sci Rep*Scientific Reports. **5**:, 9110., doi: 10.1038/srep09110 (2015).
33.- Montal, M,., Mueller, P (1972). Formation of bimolecular membranes from lipid monolayers and a study of their electrical properties. *Proc Natl Acad Sci U S A* 69(12):3561–6.*Proceedings of the National Academy of Sciences of the United States of America*. **69** (12), 3561–3566, (1972).
34.- Petelska AD (2012), A.D. Interfacial tension of bilayer lipid membranes. *Cent Eur J Chem*Central European Journal of Chemistry. **10** (1):), 16–26., doi: 10.2478/s11532-011-0130-7 (2012).
35.- Benz, R,., Conti, F (1986). Effects of hydrostatic pressure on lipid bilayer membranes. I. Influence on membrane thickness and activation volumes of lipophilic ion transport. *Biophys J* 50(1):91–98.*Biophysical Journal*. **50** (1), 91–98, doi: 10.1016/S0006-3495(86)83442-7 (1986).

書式変更: フッター

書式変更: フォント : Times New Roman, 12 pt

36. Meryman HT, H.T., Kafig, E (1955). *The study of frozen specimens, ice crystals and ice crystal growth by electron microscopy*. Naval Medical Research Institute, National Naval Medical Center. (1955).
37. Steere RL (1957), R.L. Electron microscopy of structural detail in frozen biological specimens. *J Biophys Biochem Cytol The Journal of biophysical and biochemical cytology*. **3** (1):), 45–60., doi: 10.1083/jcb.3.1.45 (1957).
38. de Gennes, P.-G., Brochard-Wyart, F., Quere, D (2003). *Capillarity and Wetting Phenomena: Drops, Bubbles, Pearls, Waves*. Springer. (2003).
39. Butt, H.-J., Kappl, M (2018). *Surface and Interfacial Forces*. Wiley-VCH. Weinheim). 2nd Ed.. (2018).
40. Requena, J., Haydon DA (1975), D.A. The lippmann equation and the characterization of black lipid films. *J Journal of Colloid and Interface Sci Science*. **51** (2):), 315–327., doi: 10.1016/0021-9797(75)90119-8 (1975).
41. Taylor GJ, G.J. *et al.* (2015) Direct in situ measurement of specific capacitance, monolayer tension, and bilayer tension in a droplet interface bilayer. *Soft Matter*. **11** (38):), 7592–7605., doi: 10.1039/C5SM01005E (2015).
42. Dixit SS, S.S., Pincus, A., Guo, B., Faris GW (2012), G.W. Droplet shape analysis and permeability studies in droplet lipid bilayers. *Langmuir*. **28** (19):), 7442–51.7451, doi: 10.1021/la3005739 (2012).
43. White SH (1972), S.H. Analysis of the torus surrounding planar lipid bilayer membranes. *Biophys. J.* **12** (4), 432–445 (1972).
44. White SH (1986), S.H. The physical nature of planar bilayer membranes. *Ion Channel Reconstitution* (Plenum, New York1986).
45. Iwamoto, M., Oiki, S. Membrane Perfusion of Hydrophobic Substances Around Channels Embedded in the Contact Bubble Bilayer. 45. Marangoni C (1865) *Sull'espansione delle goccie d'un liquido galleggianti sulla superfice di altro liquido* (Pavia tipografia dei Fratelli Fusi). *Scientific Reports*. **7** (1), 6857, doi: 10.1038/s41598-017-07048-4 (2017).
46. Velarde MG, M.G., Zeytounian RK, R.K., others (2002) *Interfacial phenomena and the Marangoni effect*. Springer. (2002).
47. Ryazantsev, Y.S., Velarde, M.G., Rubio, R.G., Guzmán, E., Ortega, F., López, P. Thermo- and soluto-capillarity: Passive and active drops. *Adv Colloid Interface Sci*. **247**, 52–80 (2017).
48. Kornberg RD, R.D., Mcconnell HM (1971), H.M. Inside-Outside Transitions of Phospholipids in Vesicle Membranes. *Biochemistry*. **10** (7):), 1111–1120., doi: 10.1021/bi00783a003 (1971).
48. 49. Wimley WC, W.C., Thompson TE (1990), T.E. Exchange and Flip-Flop of Dimyristoylphosphatidylcholine in Liquid-Crystalline, Gel, and Two-Component, Two-Phase Large Unilamellar Vesicles. *Biochemistry*. **29** (5):), 1296–1303., doi: 10.1021/bi00457a027 (1990).
49. 50. Nakao, H., *et al.* (2015) pH-dependent promotion of phospholipid flip-flop by the KcsA potassium channel. *Biochim Biophys Biochimica et Biophysica Acta – Biomembr (BBA) - Biomembranes*. **1848** (1):), 145–150., doi: 10.1016/j.bbamem.2014.10.001 (2015).

書式変更: フッター

書式変更: フォント : Times New Roman, 12 pt

50. Venkatesan GA, et al. (2016) Adsorption kinetics dictate monolayer self-assembly for both lipid-in and lipid-out approaches to droplet interface bilayer formation. *Langmuir* 31(47):12883–12893.
51. Silvius JR (1982) Thermotropic phase transitions of pure lipids in model membranes and their modifications by membrane proteins. *Lipid-protein Interact* 2:239–281.
52. Lindsey H, Petersen NO, Chan SI (1979) Physicochemical characterization of 1,2-diphytanoyl-sn-glycero-3-phosphocholine in model membrane systems. *Biochim Biophys Acta - Biomembr* 555(1):147–167.
53. Iwamoto M, Oiki S (2017) Membrane Perfusion of Hydrophobic Substances Around Channels Embedded in the Contact Bubble Bilayer. *Sci Rep* 7(1):6857.
54. Matsuki Y, et al. (2016)51. Matsuki, Y., Iwamoto, M., Mita, K., Shigemi, K., Matsunaga, S., Oiki, S. Rectified Proton Grotthuss Conduction Across a Long Water-Wire in the Test Nanotube of the Polytheonamide B Channel. *J Am Chem SocJournal of the American Chemical Society*. **138** (12):), 4168–4177., doi: 10.1021/jacs.5b13377 (2016).
55. 52. Iwamoto, M., Shimizu, H., Muramatsu, I., Oiki, S (2010). A cytotoxic peptide from a marine sponge exhibits ion channel activity through vectorial-insertion into the membrane. *FEBS Lettletters*. **584** (18):), 3995–3999., doi: S0014-5793(10)00644-7 [pii] 10.1016/j.febslet.2010.08.007 (2010).
56. 53. Iwamoto, M, et al. (2017)., Sumino, A., Shimada, E., Kinoshita, M., Matsumori, N., Oiki, S. Channel Formation and Membrane Deformation via Sterol-Aided Polymorphism of Amphidinol 3. *Sci RepScientific Reports*. **7** (1):), 10782., doi: 10.1038/s41598-017-11135-x (2017).
57. Iwamoto M, Elfaramawy MA, Yamatake M, Matsuura T, Oiki S (2018) Concurrent in Vitro Synthesis and Functional Detection of Nascent Activity of the KcsA Channel under a Membrane Potential. *ACS Synth Biol* 7(4):1004–1011.
58. Cortes DM, Perozo E (1997) Structural dynamics of the Streptomyces lividans K+ channel (SKC1): oligomeric stoichiometry and stability. *Biochemistry* 36(33):10343–10352.
59. MacKinnon R, Cohen SL, Kuo A, Lee A, Chait BT (1998) Structural Conservation in Prokaryotic and Eukaryotic Potassium Channels. *Science (80-)* 280(5360):106–109.
60. LeMasurier M, Heginbotham L, Miller C (2001) KcsA: it's a potassium channel. *J Gen Physiol* 118(3):303–14.
61. Heginbotham L, LeMasurier M, Kolmakova-Partensky L, Miller C (1999) Single streptomyces lividans K(+) channels: functional asymmetries and sidedness of proton activation. *J Gen Physiol* 114(4):551–60.
62. 54. Barry PH, P.H., Lynch JW (1991), J.W. Liquid junction potentials and small cell effects in patch-clamp analysis. *J Membr BiolThe Journal of membrane biology*. **121** (2):), 101–17.117 (1991).
63. 55. Barry PH (1994), P.H. JPCalc, a software package for calculating liquid junction potential corrections in patch-clamp, intracellular, epithelial and bilayer measurements and for correcting junction potential measurements. *J NeurosciJournal of Neuroscience Methods*. **51** (1):), 107–116., doi: 10.1016/0165-0270(94)90031-0 (1994).
64. Iwamoto M, 56. Oiki, S (2015) Contact Bubble Bilayers with Flush Drainage. *Sci Rep* 5:9110.

書式変更: フッター

書式変更: フォント : Times New Roman, 12 pt

65. Oiki S, Muramatsu I, Matsunaga S, Fusetani N (1997). A channel-forming peptide toxin: polytheonamide from marine sponge (Theonella swinhoei). *Nihon Yakurigaku Zasshi*. **110** (Suppl. 1): 195P–198P. (1997).
57. Heginbotham, L., LeMasurier, M., Kolmakova-Partensky, L., Miller, C. Single streptomyces lividans K(+) channels: functional asymmetries and sidedness of proton activation. *The Journal of General Physiology*. **114** (4), 551–560 (1999).
58. Cortes, D.M., Perozo, E. Structural dynamics of the Streptomyces lividans K+ channel (SKC1): oligomeric stoichiometry and stability. *Biochemistry*. **36** (33), 10343–10352, doi: 10.1021/bi971018ybi971018y [pii] (1997).
59. MacKinnon, R., Cohen, S.L., Kuo, A., Lee, A., Chait, B.T. Structural Conservation in Prokaryotic and Eukaryotic Potassium Channels. *Science*. **280** (5360), 106–109, doi: 10.1126/science.280.5360.106 (1998).
60. LeMasurier, M., Heginbotham, L., Miller, C. KcsA: it's a potassium channel. *J Gen Physiol*. **118** (3), 303–314 (2001).
61. Oiki, S. Planar lipid bilayer methods for channel study. *New Patch-Clamp Experimental Techniques*. 208–244 (2001).
62. Iwamoto, M., Elfaramawy, M.A., Yamatake, M., Matsuura, T., Oiki, S. Concurrent in Vitro Synthesis and Functional Detection of Nascent Activity of the KcsA Channel under a Membrane Potential. *ACS Synthetic Biology*. **7** (4), 1004–1011, doi: 10.1021/acssynbio.7b00454 (2018).
63. Venkatesan, G.A., Lee, J., Farimani, Amir Barati Heiranian, M., Collier, C.P., Aluru, N.R., Sarles, S.A. Adsorption kinetics dictate monolayer self-assembly for both lipid-in and lipid-out approaches to droplet interface bilayer formation. *Langmuir*. **31** (47), 12883–12893, doi: 10.1021/acs.langmuir.5b02293 (2016).
64. Silvius, J.R. Thermotropic phase transitions of pure lipids in model membranes and their modifications by membrane proteins. *Lipid-protein interactions*. **2**, 239–281 (1982).
65. Lindsey, H., Petersen, N.O., Chan, S.I. Physicochemical characterization of 1,2-diphytanoyl-sn-glycero-3-phosphocholine in model membrane systems. *Biochimica et Biophysica Acta (BBA) - Biomembranes*. **555** (1), 147–167, doi: 10.1016/0005-2736(79)90079-8 (1979).
66. Moore JW, J.W., Hines M, Harris EM (1984), E.M. Compensation for resistance in series with excitable membranes. *Biophys J*. **46** (4): 507–514. (1984).
67. Armstrong CM, C.M., Chow RH (1987), R.H. Supercharging: a method for improving patch-clamp performance. *Biophys. J*. **52** (1): 133–136. (1987).
68. Armstrong CM, C.M., Gilly WF (1992), W.F. Access resistance and space clamp problems associated with whole-cell patch clamping. *Methods Enzym Enzymol*. **207**: 100–122. (1992).
69. Kojima S, Iwamoto M, Oiki S, Tochigi S, Takahashi H (2018). Thylakoid membranes contain a non-selective channel permeable to small organic molecules. *J Biol Chem Journal of Biological Chemistry*. **293** (20): 7777–7785, doi: 10.1074/jbc.RA118.002367 (2018).

書式変更: フッター
書式変更: フォント : Times New Roman, 12 pt

70.- Winterstein ~~LM,~~, L.M. *et al.* ~~(2018)~~ Reconstitution and functional characterization of ion channels from nanodiscs in lipid bilayers. ~~*J*~~*Journal of genaral* ~~*Physiol*~~*physiology*. **150** (4~~)~~:), 637–646~~.~~ (2018).

71. Iwamoto, M., Sumino, A., Shimada, E., Kinoshita, M., Matsumori, N., Oiki, S. Channel Formation and Membrane Deformation via Sterol-Aided Polymorphism of Amphidinol. *Scientific Reports*. **7** (1), doi: 10.1038/s41598-017-11135-x (2017).

書式変更: フッター

書式変更: フォント : Times New Roman, 12 pt