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ORIGINAL PAPER

Storable droplet interface lipid bilayers for cell-free ion channel studies

Sung-Ho Jung · Sangbaek Choi · Young-Rok Kim · Tae-Joon Jeon

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Abstract An artificially created lipid bilayer is an important platform in studying ion channels and engineered biosensor applications. However, a lipid bilayer created using conventional techniques is fragile and short-lived, and the measurement of ion channels requires expertise and laborious procedures, precluding practical applications. Here, we demonstrate a storable droplet lipid bilayer precursor frozen with ion channels, resulting in a droplet interface bilayer upon thawing. A small vial with an aqueous droplet in organic solution was flash frozen in −80 °C methanol immediately after an aqueous droplet was introduced into the organic solution and gravity draws the droplet down to the interface upon thawing. A lipid bilayer created along the interface using this method had giga-ohm resistance and typical specific capacitance values. The noise level of this system is favorably comparable to the conventional system. The subsequent incorporation of ion channels, alpha-hemolysin and gramicidin A, showed typical conductance values consistent with those in previous literatures. This novel system to create a lipid bilayer as a whole can be automated from its manufacture to use and indefinitely stored when frozen. As a result, ion channel measurements can be carried out in any place, increasing the accessibility of ion channel studies as well as a number of applications, such as biosensors, ion channel drug screening, and biophysical studies.

S.-H. Jung · S. Choi · T.-J. Jeon (✉)
Department of Biological Engineering, Inha University, Incheon, Korea
e-mail: tjjeon@inha.ac.kr

Y.-R. Kim
Institute of Life Science and Resources and Department of Food Science and Biotechnology, Kyung Hee University, Yongin, Korea

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Abbreviations

HTS	High-throughput screening
hERG	Human ether-á-go-go related gene
DPhPC	1,2-Diphytanoyl-sn-glycero-3-phosphocholine

Introduction

Membrane proteins have been explored to elucidate their unique characteristics. These proteins transport particular ions and molecules across a plasma membrane in response to chemical, electrical, or mechanical stimuli, and bind to ligands in highly specific manner. Of particular interest among the membrane proteins ion channels play important roles in cell-to-cell communications and often become drug targets. In the late 1990s, a number of drugs were withdrawn from the market and new drugs in the pipeline were postponed due to an interaction with a K^+ ion channel coded by hERG [1]. Since many drug family including antihistamines, antidepressant, and antibiotic compounds were found to interact with hERG-coded ion channels [2], there has been a great demand for novel electrophysiology techniques for drug screening. Based on these characteristics, ion channels are utilized for the practical applications in pharmaceutical assays and molecular diagnostics.

To study ion channels conventional techniques utilize patch clamp, in which cells containing a target ion channel is tightly held against a micron-sized glass pipette by suction [3]. Using a low-noise amplifier, any ionic currents flowing across the membrane patch can be directly

measured. Although the patch clamp is often considered as gold standard, providing high-information content during the electrophysiological recording of transmembrane currents, this technique has high-barrier to entry for equipment and expertise as well as its maintenance [4, 5]. As a result, an artificial lipid bilayer integrated with isolated ion channels has been suggested as an alternative platform to study membrane based electrophysiology [6–8]. The electrical characteristics of ion channels incorporated in artificial bilayers can be determined as in patch clamp. More importantly, the artificial bilayer is a versatile platform, enabling variations of experimental conditions. Artificial bilayers, in which the function of ion channels can be reconstituted, have been employed in engineered ion channel sensors [9, 10].

Despite a number of advantages artificial lipid bilayers suffer from several technological shortcomings including their fragility and short-lifetime, and the requirement of operator's expertise limiting their potential applications [11, 12]. Following early efforts to advance lipid bilayer platforms on-demand bilayer formation have been explored to address these concerns [12, 13]. A lipid bilayer was formed in a microfluidic device, in which two leaflets of lipid monolayers at aqueous/organic fluid interfaces were mechanically joined, resulting in a lipid bilayer [12, 13]. A lipid bilayer forms in a microfluidic device by contacting two leaflets of lipid monolayers along the interface between aqueous phase and oil phase that contains lipid molecules. Funakoshi et al. [14] created a microfluidic device in which bilayers were formed by precise manipulation of two aqueous droplets in organic solvent. This technique has also been used to create droplet networks [15, 16] for electrical and optical tracking of lipid bilayers, ion channel studies [17–19], and other engineered applications [20, 21]. Recently, this method was used for automated high-throughput bilayer formation using a liquid handling robot, creating >2,200 lipid bilayers in a 3 h period [22]. However, these bilayers must be prepared immediately before measurement due to the bilayers' low mechanical stability and longevity. Therefore, this technique is only the purview of big pharmaceutical companies, in which expensive high-throughput robotic systems are available. Interest in drug discovery and screening is further expanded to small companies and laboratories since ion channel drug screening in a drug development process has become more important [23].

More recently, bilayer formation technique from a frozen membrane precursor was demonstrated to eliminate the requirement of membrane formation [24]. The membrane precursor was created using high-freezing point solvent mixture. A lipid bilayer is formed from the frozen membrane precursor upon thawing. The frozen membrane precursor enables long-term storage and shipping, widening the use of an artificial membrane platform. However, membrane precursors deposited on a thin film had a wide range of membrane formation time from 30 min to 24 h and the success rate was low. Later work with a pin tool further reduced the membrane formation time and improved the success rate by precise volume control [25].

In this work, we demonstrate a technique that utilizes a freezable droplet membrane system, in which lipid bilayer forms in consistent manner-formation time and success rate. We flash froze vertically oriented membrane system using −80 °C methanol, enabling long-term storage and shipping. When a lipid bilayer is needed, an aqueous droplet resumes to fall to the interface, resulting in the formation of lipid bilayer on the organic/aqueous interface. To measure the specific conductance of the lipid bilayer, electrical and optical measurements were made simultaneously. Ion channels frozen together with the membrane precursor were subsequently incorporated in the bilayer, showing their typical conductance levels as measured in a conventional lipid bilayer system. With a preassembled and indefinitely storable bilayer integrated with ion channels, ion channels measurement and applications with ion channels will be no longer limited to trained workers at the time and place of bilayer formation.

Material and methods

Chamber preparation and electrode placement

To show the feasibility of a droplet membrane formation from a frozen membrane precursor, initial experiments were done in a 4 mL glass vial (Fisher Scientific) with a Ag/AgCl (World Precision Instruments) electrode placed in the bottom of the vial. This frozen droplet system was also demonstrated in a commercially available 96-well plate. Another Ag/AgCl electrode was made from a 208 μm silver wire which was activated by placing it in Clorox bleach for >1 min.

Frozen droplet precursor preparation

One milliliter of 1 M KCl with 1 mM EDTA solution buffered with 10 mM Tris–HCl at pH 8.0 was added to the glass vial containing the electrode. The buffer was degassed before use. 300 μL of a 4:1 mixture of hexadecane (Sigma-Aldrich) and *n*-decane (MP Biomedical) containing 3% (w/w) asolectin (Sigma-Aldrich) was added atop the aqueous phase. After ~2 h monolayer stabilization time a lipid monolayer formed on the bottom interface. After this stabilization time, a 0.5 μL aqueous droplet, composed of the same solution as the lower aqueous phase, was dispensed into the solvent layer. This solution was

immediately submerged in −80 °C methanol to flash freeze all solutions. Both the aqueous and organic phases were frozen in <1 min and stored in a freezer until use.

Lipid bilayer formation

Prior to measurement, the frozen droplet membrane system was brought to room temperature, where both the aqueous and organic phases melted. Since oil phase in the top layer thaws at higher temperature than the aqueous phase, the aqueous droplet melted first and remained securely in its original position. When the organic solvent thawed the aqueous droplet fell on the aqueous/organic interface and formed a bilayer.

Electrical and optical measurement

A Ag/AgCl electrode was placed into the aqueous droplet to measure current across the membrane. All data were recorded using an Axopatch 200B amplifier and a DigiData 1332 DAQ system (Axon Instruments). To measure membranes' capacitive characteristic optical measurement using a digital microscope (Prime Entertainment) with transmitted light source was conducted simultaneously.

Ion channel reconstitution

Alpha-hemolysin (αHL) and gramicidin A (Sigma) were used for single channel measurements without further purification. The aHL stock solution was made at 1.7 ng/μL concentration in 200 mM NaCl buffered with 100 mM Tris–HCl at pH 8.2. For protein activity test, 1.7 ng of αHL was added in the aqueous droplet. Upon membrane formation, the subsequent insertion of αHL was electrically measured. Gramicidin A was resuspended in ethanol at 1 μg/mL concentration and stored in a −80 °C freezer. For gramicidin reconstitution, the stock solution was resuspended in ethanol at 1 ng/mL concentration and 1 μL of the gramicidin solution was then added to the organic phase. In all cases, electrical measurements were made using an Axopatch amplifier described above. Data were acquired using pClamp software (Axon Instruments) and further analyzed using Clampfit software.

Results and discussion

Frozen droplet membrane precursor

Introduction of an aqueous droplet to the upper organic phase will result in a lipid bilayer at the aqueous/organic interface as reported in the previous literature [22]. To make this system freezable a mixture of solvent consisting of hexadecane and *n*-decane was used. Hexadecane freezes at 18 °C just below room temperature. Initially, we attempted to freeze the system at 4 °C, as to cause the solvent to freeze, leaving the aqueous droplet fluid [24]. However, no droplet was noticed upon thawing. It is most likely due to the mixing of the two aqueous phases that were separated by a porous solvent ice barrier. This was also noticed when a freezing process was done slowly at −20 °C. The upper aqueous droplet may have been extruded to the lower aqueous phase through the thin crystalline structure of the organic phase before and after the complete freezing of the water. We consequently found the flash freezing of the system is important to maintain the aqueous droplet for subsequent bilayer formation. With the rapid cooling method by contacting ∼−80 °C methanol solution we were able to freeze both phases nearly instantaneously and immobilize an aqueous droplet in its initial position.

Lipid bilayer formation

The aqueous droplet frozen in the organic phase will fall on the aqueous/organic interface upon thawing, resulting in a lipid bilayer. Figures 1 and 2 show the subsequent droplet membrane formation process from the frozen droplet membrane precursor by cooling. The aqueous droplet was vertically positioned in the middle of concave meniscus created by the physical affinity contact angle of lower aqueous phase when contacted with the hydrophilic surface of the small chamber. Upon thawing a lipid bilayer is formed by the spontaneous falling of the aqueous droplet onto the monolayer. It was also observed that after thawing the droplet appeared more elliptical than before freezing. This is most likely due to the reduction in surface tension caused by the presence of lipids and the contacting of the droplet with the lower phase. Since the membrane is horizontally located, we can make optical and electrical measurement simultaneously, enabling electrical measurement together with the optical tracking of lipid bilayers and ion channel activities [17–19]. This was previously done with supported lipid bilayers [18].

When the thawing process is initiated air bubbles were found in the system, which may interfere with both optical and electrical measurement. While the droplet bilayer system was flash frozen, gas dissolve in the aqueous phase was trapped within ice crystalline structure, making the ice cloudy. Upon thawing we found air bubbles, most of which were located along the upper/lower phase interface. To eliminate air bubbles we degassed the aqueous solution, minimizing gas dissolved in the lower aqueous phase prior to adding organic phase [26]. In general, the solubility of gas follows Henry's law, in which the amount of a dissolved gas in a liquid is proportional to its partial pressure,

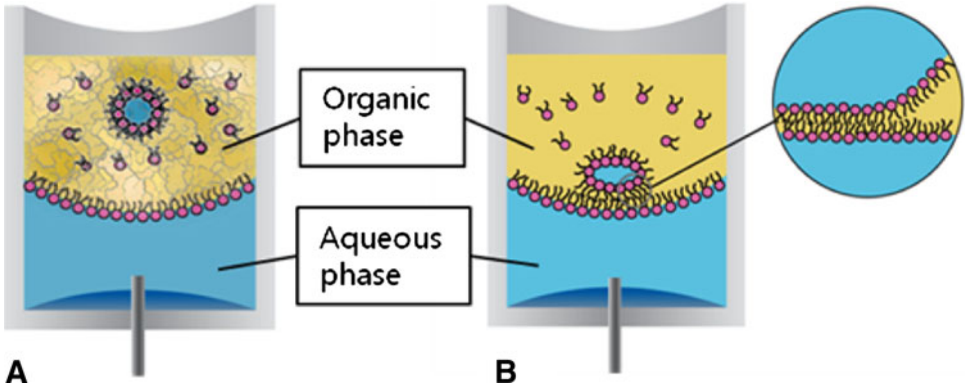

Fig. 1 Schematic illustration: **a** frozen droplet membrane precursor in a vial. **b** Formation of a droplet interface bilayer (DIB) after thawing

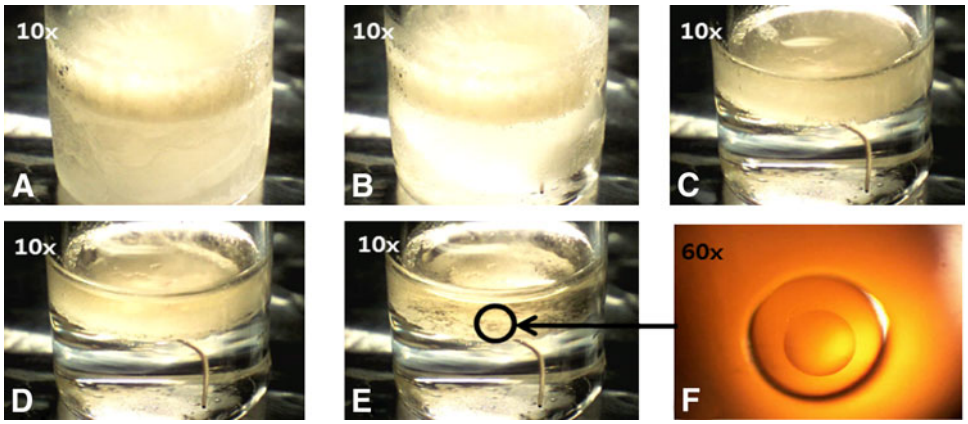

Fig. 2 The membrane formation process **a**–**e**. Flash frozen in −80 °C methanol. Upon thawing gravity drives membrane formation as the immobilized droplet moves down to the interface. Both phases thawed and Ag/AgCl electrode is placed into the droplet to measure ion channel activities. The aqueous droplet in **f** is magnified by ×60. The magnified image shows the typical shape of a lipid bilayer

and therefore minimizes the air bubbles in the system by degassing the aqueous solution.

To make well-formed lipid bilayers, sufficient monolayer stabilization time to self-assemble is required. Otherwise, the aqueous droplet will fuse with lower aqueous phase, resulting in no membrane formation. Lipid bilayers formed using this method had typical lifetimes of >7 h, far sufficient to measure ion channel activities and other associated events. We also found that the proper volume of an aqueous droplet facilitate bilayer formation and had the highest success rate with 0.8 μL of a droplet in our system.

Optical and electrical measurement of a droplet lipid bilayer

To measure a thinning-out process (i.e. lipid bilayer formation) and measure the specific capacitance of the resulting bilayers we measure the optical and electrical property of the bilayer simultaneously (Fig. 3b). Bilayer formation was monitored by measuring the capacitive current upon the application of 8 Hz 20 mV peak-to-peak triangle wave. Figure 3 shows increasing capacitance resulted from a thinning-out process, showing the membrane formation. The membrane formation could be confirmed both optically and electrically, and the measurement of single channel events of ion channels were made (discussed below). After successful membrane formation, membranes' resistance was measured greater than giga-ohm seal, necessary to measure incorporated ion channels and other associate events, and the thickness of bilayers was measured ~4 nm. Together with simultaneous measurement of bilayer capacitance and area, the specific capacitance of bilayers were calculated ~480 nF/cm^2, similar to the value of a lipid bilayer membrane in the previous literature [27]. In addition, with the analysis of power spectrum, the noise level of this system was favorably comparable to the conventional system, which is critical to measure ion channels with small ionic currents.

Ion channel reconstitution and measurement

Ion channel reconstitution and measurement across a lipid bilayer is a straightforward method to demonstrate the feasibility of a membrane platform. We incorporated αHL, most widely used in a number of engineered sensor applications including a small molecule detection, biopolymer sensing, and potentially DNA sequencing [9, 28]. αHL is originated from a bacterium, *Staphylococcus aureus*, and secreted as a monomer which can be incorporated

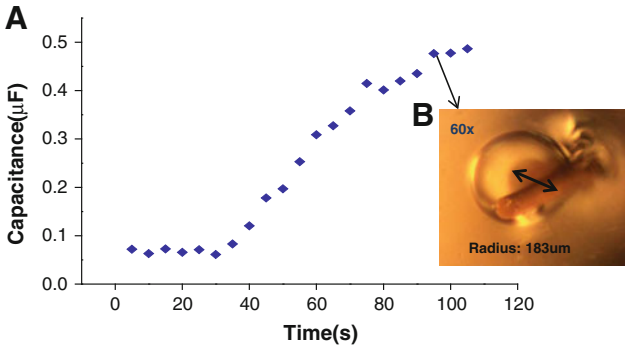

Fig. 3 **a** Electrical property of a lipid bilayer formed after thawing. To calculate specific capacitance, electrical recording was simultaneously done with optical measurement **b**

into the cell membranes. The monomers oligomerize to form a heptameric nanopore structure. αHL creates nanopores in a lipid bilayer in a very controlled manner, thus each αHL pores has a conductance of ~0.8 nS, making very discrete current increase by subsequent insertion of αHL pores as shown in Fig. 4. Since αHL is added to the lower aqueous solution or aqueous droplet in our experiment αHL can be frozen and stored together with droplet bilayer system. When a lipid bilayer is formed, αHL pores were subsequently formed in the bilayer within <1 h.

The frozen droplet bilayer system also enables the storage of channel proteins alongside of the membrane precursor. Similar to water soluble protein α-HL, water insoluble ion channels compatible with organic phases can be also frozen with the system by dissolving them in the membrane precursor solution. Since both peptides and precursor are stored in solid phase, the peptide can be indefinitely stored along in the frozen droplet bilayer precursor. Gramicidin A (gA) channel activities began upon membrane formation from frozen membrane precursor containing gA molecules. Discrete channel conductances due to the association/dissociation of gA dimers were shown in Fig. 4, consistent with previous literature [29]. Our system allows the fixed copies of gramicidin molecules present in the precursor solution, whereas, in conventional single channel experiment, the proteins/peptides were added directly to the aqueous phase, resulting in uncontrollable channel insertion. Furthermore, the measurement of gramicidin A channels proves that our droplet lipid bilayer platform also supports low-noise measurement since the gramicidin A has relatively small conductance through its channel.

Conclusion

In summary, we have successfully developed a novel platform preassembled and indefinitely storable to conduct ion channel experiment. In this work, we adopted a frozen membrane precursor using high-freezing point solvent mixture. Upon thawing gravity spontaneously drives membrane precursor to form lipid bilayer at the aqueous/organic interface. The significance of this system is that no expertise and laborious works are required for bilayer formation. The user only needs to warm the bilayer vial and insert an electrode for use. Various bilayer compositions can be used by mixing individual components into the organic solution. From a pharmaceutical stand point this system can be scaled up by using low temperature compatible 96 or 384-well plates. Ion channels stored in frozen membrane precursor enables that ion channel measurements can be carried out in any place. The platform described in this work will improve the accessibility to bilayer measurements, widening the applicability and users. Furthermore, this will significantly improve drug development with high-throughput ion cannel drug

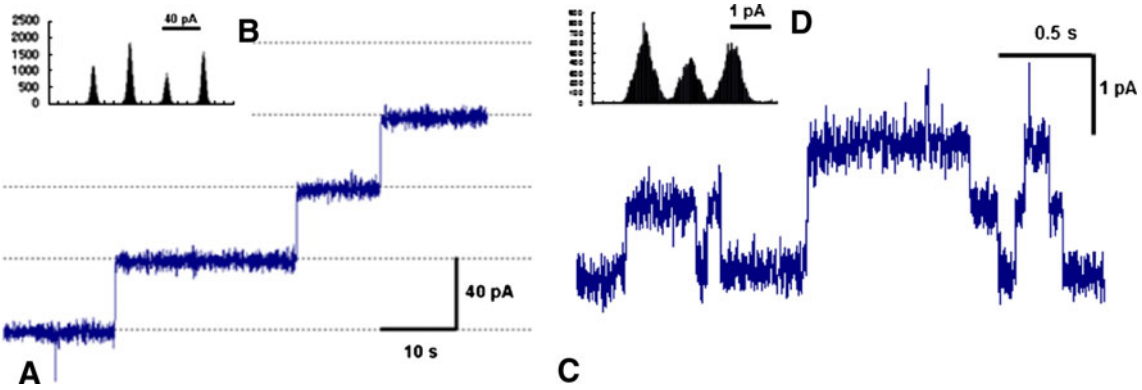

Fig. 4 **a**, **c** Ion channel activities of alpha-hemolysin (αHL) and gramicidin A were shown after a bilayer is formed. Applied voltage across the droplet membrane is 50 and 40 mV, respectively. **b** Histogram of **a**. Characteristic conductance of alpha-hemolysin is shown. Each current jump corresponds to ~0.8 nS. **d** Histogram of **c**. The typical association and dissociation events of gramicidin A were shown

screening in the pharmaceutical industry and broaden the range of practical applications as well as scientifically engineered biosensor components.

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