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Pseudo painting/air bubble technique for planar lipid bilayers

Christian J. Braun[a,*], Tom Baer[a], Anna Moroni[b,c], Gerhard Thiel[a]

[a] Membrane Biophysics, Technical University of Darmstadt, Schnittspahnstrasse 3, 64287 Darmstadt, Germany
[b] Department of Biosciences, University of Milan, Via Celoria 26, 20133 Milano, Italy
[c] CNR-IBF, Via Celoria 26, 20133 Milan, Italy

HIGHLIGHTS

- We improved the creation of stable planar lipid bilayers with the help of an air bubble.
- The improved method is less time consuming and easy to handle.
- The method allows formation of bilayers from phospholipids with short acyl chain-length.
- When too many channels are inserted in a bilayer the air bubble can remove access channels proteins in order to achieve single-channel recordings.
- The improved technique can be used for a wide range of planar lipid bilayer experiments.

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ABSTRACT

Background: A functional reconstitution of channel proteins in planar lipid bilayers is still very versatile to study structure/function correlates under well-defined conditions at the single protein level.
New method: In this study we present an improved planar lipid bilayer technique in which an air bubble is used for stabilizing unstable/leaky bilayers or for removing excess lipids. The bubble can also be used as a tool for reducing the number of channels in the bilayer with the goal of having only one active channel in the membrane.
Results: Stable planar lipid bilayers are formed within seconds to minutes. In the case of multiple channel insertion the air bubble can be used to reduce the number of channels within minutes.
Comparison with existing method(s): The simple improvement of the classical folding technique guarantees a very fast creation of stable bilayers even with difficult phospholipids in a conventional vertical bilayer set-up; it requires no modifications of the existing set-up.
Conclusions: This technique is very easy to handle and guarantees successful single channel recordings for any kind of planar lipid bilayer experiment.

1. Introduction

Many techniques are currently available for studying at the single-protein level the structure/function correlates of ion channels and their interactions with the lipid membrane (Coronado and Latorre, 1983; Fertig et al., 2003; Hamill et al., 1981; Mueller et al., 1962; Syeda et al., 2008). One of the most reduced and defined experimental systems among the many methods with the best control over the experimental parameters are planar lipid bilayers (BLM; also known as black lipid membranes). In this method the experimenter controls the channel protein of interest, the composition of the lipids and the simple buffers on both sides of the membrane. Bilayers, which are among the oldest methods for channel recordings (Mueller et al., 1962) are generally formed by folding bilayers from monolayers (Montal and Mueller, 1972) or by painting a bilayer over a hole in a septum (Mueller et al., 1962). A disadvantage of the 'painting' technique is that the bilayer may still contain solvents, which are used for dissolving the lipids. This may alter the physical properties of the membrane (Benz et al., 1975, 1973). Planar lipid bilayers, which are folded from monolayers in contrast can have a lower solvent content (White et al., 1976) and depending on the solvent they are even considered as virtually solvent free (Benz et al., 1975); this method may be more suitable for single-channel recordings when the properties of the bilayer are important for channel function. Both the painting and the folding technique generally create very stable bilayers with a high electrical

Abbreviations: BLM, black lipid membrane; DPhPC, 1,2-diphytanoyl-sn-glycero-3-phosphocholine; DMPC, 1,2-dimyristoyl-sn-glycero-3-phosphocholine.

* Corresponding author. Tel.: +49 6151 16 6083.
E-mail address: braunc@bio.tu-darmstadt.de (C.J. Braun).

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resistance; this makes them suitable for long lasting single-channel measurements. A problem with both methods is that the formation of a suitable bilayer can be time consuming. Often the bilayer is either leaky, unstable and breaks easily or the hole in the septum is covered with too much lipids, which do not thin out into a bilayer. A further problem, which is known to every user of bilayers, is that frequently too many channel proteins insert into the membrane; this limits the use of data for single channel analysis.

Here we present an improvement of the classical planar lipid bilayer technique. The use of an air bubble, a technique which has been occasionally mentioned in literatures (Langford et al., 2011; Haque et al., 2013; Rosenstein et al., 2013), makes it easy to combine the advantages of the folding and painting technique; it allows a very rapid formation of stable bilayers and even more important a fast lowering of the number of active channels in a bilayer.

2. Materials and methods

Planar lipid bilayer experiments were done with a conventional, vertical bilayer setup (IonoVation, Osnabrück Germany) (Bartsch et al., 2013) and a stereomicroscope (Novex, Netherlands) for optical monitoring. A 1% hexadecane solution (MERCK KGaA, Darmstadt, Germany) in *n*-hexane (Carl ROTH GmbH, Karlsruhe, Germany) was used for pretreating the Teflon foil. The hexadecane solution (ca. 0.5 μl) was pipetted onto the hole (100 μm in diameter) in the Teflon foil with a bent Hamilton syringe (Hamilton Company, Reno, Nevada, USA). It was waited until the solvent was evaporated. The experimental solution contained of 100 mM KCl and was buffered with 10 mM HEPES/KOH to a pH of 7. The lipids 1,2-diphytanoyl-*sn*-glycero-3-phosphocholine (DPhPC) and 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) (both from Avanti Polar Lipids, Alabaster, AL, USA) were used at a concentration of 0.15–25 mg/ml in *n*-pentane (MERCK KGaA, Darmstadt, Germany).

The potassium channel Kcv_{NTS} (K^+ channel *c*hlorella virus-isolate Next-to-Smith), which was used for single-channel measurements, was synthetized cell-free in nanolipoprotein discs (Katzen et al., 2008). The channel was purified indirectly over a Ni-NTA column using a His-tag attached to the nanolipoprotein. The recombinant channel protein, which is embedded in the isolated nanodisc, was therefore first centrifuged (12 min, 16 060 × *g*, RT) to remove insoluble material. The supernatant was loaded onto a Ni-NTA column and incubated for 30 min. Later the column was washed twice with 20 mM imidazole. The channel protein was then eluted by 250 mM imidazole (fractions a 100 μl). The recombinant channel protein in the nanodiscs dissolved in 250 mM imidazole was directly added to the bilayer. This resulted in a spontaneous insertion of channel protein into the bilayer; an osmotic gradient between the chambers was not required for protein insertion. Reconstitution was best when small volumes of protein in imidazole were administered with a Hamilton syringe directly below the bilayer (Braun et al., 2013). In control experiments we found that the small volume of imidazole alone had no effect on the bilayer. Once inserted into the bilayer the channel protein must be released from its nanolipoprotein scaffold because channel function was determined by the physicochemical properties of the bilayer. In additional experiments we also reconstituted the same channel protein in detergent after recombinant production of the channel in *Pichia pastoris* cells. The behavior of the channel in the bilayer was independent on the method of purification or whether solubilization in detergent or dissolving in imidazole (Braun et al., 2013).

All experiments were done at room temperature between 20 and 25 °C. The Ag/AgCl electrodes were connected to a head-stage of a patch-clamp amplifier (L/M-EPC 7, List-Medical, Darmstadt). Single-channel currents were filtered at 1 kHz and digitized with a sampling interval of 280 μs (3.57 kHz) by an A/D-converter (LIH 1600, HEKA Electronik, Lambrecht, Germany).

3. Results

The formation of planar lipid bilayers by the monolayer folding technique is described elsewhere (Montal and Mueller, 1972) and schematically shown in Fig. 1A. Lipids, which are dissolved in an organic solvent, are spread as monolayers onto an experimental solution. After evaporation of the solvent two monolayers are folded over the hole in the septum by raising the experimental solution in both chambers; this eventually creates a bilayer. Frequently the bilayer folding method is time consuming and not every membrane is suitable for bilayer measurements. Often the bilayer is leaky or the hole in the septum contains too much lipid, which does not thin out into a suitable bilayer. In our approach we start the procedure with a conventional formation of a lipid bilayer from folding of monolayers (Montal and Mueller, 1972) as in Fig. 1 (step 1, A and B). In preparation of this procedure the edges of the hole are first pretreated with 1% hexadecane solution, which creates a very hydrophobic area around the hole. The bilayer is then formed as shown in the sketch of Fig. 1. In the case that this procedure already results in a perfect bilayer channel proteins can be inserted in the bilayer and the channel recordings can be started directly. If the bilayer is unstable, leaky or too thick we can apply a "pseudo painting" with an air bubble. Because of the hexadecane pretreatment the edges of the hole presumably keep a reservoir of lipids. These lipids from the reservoir can now be spread over the hole. For this purpose we use a bent Hamilton syringe and create a small air bubble under water Fig. 1B (step 2). A photo with the real dimensions of the syringe with the bubble in front of the hole in the Teflon septum is shown in Fig. 2. The air bubble can be spread over the hole with the syringe under visual control through the stereomicroscope. In the case of leaky/unstable bilayers the movement of the air bubble from the edges over the hole transfers lipids from the aforementioned reservoir over the hole and rapidly creates a new bilayer (Fig. 1B, steps 3 and 4). In the case of a thick lipid deposit, the air bubble is moved right in front of the hole; in this position the bubble attracts with the water/air interface lipids and moves them out of the hole. Also with this procedure it is possible to rapidly create suitable stable bilayers.

Fig. 3 demonstrates in an example how rapid bilayers can be formed with this method. The representative recording shows the measurement of currents across a stable bilayer, which was created by folding. The bilayer was then destroyed with the air bubble. By spreading the air bubble over the hole in the aforementioned fashion, a new stable bilayer was formed in less than 10 sec. The destruction and reformation of the bilayer was repeated once more and a bilayer was again formed within less than 10 sec. This operation can be frequently repeated until the reservoir runs out of lipids.

In the context of questions on protein/lipid interactions it is often desirable to measure the activity of a channel in different kinds of pure lipid bilayers. This includes bilayers with different chain length. An interesting lipid is 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC). This lipid is frequently used in molecular dynamic simulations (Braun et al., 2013; Tarek et al., 2003; Tayefeh et al., 2007; Woolf and Roux, 1994) and it is hence interesting to compare the data from simulations and experiments in the same lipid. The problem however is that DMPC forms very unstable planar lipid bilayers (Schmidt et al., 2006). This makes it difficult for single-channel recordings. For this reason it is not surprising that there are only very few reports in the literature in which this lipid was successfully used for bilayer measurements. Indeed when we attempted to create bilayers with the conventional