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SOLVENT-FREE LIPID BIMOLECULAR MEMBRANES OF LARGE SURFACE AREA

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The formation of a solvent-free lipid bimolecular membrane of large surface area (approx. 2 mm^2) by the successive transfer of two monolayers upon an aperature of a closed chamber has been demonstrated. The electrical parameters of the membrane appear to be similar to the conventional Montal-Mueller solvent-free membrane. The use of a closed chamber greatly increases the stability of the membrane to mechanical disturbances and produces hydrostatic equilibrium necessary for electrical measurements.

Introduction

The application of solvent-free bimolecular lipid membranes to the reconstitution of membrane-associated biochemical systems has in recent years become an area of considerable interest. This is principally a result of the studies of White [1,2] and others [3,4] who have shown that residual solvent present in bilayers formed by traditional Mueller-Rudin methods [5] serves to considerably modify the physical properties of the resultant membrane. In particular, the increased thickness of such solvent-containing membranes over solvent-free systems is evidenced by the lowered capacitance of the former [6]. This suggests that solvent-containing bilayers may not be desirable as models for the cell plasma membrane.

There are several general approaches by which functionally 'solvent-free' lipid bimolecular membranes can be prepared. Conventional Muller-Rudin techniques can be applied, except that a solvent with a very limited solubility in the lipid of the membrane may be used [21]. Alternatively, the temperature of the membrane may be lowered after its conventional formation to a point below the melting point of the solvent. This causes the torus of solvent supporting the membrane to freeze, allowing the solvent to be functionally removed from the bilayer [22]. Alternatively, solvent can be totally excluded from the organic phase by this method [23]. Other more general approaches are based upon the apposition of two monolayers using the technique of Montal and Muller [6,24]. Although the membrane which results from this procedure is generally stable, the protocol which is required is in practice rather complex, and such membranes have not had areas much in excess of 0.1 mm^2. Additionally, it is difficult to control small differences in hydrostatic pressure between the two chambers which can lead to mechanical distortion of the membrane.

We have recently prepared solvent-free membranes of area of about 2 mm^2 using a simple modification of the Montal-Mueller method for the construction of a bilayer from a monolayer by passing a hydrostatically closed chamber successively through a single monolayer interface.

Historically, Hardy in 1925 [7] was probably the first to produce a water film bounded on both sides by monolayers. Then Langmuir and Waugh, in 1938 [8], repeated this experiment. They dipped a platinum plate which was perforated with small holes through a monolayer surface and produced a membrane. At about the same time, Bikerman [9]

carried out a series of experiments of the same general nature, using the Langmuir-Blodgett method [10,11] for the preparation of multilayer films upon metallic surfaces. In particular, he constructed a multilayer film upon a grooved metallic plate, each groove being prefilled with aqueous electrolyte. The result of dipping such a grooved plate repeatedly through a monolayer at the air-water interface was an isolated multilayer membrane located upon each groove.

We investigated the application of this approach to the construction of a bimolecular lipid membrane upon an aqueous surface. The best way to imagine the idea is to conceive of a plate possessing a hydrophyllic surface which is withdrawn from below a lipid monolayer located at the air-water interface and is then dipped through it again. Clearly, a bimolecular lipid membrane should then cover the surface of the plate, if the dipping operation is carried out at a rate commensurate with deposition. Now, let us imagine that a small hole is drilled through the plate, and that this hole is filled with aqueous solution. If one end of this orifice is now closed with an electrode the only means by which the aqueous contents of the hole can communicate with the surroundings is though the front of the opening. Now, if such a plate is withdrawn from below the monolayer, and subsequently dipped through it, a bimolecular lipid membrane will form upon the surface of the 'chamber' which we have created within the plate.

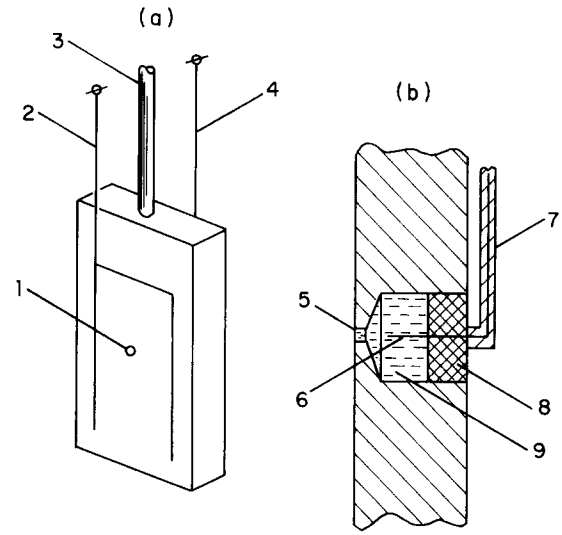

Fig. 1. Chamber for the preparation of solvent-free bilayers from a monolayer. (a) The general appearance of the chamber. 1, aperture; 2 and 4, electrodes; 3, holder. (b) Cross-section of the chamber perpendicular to the front side. 5, aperture; 6 and 7, internal electrode; 8, stopper; 9, internal electrolyte.

Materials and Methods

Phosphatidylcholine was obtained from Sigma (Type E, from egg yolk). Gramicidin was a gift of Burroughs Wellcome Co. All other chemicals were of reagent grade. The electrolyte used for all studies is 20 mM in KCl, and 20 mM in NaCl, with the pH adjusted to 7.40 with $NaHCO_3$.

Two bilayer chambers were constructed of teflon sheet 7.0 mm thick, and were machined to form a rectangular plate $20 \times 14 \times 7$ mm. The construction of the chamber is illustrated in Fig. 1. A hole 2.0 mm in diameter was drilled into the plate almost completely through it, such that a septum about 15 μM in thickness remained. These septums were pierced with a steel surgical needle or drill to yield the apertures of areas 0.14 and 1.93 mm^2, measured optically [1]. This hole served as the surface for the construction of the bilayer membrane. The anterior end of the hole was sealed with a polyethylene stopper interference fitted to it, containing a Ag/AgCl wire. The interior of the chamber was filled with electrolyte prior to seeling, and it was carefully freed of air bubbles prior to its use. The front surface of the chamber was polished with fine alumina prior to its use. The second electrode, a Ag/AgCl wire, was attached to the front of the plate containing the chamber. The volume of the chamber was about 10 μl. The plate containing the chamber was attached to a micromanipulator (Brinkman 8234) which was utilized for the dipping operations.

The trough for the formation of the monolayers was constructed of teflon and was $110 \times 80 \times 25$ mm. The effective surface area of the trough was 40 cm^2, which would be decreased with the aid of a teflon barrier. The shallow portion of the trough was 5 mm in depth, and the well, designed for containing the bilayer chamber, was 20 mm in depth. The trough was equipped with a floating barrier, constructed from Mylar 120 μm in thickness, which served to keep the surface pressure of

the monolayer constant [12,13]. The entire trough was contained within a massive metal block which was thermostatically controlled to ±0.01°C with a YSI 72 temperature controller. The temperature was monitored to a precision of ±0.02°C with a Cole-Parmer 8502-25 digital thermometer.

The electrical properties of the bilayer system were measured with the aid of a computer based system. Briefly, current-voltage information from a voltage clamp of standard design [6] flows through a digital voltmeter (Hewlett-Packard 3544A) into the IEEE 488 bus structure, where it is processed by a 32 K PET computer. The AC capacitance and DC conductance can be quantitated simultaneously by inititating under computer control a burst of AC signal between the discrete DC voltage steps applied to the system, from a Hewlett-Packard 203A Digital to Analog Converter. A PAR 128A lock-in amplifier is used for the AC measurements. Data are output to a teletype, or to an external computer (Hewlett-Packard 3000) for graphical output and further processing.

Procedure. Prior to the formation of a monolayer in the trough the internal compartment of the chamber was filled by electrolyte, and the internal electrode fitted into the rear hole such that a small drop of solution was forced out the aperture hole. Then, the aqueous surface of the monolayer trough was cleaned by means of a teflon brush, and the chamber was placed below the water surface into the well with the micromanipulator, such that the aperture was just below the water level. The phosphatidylcholine was added to the surface of the trough as a 1% (w/v) solution in *n*-hexane. The amount of the lipid was in excess compared with the minimal amount required to form a condensed monolayer. After the solvent had evaporated, the chamber was slowly withdrawn, allowing the chamber aperture to just cross the air-water interface containing the monolayer. Then the chamber was slowly dipped again through the monolayer, such that the aperture was located just below the air-water interface. The formation of a membrane was continuously examined by monitoring the electrical capacitance. The surface pressure of the monolayer during the formation of the bimolecular membrane was measured by means of the method of Wilhelmy [14] to be about 30 dyn/cm.

After formation of a stable membrane the stationary current dependence upon the voltage $I(V)$ and conductivity and capacitance at the frequencies 400, 800, and 1600 Hz were measured. Conductivity and capacitance were calculated by the substitution method [15] allowing for the contribution of the electrode system parameters.

Results

When the phosphatidylcholine solution was added to the water surface of the monolayer trough, and the accompanying solvent was allowed sufficient time to completely evaporate, the formation of a lipid bimolecular membrane was accompanied by a sharp increase in electrical capacitance. Typically, the value reached was 0.8 ± 0.1 $\mu F/cm^2$. However, the rate of membrane formation, as evidenced by the rate of rise of the capacitance, and the limiting capacitance reached, depended upon the completeness of evaporation of residual solvent. Fig. 2 illustrates the capacitance dependence upon time for various periods of solvent evaporation. If the membrane contains a considerable amount of residual solvent, the capacitance increases very slowly to its limiting value, as seen in Fig. 2. If on the other hand the monolayer is completely solvent free, the limiting value is attained within a few seconds. This is completely

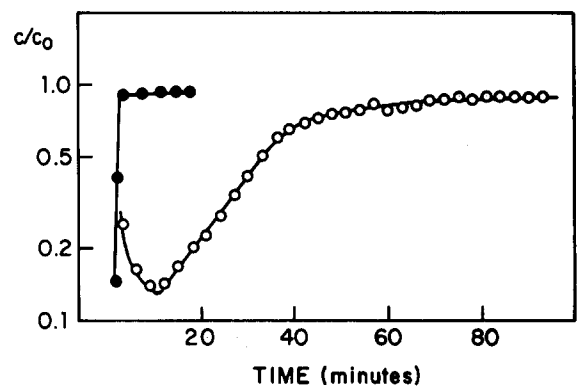

Fig. 2. Time dependence of the relative capacity C/C_0. C_0 is the stationary value of the capacitance. A membrane formed immediately after the addition of a solution of phosphatidylcholine in *n*-hexane to the electrolyte surface (O——O). A membrane formed 3 min after the addition of a solution of the phosphatidylcholine (●——●).

analogous to the dependence of capacitance upon evaporation time observed by Benz et al. [16] for solvent free bilayers constructed by the method of Montal and Mueller [6], with the exception of the capacitance minima we observe in Fig. 2.

The lifetime of the lipid bimolecular membrane also appears to depend upon the completeness of the solvent evaporation. Decreasing the residual solvent in the monolayer decreases the lifetime of the bilayer which is formed from 2–3 h to several minutes. The pretreatment of the aperture of the bilayer chamber with a dilute solution of vaseline in *n*-hexane or phosphatidylcholine in *n*-hexane increases the effective lifetime of the membrane without marked effect upon the value of the limiting membrane capacitance attained. A mean lifetime of the membranes after pretreatment of the aperture with phosphatidylcholine is 2 h.

The DC resistivity of the membrane at 25.0°C is in the range of 10^8 to 10^9 ohm · cm^2, and decreases to about 10^5 ohm · cm^2 when gramicidin of concentration $1.5 \cdot 10^{-3}$% (w/v) is present in the lipid solution used to form the monolayer. The current voltage dependence of the membranes which are formed is linear over the range of applied voltage 0 to 150 mV (correlation coefficient 0.85 ± 0.05).

As a final test of the hypothesis that membranes prepared by the present protocol were functionally identical to conventional Montal-Muller solvent-free membranes, the voltage dependence of the specific capacitance was examined. Benz et al. [16] have reported that the capacitance of glycerol monooleate bilayers formed by the method of Montal and Muller [6] was independent of the applied voltage. It was shown that regardless of the duration of the applied voltage steps that the change in the capacitance of the Muller-Rudin membranes with the applied voltage could be well represented by a quadratic function. This is in sharp contrast to the behavior observed in solvent-free membranes prepared from phosphatidylcholine monolayers by means of the present method. These latter membranes did not evidence a change in capacitance with voltage within the limits of experimental precision [25]. It therefore appears that by these criteria the membrane is functionally 'solvent-free' also.

We did not observe noticable dependence of the capacitance upon frequency over the range of frequencies which were applied.

Discussion

In the present study we have demonstrated that a solvent-free lipid bimolecular membrane of large surface area can be formed by the successive transfer of two monolayers upon an aperture of a closed chamber. Two results indicate that the membrane is indeed bimolecular in nature: (a) The resistance is decreased three orders of magnitude upon the addition of gramicidin, which is known to only so influence membranes of bimolecular thickness [17], (b) The observed value of the capacitance is close to the observed values for solvent free lipid bimolecular membranes [6,16]. The capacitance, 0.8 μF/cm^2, is just above the value reported by Benz et al. [16] of 0.76 μF/cm^2. The small differences in the capacitance could be due to our errors in the measurement of the aperature area, or the membrane area itself may not be precisely equal to that of the aperture [16].

We pose the question: is the membrane constructed by our method identical with conventional solvent-free lipid bimolecular membranes?

Our approach involves the successive transfer of the monolayer, formed upon the aqueous surface of the trough, to the liquid surface of the closed compartment of the chamber, such that the first monolayer membrane appears upon the water aperture with its hydrophobic groups oriented toward the air. Then, the second monolayer is transferred upon the top of the first, and junction of the hydrophobic groups occurs to form a lipid bimolecular membrane.

In the method of Montal and Mueller, the aperture is stationary, and the monolayers are successively lifted even with it, covering the aperture first in one compartment, and then in the second. The identical process of hydrophobic interaction of the two monolayers occurs, so superficially it would appear that the membrane prepared by the present method is identical with that of Montal and Mueller.

However, our method differs in several important respects from the conventional approach. Firstly, the initial monolayer is transferred to the surface of the electrolyte in the bilayer chamber.

In conventional methods, the first monolayer remains in contact with the solution upon which it was formed, and the solution can be exchanged only after the membrane is formed.

Secondly, after formation of the bimolecular lipid membrane the hydrostatically separated internal compartment of the chamber is closed. This results in an increase in the stability of the membrane to various mechanical disturbances [18]. This is a result of the fact that a pressure drop in the open compartment is equilibrated by the pressure in the closed compartment, as water may be considered essentially incompressible at these low hydrostatic pressures.

A further advantage of this method is that at least one of the aqueous compartments bathing the membrane could be exceedingly small which will be very useful when using substances which are not in abundance.

The closed chamber design presents other more fundamental advantages. A membrane formed in a chamber with conventional open compartments is usually under some small difference in hydrostatic pressure across the two compartments, unless special precautions are taken [16,19]. This difference perturbs the membrane surface tension, resulting in an increase in the radius of curvature which is a function of the pressure differential as well as the tension. When a voltage is applied to such a membrane, its tension is inevitably changed [20], and this change is then equilibrated by the pressure difference to yield a membrane with a different total surface area. Therefore, in an open chamber the surface area of a membrane is fundamentally indeterminant. In a chamber with one closed compartment, the hydrostatic pressure differential is very close to zero. Consequently, although the tension of the bimolecular lipid membrane will still vary as a function of the applied voltage, this will not result in a perturbation in the membrane curvature of surface area. This becomes extremely important when one measures membrane parameters which depend upon the geometrical surface area under transmembrane voltage conditions.

A further advantage of this approach as well as the Montal-Mueller method is that if a membrane is broken, a new membrane can be rapidly formed from the initial monolayer or a new monolayer. The chamber is simply withdrawn, and dipped through the monolayer, which only requires a few seconds to complete. By the use of new monolayer it is also possible to form asymmetric lipid bimolecular membranes as conveniently as by the Montal and Mueller method, as the electrolyte contained in the bilayer chamber does not flow out of the chamber when it is raised above the monolayer surface. While our studies were in progress, Korenbrot and Hwang [26] reported a dipping method for the preparation of lipid bimolecular utilizing an open chamber along with a supporting membrane. This approach does not employ the advantages of a hydrostatically closed chamber or an unsupported membrane.

In summary, we have demonstrated that a method involving successive transfer of monolayers onto the aqueous surface of a closed bilayer chamber represents a simple technique for the construction of stable, solvent-free lipid bimolecular membranes of large surface area. We believe that this approach will prove of particular utility for the functional reconstitution of a wide variety of membrane-associated biological macromolecules.

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