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Reversible bilayer junction of lipid monolayers: free mono-bi-monolayer contact

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A stable lipid bilayer is formed from two lipid monolayers at the air/water interface such that the three-phase contact monolayer-bilayer-monolayer is free from any support ('MBM technique'). The bilayers are made from cardiolipin and glycerin monooleate spread from a volatile solvent. Joining and disjoining of the monolayers to a quasi-solvent-free bilayer is reversible. The process is characterized by video and by recording capacitance. The new method provides the basis for a formation of bilayers in equilibrium with monolayers on two Langmuir troughs in a controlled state.

Introduction

The two lipid systems, planar bilayer and unilamellar vesicle, as introduced 20 years ago [1–4] are still of topical interest with respect to the simulation and reconstitution of biomembranes [5–7]. Their preparation, however, still implies some obscure operations which may reduced only by a thorough physical characterization of the assembly and stability of the systems. The crucial processes involved are on one hand the closure and opening of bilayer fragments [8] and on the other hand the joining and disjoining of monolayers.

After the initial attempts of Langmuir [9], a series of methods have been applied successfully to join two monolayers from an oil/water or an air/water interface, to mention in particular those of Van den Bergh [10] and of Montal and Mueller [11–13]. However, despite recent progress [14,15], no system has been developed in which the monolayer/bilayer junction is under complete control in the sense that the two monolayers are in adjustable states with respect to molecular packing, that the three-phase contact mono-bi-monolayer is free and accessible and that the joined bilayer may be studied by standard electrochemical and spectroscopic techniques. Such a system could help to determine the disjoining energy [16]; it could provide a direct relation of packing in monolayer and bilayer [17] and it could guide the selection of an optimal membrane support [18] such that reproducible and stable membranes are formed and can be applied for biochemical studies.

As a first step towards such a system, we present in this paper the realization of reversible joining and disjoining of two monolayers at an air/water interface to a quasi-solvent-free bilayer between electrically isolated compartments with a free three-phase contact line (Fig. 1, inset).

Materials and Methods

As lipids we have used cardiolipin, glycerin monooleate, glycerin monoeruceate and egg phosphatidylcholine/cholesterol (molar ratio 2 : 1) (all from Sigma). The lipids are spread from a 1 mM solution in chloroform/methanol (10 : 1). As contact oils [18] with low solubility in lipid we have applied squalane (Fluka) [19,20] dissolved in chloroform at a molar ratio 1 : 10 and perfluorokero-

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sene (Fluka, high boiling point). Sodium chloride (Merck, p.a.) was used as an electrolyte at a concentration of 0.2 M after roasting it at 500°C for at least 5 h. Water was taken from a Milli-Q apparatus (Millipore).

The overall arrangement is shown in Fig. 1. Two troughs (volume 4 ml) are milled from poly(tetrafluoroethylene) (PTFE) (Hit Huth, Starnberg). They are pressed onto a disk of PTFE (2 mm thick) with soft double-lips turned into their circular edges. Two Wilhelmy balances are connected to the double trough to measure the surface tension [21,22]. The central disk may be rotated by hand or by a cog-belt and motor coupled to a potentiometer. The latter arrangement allows a slow, steady rotation with registration of the angle of rotation in analogy to the deposition of Langmuir-Blodgett films [22].

An excentric opening (diameter 4 mm) in the disk is closed by a thin (6 μm) PTFE-sheet (Hit Huth Starnberg). The sheet is welded to the disk at 350°C (15 min) under spring loading using a sheet of tetrafluoroethylene-perfluoroalkyl ether (60 μm) (Hoechst AG, Gendorf). Before welding, the PTFE sheet is cut with a scalpel. The heating stretches the PTFE sheet and opens the cut to a fish-like slot which is 0.4–1 mm long and 0.05–0.4 mm wide. The edge of this slot is shown in Fig. 2a and b. The cut is oriented such that a rotation of the disk leads to orthogonal dipping of the slot across the air/water interface. An open U-shaped slot – mounted to a disk with a open sector – is used for visual inspection from top (cf. Fig. 2c).

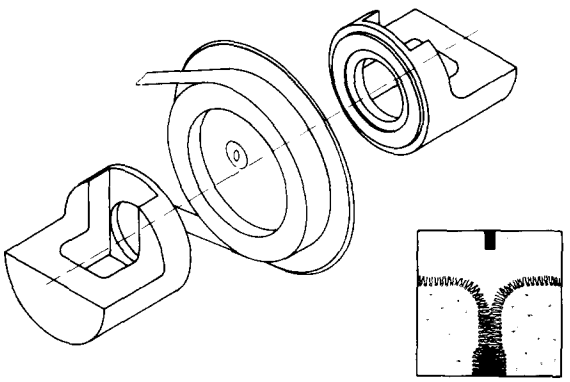

Fig. 1. Arrangement of the mono-bi-monolayer trough used to join two monolayers to a bilayer. The two semi-troughs are pressed onto the central disk by means of springs. A thin PTFE sheet perforated by a slot (length 0.4–1 mm, width 0.05–0.4 mm) spans the excentric opening in the central disk. The slot is dipped across the water surface by rotation of the disk. The inset is a rough sketch of the geometry of the monolayer-bilayer-monolayer junction with free three-phase contact line. (Note the radically different scale for the bilayer (thickness 5 nm) and for the PTFE sheet (thickness about 5 μm).)

The aqueous phases of the two troughs are contacted by Ag|AgCl electrodes inserted into the bottom of the troughs. The troughs are electrically isolated with a contact resistance of more than 10^{12} Ω. AC and DC voltage are applied using an inverting operational amplifier with various feedback resistors (Burr & Brown 3528). Usually during the formation of a bilayer a square-wave (amplitude 5 mV) is applied and the current is observed on an oscilloscope. The capacitance is obtained by application of a sine voltage of 1 kHz (amplitude 5 mV) and observation of the phase-shifted current by a lock-in amplifier (Princeton Applied). The capacitance of the PTFE sheet itself is subtracted from the total capacitance of the system. The conductivity is obtained by application of a DC voltage.

One side of the PTFE sheet is illuminated by a tungsten lamp using a light guide (Schott). The dipping of the slot is observed through a stereo microscope (Zeiss) usually with the help of a video system (Hitachi).

We clean the three parts of the trough with cold chromic acid for at least 24 h, rinse them extensively with pure water and keep them under water until use. We fill the troughs with electrolyte (0.2 M NaCl) up to an identical level as adjusted by a syringe. As the PTFE sheet is transparent we can control both water levels by video. We spray an excess of squalane or perfluorokerosene to both sides of the PTFE sheet or we apply about 20 μl near the slot using a Hamilton syringe with subsequent distribution of the oil by rotating the disk several times up and down. Finally we clean the water surface by suction.

We spread the lipid at the two air/water interfaces using a Hamilton syringe at a large excess or just until a lens of the solvent appears. The surface pressure is around 46 mN/m. Then we dip the slot across the air/water interface. During the dipping process we control the region of contact

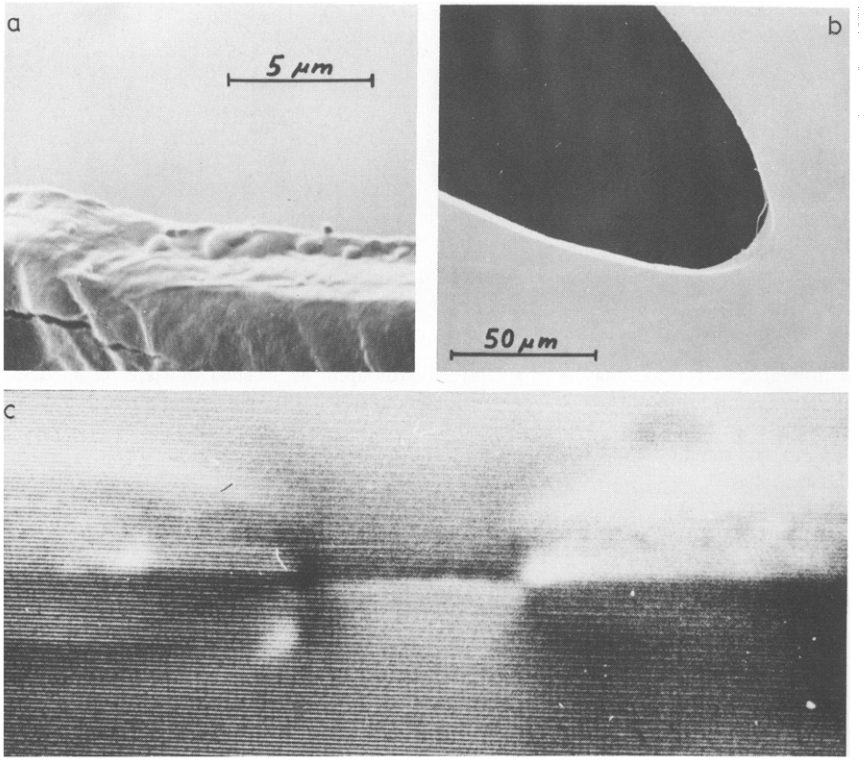

Fig. 2. (a,b) Shape of a slot cut into a sheet of PTFE (6 μm) seen in the scanning electronmicroscope. (c) View on the three phase contact of the mono-bi-monolayer from top (dark line in the center) during dipping in an open U-shaped slot (width 0.15 mm).

of water and PTFE sheet visually on the monitor and check the capacitance continuously on the oscilloscope by the exponential decay of the signal with the applied square-wave voltage.

Results and Discussion

The joining and disjoining of two monolayers of cardiolipin to a bilayer by the mono-bi-mono technique is shown in Figs. 3 and 4 as a series of photographs taken from the monitor and as a record of the capacitance. The total area of the slot is $A_{tot} = 0.25$ mm^2 (length 1 mm, width 0.25 mm). The change of capacitance due to the formation of the bilayer correlates with the motion of the free three-phase contact mono-bi-monolayer along the slot in the PTFE sheet. The top view of the mono-bi-monolayer junction is shown in Fig. 2c during a similar experiment with an open U-shaped slot.

We also obtain results such as that shown in Figs. 3 and 4 with perfluorokerosen instead of squalane. In this case the contact of wetting at the PTFE sheet is smoother. A certain disadvantage of that oil is a softening of the PTFE sheet with concomitant deformation of the slot. The formation of a mono-bi-mono-junction 'on sight' immediately after spreading of the monolayers is not guaranteed. In many cases several trials are required. A drop in surface pressure as due to transfer of monolayers to the PTFE is compensated by additional spreading.

The capacitance of the bilayer during steady slow dipping (rate 1 mm/min) is shown in Fig. 5 as a function of the area of the slot submerged in water. In this experiment the total area of the slot is $A_{tot} = 0.026$ mm^2 (length 0.5 mm, width 0.05 mm). We have obtained the data from a continuous record of the capacitance and of the angle of rotation using a slot with a geometry well characterized in a microscope. The linear relation of capacitance and area corresponds to a specific

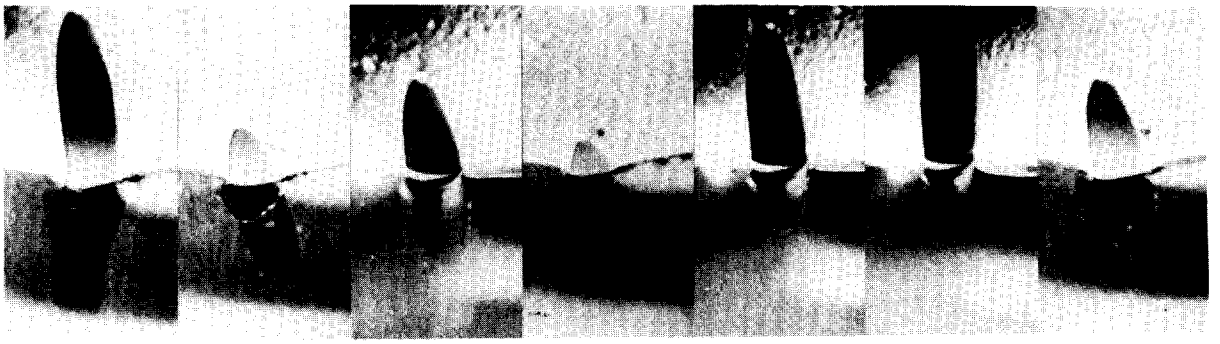

Fig. 3. Sequence of stages of joining and disjoining of two monolayers to a bilayer (cardiolipin) as attained by stepwise manual dipping of a slot across two spread monolayers. The meniscus of the two monolayers joining to the bilayer is apparent (cf. Fig. 2c). The dark part of the oval is the slot above the air/water interface. The width of the slot is 0.25 mm. (The dark oval on the water surface is the shadow of reflectance of the slot.) The stability and reversibility of bilayer formation is seen by comparison with the time-course of capacitance as shown in Fig. 4.

capacitance of 0.76 $\mu F/cm^2$.

The reversibility of bilayer formation is shown in Fig. 6 for cardiolipin. The increase and decrease in capacitance correlate with the oscillation of the slot. In this experiment, the total area of the slot is $A_{tot} = 0.091$ mm^2 (length 0.73 mm, width 0.125 mm). The change in capacitance in such an experiment, however, is far lower than we would expect from the amplitude of dipping. The dashed line in Fig. 6 indicates the motion to be assigned to the capacitance according to the geometry of the slot used. Thus, joining and disjoining of the monolayers follow the motion of the support with some hysteresis. At present moment we do not know whether this hysteresis is due to the mutual adhesion of the monolayers themselves or to the wetting of the support i.e., to the adhesion of monolayers and water surfaces to PTFE.

We have determined the specific capacitances of mono-bi-mono membranes by turning the slot by a step and measuring the increment of capacitance and evaluating the increment of membrane

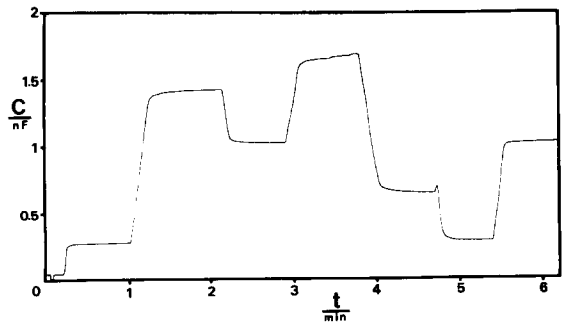

Fig. 4. Capacitance of the monolayer/bilayer system (cardiolipin) as a function of time during a sequence of joining and disjoining as illustrated in Fig. 3. The quasi-stationary states correspond to the seven stages shown in Fig. 3. The stability of the monolayer/bilayer junction with free three-phase contact mono-bi-monolayer is apparent. (The little peak at the end of phase five is due to a short motion downwards.)

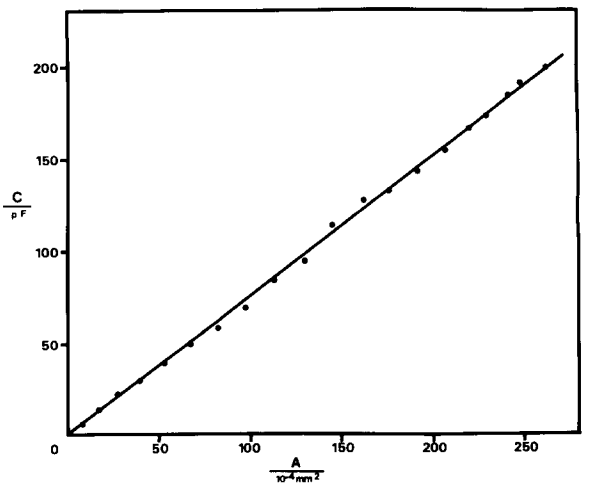

Fig. 5. Capacitance of a bilayer of cardiolipin as a function of the area of the slot beneath the air/water interface. The measurement is obtained with steady slow dipping (1 mm/min). The area is determined for a selected set of stages from the photograph of the slot correlated to the stage of the dipping process. The width of the slot is 0.05 mm.

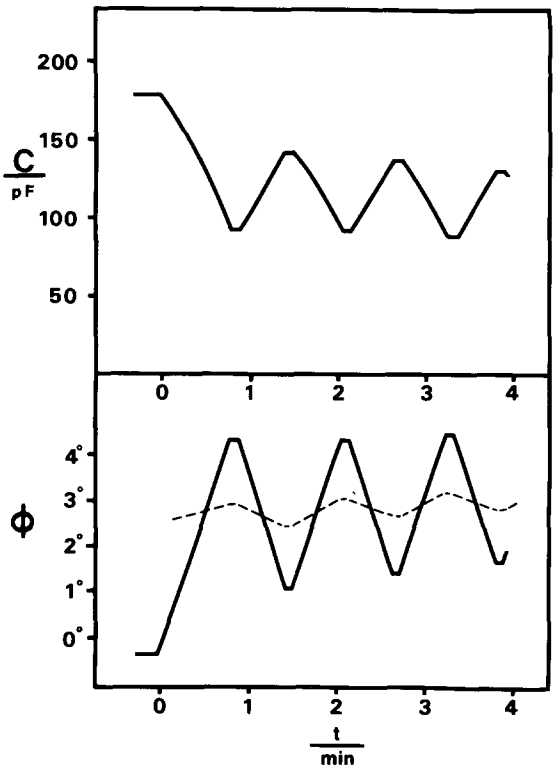

Fig. 6. Capacitance of a bilayer of cardiolipin (top) and rotation angle of the support (bottom) as a function of time. The correlation is apparent. The angle corresponding to the measured capacitance as expected from the geometry of the slot is indicated as a dashed line. The actual motion of the support exceeds the motion of the mono-bi-monolayer contact (hysteresis). The width of the slot is around 0.12 mm.

area from photographs taken during the motion. The capacitances found are: cardiolipin $C_{MBM} = 0.72 \pm 0.05$ $\mu F/cm^2$, glycerin monooleate $C_{MBM} = 0.82 \pm 0.08$ $\mu F/cm^2$, glycerin monoeruceate $C_{MBM} = 0.43 \pm 0.09$ $\mu F/cm^2$ and egg phosphatidylcholine/ cholesterol $C_{MBM} = 0.62 \pm 0.08$ $\mu F/cm^2$. (The errors are standard deviations for ten mono-bi-monolayer membranes.) The capacitance of glycerin monooleate – used often as a standard – corresponds to that found by several authors for quasi-solvent-free bilayers [13,23–25].

Controlling the resistance of the bilayers, we found it in most cases to be lower than reported values. We have observed values from 10^{-3} to 0.1 $M\Omega \cdot cm^2$ whereas in the literature the resistance of quasi-solvent-free bilayers of various compositions is found to be above 1 $M\Omega \cdot cm^2$ [12–14,25]. The origin of this discrepancy is unknown at present.

Mono-bi-mono junctions made from cardiolipin and glycerin monooleate were frequently stable for hours. Rupture occurred preferentially during the joining or disjoining process, often when the contact line passed a certain site of a particular slot. The nature of a possible mechanical defect of the PTFE edge (cf. Fig. 2a, 2b) inducing such an instability is unknown. Membranes of egg phosphatidylcholine/ cholesterol and glycerin monoeruceate were found to be stable for only 2–3 min.

Conclusions

At present, the immediate advantages of the mono-bi-monolayer technique as compared to other methods for the formation of solvent-free bilayers are: (i) direct visual control of the process of bilayer formation; (ii) preparation of monolayers under controlled conditions according to conventional procedures; (iii) steady reversible joining and disjoining of the monolayers with continuous control of capacitance and conductivity.

The main issues of the mono-bi-monolayer technique are the prospects for apparent application. (i) Preliminary experiments show that the spreading pressure may be replaced by the pressure of barriers of two Langmuir troughs. Thus the relation of the packing of the monolayer and of the properties of the bilayer (stability, capacitance, resistance, packing, phase transition) may be studied directly. (ii) As the bilayer is laterally open due to the free three-phase contact line an optical coupling into the interior of the solvent-free bilayer is feasible for spectroscopic studies. (iii) If the formation of mono-bi-mono junctions 'on-sight' can be improved, the design of asymmetric bilayers from well-characterized monolayers may become routine. The size, the geometry, the possible asymmetry and the control of surface pressure make it worthwhile to build up an instrumentation for fluorescence and absorption spectroscopy to be applied to dyes probing electrical potentials and phase transitions as well as to membrane proteins as reaction centers and labelled channels.

Acknowledgments

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