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Asymmetric black membranes formed by one monolayer of bipolar lipids at the air/water interface

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In this work a new technique is presented for the formation of black lipid membranes from a single monolayer of bipolar lipids at the air/water interface. The lipid, extracted from the thermophilic archaeobacterium *Sulfolobus solfataricus*, is characterized by two different polar heads. The membrane is formed with a technique similar to that introduced by Montal and Mueller; however, the lipid is spread only on one side of the teflon partition. Conductance in the presence of valinomycin, voltage-dependent capacitance, current–voltage measurements and electroporation indicate that, as expected, the membrane is asymmetric.

Introduction

The membrane of the thermophilic archaeobacterium *Sulfolobus solfataricus* is organized in a simple monolayer [1,2]. This is possible because the lipids consist of a polar headgroup at each end of a double C_{40} hydrocarbon chain with variable degree of cyclization. Several fractions of the membrane bipolar lipids have been isolated and characterized [3]. Studies on monolayers at the air/water interface have shown that at high pressures most of these compounds are oriented in an upright position, with one polar head anchored to the aqueous subphase [4]. In the case of the hydrolytic fraction glycerol dialkyl nonitol tetraether (GDNT), shown in Fig. 1a, it has been shown that besides an upright position, possibly with some tilt with respect to the normal to the water surface, the molecules are also able to adopt an arch-shaped configuration with the two polar groups in contact with water [4–8].

In earlier studies we formed black lipid membranes (BLM) of GDNT at high temperatures (≥ 40°C), using the conventional technique introduced by Mueller, Rudin, Tien and Wescott (MRTW) [9]. A subsequent decrease in temperature made it possible to study the membrane behaviour over a wide temperature range (6–80°C) [2]. BLMs of GDNT were also formed to study electromechanical stability [10]. It was shown that the electroporation process occurs even in this more rigid system. It is suggested that, under electric field, molecules should bend to form aqueous pores and the polar headgroups of such molecules cover the pore's interior.

More recently, BLMs of several bipolar lipids were formed at room temperature, pretreating the hole of the teflon partition with small amounts of diphytanoylphosphatidylcholine (DPhPC) in n-hexane, to favour the formation of the torus [11]. In the present work we utilized a technique similar to that introduced by Montal and Mueller [12]. The novelty of our method is to form the monolayer only on one side of the teflon partition. In this way it is possible not only to form membranes at room temperature without any DPhPC treatment, but also to obtain 'solvent free' planar lipid films formed by a single monolayer. It will be shown that carrier mediated transport properties, voltage-dependent capacitance and electric breakdown are typical of a highly asymmetric system.

Materials and Methods

The hydrolytic fractions GDNT and GDGT (glycerol dialkyl glycerol tetraether) were extracted from the membrane of the archaeobacterium *Sulfolobus solfataricus* as described previously [13]. The structure of these compounds is shown in Fig. 1a. Aristar chloroform (purity 99.4%) was purchased from BDH, England. Water was purified by means of a Millipore Milli-Q system, including a terminal 0.22-μm filter. Valinomycin (Aldrich, USA) was added at a concentration of 10^{-7} M from an ethanolic stock solution to the preformed BLM.

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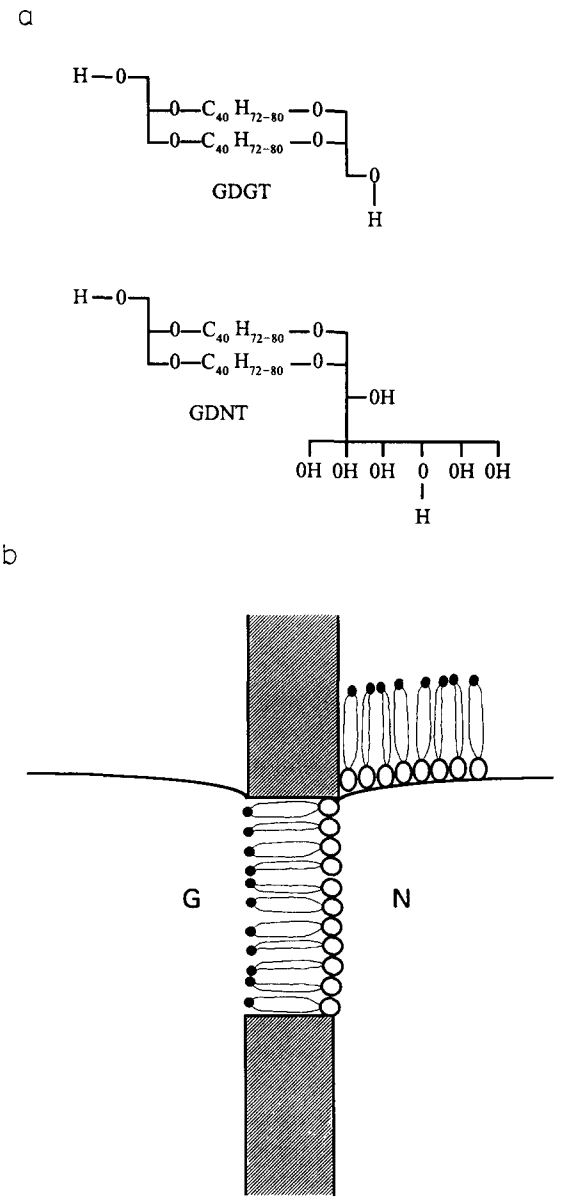

Fig. 1. (a) Structure of isoprenoid ethers, backbone of complex lipids of thermophilic archaeobacteria. (b) A sketch of the nonitol (N) and glycerol (G) side of the asymmetric membrane.

The membranes were formed at room temperature with a modified version of the technique introduced by Montal and Mueller [12]. The thin teflon film separating the aqueous compartments of the two half-cells (2 ml) had a circular hole 160 μm in diameter. No hydrophobic preconditioning of the teflon partition was required. The two compartments were filled below the aperture with a 0.1 M non-buffered KCl solution (pH 5.5). Bipolar lipids were dissolved in chloroform at a concentration of 10 mg/ml and 10 μl of this solution were spread on a surface of about 1 cm^2 to form a monolayer only on one side of the two air/water interfaces. Raising simultaneously the water level of the two half-cells above the aperture led to membrane formation (Fig. 1b), which was checked by a subsequent measurement of the membrane capacitance.

The electric signals were recorded via a voltage clamp circuit according to the usual two electrodes configuration using a high-impedance (10^{13} Ω) operational amplifier (Burr Brown 3528 CM). Ag/AgCl electrodes were employed for applying and recording potentials. Membrane conductance was determined using a 40 mV peak-to-peak square wave at $\nu = 0.5 \cdot 10^{-2}$ Hz. The capacitance–voltage measurements were obtained after sending a 10 mV peak-to-peak and 10 kHz input sinusoidal signal through an adder circuit. A decoding circuit gave an output signal proportional to the membrane capacitance. The sensitivity was on the order of 1%. The electroporation measurements were performed under current clamp conditions with the membrane in a feedback network of the high impedance operational amplifier, as previously described [14].

Results and Discussion

Membranes of GDNT formed quite easily after the lipid spreading, while after longer periods (30 min) membrane formation was very difficult. No membranes were formed when the two levels were raised independently. By contrast, we were unable to form BLMs with the GDGT fraction, as already found previously using the MRTW technique [15]. Only occasionally 'thick membranes' characterized by a capacitance C_s = 0.1 $\mu F/cm^2$ and a dielectric thickness of 180 Å were formed.

The specific membrane conductance of GDNT membranes in 0.1 M KCl was on the order of 10^{-8} $\Omega^{-1}\,cm^{-2}$, a value which agrees with that obtained by the MRTW technique, and indicates that this membrane is a very good insulator. The specific capacitance is $C_s = (0.70 \pm 0.08)$ $\mu F/cm^2$ and the dielectric thickness (considering a dielectric permittivity $\epsilon_r = 2.2$) is d = 28 Å, in agreement with previous findings [2,11,15]. This value indicates that both techniques allow the formation of 'solvent-free' membranes and that the molecules are spanning the membrane. Since the length of the fully extended C_{40} hydrocarbon chain, with a mean value of 2.3 cyclopentane rings, is $d \simeq 37$ Å [16], a thickness of 28 Å indicates that molecules tilt with respect to the normal of the membrane plane at an angle of approx. 41°.

Occasionally, membranes with a lower specific capacitance $C_s = 0.46 \pm 0.05$ $\mu F/cm^2$, corresponding to a mean dielectric thickness d = 43 Å, were also formed. This value is higher than the fully extended length of the hydrocarbon chain. Therefore it is difficult to imagine that the membrane is formed by a simple monolayer. A more reliable model is one in which a U-shaped configuration of molecules (which would form a double

layer, or an interdigitated structure with glycerols partitioning in the apolar core) coexists with molecules in an upright position (which form a single monolayer with the polar headgroups segregated on opposite sides of the membrane). This membrane would be a heterogeneous structure formed by a patchwork of the two types of domain. The molecular arrangement and the transport properties of these thicker membranes will be examined in a future study.

One of the attractive features offered by this method is the possibility to form asymmetric membranes. For instance, in a GDNT monolayer at the air/water interface, the larger polar head nonitol (N) is more firmly anchored to the aqueous subphase than the glycerol (G) for both configurations of the molecules (upright and arch-shaped). Thus, once the membranes are formed, the glycerol will be generally projected on the other aqueous side so that the two different polar heads will be located at the two opposite sides of the membranes, named N and G, as sketched in Fig. 1b. The N side is the one containing the monolayer at the air/water interface.

Four independent series of experiments have been performed in order to test the asymmetry in membranes with high as well as low capacitance. The first consisted in measuring the increase in membrane conductance induced by valinomycin when the carrier was added separately to the N or G side. The experiment was performed by spreading (after membrane formation) a lipid monolayer on the aqueous solution also on the G side to avoid asymmetric partitioning of the valinomycin. Valinomycin was first added to the N side and then, after measuring the conductance, to the G side. Fig. 2 depicts the time behaviour of a typical experimental run. As expected, a much smaller increase in conductance is observed when valinomycin is added to the N side. This result indicates a much lower partition coefficient of valinomycin at the nonitol with respect to the glycerol side, due to the network of hydrogen bonds among the nonitol polar heads [17].

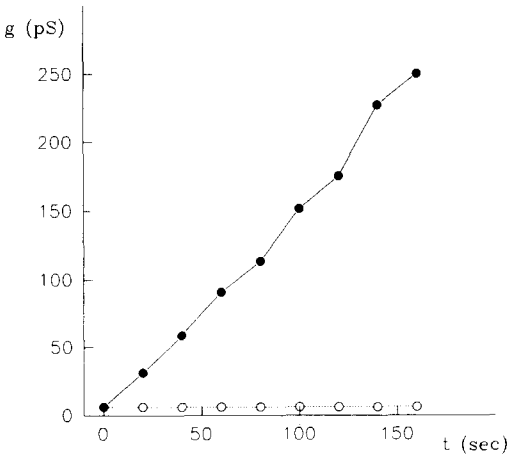

Fig. 2. A typical experimental run indicating the time-dependent conductance, g, after addition of valinomycin (final concentration 10^{-7} M) in two different ways: only on the N side (○); also on the G side (●). Ionic solution: KCl 10^{-1} M. Specific membrane capacitance $C_s = 0.75\ \mu F/cm^2$.

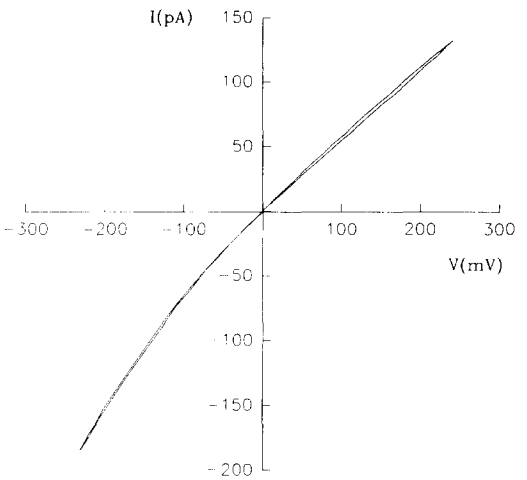

Fig. 3. Current–voltage characteristic performed under voltage clamp conditions by applying a voltage triangular wave to the membrane at a frequency $\nu = 0.3 \cdot 10^{-2}$ Hz. The membrane was unmodified. Ionic solution: 10^{-1} M KCl. Specific membrane capacitance $C_s = 0.73\ \mu F/cm^2$.

In the second set of experiments the current–voltage characteristics have been recorded, as shown in Fig. 3. Also in this case a clear asymmetry can be observed, due to the internal field inside the membrane, which is positive on the nonitol side. In all these experiments control measurements were performed (to avoid artifacts due to asymmetry of the system) by spreading the lipid in the opposite half-cell.

The third and fourth experiments were performed on thicker membranes, which could withstand very high electric fields. The third experiment involved capacitance–voltage (C–V) measurements. It has been shown that the capacitance C varies as a function of V, according to the relationship

$$C(V) = C(0)\left[1 + \alpha(V + \psi)^2\right] \tag{1}$$

where $C(0)$ is the capacitance at zero potential, α is related to the thickness elastic modulus and ψ is the internal potential across the membrane. This relationship has been shown to be valid for membranes with and without solvents. Thus, if membrane asymmetry exists, an internal potential arises owing to the difference in the dipole potentials of the two surfaces [18]. In this case, the $C(V)$ curve shown in Fig. 2 gives $\alpha = 3 \cdot 10^{-3}\ V^{-2}$ and $|\psi| = 70$ mV, the internal potential being more positive on the nonitol side. This result indicates that the thicker membranes are not only asymmetrical, but that they are also characterized by extremely stiff chains. In fact, the value of α for

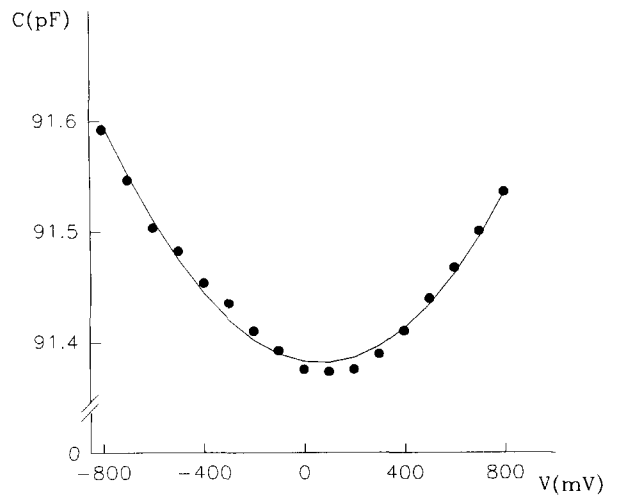

Fig. 4. A typical plot of changes in capacitance vs. the external applied potential. The solid curve is the best fit of the experimental points with Eqn. 1. The fitting parameters are $\alpha = 3 \cdot 10^{-3}$ V^{-2}, $\psi = -70$ mV. Ionic solution KCl 10^{-1} M. Specific membrane capacitance $C_s = 0.45$ μF/cm^2.

'solvent-free' bilayers of glycerol monooleate is $\alpha \simeq 3 \cdot 10^{-2}$ V^{-2} [19] while for GDNT at $T \simeq 42$°C only an upper limit $\alpha < 5 \cdot 10^{-2}$ V^{-2} had been estimated [15]. Fig. 4 also indicates that these thicker membranes can withstand very high potentials (around 800 mV) without breaking.

Finally, the last experiment is related to the electromechanical stability of the membrane under current clamp conditions. It has been shown that the current–voltage characteristic curve of the membrane displays a reversible conductance transition to a higher level. This transition, which occurs above a critical potential V_c [14,18] is interpreted, according to the electroporation theory, as due to the formation of aqueous pores. While in a symmetric membrane the critical potential does not depend on the field direction, this is not the case for an asymmetric membrane. Indeed Fig. 5a shows that the transition occurs at quite different values, when the electric field is reversed. This behaviour provides a further evidence of the membrane asymmetry. Repeating the I–V cycles the degree of asymmetry decreases, as shown in Fig. 5b. The latter cycle corresponds to a lower frequency and therefore the capacity current and the hysteresis effects are reduced. The hysteresis of the cycles is related to the relaxation time of the membrane, which slowly recovers its initial conductance state. During this process the potential returns to zero within some minutes, and only at this stage was the cycle repeated. In order to explain the process of symmetrization observed after the first I–V cycle, one would expect molecules to flip across the hydrophobic core. This behaviour is consistent with the model proposed for electroporation in bipolar lipid membranes [10], which predicts the formation of hydrophilic pores covered by the bent U-shaped molecules.

Concluding remarks

It can be concluded that this new membrane formation method can be used to prepare stable and stiff membranes. Moreover, this method provides direct evidence that the lipids are really spanning the membrane. The thinner membranes exhibit a higher asymmetry than that obtained by 'conditioning' the membrane at high temperatures (approx. 80°C) [2]. This

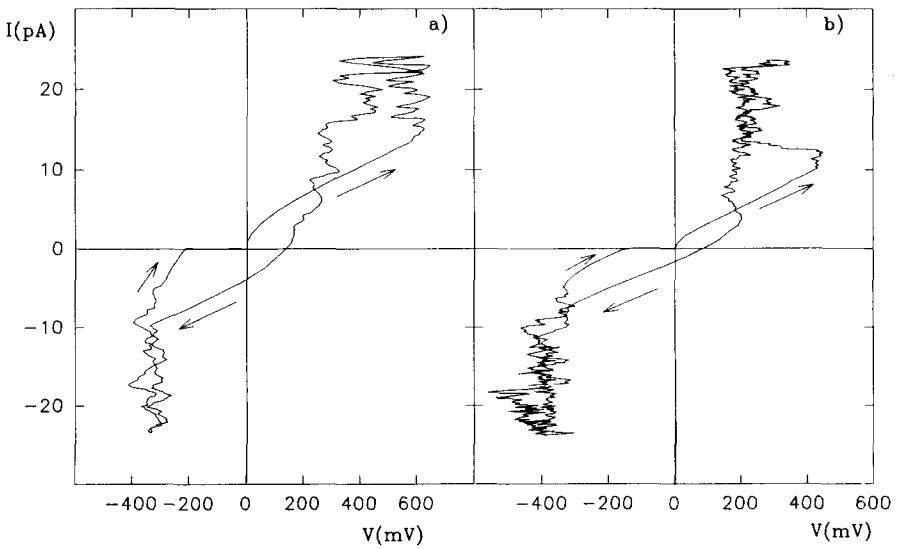

Fig. 5. Current–voltage characteristics of a GDNT membrane under current clamp conditions in the presence of valinomycin 10^{-7} M. (a) First I–V cycle. Frequency $\nu = 10^{-2}$ Hz. The capacity current in the linear region is 2.4 pA. (b) Eighth I–V cycle. $\nu = 3 \cdot 10^{-3}$ Hz. The capacity current in the linear region is 0.6 pA. Ionic solution KCl 10^{-1} M. Specific membrane capacitance $C_s = 0.35$ μF/cm^2.

asymmetry gives rise to a dipole potential higher on the nonitol side, which could constitute a barrier to the diffusion of H^+ ions. Since membrane asymmetry has also been shown to exist in vivo [20], it is likely that the physiological conditions of high temperature and high pH gradient can be substained by the membrane of *Sulfolobus solfataricus* not only due to the absence of a fracture plane in the middle region of the membrane, but also because of the dipole potential due to the asymmetric disposition of molecules.

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