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Alkylated glass partition allows formation of solvent-free lipid bilayer by Montal–Mueller technique

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ABSTRACT

Formation of bilayer lipid membrane (BLM) by Montal–Mueller technique across a small aperture in a partition film traditionally requires coating of the aperture with a hydrophobic substance, often just an organic solvent. However, we demonstrate here that the most effective coating is not strictly hydrophobic but rather provides water/oil repellent properties. BLM were formed from diphytanoylphosphatidylcholine (DPhPC) on small 0.1–0.8 mm apertures made in specially prepared alkylated glass coverslips. The coverslips were either fluorosiliconized by 3,3,3-Trifluoropropyl-trimethoxysilane, which reduces adsorption of DPhPC in addition to creation of hydrophobic surface, or silanized, which promote adsorption of DPhPC. At fluorosiliconized surfaces stable BLM were formed. Specific capacitance of these BLM was 0.86 μF/cm^2 ± 5%, while their lateral tension was estimated as 4.3 ± 0.4 mN/m. BLM were stable for hours under moderate voltage applied. At silanized surfaces stable BLM were formed only in acidic medium (3 < pH < 4), while at higher pH the membranes could cover the aperture only partially. Thus, apertures in fluorosiliconized glass can be robustly used for formation of model lipid membranes under physiological conditions.

1. Introduction

Two basic techniques of planar bilayer lipid membranes (BLM) formation are presently known. The first one (Mueller–Rudin technique) consists in placing a droplet of lipid solution in a non-polar organic solvent at an aperture in a Teflon film [1]. The second one (Montal–Mueller technique) consists in folding two lipid monolayers at an aperture in a Teflon partition placed between two half-cells [2,3]. It is evident that some amount of the solvent remains in the central hydrophobic area of the membrane formed by the first technique. This was confirmed by direct measurements of specific BLM capacitance depending on the solvent used [4]. Presence of the solvent in microlenses and membrane meniscus does not allow obtaining adequate data of lipid bilayer tension and its influence on membrane fusion. The fusion kinetics is strongly dependent on the solvent type, namely the size of its microlenses [5,6]. Clearly, fusion of such BLM is not an adequate model of biomembrane fusion. At first blush, the Montal–Mueller technique of monolayer folding appears to be free from the above-mentioned disadvantages. However, White et al. [7] has shown that the stable membranes are formed by this technique only after pretreatment of the aperture edges by high weight hydrocarbons (squalene, squalane, etc.) or before total evaporation of solvent from monolayers. Unfortunately, these substances are contained in membrane meniscus and in small amounts in the bilayer [8]. Thus, neither of the techniques allows forming of absolutely solvent-free BLM (without solvent in bilayer and meniscus).

Why is the presence of solvent so important for membrane formation? It creates a range of essential conditions. Firstly, the solvent increases hydrophobicity of the partition (reduced by amphiphilic lipid molecules) and provide proper orientation of lipids near the edge of the aperture. Secondly, the meniscus with solvent serves as a vibration absorber improving the membrane stability. Thirdly, non-polar solvent performs a function of insulator and sealing material preventing electric leakage in the membrane edge area. Therefore, it would take a very specific surface to create a truly solvent-free BLM.

Recently alkylated inorganic materials (glass or silicon) were proposed for membrane formation instead of polymer films [9]. Although BLM were formed by painting (Mueller–Rudin) technique, it is principally possible to form membranes by other techniques. These new materials differ from polymers in surface stability due to their hardness. Moreover, by binding different chemical agents with long flexible hydrophobic residues the surface properties can be easily varied.

It is clear that a solvent-free BLM should be less stable than ordinary artificial membranes. It is known, that physical properties of diphytanoylphosphatidylcholine (DPhPC) provide very high stability of planar bilayers formed from this lipid. DPhPC has pK_a of 2.1 and 13.9, so it remains neutral in a wide range of pH [10]. Moreover, it does not have a phase transition from −120 °C to 80 °C [11]. These properties of DPhPC make formation of membranes without solvent more convenient.

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The aim of the work was to develop a technique of truly solvent-free BLM formation by bringing monolayers at partitions with specific grafted surface together. Evidently, the interaction between lipids and alkylated surface plays a key role in this process. Optimization of the surface treatment by different alkylating agents as well as variation of lipid adsorption properties should allow maximally compensate the absence of solvent and choose the best conditions for stable membrane formation.

2. Materials and methods

2.1. Alkylation of glass partitions

Bilayer lipid membranes were formed at apertures in glass coverslips (#0, Thomas Scientific, USA). The apertures with the diameter of 100–400 μm were produced by etching in HF (Chimmed, Russia). A slot aperture was used in some experiments. The coverslips were treated by silanization in 2% solutions of trichlorooctadecylsilane, decyl-dimetyl-chlorosilane, dichlorodimetylsilanesolutions (all from Fluka Chemie, Germany) in toluene (for UV spectroscopy, Chemapol, Czech Republic) or by fluorosiliconization in 2% toluene solution of 3,3,3-Trifluoropropyl-trimetoxysilane (Fluka Chemie, Germany) to make the surface hydrophobic or amphiphobic (oil/water repellent), correspondingly.

2.2. Contact angle measurements

Measurements of contact angle were performed on sessile water drops (10 μl droplets) by measuring the tangent to the drop at its contact with the coverslip surface. The droplet image was obtained by microscope (MBS-2, "LOMO", Russia) with digital camera («Nikon», model Coolpix 4500, Japan) and observed at monitor display («TVS», model MM-15, Taiwan). To study the effect of lipid monolayer on the contact angle, 0.5 μl 0.1 mg/ml solution of 1,2-Diphytanoyl-*sn*-Glycero-3-Phosphocholine (DPhPC) (Avanti Polar Lipids Inc., USA) in hexane (for chromatography, Reachem, Russia) was placed on the surface of the water droplet.

2.3. Membrane formation

Solvent-free membranes were formed by folding two lipid monolayers at the aperture in a partition placed between two Teflon half-cells. In contrast to usual Montal–Mueller technique [2,3,7], we did not perform any partition pretreatment by hydrocarbon solvent. Each half-cell contained 0.1 M solution of KCl (99.8% grade, Reachem, Ukraine) in distilled water. Solution pH was varied by adding HCl (Chimmed, Russia). The pH values were measured by digital pH-meter ("Ecotest-120", Econix, Russia). A droplet (near 2 μl) of 0.5 mg/ml DPhPC solution in hexane was placed onto the surface of the aqueous solution. This amount of lipid was two times greater than it was necessary for monolayer formation. After 15 min, hexane evaporated and monolayers were folded by sequential lifting water levels in both half-cells. The half-cells were filled with the solution by two syringes with micro-supply through Teflon microtubes (inner diameter of 1 mm). The level was elevated with the average velocity of about 0.1 mm/s and stopped near the lower edge of the aperture. Membranes were formed spontaneously within several minutes. To measure membrane conductivity in the presence of nystatin («Sigma–Aldrich», USA), 2.5 mol. percents of cholesterol («Avanti Polar Lipids», USA) was added to DPhPC.

The process of membrane formation was optically controlled by microscope (MBS-2, "LOMO", Russia) with digital camera («Nikon», model Coolpix 4500, Japan) and observed at monitor display («TVS», model MM-15, Taiwan). The membrane formation was detected by abrupt increase of the measured electrical capacitance.

2.4. Electrical measurements

All electrical measurements were performed by potentiodynamic technique. Triangular wave signal (100 mV peak-to-peak, frequency 10–1000 Hz) was applied to the membrane from a generator («EG&G Parc», model 175, USA) by a pair of Ag/AgCl electrodes. Current response was measured by current amplifier («Keithley», model 427, USA) at digital oscilloscope («Gould», model 1425, UK). Membrane conductance and capacitance were calculated assuming that the membrane conductance is negligible compared to the conductance of the bulk solution.

3. Results and discussion

3.1. Preparation of alkylated glass

When forming BLM by Montal–Mueller technique, the partition with aperture dividing two half-cells should have very hydrophobic surface. Otherwise, the orientation of the lipid molecules will not allow forming the bilayer by folding two lipid monolayers. Besides, partition wettability in the presence of amphiphilic lipid molecules increases.

In order to provide proper orientation of the lipids, a technique of glass surface preparation to subsequent alkylation was used [12]. Glass treatment was performed as follows:

1) 30 min incubation in mixture of methanol with hydrochloric acid (1:1);
2) 30 min incubation in concentrated sulfuric acid;
3) alkylation in solution of chlorosilane (silanization) or siloxane (fluorosiliconization).

Silanization technique proposed in [12] was modified by sequential treatment of the samples by three chlorosilanes (trichlorooctadecylsilane, decyldimethylchlorosilane, dichlorodimethylsilane) in toluene in decreasing order of their hydrocarbon tail length [13]. The procedure should promote occupation of vacant surface by shorter silane molecules after adsorption of the longer ones.

Fluorosiliconization was made by treatment of the freshly washed and dried glass coverslips in 2% solution of 3,3,3-Trifluoropropyl-trimetoxysilane in toluene. During the treatment, chemical binding and subsequent two-dimensional polymerization of the siloxane occurred. Owing to CF_3 groups at the end of the siloxane molecule, the fluorosiliconized glass surface became inert with low surface free energy. It is known that monolayer of CF_3 groups is a surface with the lowest free energy of about 6 mN/m versus 18 mN/m for Teflon [14]. Thus, the fluorosiliconized surface became amphiphobic (exhibiting a water/oil repellency).

After triple rinse of each coverslip in toluene (to remove unbound silane or siloxane), they were dried and stored dry under Ar. The samples were used within 1–3 days after glass treatment.

High value of contact angle served as a criterion for successful treatment. Pure water droplet on this surface had a contact angle of about 110°. Thus, the hydrophobicity of the treated coverslips was close to that of Teflon surface (contact angle 110°–120°).

3.2. Formation of bilayers at silanized partitions

After studying surface properties of the alkylated glass coverslips we proceeded to their application for forming BLM at an aperture in a partition between two half-cells. The water level in the first half-cell was higher than in the second one before and during elevating the second monolayer, i.e. the solution of the first half-cell filled the aperture cavity completely. Indeed, focusing at the nearside of the partition through the second half-cell before elevating the level there, we could sharply see the flat mirror-like monolayer surface. We succeeded in formation of solvent-free membranes by monolayer

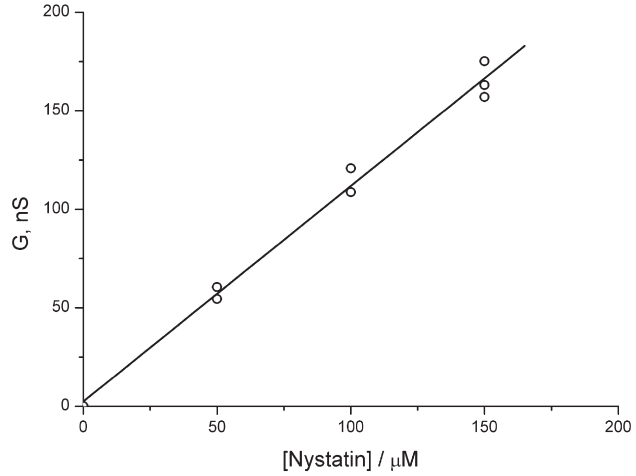

Fig. 1. Membrane conductivity as a function of nystatin concentration. The aperture diameter was equal to 277 μm. The straight line corresponds to linear regression of experimental data. Coefficient of determination r^2=0,995.

folding at low pH (from 3 to 4). Lifetime of the membranes reached 2 h. BLM capacitance did not change with frequency of voltage pulses in the range of 10–1000 Hz. The membrane occupied aperture in the partition incompletely when pH was higher than mentioned values. It was observed optically and by membrane capacitance value. In this case, after elevating the water level in the second half-cell the membranes broke inevitably. The same results were obtained at apertures in Teflon films (thickness 15 μm).

To confirm bilayer structure we have studied dependence of the membrane conductivity on nystatin concentration in water solution. This antibiotic can make channels only in cholesterol containing lipid bilayers when it is added to one side of the membrane [15,16]. We observed that membrane conductivity grew proportionally to nystatin concentration (see Fig. 1). The conductivity was negligible without nystatin and increased by 57 nS per 50 μM of its concentration. This unambiguously proved bilayer structure of our membranes.

As at pH from 3 to 4 the membranes occupy the entire area of the aperture, its area was used to calculate specific capacitance of the membranes, yielding the value of 0.86 μF/cm^2±5%. The value coincides with capacitance of membrane formed my Montal–Mueller technique from DPhPC solution in squalene [8]. Incomplete occupation of partition aperture by BLM at rather high pH can be explained by lipid adsorption at hydrophobic substrate, which increases its wettability.

3.3. Formation of bilayers at fluorosiliconized partitions

Alkyl residues of silane molecules have high affinity to hydrocarbon tails of lipids. Water/oil repellency of fluorosiliconized coating on glass and slight adsorption of DPhPC at any pH should allow obtaining stable solvent-free membranes occupying all aperture area. The membranes formed on such partitions had high capacitance independent of pH. They were stable and did not change its area (controlled by capacitance) both with alignment of the levels and increase of hydrostatic pressure (up to 15 mm of water column) at that side of BLM where the first monolayer was folded (similar to membranes formed at apertures in silanized partitions). These membranes did not break after elevating the second level above the aperture resulted in their area increase (controlled by capacitance). This fact allowed us to measure membrane tension.

According to Laplace's Eq. (1) the pressure difference across membrane is inversely proportional to its curvature radius with aspect ratio equal to 2σ, where σ is the membrane tension:

$$\Delta P = \frac{2\sigma}{R} \tag{1}$$

where ΔP is a pressure difference across the membrane and R is the radius of curvature of the bilayer membrane. Since it is very difficult to estimate R by conventional optical techniques, we calculate it by measurement of membrane capacitance as follows.

The area of bowed membrane is given by simple geometric relation:

$$S = 2\pi R\left(R - \sqrt{R^2 - r^2}\right) \tag{2}$$

where r is a radius of aperture on which BLM was formed. It is supposed that membrane bows only up to the half sphere. Since the membranes formed by our technique are absolutely free of solvent we assumed that the area of flat BLM is equal to the area of aperture.

Hence,

$$R = \frac{S}{2\pi\sqrt{\frac{S}{\pi} - r^2}} \tag{3}$$

The membrane area can be obtained from measurements of its capacitance as

$$S = \frac{C}{C_0}\pi r^2 \tag{4}$$

where C_0 is an area of flat BLM.

Therefore, membrane capacitance as a function of pressure difference across the membrane should be following:

$$C = \frac{4\sigma}{r^2(\Delta P)^2}\left(2\sigma - \sqrt{4\sigma^2 - r^2(\Delta P)^2}\right)\cdot C_0 \tag{5}$$

The membrane capacitance was half as much again when pressure increased by 74±3 Pa (see Fig. 2). This essential membrane area increase was probably provided by lipid excess in membrane edge region. Experimental data were extrapolated by second-order polynomial and the capacitance of planar membrane was taken at the minimum of the curve. It was 69 pF. This value differs from the initial membrane capacitance (66 pF) by less than 5%, so BLM was flat when formed. Since the diameter of aperture was 100 μm, the specific membrane capacitance was estimated from minimum capacitance as 0.86 μF/cm^2±5% (equal to specific capacitance of BLM formed on silanized partitions).

Using Eqs. (1)–(4) and the value of C_0 we can estimate the membrane tension. The pressure difference was proportional to inverse radius of membrane curvature. Experimental data in

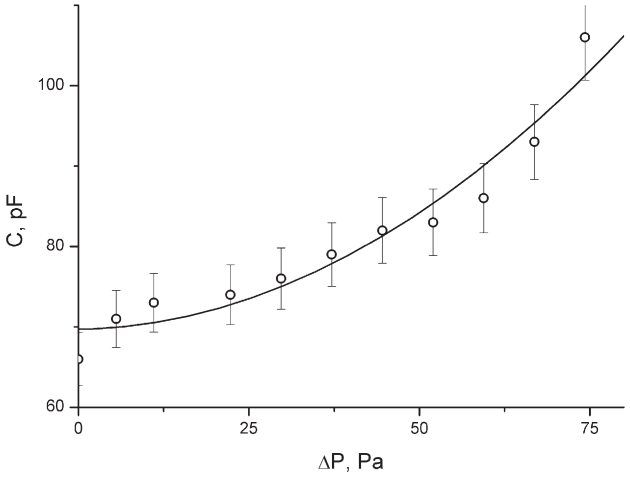

Fig. 2. Membrane capacitance as a function of pressure difference across the membrane. Membranes were formed at pH=6,53 at aperture with diameter of 100 μm in fluorosiliconized glass coverslip. The curve corresponds to the second-order polynomial interpolation of the data.

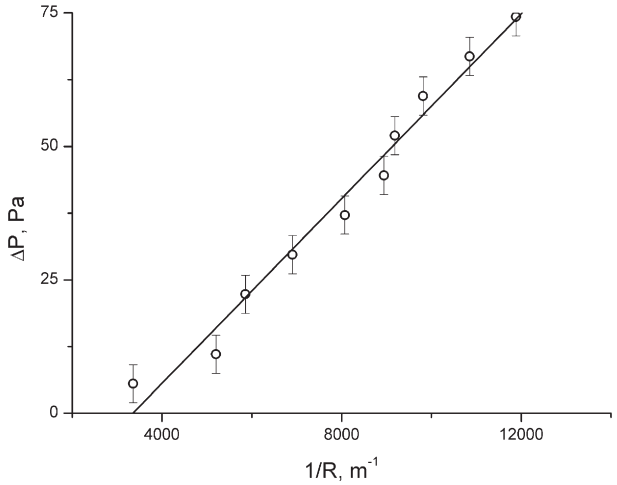

Fig. 3. Calculated from membrane capacitance inverse radius of curvature as a function of the pressure difference across the membrane. All conditions are the same as for Fig. 2. The straight line corresponds to linear regression of experimental data. Coefficient of determination r^2=0,989.

corresponding coordinates are shown in Fig. 3. Based on the slope, the membrane tension can be estimated as σ=4.3±0.4 mN/m. For solvent-containing membranes, lower values are typical. For example, σ=3.4 mN/m for egg lecithin membranes [17]. High tension of our membranes is presumably caused by absence of solvent. Indeed, the solvent facilitates building up of the bilayer under pressure difference, but can produce defects (microlenses) in membrane.

4. Conclusions

We present the method of creation of solvent-free bilayer lipid membranes by Montal–Mueller technique [2,3] at the aperture in alkylated glass partitions. Formation of the bilayer films was confirmed by high value of their specific capacitance (0.86 μF/cm^2) and linear increase of their conductivity in presence of nystatin. On silanized surfaces DPhPC adsorption increased wettability of the partitions that caused difficulties in membrane formation. Contrary, siliconization of glass coverslips by fluorosiloxane in addition to hydrophobization reduced lipid adsorption owing to property of oil repellency. Siliconization allowed forming stable BLM in wide range of pH. BLM can exchange and retrieve lipids from the lipid layers (reservoirs) on siliconized partition that, in particular, allows blowing of BLM to measure its lateral tension. High values of the tension (σ=4.3±0.4 mN/m) indicate absence of solvent in the membrane and meniscus. Overall, fluorosiliconized glass partitions hold much promise as a support for truly solvent-free membranes suitable for membrane studies.

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