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Hydrophobic thickness of fluid planar monooleylglycerol membranes maximally thinned by inversed micellisation

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Abstract

A procedure of making membranes of amphiphilic materials at the bottom of a U-shaped flexible plastic tube within an aqueous medium is described. The membranes were made sufficiently large in order for the annulus area to be neglected. Consequently the hydrophobic thickness of the membrane could be measured by a capacitance technique assuming the relative permittivity of the hydrophobic part of the bilayer. Introduction of an AC microvolt technique allowed manufacture of stable thick membranes by quenching the electroconstriction observed when DC electrical potentials in the millivolt range are used. By continuously monitoring the hydrophobic thickness and by use of the AC microvolt technique the membrane-thinning process by chemical means could be studied in isolation because the electroconstriction was quenched. The maximally thinned hydrophobic thickness of a monooleylglycerol membrane measured at 38°C was found to be 25 ± 1.2 Å. Criteria and argumentation for maximal thinning of the membrane are put forward. A distinction between genuine and modified cholesterol was demonstrated to be possible by the described method.

Keywords: Lipid bilayer; Hydrophobic thickness; Capacitance; Microvolt technique; Maximal thinning; Spontaneous emulsification; Modified cholesterol

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1. Introduction

Artificial planar fluid lipid membranes have been studied since 1938 [1] mainly by two classical techniques: from two monolayers using the dipping technique [2]; and since 1957–1960 by the BLM or the brush technique [3]. The patch-clamp- and the vesicle-derived techniques [4] are modifications of the dipping technique. When using a parallel plate capacitor as a model of fluid lipid membranes a reliable determination of the membrane area is essential. Furthermore, in order to determine the real membrane area, planarity and area subtraction of the part of the membrane occupied by the annulus (torus, Gibbs-Plateau border) is required. It is, however, difficult by the classical techniques to make artificial fluid membranes large enough, so that the area of the annulus is relatively small enough for the geometric area of the membrane to be used in the calculation of the membrane thickness.

With the method presented in the present paper, large fluid monooleylglycerol membranes are made by fast expansion of the membrane area. During this fast expansion the membrane stability is secured by the Marangoni effect, and afterwards the membrane thinning by inversed micellisation is monitored by continuous measurement of the membrane capacitance by a microvolt AC technique.

Besides a detailed description of the method (apparatus and procedure), a preferred thickness of 25 ± 1.2 Å of the stable maximally thinned membranes is found. An extended description is given of the membrane thinning process and the different possibilities for drainage of the membrane-forming materials during the membrane-thinning process.

In the present paper only fluid lipid membranes are considered, and a maximally thinned membrane is defined as a lipid bilayer consisting of two apposed, non-interdigitating monolayers without support, neither adsorbed at a rigid surface, nor made rigid by polymerisation of membrane-forming components. Furthermore, the two monolayers are apposed to each other without any detectable amounts of membrane-forming components in between them.

2. Materials and methods

2.1. Membrane-forming components

Paraffin dünnflüssig, Merck 7174 (par-oil); 1-monooleyl-rac-glycerol; C18:1; cis 9; Sigma 7765, relative permittivity = 2.2 (GMO); 5-cholesten-3-ol, Merck 3670 (chol.); and oxidised cholesterol (ox. chol.) was prepared from freshly re-crystallised cholesterol that was dissolved in *n*-octane and refluxed in oxygen at 126°C for 6 h. After lyophilization the white fluffy powder [5] was added to a solution of GMO in low viscosity paraffin oil (par-oil).

2.2. The aqueous medium

Sodium chloride 154 mM and boric acid 100 mM was dissolved in distilled water and pH was adjusted to a value between 6.5 and 7.0. The specific conductivity of this solution was found to be 16 mS/cm at 26°C. The urea added to the aqueous medium in certain experiments was urea, Merck 108487, 5 mM dissolved in the aqueous medium.

2.3. Preparation of membrane-forming liquids

GMO, 150 mg of GMO dissolved in 5 ml of par-oil; GMO + ox. chol. dispersion, 150 mg of GMO was dissolved in and 250 mg of ox. chol. and added to 5 ml of par-oil. After pre-thermostating at 60°C the GMO + ox. chol. dispersion was made by stirring for 1 min and centrifuged at $400 \times g$ for 5 min. The supernatant, a turbid emulsion, was used as membrane-thinning agent. GMO + chol. solution: 150 mg of GMO and 250 mg of chol. were dissolved in 5 ml of par-oil. The three membrane-forming liquids were divided into 300-μl specimens (Sanz-tubes) and kept in a dark place at 5°C until use. Before application in the apparatus (see Section 2.4), the specimens were stirred, centrifuged and thermostated at 45°C for at least 1 h.

2.4. Apparatus

The measuring tube (see Fig. 1) consisted of

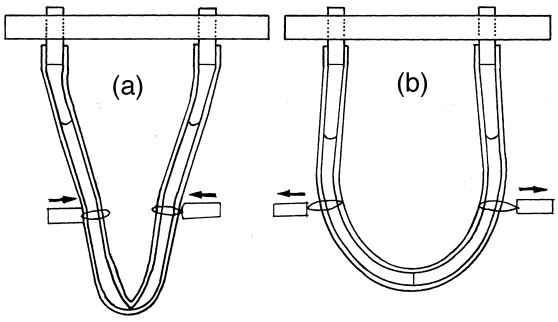

Fig. 1. The U-shaped measuring tube in two positions: A, bent, making a virtual hole at the bend; and B, expanded with re-establishment of the full bore of the tube. At the bottom of the U-shaped tube, the fluid lipid membrane is depicted as a vertical line.

flexible pellucid silicone rubber, 17 cm long, 4 mm bore and with an outer diameter of 7 mm. The measuring tubes were stored in par-oil. In addition to the measuring tube, the thermostat (38°C) contained an equally dimensioned tube for calibration purpose. In the calibration tube the lipid membrane was mimicked by fixed platinum electrodes connected to three known capacitances of 47, 68 and 100 nF parallel to resistances of 50, 100, 500 and 1000 kΩ. The three capacitances at 500 kΩ were measured at every thickness determination as part of the standard recording (see Section 2.5).

Another set of platinum electrodes could be moved by hand between the two tubes and a resting position outside the thermostat. This set of electrodes was connected to a two-phase lock-in amplifier EG and G PARC model 5210. Between the oscillator output of the amplifier and one of the electrodes, an ohmic voltage divider (1000–1) was inserted, securing a microvolt potential over the lipid membrane. A potential of 9 μV, a reference oscillator frequency of 21.3 Hz and a time constant of 10 s were chosen. The phase-sensitive detector was tuned for capacitance measurements and connected to an IBM compatible computer and screen.

The measuring tube could be bent and expanded from outside the thermostat by means of two arms mounted on a Faraday cage.

2.5. Standard procedure and standard recording

Initially the three calibration capacitances were registered in the computer and checked on the screen. Then 0.5 ml of aqueous medium was added to the measuring tube and the moveable set of electrodes was transferred from the calibration tube to the measuring tube. After the tube was bent (Fig. 1A), 10 μl of GMO solution were placed at the bend. Then the full bore of the measuring tube was quickly re-established (Fig. 1B), leading to the formation of the membrane. Immediately the recording was started and monitored over 80 s, after which the recording was stopped. After the measuring tube was bent, 2 μl of the membrane-forming liquid to be analysed (e.g. GMO + ox. chol. dispersion or GMO + chol. solution) was added at both sides of the bend. Immediately after the membrane was made, the recording was started and monitored without interruption for 176 s for detection of a possible membrane thinning.

In the following sections we shall, by the standard recording procedure, refer to the entire recording (256 s) consisting of 80 s for calibration and recording the thick membrane of the GMO solution, and 176 s for detection of a membrane-thinning effect of a given agent.

2.6. Capacitive membrane thickness determination and calculation of the hydrophobic thickness

When a parallel plate capacitor is used as a model for the bilayer area, planarity and area subtraction of the part of the membrane occupied by the annulus (torus, Gibbs–Plateau border) are essential. Circular membranes with diameters of 3, 4 and 5 mm were made, and it was found that membranes with diameters of 4 and 5 mm yielded identical membrane thickness by the capacitive membrane thickness determination. Therefore a diameter of 4 mm was chosen for the tubes used in the apparatus, indicating that the area of the full bore of the tube could be used as a valid approximation to the true membrane area, which is essential for the capacitive thickness determi-

nation. Planarity was automatically secured by the way the membrane was made.

When calculating the membrane thickness by use of membrane capacitance, only the hydrophobic thickness of the lipid membrane (h) is determined,

$$h = \varepsilon_o \varepsilon_r A C^{-1} \quad (1)$$

where ε_o and ε_r are the absolute and relative permittivities and C is the capacitance. The h is measured in angstroms when $\varepsilon_o = 0.08859$ pF, $\varepsilon_r = 2.2$ (assumed), $A = 0.125$ cm^2 (calculated from tube dimensions) and C was recorded in nF on the phase-sensitive detector.

Determination of membrane area and capacitance is fairly accurate, the relative permittivity, however, can only be assumed. In the calculations the actual value for ε_r was chosen because the relative permittivity of the used low-viscosity paraffin oil is 2.2 and the relative permittivity of GMO is 2.200 [6].

When electrical potentials in the microvolt range are used, the annoying electrode polarisation is omitted, normally experienced when electrical potentials in the millivolt range are used. The introduction of the phase-sensitive detector offers the possibility of continuous recording of membrane capacitances. It was found that an established lipid membrane by direct application of a membrane-thinning agent in excess was maximally thinned within seconds and shown subsequently to be stable for hours.

Some relevant observations made during the development of the method, including membrane thinning by electroconstriction, are reported in the Appendix.

3. Results

The results are divided into two groups, depending on whether they are collected by use of the standard procedure and recording, or by application of the membrane-thinning materials directly upon the established thick membrane.

3.1. Results obtained by use of the standard procedure and recording

Fig. 2 shows a curve obtained by this technique. After recording of the three known capacitances 10 μl of GMO solution were added to the bend of the measuring tube (see Fig. 1A) followed by quick establishment of the full bore of the tube (Fig. 1B).

The thick membrane was so thick that the

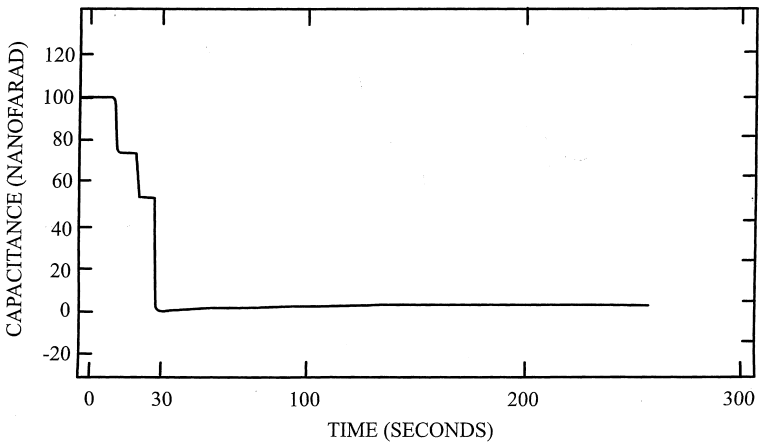

Fig. 2. Standard recording of GMO solution (see text); 0–30 s, three known capacitances (47, 68 and 100 nF); and 30–256 s: recording of membrane thickness after application of 10 μl of GMO solution at the bend of the U-shaped tube and re-establishment of the full bore of the tube. Time constant is 3 s. No membrane-thinning was observed. The thick membranes were stable with membrane capacitances of effectively 0 nF.

phase-sensitive detector tuned for capacitance measurement could not measure the thickness. Zero capacitance means that the thick membranes were over 600 Å thick. These thick membranes were stable for hours. Fig. 3 shows an example of the standard recording of 10 μl of GMO solution to which 2 μl of GMO + ox. chol. dispersion was added at time 80 s. The membranes thinned within 20–30 s and remained thin for hours. The choice of a time constant of 10 s depressed the background noise, but extended the recording time of the thinning process to 20–30 s. With a time constant of 3 s this time lag was reduced to approximately 10 s. With time constants of 3 and 10 s the real thickness were averaged over periods of 3 and 10 s, so it can be deduced that if the thinning process could be depicted in real time, this process could be estimated to last for approximately 1 s.

The use of a time constant of 10 s also blurred the stepwise mode of thinning, which was recognised to the full extent when the membrane-thinning was followed by recording of the membrane resistance in real time, using a membrane potential of 30 mV (see Appendix A).

When the capacitance had reached a constant value within seconds, and this value remained constant for the rest of the measuring time (176 s), it was assumed that the membrane would not thin further. Since the thin membranes were stable for hours, and the thickness of the single thin membrane could be monitored for many minutes without a change in thickness, it was assumed that the membranes were maximally thinned.

The standard procedure and standard recording shown in Fig. 3 were repeated many times, typically once per week as a control of the apparatus and the liquids used. These repeated checks were performed without any preconceived idea of a large reproducibility of the membrane thickness under these standardised conditions. However, in fact a high degree of reproducibility turned out to be the case.

During the study three batches of oxidised cholesterol were made and chronologically marked batch 1, 2 and 3 in total, corresponding to 90 samples. From Table 1 it can be seen that batch 2 and 3, with regard to average thickness, are the same differing from batch 1 by a 1.6-Å lower value. The standard deviations of the normal distributions, however, were the same, so it can be claimed that the present method by use of the standard procedure and the standard recording was precise enough to distinguish batch 1 from batch 2 and 3.

Therefore, because the standard deviations for all three batches were the same, it can be as-

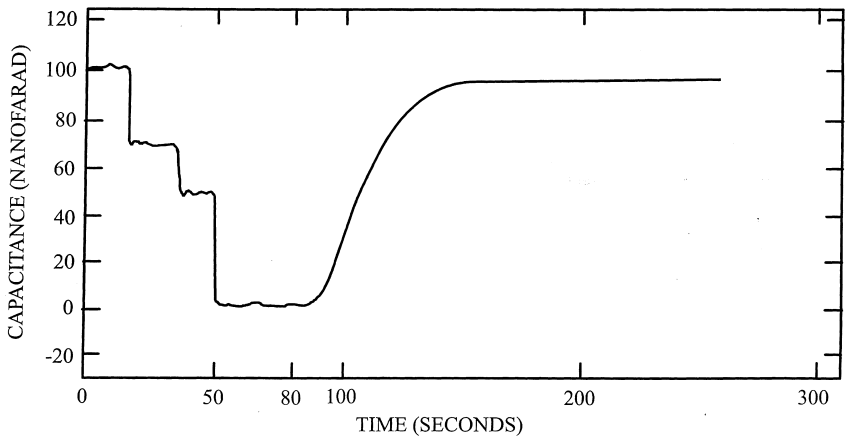

Fig. 3. Standard recording with membrane-thinning material (see text): 0–50 s, three known capacitances; 50–80 s, GMO solution; and 80–256 s, GMO + ox. chol. dispersion. Time constant is 10 s. The actual capacitances with one decimal were recorded every 10 s. Average of capacitances at 230, 240 and 250 s is 101.1 nF. Membrane thickness (2436/101.1) is 24.1 Å, cf. Eq. (1).

Table 1
Hydrophobic thickness of maximally thinned fluid monooleylglycerol membranes at 38°C.

	Batch 1	Batch 2	Batch 3	Batch 2 + 3	Batch 1 + 2 + 3
Number	28	27	35	62	90
$\bar{h}$Å[a]	26.1	24.4	24.6	24.5	25.0
S.D.	1.26	1.25	1.24	1.24	1.44

[a]Average hydrophobic thickness, $\bar{h}$, in angstrom at 38°C of maximally thinned fluid membranes of monooleylglycerol (GMO), made by three different batches of oxidised (modified) cholesterol (see text). *Abbreviations*. 'Number' denotes the number of samples used from each batch. $\bar{h}$ refers to the average thickness in angstrom of the samples in each batch. S.D. denotes standard deviation.

sumed that all the 90 values of hydrophobic membrane thickness reflect maximal thinning of the membranes, suggesting the hydrophobic thickness of a GMO bilayer at 38°C to be 25 ± 1.2 Å.

The amount of membrane-forming fluid that initially exists in the thick membranes was supposed to be drained quantitatively by inversed micellisation (see Section 5). As a sign of inversed micellisation occurring when the GMO + ox. chol. dispersion contacts the aqueous medium, a bright glimpse of light was observed at the bend of the measuring tube (Fig. 1A) which was strongly illuminated. The instantaneous glimpse was caused by a quick change in the refractive index at the interface between oil and water. This phenomenon can be compared to the momentaneous interfacial turbulence described when spontaneous emulsification takes place [7]. When urea (5 mM) was added to the aqueous medium, no fast glimpse of light, nor any thinning of the membrane was observed (see Section 3.2).

Fig. 4 shows an example of a standard recording of 10 μl of GMO solution to which 2 μl of GMO + chol. solution were added at time 80 s. No increase in capacitance (membrane-thinning) at all was registered, neither a glimpse of light in connection with the application. By modification of the membrane-forming materials — adding genuine cholesterol in molar fractions of 0.125, 0.25 and 0.5 to the GMO solution — only thick and stable membranes were produced.

Stable thin membranes as the one depicted in Fig. 3 could be destroyed by bending the measuring tube (Fig. 1A), and then remade by re-establishment of the membrane (Fig. 1B) several times after one application of 2 μl of GMO + ox. chol. dispersion to 10 μl of GMO solution by the standard procedure. The first three to four of the

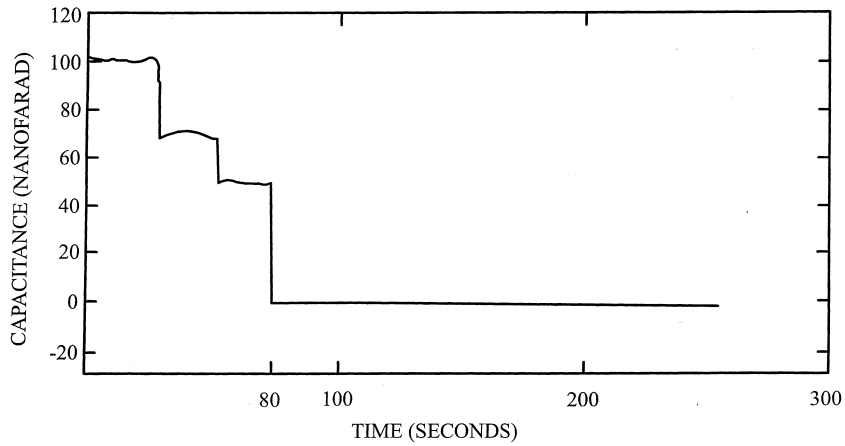

Fig. 4. Standard recording of genuine unmodified cholesterol (see text): 0–80 s, three known capacitances; (see text). Time constant is 10 s. No membrane-thinning was observed.

reformed membranes showed maximal thinning. Then afterwards only membranes with full thickness (zero capacitance), as the one depicted in Fig. 2 could be made-until no more membrane-forming fluid was left in quantities sufficient for making the thick membranes.

The oily membrane-thinning material initially placed at the bottom of the aqueous medium eventually gathered at the top of the two columns of aqueous medium. When the membrane-forming liquid was collected from the two aqueous surfaces and then added to the GMO solution in a new experiment, no membrane thinning could be detected, only stable thick membranes could be made. This means that the modified cholesterol was separated from the membrane-forming fluid before transport up along the hydrophobic inside of the measuring tube.

When modified cholesterol was present in excess, the intermonolayer membrane-forming fluid was evacuated from the membrane, but enough excess modified cholesterol still remained in the annulus fluid for two to three maximally thinned membranes to be made before only stable thick membranes could be formed.

Repeated experiments with urea added to the aqueous medium produced a monotonous response. Addition of urea (5 mM) to the aqueous medium completely prevented the membrane thinning observed when addition of the GMO + ox. chol. dispersion to the GMO solution was made according to the standard procedure (see Fig. 3). In all these experiments, only stable thick membranes as the one shown in Fig. 2 were obtained.

3.2. Results obtained by direct application on established thick membranes

First of all, it was possible to demonstrate the quick thinning of the membranes as seen by use of the standard procedure by addition of 2 μl of GMO + ox. chol. dispersion directly to the preformed thick GMO solution membrane. Secondly, the urea experiments were repeated by use of direct application on the established thick membrane. Two ways of adding urea to the membrane were investigated. In the first way, 10 mM of urea was added to the aqueous medium and no thinning, even after hours, could by observed by direct application of modified cholesterol on the existing thick membrane.

The other way of adding urea to the membrane involved application of a few microlitres of 10 mM urea dissolved in the aqueous medium next to the established thick membrane. After application of 2 μl of GMO + ox. chol. dispersion onto the same side of the thick membrane, initially no membrane thinning could be observed, but after approximately 20 min the membrane began thinning, and after a further 2–3 min the thinning membrane broke when a thickness of approximately 35 Å was reached. Maximally thinned GMO membranes could not be made in this way.

The experiments show that if the urea could be removed by diffusion away from the membrane into the bulk of aqueous medium, the membrane-thinning effect of modified cholesterol returned gradually, no doubt effected by an inversed micellisation at the oil–water interface, as described by Davies and Rideal [7].

When urea was present on the aqueous side of the oil–water interface, inversed micellisation was prevented, and only thick membranes could be made. When urea was present, modified cholesterol behaved as genuine cholesterol. Presumably, the chaotropic urea molecule breaks the hydrogen bonds of structured water that is necessary for the inversed micellisation at the oil–water interface to be provoked.

4. Membrane thinning

The study of the process of membrane thinning is obviously facilitated by easy access to stable thick membranes, and by the option of continuous recording of the membrane thickness. Both are possible by the described method. Thick membranes as used in this method are stable planar, vertical oily structures in an aqueous medium consisting of GMO dissolved in thin paraffin oil between two monolayers of GMO at the two oil–water interfaces. The thinning process is then effected by drainage of the liquid between these two monolayers.

Other options of the present method is quenching of electroconstriction by use of potentials in the microvolt range, necessary for studying the effect of chemical membrane-thinning agents. Finally, there is the option of applying these membrane-active chemicals in two ways by manufacture of the membranes: (1) by application of the chemicals to the GMO solution as a GMO + ox. chol. dispersion before manufacture of the membrane; and (2) by application of the GMO + ox. chol. dispersion directly onto the established thick membrane made of GMO solution. Neither of these options appears to have been reported in the literature before.

During the work with the present method it was experienced that the stability of the membranes depends on the hydrophobic thickness. Only thick membranes with capacitances measured to 0 nF, which means membrane hydrophobic thickness > 600 Å, and very thin membranes with a thickness < 30 Å, were stable, which means that they lived longer than the duration of the standard recording of the present method (256 s). The stable thick and very thin membranes, on the other hand, could be observed for hours, even days, without rupturing.

Another lesson of the present work with artificial fluid lipid membranes is that the membrane-thinning chemical has to be present in excess. The continuous recording of membrane thickness was started within 1/10th of a second after the manufacture of the membrane, so the thinning process was recorded from the very beginning of the formation of stable thick membranes. Only if the thinning process starts within a second and is completed within seconds, can the stable thin membranes can be made. Because the final thickness of these stable thin membranes is constant and reproducible, all the stable thin membranes made by the standard procedure and recording are claimed to be maximally thinned.

By the other option of applying the membrane-thinning chemical agent upon the already made thick membrane, it is also possible to make maximally thinned stable membranes. However, also by this way of application, the membrane-thinning agent has to be present in excess; and then, as observed by use of the standard procedure, the thinning process is complete within seconds.

If smaller amounts of the membrane-thinning agent — not enough to be present in excess — were added to the thick membrane, only metastable membranes could be made. They thinned slowly within minutes until a thickness of approximately 35 Å was reached; then they ruptured. One of the aims of the present work with membranes was to make stable membranes with any prescribed hydrophobic thickness. This aim could not be obtained. It was never possible to make stable membranes with a thickness within an interval of 30 to approximately 600 Å by any of the two ways of applying the membrane-thinning chemicals.

Immediately after establishment of the full bore of the measuring tube, a thick membrane was always recorded, when 1 or 2 s have elapsed between the application of the membrane-thinning agent and expansion of the membrane. Even when the membrane-thinning chemical is present in excess, the expansion of the membrane did not rupture the membrane, probably because of the Marangoni effect, by which the interfacial tension in an oil–water interface is substantially increased during expansion and only during the expansion. This effect is due to transient dilution of the surface-tension-lowering agents at the interface, by which the high surface tension of water is temporarily approached. This also implies a transient increase in free water activity at the interface, which has been shown to be able to stop or quench spontaneous emulsification [8].

The state of the water molecules of the aqueous medium at the interface also seems to be of crucial importance for the membrane-thinning process of the present method. At fully equilibrated oil–water interfaces, the water molecules are structurally arranged as hydrate or vicinal water [9]. A perturbation of this equilibrated water configuration at the interface, for example by addition of a chaotropic agent breaking the hydrogen bonding of the water molecules near the oil–water interface, could be expected to interfere with the membrane-thinning process, in accordance with the observation made in the present work. As an example of this phenomenon it

was found, as described in Section 3, that the presence of urea at the oil–water interface prevents the membrane-thinning process.

5. Drainage of membrane-forming liquid from the thick membrane

When a thick planar fluid lipid membrane becomes thin, the amount of membrane-forming fluid between the two monolayers has to be diminished. If only a little fluid is removed, the thick membrane stays thick, and its thickness cannot be measured with the phase-sensitive detector used in the present work.

At the other extreme, at the conditions of maximal thinning and with maximal performance of the phase-sensitive detector, the intermonolayer membrane fluid is evacuated quantitatively, leaving the two monolayers apposing each other in the middle of the hydrophobic region. The hydrophobic thickness is then determined by two times the average length of the hydrophobic tails of the amphiphiles, under the assumption that the hydrophobic tails do not interdigitate, and that they are arranged in parallel to each other and to the normal of the surface of the membrane.

With the present method there are six possibilities for drainage of the intermonolayer membrane-forming components from a thick membrane:

1. Drainage away from the planar membrane to the annulus of the membrane (the Plateau border), which is facilitated by suction effected by the annulus, as a consequence of the first law of Laplace.
2. Drainage away from the annulus of the membrane by capillary suction between the aqueous medium and the hydrophobic inside of the measuring tube. This kind of drainage involves the hydrophobic GMO solution, spreading up along the hydrophobic inside of the tube. When the GMO + ox. chol. dispersion is present, only the GMO solution part of this dispersion is drained, leaving the more polar modified cholesterol up-concentrated in the annulus and membrane fluid. The fast spreading of amphiphiles with long aliphatic carbon chains at an oil–water interface has been described as a discontinuous, stepwise event. Biebuyck and Whitesides [10] explain this phenomenon as discontinuous spreading of self-assembled monolayers and a bulky part of long-chain amphiphiles, and Chan and Horn [11] conclude, that the continuum Reynolds theory breaks down when the drainage of very thin films (> 50 Å), made of, e.g. *n*-tetradecane and *n*-hexadecane, occurs in a series of abrupt steps, whose size matches the thickness of molecular layers in the liquid. Bhushan et al. [12] explain the discontinuity in sliding between surfaces of liquids and solids by shear-induced difference in ordering of the liquid molecules, effected by the time–temperature superposition, creating periodic dynamic first-order transitions from high friction states to low friction states. This phenomenon has been treated theoretically by Weber [13].
3. Drainage due to the pressure heads of the two water columns of 2.5 cm of aqueous medium on both sides of the vertical planar membrane made in the bottom of the U-shaped measuring tube. Another possibility of drainage due to gravity is effected by the difference in density between oil and water. These three possibilities of drainage, all tend to diminish excess intermonolayer membrane-forming fluid. However, as stable thick membranes could be made by the AC microvolt technique by use of GMO solution and the GMO + chol. solution, but not by use of the GMO + ox. chol. dispersion that is a turbid solubilization, it is obvious that the three possibilities of membrane drainage are not the only mechanisms capable of thinning the membrane. The membrane thinning observed by use of the DC millivolt technique without addition of modified cholesterol, and the thinning observed by use of the AC microvolt technique with addition of modified cholesterol have to be explained by other possibilities of membrane drainage. Three other possibilities exist:

4. Drainage by electroconstriction [14,15]. At constant area, the measured capacitance is inversely proportional to the membrane hydrophobic thickness, and therefore a measure of this membrane parameter. The size of the electrical potential used is important: if this is in the millivolt range the potential itself thins the membrane [15,16]. Therefore, if one wants to study membrane-thinning by non-electrical, e.g. chemical means, the electroconstriction has to be quenched. This can be done by use of potentials in the low microvolt range, and by use of high carbon lipid solvents in the preparation of the membrane-forming liquid, securing a substantial decrease in the coefficient, α [cf. Eq. (2) below], determined by the choice of lipid solvent. The influence of the electrical potential, E, and α can be evaluated by the empirical equation

$$C_m = C_o(1 + \alpha E^2) \quad (2)$$

where C_m and C_o are the two capacitances (membrane thickness) with and without application of the potential. α is 10, when low carbon solvents (e.g. methanol, hexane and decane) are used, and α is 0.02, when high carbon solvents (e.g. fluid hydrocarbons with 14 or more carbon atoms) are used for the preparation of membrane-forming fluids [15]. By use of low-viscosity paraffin oil (containing straight and branched hydrocarbons with 18 or more carbon atoms) and an electrical potential of 9 μV as used in the present work, quenching of the electroconstriction was secured, and the exact value of α could be ignored. In that case, stable thick membranes could be made when membrane-forming fluid without addition of modified cholesterol was used.
5. Drainage by lowering of the interfacial tension of the oil–water interface. The surface tension of water is decreased by the water-soluble components of the aqueous medium. At the oil–water interface between the headgroups of the monolayer-forming amphiphiles, the interfacial tension is further decreased by the long aliphatic hydrocarbon tails of the amphiphiles. With a probable interfacial tension of 50–60 dyn/cm at the oil–water interface in the system used in the present work, a decrease in interfacial tension will promote a drainage of the membrane-forming liquid away from the membrane by the drainage possibilities 1, 2 and 3. In connection with formation of microemulsions [17] or with initiation of spontaneous emulsification [7], very low oil–water interfacial tensions have been reported, when an additional, more polar surface-tension-lowering agent was added to a solution of high carbon solvents. In the present work the more polar surface-tension-lowering agent is modified cholesterol. Genuine cholesterol is fully soluble in the GMO solution, and does not thin the thick membrane. The lowering of the interfacial tension could account for drainage of most of the intermonolayer fluid by the drain possibilities 1, 2 and 3, when the fourth possibility electroconstriction was quenched. However, there would be some membrane-forming fluid left centrally in the membrane caused by the capillary suction of the membrane itself. Hence, for quantitative evacuation of the intermonolayer membrane fluid still an additional possibility of drainage is needed.
6. Drainage of membrane fluid through the oil–water interface. This possibility could be effected by spontaneous emulsification caused by one or more of the following three items: (i) negative interfacial tension; (ii) interfacial turbulence; and (iii) 'diffusion and stranding' into the aqueous medium in the form of fine drops [7]. These fine drops will in the present work be called inverse micelles.

For initiation of the spontaneous emulsification in the present method, a third inter-facial-tension-lowering agent, e.g. a second amphiphile, is required, which has to be only little soluble in oil and water, but more soluble in water than in oil. This third agent was primarily solubilized in oil as agent-in-oil particles,[1] and fast spontaneous emulsification takes place when these agent-in-oil micelles were exposed to a water phase with

highly structured water molecules at the interfaces. The agent-in-oil micelles convert to agent-in-water micelles according to Bancroft's rule, which states, that the phase in which the emulsion-stabilising agent is more soluble will be the continuous phase. This process has been described as going on until only little oil as continuous phase is left [18] and as a fast reaction lasting for seconds [7].

In the present work, modified cholesterol has been used as the additional interfacial-tension-lowering agent. Genuine cholesterol is fully soluble in thin paraffin oil, and therefore spontaneous emulsification cannot be provoked. The modified cholesterol, however, can only be solubilized in thin paraffin oil, because it is more polar than genuine cholesterol. Therefore, modified cholesterol fulfils the requirements for an additional agent capable of initiating spontaneous emulsification.

It is here assumed that parts of the other intermonolayer membrane-forming components (GMO dissolved in thin paraffin oil) are taken along with the agent-in-oil micelles, when they were converted into agent-in-water micelles at the oil–water interface.

Finally, the quantitative removal of the residual membrane-forming fluid between the two monolayers can be effected by demolition of the capillary suction of the membrane itself by interfacial turbulence [7] in the 'walls', constituting the two oil–water interfaces. When the distance between the two interfaces in apposition across the hydrocarbon region is within the distance allowed by a balance between universal attractive and repulsive forces between the two interfaces, and this distance is accommodated by two times the average length of the acyl chains of the used amphiphiles, the thin fluid membrane is stable and maximally thinned. A maximally thinned membrane is a fluid lipid membrane with no measurable hydrophobic fluid between the two monolayers.

[1]Because these particles added to the GMO solution are big enough to make a turbid solubilization, the agent-in-oil particles are referred to as agent-in-oil micelles.

By the 6th possibility of drainage, the quantitative evacuation of the intermonolayer membrane fluid is then possible if the modified cholesterol is present in excess, and the conditions for inversed micellisation are present.

6. Discussion

The characteristic magnitude of approximately 25 Å of the hydrophobic thickness (the hydrocarbon layer) of stable and reproducible planar fluid lipid membranes made by C18:1 acyl chains at 38°C is determined by different forces and molecular interactions within the membrane. Besides the common intermolecular forces, the attractive and repulsive forces specific for surfaces (e.g. oil–water interfaces) have to be considered, especially when they are in close apposition across water or hydrocarbon.

These surface-specific universal attractive and repulsive forces have not been measured across hydrocarbon, but are well characterised across water [19,20]. Upon approaching polar surfaces across water, the long-range attractive van der Waals force and the strong repulsive hydration force begin to be of importance when the separation between the interfaces decreases beneath 30 Å. From 30 to 20 Å the two forces increase, but the repulsive force increases more steeply than the attractive force, and at a given distance (approx. 25 Å), the repulsive and the attractive 'inter-surface' forces counterbalance.

The possibility exists that these universal forces acting between surfaces (here defined as interfaces between oil and water) across water, also exist across hydrocarbon. If this could be experimentally verified, it would offer an explanation of the 25-Å thickness of the GMO membranes of this study being stable and reproducible because they cannot be further thinned, and because the actual and average length of the acyl chains at 38°C is 12.5 Å. This length of the acyl chains of a monolayer secures a 25-Å thickness of the hydrophobic part of a bilayer, where the acyl chains are arranged parallel to the bilayer normal without any interdigitation or intercalation of any

hydrophobic material in between the two monolayers.

In mammalian cell membranes membrane thinning is not needed, because the daughter cell membranes made by mitotic division show the same hydrophobic thickness as that of the parent cells, and the common cell membrane potential in the millivolt range secures maximal thinning by electroconstriction.

Two important questions now arise: (1) is the mammalian cell membrane maximally thinned with no hydrophobic material between the two monolayers; and (2) if it is, we need an explanation of the great diversity in acyl-chain length observed in the hydrophobic part of mammalian cell membranes.

In fact it can be shown that 95–98% of the acyl chains of a broad section of mammalian plasma cell membrane lipids do have an average length which multiplied by two yields 25 ± 1.5 Å (Knudsen, unpublished) securing a maximally thinned hydrophobic phase and simultaneously the energenetically cheapest membrane configuration in this layer. The shortest possible distance between the two water phases across the hydrocarbon phase of the cell membrane is also secured.

Hence by the method of the present study using membrane potentials in the microvolt range electroconstriction was quenched which made the study of membrane thinning by chemical means possible. By this new approach, it was possible to point at a preferable hydrophobic thickness of artificial and mammalian cell membranes of 24 ± 1.5 Å. A hydrophobic thickness of this magnitude of artificial and mammalian cell membranes secures stability and reproducibility, which by nature is used in mammalian plasma cell membranes by the choice of amphiphiles with acyl chains of the proper average length.

Another achievement of the present work is that it has proven to be possible — by the ability to thin a thick membrane — to distinguish between genuine and modified cholesterol. This observation may probably be of interest in the study of cholesterol and cholesterol derivatives involved in development of atheromatous diseases of the heart and the blood vessels.

For the future use of the method, it is possibly of interest that very large circular (4–5 mm in diameter) and maximally thinned fluid lipid membranes can be easily produced. If these large and well-characterised membranes are made permanently stable, ultra-thin semiconductors could be made with a transmembrane energy dissipation comparable to that of the brain cells.

7. Nomenclature

BLM: Black lipid membrane
GMO: Monooleylglycerol
Chol.: Cholesterol
Ox. Chol.: Oxidised cholesterol

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Appendix A

In the course of developing an operational version of the method described in this paper a number of observations were made.

Initially, the tube within which the vertical membranes were made was rotated in order to counteract the difference in density between oil and water. Since it was found that very thin membranes of GMO were stable, the rotation was omitted. Thinning of the membranes was established by application of a DC electrical potential in the millivolt range, and stabilisation of the very thin membranes was secured by addition of oxidised cholesterol, recommended by Tien [5].

The capacitance ($C = \tau R$) was determined by measuring the time of 62.3% of full deflection (τ) by loading and unloading the membrane with 30 mV. The source of current was a DC electrical current in the nanoamp range. The time of 62.3% of full deflection was measured on photographs of the loading and unloading the membrane, reproduced from the screen of a storage oscilloscope. Membrane thicknesses of approximately 300–23 Å where observed; the more thin the more stable membranes. However, membranes with stable thick membranes or membranes with reproducible thickness could not be made by this DC millivolt technique.

When the set-up was turned upside down, so the membrane was made not at the bottom, but at the top of the U-formed tube filled with aqueous medium, the membrane-thinning could still be demonstrated. Under these conditions, the drainage away from the annulus by the difference in density between oil and water of the excess membrane fluid was not possible.

The membrane electrical resistance (R) was measured by an electrometer. The membrane-thinning process was followed in real time, and it was found to occur stepwise. It was, however, not possible to detect any systematics in the size of the jumps.

With the DC millivolt technique both oxidised cholesterol [5] and genuine re-crystallised cholesterol stabilised the thin membranes, and no discrimination between the two compounds could be made with this technique. Neither was manufacture of stable thick membranes possible.

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